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# OBTENCIÓN DE BIOPÉPTIDOS REGULADORES DEL ÍNDICE GLUCÉMICO PARA ALIMENTACIÓN FUNCIONAL

**TESIS DOCTORAL**

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*“Los humanos son extremadamente buenos para adquirir nuevo poder, pero no son muy buenos para traducir este poder en una mayor felicidad.”*

Yuval Noah Harari

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# RESUMEN

# 1. INTRODUCCIÓN

## 1.1 Proteínas, hidrolizados de proteína y péptidos

Las proteínas son uno de los principales componentes de la dieta humana. Estas biomacromoléculas están formadas por la asociación de aminoácidos, a través de uniones covalentes denominadas enlace peptídico entre el grupo amino de un aminoácido y el grupo carboxilo del siguiente aminoácido de la cadena (Nelson & Cox, 2008). La figura 1 muestra la estructura química de un aminoácido, siendo R el radical, que determinará de qué aminoácido se trata. Este radical R puede contener un grupo con características altamente variables: ácido, base, hidrofílico, hidrofóbico, etc., que determinará el comportamiento del aminoácido en sí, sus posibles interacciones, y las características de los péptidos como conjunto de aminoácidos con distintos radicales. La asociación de aminoácidos en una cadena lineal se denomina estructura primaria. Cuando los aminoácidos de la secuencia interactúan a través de enlaces de hidrógeno, se denomina estructura secundaria, y se forman hojas plegadas o hélices alfa. Cuando existen interacciones entre esas estructuras previamente formadas, se denomina estructura terciaria, y finalmente la estructura cuaternaria de las proteínas es una proteína formada por la unión mediante enlaces débiles de más de una cadena polipeptídica en estructura terciaria.

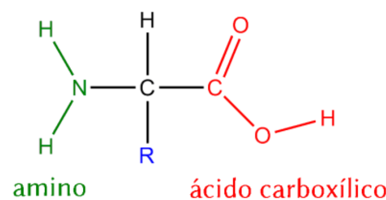


**Figura 1**: Estructura química un aminoácido.

La digestión gastrointestinal de las proteínas conduce a la formación de péptidos en el tracto humano debido a la acción de las proteasas digestivas, y estos péptidos tienen efectos beneficiosos. Se ha demostrado que los péptidos derivados las proteínas alimentarias, generalmente entre 2 y 20 aminoácidos, tienen propiedades bioactivas por la similitud con la estructura de los péptidos reguladores humanos, ya que pueden interactuar con algunas enzimas y receptores implicados en el metabolismo humano. Además, esta hidrólisis de proteínas proporciona los aminoácidos necesarios para la síntesis de las proteínas endógenas en los ribosomas. Análogamente, la hidrólisis

enzimática de proteínas alimentarias mediante proteasas de grado alimentario, cuya especificidad es diferente a la de las enzimas gastrointestinales, ofrece la posibilidad de obtener lo que se denomina hidrolizado de proteína, es decir, el conjunto de péptidos que conformaban la proteína nativa (McDonald, 1985).

La reacción de hidrólisis consiste en romper los enlaces peptídicos y liberar cadenas peptídicas de diferentes tamaños, cuyas propiedades pueden variar en función de las propiedades de los aminoácidos incluidos en cada péptido (Adler-Nissen, 1986). De esta manera, a partir del mismo sustrato, el conjunto de péptidos producidos sería diferente y mostraría propiedades diferentes según el tratamiento enzimático que se aplique a la proteína, y los péptidos resultantes pueden ser más activos y por ende beneficiosos, de lo que lo serían los péptidos liberados por la digestión gastrointestinal de la proteína si se ingiere en su forma nativa. Esta modificación de las proteínas conduce a modificaciones de sus propiedades tecnológicas (solubilidad, poder emulsificante, etc) y biológicas.

A nivel nutricional, la rotura de proteínas en fragmentos peptídicos más pequeños incrementa la digestibilidad de éstas (Acquah, Di Stefano, & Udenigwe, 2018; Hannu & Pihlanto, 2006) y conduce a la pérdida de antigenicidad, reduciendo la inmunoreactividad de la proteína nativa (García-Moreno et al., 2017). Como ventaja adicional, dadas las condiciones en las que se realiza la reacción, no existe pérdida del valor nutricional. Sin embargo, los péptidos suelen tener amargor, por la exposición de residuos hidrófobos. Este amargor puede aminorarse mediante la incorporación o encapsulación en matrices o mediante la adición de proteasas dan lugar a desarrollo de sabor (i.e Flavourzyme ®).

La hidrólisis enzimática de proteínas consiste en la fragmentación de éstas en péptidos y aminoácidos mediante el uso de proteasas, consumiendo una molécula de agua por cada enlace peptídico roto.

$$P1 - CO - NH - P2 \Rightarrow P1 - COOH + P2 - NH2 \qquad (1)$$

La reacción requiere condiciones de reacción suaves y es específica y controlable, además de no implicar pérdida del valor nutricional. La reacción en sí es simple, necesita el sustrato (proteína) y la enzima (proteasa), que puede ser una o varias.

Las condiciones de reacción (pH y temperatura) están determinadas por las condiciones de actividad y estabilidad de la proteasa, y también deben tenerse en cuenta otros factores como la relación enzima/sustrato y el tiempo de reacción. Las condiciones óptimas para la obtención de hidrolizados altamente bioactivos se suelen conseguir mediante diferentes tipos de diseños experimentales (Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2018;

Nongonierma et al., 2019; Pérez-Gálvez, Morales-Medina, Espejo-Carpio, Guadix, & Guadix, 2016).

Al romperse el enlace peptídico, los grupos amino y carboxilo terminal formarán los equilibrios ácidos bases con sus formas $NH3^+$ y $COO^-$, respectivamente. La disociación de los grupos dependerá del pH del medio y así, durante la hidrólisis ocurre una variación del pH por la liberación o captación de los protones, que suele implicar la adición de base o ácido para mantener el valor de pH constante. Esta característica se utiliza para determinar la extensión de la reacción de hidrólisis, mediante el cálculo del denominado grado de hidrólisis (DH, *degree of hydrolysis*), por el método del pH-stato (Camacho, González-Tello, Páez-Dueñas, Guadix, & Guadix, 2001). El DH es la relación entre el número de enlaces rotos respecto al número de enlaces totales.

Las enzimas responsables de la hidrólisis de proteínas se denominan proteasas (EC 3.4.X.X) y según su acción catalítica se distinguen:

(a) endopeptidasas, si el sitio de escisión está dentro de la proteína.

(b) exopeptidasas, si los sitios de escisión están ubicados en los extremos de las cadenas o cerca de ellos.

El sitio activo de la proteasa determina su especificidad de sustrato, es decir, la posición donde tendrá lugar la hidrólisis (Turk, 2006). La elección de la proteasa empleada es fundamental, ya que definirá el grado de hidrólisis y el perfil de péptidos liberados (Tavano, 2013).

Las endopeptidasas se pueden clasificar en función de su mecanismo catalítico y su estructura terciaria, considerando el aminoácido o metal presente en el sitio activo, como aspartato, cisteíno metalo o serino-proteasas. Las exopeptidasas se pueden clasificar como aminopeptidasas, carboxipeptidasas o dipeptidasas (McDonald, 1985; Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018).

La elección del sistema enzima-sustrato es crucial para la obtención de péptidos bioactivos con propiedades determinadas. Respecto a la elección de la proteína alimentaria, actualmente existen multitud de posibilidades. Históricamente las más estudiadas son las proteínas lácteas (caseína y lactosuero), la soja y algunas proteínas de origen marino. En la elección de qué proteínas se utilizan para generar péptidos bioactivos entran en juego numerosos factores:

- Composición de la proteína: Es el factor más importante a nivel tecnológico. Cada proteína tiene un determinado contenido de aminoácidos, que se asocian en unas determinadas secuencias y dan lugar a estructuras específicas. Es posible

encontrar una secuencia peptídica concreta en varias proteínas de distintos orígenes, pero el producto final obtenido de la hidrólisis de cada fuente de proteína de manera global será diferente.

- Revalorización: Si es un subproducto de un proceso industrial (por ejemplo, el lactosuero) o si es un producto sin valor comercial que puede reutilizarse mediante su transformación (por ejemplo, descartes de pesca).
- Impacto medioambiental: Hay que considerar el impacto medioambiental que genera la producción del alimento o fuente de la proteína, así como de su extracción, si fuera necesaria, para obtener concentrado o aislado de proteína. Por ejemplo, la producción de un kilogramo de proteína de carne es medioambientalmente mucho más dañino que la producción equivalente de proteína de insecto.
- Impacto socio-económico: Entran en juego el arraigo a las tradiciones (reticencia a consumo de insectos), el bienestar animal, la seguridad de los trabajadores, la creación de empleo, el desarrollo de nuevas tecnologías, etc.

En cuanto a la elección de la proteasa o las proteasas, hay que considerar la especificidad de las proteasas y las condiciones de reacción, que determinarán los puntos de corte y tendrán como consecuencia la obtención de secuencias únicas de péptidos de cada proteína, que podrán ser péptidos bioactivos. Así, en la cadena productiva de estos ingredientes, han de considerarse todos estos factores para determinar el óptimo, según las necesidades que tengamos. Hasta hoy, se han ensayado determinadas enzimas y se han definido proteasas adecuadas para la obtención de estas moléculas. Se deben realizar más investigaciones, ya que diferentes proteínas pueden conducir a diferentes hidrolizados de proteínas bioactivas.

El tratamiento enzimático empleado para la hidrólisis enzimática de proteínas, junto a los diversos factores que se derivan de éste, son clave para optimizar el proceso. A mayor reacción de hidrólisis, los péptidos tendrán de media una longitud menor dado que se habrán rotos más enlaces peptídicos. Por otro lado, la elección de la proteasa en sí, considerando que la especificidad que presentan cada una de ellas es diferente, determinará el perfil de secuencias de aminoácidos que tendrán los péptidos obtenidos. Es importante también considerar que la propia especificidad de las proteasas determinará el posible grado de hidrólisis que se puede alcanzar en la reacción. Una proteasa de alto espectro, es decir, no específica, podrá alcanzar grados de hidrólisis más altos en las mismas condiciones de tiempo de reacción y ratio proporcional al sustrato que otra más

específica solo para los enlaces peptídicos entre aminoácidos determinados. De la misma forma, el uso de exopeptidasas es recomendable de manera simultánea a una endopeptidasa, o posterior a la acción de una endopeptidasa que haya roto enlaces peptídicos previamente y haya dispuesto más cadenas terminales disponibles para el corte.

En los últimos años, la proporción de la población mundial que padece alguna enfermedad ha aumentado, y la prevención y el pretratamiento mediante distintas estrategias, incluida la alimentación funcional, parecen ser buenos salvoconductos para evitar que este problema vaya a más. Además de la evidente ventaja a nivel de salud del paciente, a nivel económico el pre-tratamiento implica un importante ahorro de costes, en comparación con los asociados al tratamiento de la enfermedad (R. Li, Zhang, Barker, Chowdhury, & Zhang, 2010).

Los péptidos bioactivos son secuencias de aminoácidos inmersas en una proteína precursora cuya liberación de ésta le permite ejercer determinadas actividades biológicas. Un ejemplo son los péptidos bioactivos que interactúan con el cuerpo humano en diferentes vías metabólicas, siendo una de las vías más usuales la de inhibir la actuación de determinadas enzimas. De esta manera, los péptidos pueden interactuar en el sitio activo y/o fuera del sitio catalítico de la misma, evitando que la enzima interactúe con el sustrato. En función de la longitud y la secuencia de aminoácidos de los péptidos, éstos podrán exhibir una o varias actividades. Se han identificado péptidos con actividad antioxidante, hipocolesterolémica antihipertensiva, anti-coagulante, de supresión del apetito, de unión a calcio, antimicrobiana, anticancerígena, anti-inflamatoria y antidiabética (regulación del índice glucémico) (Harnedy & FitzGerald, 2012; Patil, Mandal, Tomar, & Anand, 2015).

Entre los péptidos bioactivos de mayor impacto, debido a que modularían las patologías de mayor prevalencia a nivel mundial como enfermedades cardiovasculares, diabetes o hipertensión, encontramos aquellos que ejercen actividad antioxidante, antihipertensiva y antidiabética. Estudios recientes, tanto *in vitro* como *in vivo* (Crowe et al., 2018; Dale et al., 2018; Hovland et al., 2020), han demostrado la funcionalidad de estos hidrolizados de proteínas para prevenir o pre-tratar dichas enfermedades, y abre un campo de investigación muy amplio que se presenta como una alternativa o un complemento al tratamiento farmacéutico o médico, mediante la formulación de alimentos funcionales.

## 1.2 Digestión de carbohidratos y diabetes

El metabolismo de los carbohidratos es el proceso de transformar los carbohidratos ingeridos de los alimentos en moléculas de glucosa, la fuente de energía más eficiente. Los carbohidratos en los alimentos generalmente aparecen como polisacáridos (almidón o celulosa) o como disacáridos (lactosa o sacarosa). La digestión de carbohidratos involucra diferentes enzimas y una compleja serie de procesos metabólicos. Inicialmente, cuando se ingiere el bolo, las enzimas digestivas hidrolizan estos polisacáridos complejos. Las principales hidrolasas digestivas son:

La α-amilasa (EC 3.2.1.1), que hidroliza carbohidratos complejos como el almidón en oligosacáridos, que serían hidrolizados posteriormente por la α-glucosidasa. Esta enzima es secretada por las glándulas salivales y pancreáticas.

La α-glucosidasa (EC 3.2.1.20), que es una enzima unida a la membrana que se encuentra en la mucosa epitelial del intestino delgado (borde en cepillo de los enterocitos). Libera moléculas de glucosa libres de residuos de α-glucosa no reductores enlazados (1-4) terminales.

Además, la ingesta de alimentos provoca la liberación de hormonas en el intestino llamadas incretinas (polipéptido inhibidor gástrico, GIP y péptido 1 similar al glucagón, GLP-1). Estos dos afectarían a numerosos tejidos diana en el cuerpo, actuando como señales endocrinas al páncreas, lo que conduciría a la producción de insulina en las células β y la supresión de la liberación de glucagón en las células α. Estas dos incretinas son responsables de ~ 70% de la secreción de insulina en el páncreas después de la ingesta de alimentos (el efecto incretina) (Nauck, Baller, & Meier, 2004). Esto da como resultado la absorción de glucosa por los músculos, así como una menor producción de glucosa en el hígado. La consecuencia final es, por tanto, la disminución de la glucemia tras la ingestión, lo que permite una adecuada regulación de los niveles de glucemia post-prandial. La enzima dipeptidil peptidasa IV (DPP-IV) regula la degradación de las incretinas según las necesidades fisiológicas, tras su liberación en el torrente sanguíneo y unión a receptores específicos. GLP-1 y GIP tienen una vida media de aproximadamente 2 min y 5-7 min respectivamente, antes de que sean degradados por DPP-IV (Tahrani, Bailey, Del Prato, & Barnett, 2011; Yan, Zhao, Yang, & Zhao, 2019). DPP-IV es una enzima (EC 3.4.14.5) que escinde dipéptidos del extremo N-terminal de los polipéptidos, en el que la prolina está en la penúltima posición (Lammi et al., 2018). La DPP-IV se puede encontrar en gran medida en la superficie luminal de los enterocitos; por lo tanto,

puede interactuar con cualquiera de las moléculas de la ingesta de alimentos antes de su absorción, que puede metabolizarse aún más antes de la interacción de las moléculas con la DPP-IV endotelial soluble y vascular (la que afecta los niveles de GIP y GLP-1). Las hormonas intestinales liberadas por las células enteroendocrinas juegan un papel importante en la regulación de la ingesta de alimentos (Caron, Domenger, Dhulster, Ravallec, & Cudennec, 2017).

La diabetes mellitus tipo 2 es una de las enfermedades más prevalentes, afecta a más de 400 millones de personas y se estima que 700 millones de personas se verán afectadas para 2045 ("IDF Diabetes Atlas," 2017). Este trastorno metabólico se caracteriza por la resistencia a la insulina, es decir, la incapacidad del organismo para reaccionar a la acción de la insulina, o una producción insuficiente de esta hormona. Es especialmente importante controlar el nivel de glucosa post-prandial, porque las consecuencias a largo plazo de los niveles altos de glucosa en el torrente sanguíneo son diversas, desde insuficiencia renal hasta daño neurológico y trastornos cardiovasculares (L. Li & Hölscher, 2007; Patil et al., 2015). En cuanto a las causas, tanto factores genéticos como ambientales intervienen en el desarrollo de la diabetes. Se cree que la causa principal es la obesidad, que opera a través de varias vías que incluyen un desequilibrio en la concentración de hormonas, citoquinas y otras señales inflamatorias (Tahrani et al., 2011).

Se han descrito numerosas estrategias para controlar la hiperglucemia post-prandial y, en consecuencia, prevenir el desarrollo de diabetes tipo 2 (Patil et al., 2015). La inyección de insulina (Howard-Thompson, Khan, Jones, & George, 2018) es el tratamiento directo de esta enfermedad, regulando positivamente el funcionamiento del organismo. La principal desventaja es que la insulina no se puede ingerir por vía oral. Además de esto, los medicamentos que intervienen en la vía metabólica de la digestión también son opciones para prevenir y tratar la enfermedad.

## 1.3 Efecto de los péptidos en la regulación del índice glucémico

Los péptidos derivados de los alimentos a partir de proteínas alimentarias juegan un papel crucial en la regulación de la homeostasis de la glucosa, debido a su implicación a diferentes niveles y debido a su capacidad para inhibir las enzimas relacionadas con la digestión. Algunos autores han descrito los péptidos como capaces de mejorar los niveles de colecistoquinina, una hormona intestinal que regula la ingesta de alimentos (Nishi,

Hara, Asano, & Tomita, 2003; Sufian et al., 2006), así los péptidos bioactivos también pueden regular la homeostasis de la glucosa debido a su capacidad para regular las hormonas intestinales (Caron et al., 2017). Los péptidos y los aminoácidos tendrían un efecto sobre la pérdida de grasa corporal, la secreción de insulina, la estimulación de células L del intestino para producir GLP-1, la absorción de glucosa en músculo esquelético y la reducción de la glucemia, pero se necesita más investigación para desentrañar estos mecanismos.

Estudios recientes muestran la importancia del pretratamiento de la diabetes para minimizar el impacto económico del tratamiento de la enfermedad (Hewage, Wu, Neelakantan, & Yoong, 2020; Mata-Cases et al., 2020), más allá de las consecuencias para la salud que tiene sobre el paciente. Los péptidos bioactivos parecen ser una buena alternativa para su empleo en alimentos funcionales como ingredientes que promueven la salud. En la literatura se ha reportado el descubrimiento de péptidos provenientes de proteínas alimentarias capaces de inhibir las enzimas α-amilasa, α-glucosidasa y DPP-IV. Cada país o región posee marcos regulatorios para proteger a los consumidores de los riesgos y afirmaciones engañosas sobre los péptidos bioactivos. La justificación científica de la seguridad y eficacia de los péptidos bioactivos es clave en la aprobación de las declaraciones de propiedades saludables de los péptidos bioactivos para su lanzamiento al mercado. Por ejemplo, en Europa, la aprobación está regulada por el Reglamento (CE) No 1924/2006 del parlamento europeo y del consejo de 20 de diciembre de 2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos. Respecto a péptidos derivados de proteínas alimentarias, cabe destacar el producto Valtyron®, un hidrolizado de proteína de sardina con capacidad de inhibir la enzima ACE, que se relaciona con efectos antihipertensivos, aprobado por la Unión Europea (panel de productos dietéticos, nutrición y alergias de la EFSA) para ser usado en determinados productos (yogur, leche fermentada, sopa, etc) a 0.6 g/servicio. Hasta el momento, no existe a nivel europeo ningún producto compuesto por péptidos aprobado por la EFSA con demostrados efectos beneficiosos en la regulación del índice glucémico.

Los péptidos bioactivos para prevenir el desarrollo de enfermedades son un campo importante de investigación, cuyo interés está aumentando y que podría tener efectos positivos en la salud humana, además de una positiva repercusión en la disminución del coste económico de los tratamientos. En la siguiente sección se detallan los tipos de péptidos bioactivos definidos como moduladores del índice glucémico mediante la

inhibición de dos enzimas involucradas en el proceso de digestión mencionadas en la sección anterior.

## 1.4 Péptidos bioactivos moduladores del índice glucémico

### 1.4.1 Inhibidores de la enzima dipeptidil-peptidasa IV

El descubrimiento de que la enzima DPP-IV inactiva más del 95% del GLP-1 lo ha puesto en el centro de atención como terapia para el manejo de la diabetes mellitus tipo 2 (Thoma et al., 2003). Si el organismo padece resistencia a la insulina, considerando que la DPP-IV actúa degradando las incretinas (Kshirsagar, Aggarwal, Harle, & Deshpande, 2011), uno de los medicamentos antidiabéticos orales que se utilizan en la actualidad es el grupo de inhibidores de la enzima DPP-IV llamados gliptinas (Marya, Khan, Nabavi, & Habtemariam, 2018). Cuando se inhibe la DPP-IV, se suprime la acción inhibidora que tiene sobre las incretinas y aumenta la vida media de estas incretinas. La ingesta de proteínas también puede elevar los niveles plasmáticos de GLP-1 (Hutchison et al., 2015). Esto hace que se estimule la secreción de insulina, además de inhibir la liberación de glucagón (Lammi et al., 2018), y el nivel de glucosa en sangre está adecuadamente regulado, limitando la posibilidad de hipoglucemia e incrementando el peso corporal. Dado que en este caso la regulación se está ejerciendo mediante la molécula que fisiológicamente regula el proceso, el alcance que tiene es más efectivo y con menor probabilidad de contrarrestar la situación hasta el problema contrario.

La primera gliptina aprobada por la Administración de Alimentos y Medicamentos (FDA) fue la sitagliptina, en 2006; que recibió la aprobación de la Agencia Europea del Medicamento en 2009, a pesar de los efectos adversos como nasofaringitis, náuseas, hipersensibilidad, dolor de cabeza, irritaciones cutáneas y riesgo de pancreatitis aguda (Juillerat-Jeanneret, 2014; Liu, Cheng, & Wu, 2019). Además, su seguridad a largo plazo sigue siendo una incógnita. La obtención de péptidos alimentarios se presenta actualmente como una alternativa bastante plausible a estas gliptinas sintéticas, y dado su origen natural, no se ha mostrado que presenten efectos secundarios. El péptido hasta ahora descrito con mayor bioactividad es Ile-Pro-Ile, secuencia que se encuentra en diversas proteínas como las lácteas. Existen estudios *in vivo* sobre la eficacia de los hidrolizados de proteína como moduladores del estado fisiológico humano (ver capítulo I para recopilación de estudios recientes 2018-2020 sobre péptidos derivados de proteínas alimentarias con actividad antidiabética y revisión sobre estudios *in vivo*). Sin embargo,

más evidencias científicas y ensayos clínicos son necesarios para esclarecer y asegurar la eficacia de estos productos.

### 1.4.2 Inhibidores de la enzima α-glucosidasa y α-amilasa

Respecto al proceso de digestión y las enzimas involucradas en el metabolismo de los carbohidratos, el primer enfoque para prevenir un aumento del nivel de glucosa en sangre es evitar la degradación de los polisacáridos en glucosa. Por tanto, la inhibición de las hidrolasas digestivas (amilasas, glucosidasas) evitaría que los polisacáridos complejos se hidrolicen y, por tanto, se absorban en el torrente sanguíneo.
La inhibición de las amilasas se puede ejercer en la saliva y en el tracto gastrointestinal, reduciendo el nivel de glucosa en sangre (Yan et al., 2019). Por su parte, la inhibición de la α-glucosidasa esencialmente impediría la captación de glucosa en la circulación sanguínea, disminuyendo efectivamente la hiperglucemia post-prandial (Ibrahim, Bester, Neitz, & Gaspar, 2017; Konrad et al., 2014; Patil et al., 2015). Los inhibidores de glucosidasa no estimulan a las células β a secretar insulina, pero la absorción retardada de carbohidratos se considera un factor contribuyente adecuado para estimular la secreción de GLP-1, lo que finalmente conduciría al efecto incretina. Entre los principales inhibidores de la α-glucosidasa y la α-amilasa se encuentran la acarbosa, el miglitol y la voglibosa. Sin embargo, se han descrito numerosos efectos secundarios de estos fármacos, como molestias gastrointestinales, dolor de estómago y flatulencia, por lo que han limitado su uso como inhibidores (Patil et al., 2015). La obtención de inhibidores de estas enzimas sin efectos secundarios es, por tanto, un interesante tema de investigación (ver capítulo I para recopilación de estudios recientes 2018-2020 sobre péptidos derivados de proteínas alimentarias con actividad antidiabética y revisión sobre estudios *in vivo*).

### 1.4.3 Biodisponibilidad

Un aspecto importante en la consideración de los péptidos bioactivos para alimentación funcional es su biodisponibilidad, donde incluimos bioaccesibilidad (liberación, solubilización, interacción), absorción y transformación (metabolismo, degradación química). Desde la perspectiva de regulación de la glucemia, para que ejerzan un efecto en la respuesta post-prandial, los péptidos han de alcanzar el intestino y/o la circulación sanguínea en su forma activa.

La ingesta oral de estos péptidos implica su digestión mediante las proteasas gástricas (pepsina), en el estómago, e intestinales (pancreatina), además de la variación de pH. Esto puede no afectar a la estructura del péptido, o escindirlo en péptidos de menor tamaño cuya actividad puede ser mayor o menor que la que poseía el péptido ingerido. Posteriormente ocurriría la absorción, que se da mayormente en el yeyuno, donde éstos atraviesan la pared intestinal (presencia de amino-peptidasas) y alcanzan la circulación sistémica (presencia de peptidasas). Se han descrito cuatro rutas diferentes en las que los péptidos pueden ser absorbidos:

- Transcitosis: Depende de un gasto energético por la polarización. El movimiento se efectúa a través de vesículas denominadas endosomas y secreción basolateral. Se ha descrito para el transporte de péptidos de cadena larga (más de cuatro residuos de aminoácidos) e hidrófobos, con carga positiva.
- Difusión pasiva transcelular: Movimiento de moléculas a través de las membranas apical y basolateral. Considerando la bicapa lipídica, esta ruta es empleada por péptidos lipófilos.
- Difusión paracelular: Movimiento de moléculas a través de canales o poros llenos de agua entre células. Está regulado por las *tight junctions* (uniones estrechas) que separan las membranas de las células epiteliales, compuestas por proteínas, limitando el paso de macromoléculas polares. Esta ruta es la preferida por los péptidos hidrófilos de bajo peso molecular con carga negativa.
- Transporte mediado por portadores: mediado por proteínas específicas, es dependiente de concentración y específico de estructuras. El transportador PepT1 se ha descrito como transportados de péptidos de cadena corta, especialmente dipéptidos y tripéptidos, con carga neutra y alta hidrofobicidad.

La absorción de péptidos bioactivos es indispensable para que puedan interactuar con las enzimas. La DPP-IV se encuentra en distintos tejidos y fluidos, pero es la inhibición de la circulante la que impacta mayormente en la actividad de las incretinas (i.e. GLP-1), por lo que verificar la estabilidad y funcionalidad de los péptidos incluso tras la absorción es importante, y suele hacerse mediante modelos celulares como la línea Caco-2. Sin embargo, más allá de los factores de composición y estructura proteicas, entran en juego factores tecnológicos (sistemas de liberación) y genéticos individuales (interacciones dieta-genes, microbiota intestinal, ratio de vaciado gástrico, etc.), por lo que los únicos modelos realmente efectivos son los análisis *in vivo*.

## 1.5 Utilización de péptidos bioactivos como ingredientes funcionales

La modificación de las propiedades tecnológicas y biológicas de las proteínas tras su hidrólisis conduce a la posibilidad de formular alimentos con estos ingredientes, para mejorar el procesamiento o para conferir a los alimentos de una característica potenciadora de la salud. Los alimentos funcionales son los que, además de un efecto nutritivo, generan un beneficio en la salud de quien lo ingiere. El uso de péptidos con fines médicos ya se utiliza por ejemplo en productos dietéticos o para pacientes con problemas relacionados con la digestión. En la actualidad, diversas empresas comercializan productos con péptidos como ingredientes, como por ejemplo Arla Foods Ingredients, que ofrece hidrolizados de proteína de lactosuero, declarando que se absorbe más rápido en sangre, o un hidrolizado de proteína de colágeno de Abbott, en su producto Promod® Liquid Protein, para mejorar la cicatrización de úlceras (S. K. Lee, Posthauer, Dorner, Redovian, & Maloney, 2006). Respecto a hidrolizados antidiabéticos, NutripeptinTM de Copalis Sea Solutions® se describe como péptido reductor del índice glucémico extraído por hidrólisis enzimática de filetes de bacalao frescos o congelados.

Las operaciones de procesamiento de alimentos que se emplean actualmente en la industria incluyen tratamientos térmicos y no térmicos, almacenamiento, secado y separación, que pueden afectar la funcionalidad de las proteínas alimentarias debido a cambios físicos y químicos. Las proteínas y los péptidos tienden a interactuar entre sí y con otras moléculas y, en consecuencia, el procesamiento podría reducir, mantener o mejorar su bioactividad (Daliri, Oh, & Lee, 2017). Los residuos de los aminoácidos interactuarían con las moléculas de diferentes formas, dependiendo también de la ubicación de los péptidos en la matriz del alimento, afectando finalmente su estado polimérico nativo y desnaturalizado (Pathania, Parmar, & Tiwari, 2019; Van Lancker, Adams, & De Kimpe, 2011). Se ha demostrado que las matrices de alimentos con alto contenido de fibra son adecuadas para transportar péptidos bioactivos, porque es poco probable que se produzcan interacciones químicas. Además, una red de fibras evitaría el sabor amargo de los péptidos hidrófobos, mejorando la aceptabilidad sensorial de los alimentos funcionales, incluidos los péptidos (Sun, Acquah, Aluko, & Udenigwe, 2020; Ten Have, Van Der Pijl, Kies, & Deutz, 2015).

No hay demasiados estudios sobre cómo el procesamiento y/o almacenamiento de alimentos modifican la estructura de los péptidos y, en consecuencia, su funcionalidad y

propiedades bioactivas (Graves et al., 2016; Contreras et al., 2011). Algunos autores han mostrado que los tratamientos no térmicos, como los ultrasonidos o las altas presiones, potencian la hidrólisis enzimática de proteínas (Perreault, Hénaux, Bazinet, & Doyen, 2017; H.-C. Yu & Tan, 2017). Sin embargo, existe una falta de información sobre cómo estas técnicas de procesamiento afectarían a los péptidos empleados como ingredientes en la formulación de alimentos. Actualmente, estas técnicas se consideran menos agresivas en términos de pérdida nutricional de ingredientes (X. Li & Farid, 2016) y pueden ser utilizados para procesos de emulsificación o esterilización. El efecto de los tratamientos no térmicos de ultrasonidos y altas presiones sobre la actividad antioxidante y antihipertensiva de péptidos de sardina ha sido recientemente estudiado por nuestro grupo de investigación (Rivero-Pino, Espejo-Carpio, & Guadix, 2020a). Sin embargo, el procesamiento de alimentos también puede afectar la composición de aminoácidos, al formar derivados como lisinoalanina, d-aminoácidos y aminas biogénicas, que generalmente están relacionadas con consecuencias fisiológicas no deseadas en el cuerpo humano si se consumen (Korhonen, Pihlanto-Leppälä, Rantamäki, & Tupasela, 1998).

Un ejemplo importante de reacción química es la formación de compuestos de Maillard, productos de la glicosilación no enzimática de proteínas. El azúcar es un ingrediente muy utilizado en la industria alimentaria debido a su sabor dulce. La combinación de azúcares reductores con proteínas o péptidos a alta temperatura conduce a la formación de estos compuestos (Fu, Zhang, Soladoye, & Aluko, 2019), afectando a la estabilidad oxidativa (Morales & Jiménez-Pérez, 2001), pero también pudiendo provocar mejora en la actividad antioxidante y antihipertensiva de los hidrolizados de proteína (Abd El-Salam & El-Shibiny, 2018; Hong, Meng, & Lu, 2015; Rivero-Pino, Espejo-Carpio, & Guadix, 2020a; Q. Zhang, Wu, Fan, Li, & Sun, 2018; Zhao et al., 2018). No obstante, hasta donde sabemos, no hay literatura que informe sobre el aumento de la actividad inhibidora de la DPP-IV o la α-glucosidasa de los productos de reacción de Maillard procedentes de hidrolizados de proteínas. Los péptidos pueden reaccionar también con lípidos oxidados, así como con quinonas por los grupos amino o tiol.

Muchos factores también están involucrados en la posible pérdida o ganancia de bioactividad a través de la modificación de la estructura de los péptidos, o la agregación de ellos. El estado de la proteína determina sus propiedades (Wang, Sun, Pu, & Wei, 2017), pero por lo general la estructura primaria de éstas no se ve afectada por la desnaturalización causada por procesos físicos (Rahaman, Vasiljevic, & Ramchandran, 2016), mientras que estructuras más complejas y agregaciones si pueden verse afectadas.

Se espera que los hidrolizados de proteínas, como mezcla de péptidos definidos, no sufran más modificaciones, ya que las secuencias lineales se ven afectadas por procesos de descomposición de secuencias, como la propia hidrólisis o la fermentación. La composición química heterogénea de un alimento, así como su estructura molecular, está relacionada con diferentes comportamientos de reacciones químicas (Capuano, Oliviero, & van Boekel, 2017) y, en consecuencia, su funcionalidad. Recientemente, Amini Sarteshnizi et al. (2021) analizaron la interacción entre un hidrolizado de sardina y el extracto de cáscara de pistacho (por contener compuestos fenólicos antioxidantes) en la actividad inhibitoria de α-glucosidasa, que se vio afectada negativamente, y de DPP-IV, sin efectos notables. Existen algunas técnicas para evitar o ralentizar el efecto del proceso de digestión y para aumentar la estabilidad de los péptidos cuando se introducen en matrices alimentarias. La técnica más utilizada es la encapsulación (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015) con polímeros o hidrogeles (Lammi, Bollati, Gelain, Arnoldi, & Pugliese, 2019) o la modificación de la estructura de los péptidos (acetilación, amidación de los terminales, ciclación, fosforilación, etc.).

Una vez que se formula el producto que contiene péptidos y se mantiene su bioactividad, también debe asegurarse que no se pierda la bioactividad durante su vida como producto comercial. Pueden ocurrir reacciones químicas durante el almacenamiento, dependiendo de la formulación del producto y la temperatura de almacenamiento del mismo. Se ha descrito que la reacción de Maillard ocurre a altas temperaturas, pero períodos prolongados también pueden conducir a la aparición de productos de reacción de Maillard (Albalá-Hurtado, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1999; Guyomarc'h, Warin, Donald Muir, & Leaver, 2000). Harnedy-Rothwell et al. (2021) enriquecieron distintas matrices alimentarias (sopas y jugos a base de tomate) que fueron sometidos a tratamientos térmicos (esterilización y pasteurización) y almacenados a temperatura refrigerada por 30 días. No se describió ninguna modificación de la bioactividad, lo que indica el uso potencial de este tratamiento en alimentos que podrían contener hidrolizados de proteínas bioactivas. Sin embargo, los péptidos y las proteínas pueden tender a agregarse o precipitarse con el tiempo, debido a distintos tipos de interacciones (Lv, Guo, & Yang, 2009), como la interacción de van de Waals, los enlaces de hidrógeno o una interacción hidrófoba. Por lo tanto, al considerar el uso de un hidrolizado de proteína como ingrediente bioactivo, se debe establecer su estabilidad durante la formulación del alimento y su estabilidad durante el almacenamiento.

# 2. OBJETIVOS

El objetivo de esta tesis doctoral es la obtención de péptidos moduladores del índice glucémico mediante la inhibición de dos enzimas clave en la digestión, la dipeptidil-peptidasa IV (DPP-IV) y la α-glucosidasa, a partir de proteínas alimentarias procedentes de fuentes sostenibles, y especialmente en proteína de insecto dados sus beneficios y su novedad a día de hoy. Para ello se han establecido los siguientes objetivos específicos:

- Revisión bibliográfica del estado del arte de péptidos reguladores del índice glucémico (Capítulo I: **Péptidos antidiabéticos derivados de alimentos para alimentación funcional: producción, funcionalidad y evidencias *in vivo***).
- Obtención de hidrolizados de proteína con alta capacidad inhibitoria de la enzima DPP-IV de fuentes tradicionales.
    - o Péptidos procedentes de descarte de pesca de sardina (capítulo II: **Producción e identificación de péptidos inhibidores de la dipeptidil peptidasa IV (DPP-IV) procedentes de proteína de descartes de "*Sardine pilchardus*"**).
    - o Péptidos procedentes de fuentes vegetales (capítulo III: **Identificación de péptidos inhibidores de la dipeptidil peptidasa IV (DPP-IV) procedentes de fuentes de proteínas vegetales**).
- Valorización de la proteína de insecto como fuente de péptidos activos para alimentación funcional
    - o Obtención de péptidos inhibidores de la DPP-IV (capítulo IV: **Evaluación de la proteína de *Tenebrio molitor* como fuente de péptidos moduladores de procesos fisiológicos**).
    - o Obtención de péptidos inhibidores de la α-glucosidasa (capítulo V: **Efecto del pretratamiento con ultrasonido y la hidrólisis secuencial en la producción de péptidos antidiabéticos de *Tenebrio molitor***).
    - o Identificación de las secuencias de péptidos inhibidores de ambas enzimas (capítulo VI: **Identificación de nuevos péptidos inhibidores de dipeptidil peptidasa IV y α-glucosidasa de *Tenebrio molitor***)
    - o Inclusión en matrices: Análisis de funcionalidad y estabilidad (capítulo VII: **Evaluación del potencial antidiabético de un caldo de verduras enriquecido con hidrolizados de proteínas de *Tenebrio molitor***).

# 3. MATERIALES Y MÉTODOS

En esta tesis doctoral se han estudiado 9 fuentes de proteínas para la producción de péptidos bioactivos. La elección de las fuentes está basada en un criterio de revalorización de recursos de manera sostenible. Las fuentes de proteína (porcentaje de proteína indicado entre paréntesis) empleadas fueron: músculo de sardina procedente de descartes de pesca (19,6 %), harinas vegetales comerciales: Patata (81 %), guisante (80 %), soja (85 %), altramuz (32,5 %), garbanzo (50,9 %), lenteja (46,2 %) y quinoa 59,4%), y harina de gusano de la harina, *Tenebrio molitor* (55 %).

Las proteasas utilizadas fueron subtilisina (EC 3.4.21.62), serino-proteasa de alto espectro, es decir, no específica; tripsina pancreática (EC 3.4.21.4), serino-proteasa específica para los aminoácidos lisina y arginina mayoritariamente; ficina (EC 3.4.22.3), una cisteino-proteasa y Flavourzyme 1000L™ (3.4.11.1), un complejo enzimático de exo-proteasas (es decir, aminopeptidasas y dipeptidasas).

En la Tabla 1, se muestran los principales métodos que se han empleado en esta tesis doctoral. Se detalla a continuación la metodología más importante de la tesis, que se ha utilizado en la mayor parte de la investigación.

**Tabla 1**: Resumen de la metodología empleada en la tesis doctoral.

| Análisis | Descripción | Capítulo |
|---|---|---|
| **Contenido en proteína** | Análisis elemental | II, III, VI, VII |
| **Contenido en proteína** | Método Kjeldhal | II, III, V, VI, VII |
| **Contenido en grasa** | Análisis gravimétrico | IV, VII |
| **Grado de hidrólisis** | Método del pH-stato | II, III, IV, V, VI |
| **Hidrofobicidad** | Método fluorimétrico | V |
| **Solubilidad** | Determinación de la proteína soluble | IV |
| **Inhibición de la enzima DPP-IV** | Método espectrofotométrico basado en una reacción enzimática | II, III, VI VII |
| **Inhibición de la enzima α-glucosidasa** | Método espectrofotométrico basado en una reacción enzimática | V,VI, VII |
| **Digestión *in vitro*** | Hidrólisis enzimática | II, VII |

| | | |
|---|---|---|
| **Distribución de pesos moleculares de hidrolizados proteicos** | Cromatografía de exclusión molecular por tamaño (SEC) empleando cromatografía líquida rápida de proteína | II, III, IV; VII |
| **Fraccionamiento de hidrolizados proteicos** | SEC empleando FPLC acoplado un colector | II, III, VI |
| **Identificación de péptidos** | Secuenciamiento *de novo* a partir del espectro de masas de fracciones proteicas | II, III, VI |
| **Alineamiento con proteoma** | Análisis de alineamiento con el proteoma para identificar proteínas que contienen los péptidos identificados | III, VI |
| **Acoplamiento molecular *in silico*** | Análisis *in silico* mediante el software pepATTRACT | II |
| **Probabilidad de ser bioactivo *in silico*** | Peptide Ranker | III, VI |
| **Probabilidad de ser inhibidor de DPP-IV** | Análisis in silico con la herramienta iDPPIV-SCM | III |

La hidrólisis enzimática de proteínas se lleva a cabo en un reactor encamisado de 250 mL en el que se diluye el sustrato correspondiente mediante agitación magnética. El control de temperatura se realiza mediante la circulación de agua a la temperatura fijada procedente de un baño termostatizado. Una vez alcanzada la temperatura deseada, el pH se ajusta mediante la adición de NaOH 1 M por un valorador automático (902 Stat Titrando, Metrohm, AG, Herisau, Switzerland) ya que está acoplado a una sonda de pH y temperatura. En la figura 2 se representan todos los elementos del dispositivo experimental. Una vez la disolución de proteína está ajustada a la temperatura y pH deseada, se añade la enzima para iniciar la reacción. La monitorización de la reacción de hidrolisis se realiza por el método del pH-stato mediante un valorador automático. Las reacciones se finalizaron por desactivación térmica de la proteasa calentando la muestra a 90 ºC durante al menos 5 minutos.

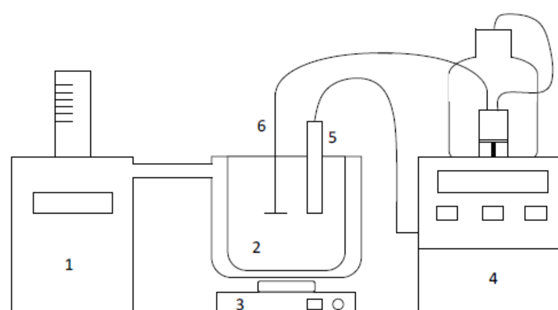


**Figura 2**: Esquema del equipamiento para llevar a cabo la hidrólisis enzimática.
1) Baño termostatizado; 2) Reactor encamisado; 3) Agitador magnético; 4) Titrador automático Titrino 902: 5) Sonda medidora de pH y temperatura; 6) Dosificador. (Espejo-Carpio, 2012).

Las medidas de inhibición de las enzimas DPP-IV y α-glucosidasa se llevaron a cabo mediante un método espectrofotométrico basado en una reacción enzimática, modificados en base a métodos descritos en literatura (Lacroix & Li-Chan, 2012; Ren et al., 2016). En la figura 3 se muestra el fundamento de la medida de inhibición de las enzimas, donde un sustrato es escindido por la enzima, liberando un compuesto detectable a 405 nm. Se evalúa el cambio de color en la reacción blanco, sin inhibidor, y se compara con la reacción llevada a cabo en presencia de la muestra, permitiendo calcular el porcentaje de inhibición a distintas concentraciones.

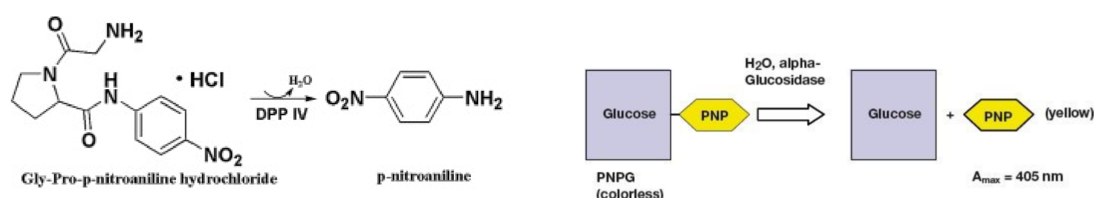


**Figura 3**: Esquema del funcionamiento del análisis espectrofotométrico para la detección de actividad inhibitoria de DPP-IV y α-glucosidasa.

Se empleó la cromatografía de exclusión de tamaño mediante un equipo de cromatografía líquida rápida de proteínas (FPLC) con una columna Superdex Peptide 10/300 GL column (GE Health-care, Uppsala, Sweden), para analizar el perfil de pesos moleculares de los hidrolizados obtenidos, así como fraccionar dichos hidrolizados acoplando el equipo a un fraccionador Frac 902 en función de esta característica, para analizar y observar en qué rango de pesos moleculares se encuentran los péptidos más bioactivos.

Las fracciones más activas recogidas del fraccionamiento por cromatografía se analizaron empleando un sistema ACQUITY UHPLC (Waters, Milford, CT, EE.UU) acoplado a un espectrómetro de masas Synapt Mass Quadrupole Time-of-Flight. Para la secuenciación *de novo*, se utilizó el programa PepSeq del software BioLynx (Micromass UK Ltd., Manchester, Reino Unido). En los distintos capítulos se incluyeron análisis *in silico* para caracterizar los péptidos identificados. Las herramientas empleadas fueron análisis BLASTp, software Peptide Ranker y la herramienta CPPpred, acoplamiento molecular con pepATTRACT y la herramienta iDPPIV-SCM.

# 4. RESULTADOS Y DISCUSIÓN

## 4.1 Producción e identificación de péptidos inhibidores de la dipeptidil peptidasa IV (DPP-IV) procedentes de proteína de descartes de "*Sardine pilchardus*"

La utilización de la sardina europea para la obtención de péptidos bioactivos ha sido altamente estudiada en los últimos años, mayormente respecto a su actividad antioxidante y antihipertensiva (García-Moreno, Espejo-Carpio, Guadix, & Guadix, 2015; García-Moreno et al., 2013; Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2016). Respecto a la actividad antidiabética, existen estudios analizando la capacidad inhibitoria de la enzima α-glucosidasa mediante péptidos de músculo de sardina (Matsui, Oki, & Osajima, 1999), pero no existe información sobre péptidos procedentes de sardina con capacidad para inhibir la enzima DPP-IV. Respecto a péptidos procedentes de otras fuentes marinas con dicha capacidad, se han analizado especies como el atún, el salmón o la bacaladilla (Harnedy et al., 2018b; Huang, Jao, Ho, & Hsu, 2012; Jin, Teng, Shang, Wang, & Liu, 2020). Sin embargo, el interés en utilizar la sardina reside en que un volumen considerable sin interés comercial por su pequeño tamaño o el contenido de aceite (variable estacionalmente) se desembarca debido a las obligaciones de desembarque (European Commission, 2020) y por tanto podrían ser revaluadas mediante la transformación en otros compuestos de mayor interés en el mercado (García-Moreno et al., 2015; Kristinsson & Rasco, 2000). En el área de la "Comisión General de Pesca del Mediterráneo", las capturas de *S. pilchardus* son las segundas más abundantes, con 188.431 toneladas en 2016 (FAO, 2018).

En este estudio, se empleó la proteína de sardina para evaluar su potencial como fuente de péptidos inhibidores de la enzima DPP-IV. Se realizaron distintos tratamientos enzimáticos utilizando tres proteasas de grado alimentario, en solitario o de manera secuencial. Inicialmente se estudió la influencia de la subtilisina, una proteasa de alto espectro, en la obtención de péptidos bioactivos según el peso molecular de éstos. Se alcanzaron grados de hidrólisis de 10, 15 y 20. Además, se estudió como la adición de una segunda proteasa puede modificar el perfil de péptidos liberados. Así, tras la hidrólisis con subtilisina, se analizó tanto la adición de tripsina, cuyo sitio activo libera péptidos con extremos con aminoácidos básicos, arginina y lisina; la adición de Flavourzyme: una

exopeptidasa que libera cadenas de 1-3 aminoácidos de los extremos de las secuencias, así como la adición de tripsina y posteriormente Flavourzyme.

El valor de bioactividad osciló entre $IC_{50}$ de 3.70 a 7.80 mg/ml, siendo todas las muestras de subtilisina, independientemente de su grado de hidrólisis, significativamente igual de inhibitorias. Este comportamiento ha sido previamente reportado para otros sustratos como el salmón (Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). Además, se demostró que la adición de tripsina o Flavourzyme después del tratamiento con subtilisina liberó nuevos péptidos con mayor actividad, ya que el valor $IC_{50}$ disminuyó significativamente. La adición secuencial de tripsina y posteriormente Flavourzyme condujo a la obtención de la muestra más activa (valor de $IC_{50}$ = 3.70 mg/ml). Los valores obtenidos en este estudio son comparables a los de otros sustratos marinos como los obtenidos para hidrolizados de barbo, con valores $IC_{50}$ de 2.21 a 3.71 mg/ml. La eficacia de la proteasa Flavourzyme para liberar péptidos inhibidores ha sido descrita para otros sustratos (Harnedy et al., 2018a), dada su especificidad que libera péptidos de pequeño tamaño, característica generalmente asociada a este tipo de péptidos inhibidores.

Mediante análisis de cromatografía se demostró que la adición secuencial de diferentes proteasas fue liberando péptidos de menos tamaño molecular con características moleculares – dada la especificidad de las proteasas- con mayor potencial bioactivo de inhibir la enzima DPP-IV. Todos los hidrolizados obtenidos fueron además sometidos a una simulación de la digestión gastrointestinal para evaluar la posibilidad de utilizar estas muestras como alimento funcional ingerido oralmente. Los resultados mostraron una pérdida parcial de bioactividad respecto a las muestras originales, pero con unos valores de $IC_{50}$ (entre 7.90 y 17.14 mg/ml), adecuados al compararlos con los disponibles en literatura para otros sustratos (Mune Mune, Minka, & Henle, 2018; Nongonierma, Lamoureux, & Fitzgerald, 2018).

Finalmente, la muestra más activa, procedente de la hidrólisis secuencial con subtilisina, trispina y Flavourzyme se fraccionó mediante cromatografía para dilucidar qué fracciones son las más activas, y se mostró que, con un $IC_{50}$ de 1.83 mg/ml, los péptidos de 800 a 1400 Dalton eran los responsables de la actividad inhibitoria del hidrolizado, además de los péptidos de 400 a 800 Da ($IC_{50}$ de 2.89 mg/ml). De la misma manera, los valores son comparables a las fracciones obtenidas para el hidrolizado de barbo, con valores $IC_{50}$ de 1.23-1.83 mg/ml (Sila et al., 2016). El análisis de estas fracciones permitió la identificación por secuenciamiento *de novo* de 19 péptidos potencialmente activos, entre ellos el péptido VGLP, reportado en literatura como péptido inhibidor de DPP-IV

previamente encontrado en la caseína. En base a un análisis de acoplamiento molecular con la enzima DPP-IV, que reportó valores similares a los obtenidos para péptidos descritos previamente en literatura como inhibidores (Hu, Fan, Qi, & Zhang, 2019) y la descripción de las características comunes encontradas en este tipo de péptidos bioactivos, se sugirieron como péptidos más activos las secuencias NAPNPR, YACSVR y CGGWLF, entre otras.

## 4.2 Identificación de péptidos inhibidores de la dipeptidil peptidasa IV (DPP-IV) procedentes de fuentes de proteínas vegetales

El consumo de proteínas vegetales en comparación con las animales tiene numerosas ventajas. Se ha demostrado que las dietas occidentales, ricas en carnes, azúcares refinados y grasas, incrementan el riesgo de desarrollar algunas enfermedades (Medawar, Huhn, Villringer, & Veronica Witte, 2019). Además, la producción de vegetales ayuda a reducir las emisiones de gases de efecto invernadero agrícolas y requiere menos recursos en comparación con la producción de la misma cantidad de proteína animal (Tilman & Clark, 2014).

La literatura disponible sobre péptidos inhibidores de la enzima DPP-IV procedente de vegetales es limitada. Hay publicaciones sobre este tipo de péptidos procedentes de soja, altramuz, quinoa o judías (Lammi et al., 2018; Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015; Vilcacundo, Martínez-Villaluenga, & Hernández-Ledesma, 2017), pero no existen publicaciones sobre obtención experimental de péptidos de patata o de lenteja, por ejemplo. El objetivo de este estudio es analizar la viabilidad de la utilización de harinas comerciales de fuentes vegetales como fuente de péptidos bioactivos en una potencial implementación industrial. Por ello, el tratamiento determinado como adecuado se empleó por igual para todos los sustratos (soja, patata, quinoa, guisante, lenteja, garbanzo y altramuz) para comparar de manera directa la actividad de los péptidos obtenidos, a nivel de hidrolizado y de sus fracciones. De esta manera, los hidrolizados de las siete proteínas presentaron un valor de bioactividad adecuado y en el rango del que presentan hidrolizados de proteínas tradicionales como lácteas o marinas. Los valores $IC_{50}$ oscilaron entre 2.39 y 5.41 mg/ml, siendo la soja hidrolizada la muestra con mayor actividad inhibitoria y la patata la menos activa. Cabe destacar el hidrolizado de lenteja,

ya que no existe ninguna información sobre sus péptidos inhibidores de DPP-IV en literatura, que presentó un valor $IC_{50}$ de 2.92 mg/ml.

Estos valores de inhibición son comparables a los encontrados en literatura para fuentes vegetales. Gonzalez-Montoya et al. (2018) reportaron un valor $IC_{50}$ de 1.49 mg/ml para un hidrolizado de soja obtenido con pepsina y pancreatina, y Nongonierma et al. (2015) obtuvieron un $IC_{50}$ de 0.88 mg/ml para un hidrolizado de quinoa obtenido con papaína. De la misma manera, están en el rango de los obtenidos para péptidos procedentes de fuentes animales, destacando su potencial respecto a ellos. Por ejemplo, hidrolizados de gelatina de piel de camello mostraron un valor de $IC_{50}$ entre 0.70 y 2.4 mg/ml (Mudgil et al., 2019) o los péptidos obtenidos para sardina con el mismo tratamiento en el capítulo II, que presentaban un valor $IC_{50}$ de 3.70 mg/ml (Rivero-Pino, Espejo-Carpio, & Guadix, 2020c).

Todos los hidrolizados se fraccionaron por cromatografía de exclusión de tamaño y la actividad y concentración de proteína de cada una de las fracciones se analizó para determinar qué péptidos eran los más activos y si el tratamiento aplicado había sido adecuado para concentrarlos, como se planteaba en la hipótesis. La fracción D resultó poseer la mayor concentración en péptidos, seguida de C y B, dependiendo de la proteína empleada. En términos generales, la fracción A (moléculas más grandes) y la fracción E (di y tri péptidos) fueron las fracciones menos concentradas. En general, los péptidos cuyo peso molecular oscilaba de 400 a 3000 Dalton fueron los más activos. Estos resultados se corresponden con los obtenidos por otros autores, por ejemplo, siendo la fracción de < 3kDa de un hidrolizado de caseinato de sodio (Lacroix & Li-Chan, 2012) o la fracción de <5 kDa en un hidrolizado de proteína de quinoa (Vilcacundo et al., 2017), más activas.

Las fracciones más activas se correspondieron respectivamente con soja, quinoa y altramuz, con unos valores $IC_{50}$ de 1.16, 1.47 y 1.73 mg/ml, respectivamente. Estas fracciones fueron analizadas por espectrometría de masas para la identificación de los péptidos. Se identificaron 9 péptidos de soja, 7 de altramuz y 4 de quinoa. De entre estos, en base a sus características moleculares y a diferentes análisis *in silico*, se propusieron como potentes inhibidores de la enzima DPP-IV, los péptidos EPAAV, NPLL y APFTVV, de soja, altramuz y quinoa respectivamente.

## 4.3 Evaluación de la proteína de *Tenebrio molitor* como fuente de péptidos moduladores de procesos fisiológicos

La proteína de insecto se considera actualmente la proteína del futuro y, de hecho, su utilización en la industria alimentaria ha crecido exponencialmente en los últimos años. Su utilización como alimento en Europa está regulada desde el 1 de enero de 2018 (European Council Regulations No. 2015/2283, 2015) al considerarlos "*novel foods*" y permitir a las empresas que producen alimentos a base de insectos poder solicitar una autorización para producir y vender sus productos. El crecimiento de este mercado es indudable y existen granjas de insectos en España que producen harina proteica y otros productos.

Hasta la realización de este estudio, tan solo un estudio sobre el potencial de la proteína de *Tenebrio molitor* como precursora de péptidos inhibidores de la enzima DPP-IV había sido reportado en literatura, y por tanto el objetivo de este estudio fue realizar un barrido de amplio rango para obtener un hidrolizado con una capacidad inhibitoria al menos comparable con los reportados para fuentes tradicionales. Para ello, se realizó un estudio con veinte tratamientos enzimáticos empleando cuatro proteasas de grado alimentario de uso. Los parámetros considerados fueron la proteasa empleada (sola o en combinación), y el grado de hidrólisis alcanzado (5, 10, 15 y 20). Las proteasas empleadas solas fueron subtilisina, tripsina y ficina, que permitieron alcanzar los grados de hidrólisis máximos de 20, 15 y 5, respectivamente, dadas en las dos últimas su limitada especificidad. Además, en combinación se consideró la enzima subtilisina, como proteasa no específica, junto a tripsina, ficina y Flavourzyme, con determinada especificidad respectivamente. No se consideró utilizar Flavourzyme como catalizador único dada su definición como exopeptidasa, al considerar que el sustrato original son cadenas proteicas con estructuras complejas a las que dicha proteasa no podría acceder para hidrolizar de manera efectiva. Los resultados mostraron valores de $IC_{50}$ entre 2.62 y 34.13 mg/ml. Indistintamente del tratamiento, se observó una correlación directa entre el aumento del grado de hidrólisis y el aumento de la inhibición ejercida por los péptidos liberados, que se explica por el aumento de la fracción de péptidos de menor tamaño molecular. Así, el hidrolizado obtenido mediante la combinación de subtilisina (endopeptidasa de alto espectro) y flavourzyme (exopeptidasa) al mayor grado de hidrólisis (DH = 20) fue el que mostró mayor bioactividad (valor $IC_{50}$ = 2.62 mg/ml), en el rango de los que se encuentran actualmente en literatura para otros sustratos como la proteína de algas marinas *(Porphyra*

*dioica)* (Cermeño et al., 2019), de jabalí (*Capros aper*) (Parthsarathy et al., 2018) o de caupí (*Vigna unguiculata*) (Castañeda-Pérez et al., 2019). Este tratamiento enzimático ya ha sido descrito para otros sustratos como adecuado para producir hidrolizados de proteína altamente activos (Harnedy-Rothwell et al., 2020), gracias a la sinergia que se produce entre ambas enzimas. La subtilisina es capaz de liberar fragmentos peptídicos que serán hidrolizados por Flavourzyme cerca de los extremos. Esto se demostró por el análisis de pesos moleculares, en los que la muestra más activa fue la que efectivamente mostró una mayor proporción de péptidos de pequeño peso molecular en comparación al resto de muestras analizadas. La fracción de péptidos <500 Da representa un 19.6% del área total, correspondiente a dipéptidos y tripéptidos, que son los que se han identificado mayormente en otras fuentes como el salmón (Harnedy et al., 2018a; Neves et al., 2017). En cuanto a las fuentes de insectos, otros autores han estudiado la actividad inhibidora de la DPP-IV mediante péptidos del gusano de la harina menor (*A. diaperninus*), reportando la actividad antidiabética máxima para el hidrolizado obtenido con termolisina con un valor $IC_{50}$ de 0.63 mg/mL (Lacroix, Dávalos Terán, Fogliano, & Wichers, 2019). Respecto al *Tenebrio molitor*, la hidrólisis de la proteína cuticular con papaína produjo un hidrolizado altamente activo, con un valor $IC_{50}$ de 0.82 mg/ml (Dávalos Terán, Imai, Lacroix, Fogliano, & Udenigwe, 2019).

## 4.4 Efecto del pretratamiento con ultrasonido y la hidrólisis secuencial en la producción de péptidos antidiabéticos de *Tenebrio molitor*

El objetivo de este estudio fue evaluar los efectos de un pre-tratamiento de la muestra de harina de insecto en la liberación de péptidos activos mediante posterior hidrólisis secuencial, siendo el tiempo de aplicación de las dos proteasas otra variable. Conceptualmente se entiende este estudio como un acercamiento al enfoque industrial con el propósito de conseguir la reducción del tiempo de reacción mediante la modificación de distintos parámetros operacionales.

Específicamente, considerando la baja solubilidad previamente analizada del sustrato y con el objetivo de aumentar la hidrofobicidad del sustrato para incrementar los enlaces peptídicos propensos a ser hidrolizados, se evaluó el efecto de la aplicación de ultrasonidos previo a la hidrólisis, en la obtención de péptidos bioactivos. De esta manera,

se realizaron tres pre-tratamientos de ultrasonido (tiempos de 0, 15 y 30 minutos), y en cada caso, se realizaron hidrólisis secuenciales con subtilisina y tripsina de una duración total fija de 7 horas, pero modificando el tiempo en que se añade la tripsina. Se consideró el uso de subtilisina por su alto espectro de corte y la tripsina por su limitada especificidad, que, en base a literatura, promueve la liberación de péptidos inhibidores de α-glucosidasa (Ibrahim et al., 2017; Z. Yu et al., 2011).

Se consideró la adición de tripsina en los tiempos = 0, 1, 2 y 3 horas, siendo el t = 0 h el caso donde no se añade la subtilisina, solo hidrólisis de tripsina. De esta manera, el caso t = 1 h, se traduce como 1 hora de hidrólisis de subtilisina exclusivamente, adición de tripsina, y continuación de la reacción durante 6 horas más. El número de reacciones evaluadas por tanto fue, considerando pretratamientos y tiempos de acción de proteasas, de 12 reacciones. Estas reacciones se realizaron a 15 g/L de proteína, concentración previamente optimizada para observar modificaciones de inhibición de la enzima objetivo a lo largo de las reacciones, y se tomaron muestras cada treinta o sesenta minutos para observar la variación en el porcentaje de inhibición sobre la enzima α-glucosidasa durante el transcurso de la reacción.

Se demostró que el tratamiento con ultrasonidos incrementó la hidrofobicidad de la muestra alrededor de un 50% y afectó posteriormente a la liberación de péptidos. La inhibición de la enzima, considerando mismo tratamiento enzimático pero distinto tiempo de pre-tratamiento, fue variable de manera que periodos cortos favorecieron la liberación temprana de péptidos activos mientras que altos tiempos de ultrasonido lo limitaron, probablemente debido a la agregación de cadenas peptídicas en la muestra original. Se ha demostrado que el tratamiento con ultrasonidos puede provocar tanto un aumento de la hidrofobicidad superficial de las muestras como la modificación de la estructura secundaria y terciaria de las proteínas (Jiang et al., 2014; Wali et al., 2017). Mir, Riar & Singh (2019) observaron que la proteína de quinoa tratada con ultrasonidos hasta 25 minutos provocó la escisión de algunos aminoácidos y la mejora de la solubilización de la proteína, mientras que cuando el pre-tratamiento tenía una duración de 35 minutos, se originaban agregados de proteínas. Además, el potenciamiento de la actividad inhibidora de glucosidasa por el pretratamiento de ultrasonidos también ha sido estudiada para la proteína de soja (M. Jiang, Yan, He, & Ma, 2018).

A la concentración analizada, los péptidos liberados únicamente durante la hidrólisis con subtilisina no ejercieron inhibición sobre la enzima, y es la adición de tripsina lo que dio lugar a la liberación de péptidos con capacidad para inhibir la enzima de manera

significativa. La reacción con únicamente adición de tripsina no mostró inhibición, por lo que se propone la reacción secuencial subtilisina-tripsina como clave para una liberación de péptidos altamente bioactivos dado que la subtilisina incrementa el número de enlaces peptídicos propensos a ser rotos, y la tripsina libera péptidos con características moleculares adecuadas. Finalmente, el pre-tratamiento con ultrasonidos durante 15 minutos, seguido de una hora de reacción con subtilisina y posteriormente 90 minutos con tripsina fue el punto en el que se alcanzó un alto nivel de inhibición de la enzima en el menor tiempo, comparando todas las reacciones ensayadas. El porcentaje de inhibición a lo largo de la reacción fue diferente para cada caso, demostrando que el grado de hidrólisis no es un factor clave en la liberación de péptidos de estas características, sino que es el tratamiento en sí el factor determinante, como mostraron previamente otros autores (Connolly, Piggott, & FitzGerald, 2014; Ren et al., 2016). Considerando los parámetros de reacción, donde la temperatura se fijó a 50 ºC, es importante considerar que se ha demostrado que la actividad proteolítica de la tripsina disminuye más de su 50% a partir de 47 ºC y es susceptible a la pérdida de actividad por autolisis, mientras que la subtilisina muestra una mayor resistencia a la degradación térmica. Esta podría ser una razón por la que, a pesar de que es la tripsina la responsable de la liberación de péptidos más activos, su disminución de actividad provoque que, en periodos de reacción prolongados, sea la subtilisina la que sea mayor responsable de la continuada hidrólisis en el reactor. La liberación de péptidos inhibidores de glucosidasa más potentes mediante la acción de tripsina en comparación con otras proteasas también fue demostrado para hidrolizados de proteína de grano de cerveza (Connolly et al., 2014) y la proteína de huevo (Z. Yu et al., 2011). Una optimización a nivel de tiempo de reacción es importante en términos de escalamiento industrial ya que permite obtener en un menor tiempo, una muestra altamente activa.

## 4.5 Identificación de nuevos péptidos inhibidores de dipeptidil peptidasa IV y α-glucosidasa de *Tenebrio molitor*

El objetivo de este estudio fue el fraccionamiento e identificación de péptidos con capacidad antidiabética en los hidrolizados más activos de los estudios previos, para la inhibición de la enzima DPP-IV y la α-glucosidasa.

El hidrolizado con menor valor $IC_{50}$ (mayor bioactividad) de la sección 4.3, correspondiente con el obtenido mediante la hidrólisis simultánea con Subtilisina y Flavourzyme a grado de hidrólisis 20, se fraccionó. Los péptidos con un peso molecular entre 500 y 1600 Dalton fueron los que presentaron mayor concentración y mayor inhibición de la enzima DPP-IV, con un valor $IC_{50}$ de 0.91 mg/ml. Kang et al. (2020) obtuvieron valores de $IC_{50}$ entre 0.79 y 0.93 mg/ml para los péptidos de menor peso molecular obtenidos con diferentes proteasas a partir de proteína de cangrejo de mar. De la misma manera, la fracción de péptidos < 1 kDa de salmón, con un valor $IC_{50}$ de 1.35 mg/ml resultó ser la más activa entre las analizadas por Li-Chan, Hunag, Jao, Ho, & Hsu (2012).

Esta fracción se analizó mediante espectrometría de masas para realizar el secuenciamiento *de novo* de los péptidos contenidos en dicha fracción. El análisis permitió secuenciar 7 péptidos que según el análisis de correspondencia al proteoma del *Tenebrio molitor*, proceden de proteínas de éste, confirmando su presencia en el hidrolizado. Entre los péptidos identificados, la secuencia APVAH se encuentra hasta siete veces en la proteína cuticular (en base al análisis de alineamiento BLASTp), y dadas sus características moleculares, se espera que sea uno de los péptidos responsables de la alta bioactividad del hidrolizado. Dávalos Terán et al. (2019) mostraron que las proteínas cuticulares presentaban mayor bioactividad que las no cuticulares. Respecto a literatura previa, péptidos cuyo N-terminal está compuesto por los aminoácidos AP o AV han sido identificados (Liu et al., 2019), pero las secuencias identificadas en esta investigación (APVAH and AVTTK) no han sido previamente reportadas. De la misma manera, el péptido PAL se ha mostrado como inhibidor de la DPP-IV (Ji, Zhang, & Ji, 2017), pero no el péptido PALLL, que dada su similitud al anterior y los prometedores valores de los análisis *in silico* podría ser un péptido altamente activo también. Las características moleculares de algunos de dichos péptidos y el análisis *in silico* efectuado permitieron sugerir cuáles de estos péptidos eran altamente probable los más activos, como APVAH.

Por otro lado, el hidrolizado de la sección 4.4 que en menor tiempo presentaba >90% de inhibición a 15 g/L de la α-glucosidasa, se obtuvo de nuevo (pre-tratamiento con ultrasonido de 15 minutos, hidrólisis con subtilisina durante una hora y posteriormente una hora y media con tripsina) y se tomó como hidrolizado de referencia, que presentó un valor $IC_{50}$ de 6.52 mg/ml. Nuestros resultados presentan un valor en el rango de los obtenidos por Yoon at al. (2019), que produjeron un hidrolizado de proteínas de larvas de gusano de la harina con Alcalasa y Flavourzyme que ejercía un 35% de inhibición a 2

mg/ml. Sin embargo, nuestros resultados preliminares mostraron que el tratamiento con alcalasa-tripsina mostraba mayor bioactividad que el tratamiento con Flavourzyme. El fraccionamiento mediante cromatografía de exclusión de tamaño permitió dilucidar que los péptidos con un peso molecular inferior a 500 Da eran los responsables de la bioactividad ejercida por el hidrolizado, presentando un valor $IC_{50}$ de 2.58 mg/ml. De la misma manera, Castañeda-Pérez et al. (2019) reportaron mayor inhibición de α-glucosidasa para la fracción <1 kDa de un hidrolizado de proteína de caupí.

Esta fracción se analizó por espectrometría de masas para realizar el secuenciamiento *de novo* de los péptidos contenidos en dicha fracción. El análisis permitió secuenciar 6 péptidos que según el análisis de correspondencia al proteoma del *Tenebrio molitor*, proceden de proteínas de éste, confirmando su posible presencia en el hidrolizado. El péptido CSR (364.4 Da), se encuentra al menos en 5 proteínas del sustrato y posee el residuo arginina, descrito como importante a la hora de inhibir la enzima α-glucosidasa (Ibrahim et al., 2017). Péptidos de bajo peso molecular con esta bioactividad también han sido encontrados en pupas de gusano de seda, como QPGR, SQSPA, QPPT y NSPR (Y. Zhang et al., 2016). Igualmente, el péptido AR se encuentra en muchas proteínas del sustrato, incluida la proteína de la cutícula LPCP-23, y, en consecuencia, se espera que este dipéptido sea responsable de la alta inhibición de la α-glucosidasa de la fracción proteica.

También se han identificado péptidos de <5 aminoácidos de longitud como péptidos inhibidores de la α- glucosidasa, GEY, GYG, LR y PLMLP (H. J. Lee et al., 2011; Ren et al., 2016). Es interesante observar cómo se identificó el péptido APYF en el hidrolizado de α-glucosidasa, pero es de esperar que también posea capacidad inhibidora de DPP-IV, debido a la presencia de prolina en su secuencia. La puntuación obtenida en el análisis *in silico* sitúa a este péptido como altamente bioactivo (puntuación = 0.949). Las características moleculares de algunos de dichos péptidos y el análisis *in silico* efectuado permitieron sugerir cuáles de estos péptidos eran altamente probable, siendo estos AR o CSR.

## 4.6 Evaluación del potencial antidiabético de un caldo de verduras enriquecido con hidrolizados de proteínas de *Tenebrio molitor*

La inclusión en matrices de los hidrolizados de proteína, el procesamiento de estos productos y su evaluación durante el almacenamiento son el paso final para poder establecer y considerar fehacientemente estos péptidos como ingredientes funcionales para alimentación. Existen algunos estudios utilizando este insecto como ingrediente, por ejemplo, remplazando parcialmente carne por escarabajos en salchichas cocinadas (Scholliers, Steen, & Fraeye, 2020) o fortificando pan con proteína de gusano de la harina (Roncolini et al., 2019). Los resultados son prometedores respecto a su uso como ingrediente para consumo humano. Respecto a péptidos, existen estudios con hidrolizados de proteína con capacidad antioxidante y antihipertensiva (Rivero-Pino, Espejo-Carpio, & Guadix, 2020b) y antidiabéticos (Harnedy-Rothwell et al., 2021) incorporados en diferentes matrices alimentarias liquidas de alimentos, resultando en bioactividad conservada o mejorada durante el almacenamiento y después de una digestión gastrointestinal simulada.

Dado que los tratamientos utilizados en la industria alimentaria, los componentes de la matriz elegida y la temperatura y tiempo de almacenamiento pueden afectar las propiedades biológicas de los péptidos, se seleccionaron los dos hidrolizados de insecto con alta capacidad inhibitoria de la enzima DPP-IV y α-glucosidasa respectivamente, para evaluar cómo se comportarían al ser incluidos como ingrediente. Ambos hidrolizados se sometieron a la misma evaluación de procesamiento y almacenamiento en una matriz líquida comercialmente disponible en supermercados (caldo de verduras), cuyo valor nutricional y contenido la hacen apropiada como vehículo para el propósito indicado de mantener la bioactividad de los péptidos como producto comercial. Así, se incluyeron los péptidos en caldo de verduras y se esterilizaron en autoclave a 121 ºC durante 21 minutos y se evaluó la bioactividad remanente. Estas muestras se conservaron en nevera durante un periodo de 40 días, tomando muestras en tiempos intermedios para evaluar la evolución del valor de $IC_{50}$ en ese tiempo. Por otro lado, los péptidos (los hidrolizados), así como la proteína original fueron sometidas a digestión gastrointestinal simulada en agua y en caldo de verduras, para evaluar la actividad de los péptidos por ingesta oral.

La sopa de verduras fue elegida como un producto familiar básico comúnmente empleado en las sociedades occidentales. La sopa de verduras enmascararía el posible sabor de los péptidos amargos, aunque los análisis sensoriales publicados en la literatura no indican gustos desagradables en productos a base de insectos. Para *Tenebrio molitor* se han descrito diferentes sabores, tales como: nuez, umami, aroma a cereal, madera, aroma a caldo, sabor a verduras y productos de reacción de Maillard (Elhassan, Wendin, Olsson, & Langton, 2019). El caldo mostró capacidad de inhibir el 40% de la actividad de la DPP-IV, pero no de la α-glucosidasa.

La capacidad de los péptidos de inhibir la enzima DPP-IV se perdió parcialmente tras la esterilización en agua (valor $IC_{50}$ de 5.07 mg/ml), pero se mantuvo cuando la muestra se encontraba incluida en el caldo de verduras. Se observa que la presencia de sopa de verduras ayuda a conservar la bioactividad. A medida que se agrega mayor proporción de sopa, hasta uno, los valores de $IC_{50}$ disminuyen hasta ser estadísticamente iguales al control. En estos casos, se sugiere que la sopa de verduras está contribuyendo a la bioactividad y/o ayudando a preservar la estructura química de los péptidos bioactivos que ejercen la bioactividad. También se observó una pérdida de actividad inhibidora de DPP-IV después de 1 hora de tratamiento térmico a 120 º C por López-Sánchez, Ponce-Alquicira, Pedroza-Islas, de la Peña-Díaz, & Soriano-Santos (2016) en un hidrolizado de proteína de amaranto, en línea con los resultados reportados para nuestra muestra. El posterior almacenamiento durante cuarenta días de esta muestra no mostró variaciones estadísticamente significativas, indicando que el ingrediente bioactivo se mantiene estable en el alcance de las condiciones del ensayo.

En el hidrolizado de proteína inhibidora de DPP-IV, la bioactividad se ve comprometida después de la digestión en agua, perdiendo ~ 40% de la inhibición ejercida. Esto es muy probable debido a la escisión específica que ejercen la pepsina y la pancreatina (sobre aminoácidos aromáticos, alifáticos y, más precisamente, arginina y lisina), ya que los péptidos liberados después de la hidrólisis tendrían características moleculares no tan adecuadas para inhibir la DPP-IV como los péptidos originales contenidos en la muestra. Sin embargo, cuando la digestión ocurre en la sopa de verduras, la bioactividad no es significativamente diferente del control. Se pueden extraer diferentes hipótesis de estos resultados: i) Los péptidos son resistentes a la digestión porque las proteasas digestivas no pueden acceder a los péptidos bioactivos con tanta eficacia como en el agua. ii) Los péptidos liberados interactúan con componentes de la matriz alimentaria, manteniendo una bioactividad global igual. iii) La matriz alimentaria mantiene los componentes que

pueden inhibir la DPP-IV. Los valores de $IC_{50}$ para la inhibición de la DPP-IV que ejerce la proteína sometida a SGID (7.23 mg/ml en agua y 8.76 mg/ml en sopa de verduras) apoyan la utilización de los hidrolizados de proteínas como ingredientes bioactivos en lugar de la ingesta de la proteína sin ningún tipo de tratamiento enzimático. Estos resultados son prometedores en la consideración de esta matriz alimentaria líquida como vehículo para transportar estos péptidos inhibidores de la DPP-IV, ya que ayudan a conservar dicha bioactividad previamente definida.

Respecto a los péptidos inhibidores de la enzima α-glucosidasa, la esterilización en agua destilada condujo a una pérdida de actividad en el rango que el método puede determinar (>15 mg/ml). Esto puede deberse a la agregación de péptidos tras el tratamiento térmico, que impide el acceso de los péptidos activos a la enzima diana. Sin embargo, al considerar el caldo de verduras, esa bioactividad, aunque disminuida, pudo ser medida (valores de $IC_{50}$ significativamente similares). Estos resultados refuerzan la hipótesis de que la estructura de los péptidos se conserva mejor gracias a la matriz vegetal, muy probablemente debido a la presencia de fibras, ya que la sopa de verduras no mostró actividad inhibidora de la α-glucosidasa como sí lo hizo para la inhibición de la DPP-IV. Según el conocimiento del autor, no hay informes disponibles en la literatura sobre cómo los tratamientos térmicos podrían modificar la bioactividad de los péptidos inhibidores de la α-glucosidasa. Respecto al almacenamiento de los péptidos en refrigeración durante 40 días, tampoco se reportó modificación de la bioactividad significativa. En general, la literatura disponible apoya la hipótesis de que estos péptidos no sufren modificaciones durante el almacenamiento refrigerado a corto-medio plazo. Este es el primer informe que demuestra que la actividad inhibidora de la α-glucosidasa de los péptidos se conserva durante el almacenamiento refrigerado.

Por su parte, la inhibición de la α-glucosidasa ejercida por el hidrolizado mejoró hasta ~ 300% tras la digestión gastrointestinal simulada. No existen diferencias significativas entre la muestra digerida en agua y en la sopa de verduras. Este tipo de péptidos inhibidores suelen tener las características moleculares de los péptidos que se liberarían de las enzimas digestivas. Por tanto, es muy probable que el producto obtenido después de la digestión esté más concentrado en péptidos inhibidores de la α-glucosidasa. El hidrolizado original fue el de alta bioactividad lograda en el menor tiempo de reacción de una batería de diferentes hidrolizados considerando dos parámetros (pretratamiento ultrasónico y tiempo de reacción de la hidrólisis secuencial con dos proteasas) (Rivero-Pino, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2020). En este sentido, es

importante ver cómo el hidrolizado siguió teniendo alta bioactividad (> 90%) al hidrolizarse por tres horas más con tripsina, resultados comparables a los resultados obtenidos en este trabajo de investigación. Se demostró que el porcentaje de péptidos con peso molecular más pequeño aumentó en la muestra digerida. La proteína nativa tras la digestión gastrointestinal simulada mostró un valor de $IC_{50}$ menor que el hidrolizado, siendo los valores de $IC_{50}$ 3.65 y 4.48 mg/ml, frente a 6.52 mg/ml del hidrolizado. Esta bioactividad informada sugiere que los péptidos naturales liberados debido a la acción de las enzimas digestivas después de la ingesta de la proteína nativa también ejercerían una adecuada actividad inhibidora de la α-glucosidasa. La menor bioactividad reportada para la proteína digerida en la sopa de verduras en comparación con la digerida en agua podría ser consecuencia de un menor grado de hidrólisis debido al impedimento de los componentes vegetales para permitir la hidrólisis de la proteína en la matriz. Sin embargo, al comparar los valores de inhibición informados, el hidrolizado ejerce una mayor inhibición después de la digestión en comparación con la proteína nativa después de la digestión. Este resultado muestra que la hidrólisis enzimática potencia la bioactividad de la muestra, debido a la liberación de péptidos específicos que ejercen una alta bioactividad, y el hidrolizado sería una mejor opción para ser empleado como ingrediente funcional para fortalecer las matrices alimentarias.

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# I. Antidiabetic Food-Derived Peptides for Functional Feeding: Production, Functionality and In Vivo Evidences[*]

Bioactive peptides released from the enzymatic hydrolysis of food proteins are currently a trending topic in the scientific community. Their potential as antidiabetic agents, by regulating the glycemic index, and thus to be employed in food formulation, is one of the most important functions of these peptides. In this review, we aimed to summarize the whole process that must be considered when talking about including these molecules as a bioactive ingredient. In this regard, at first, the production, purification and identification of bioactive peptides is summed up. The detailed metabolic pathways described included carbohydrate hydrolases (glucosidase and amylase) and dipeptidyl-peptidase IV inhibition, due to their importance in the food-derived peptides research field. Then, their characterization, concerning bioavailability in vitro and in situ, stability and functionality in food matrices, and ultimately, the in vivo evidence (from invertebrate animals to humans), was described. The future applicability that these molecules have due to their biological potential as functional ingredients makes them an important field of research, which could help the world population avoid suffering from several diseases, such as diabetes.

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# 1. INTRODUCTION

## 1.1 Proteins, protein hydrolysates and peptides

Proteins are one of the main components of human diets. These biomacromolecules are formed by the association of amino acids, through the peptidic bond between the amino group of one amino acid and the carboxyl group from the following amino acid in the chain (Nelson & Cox, 2008). Food proteins are an important topic in the research, due to their health benefits in human and their enormous variety.

At the end of the 20th century, scientists started to focus on studying the hydrolysis of food proteins in order to determine their forming-peptides, because some of them have been proven to be bioactive, and to have beneficial consequences in the organism (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). The gastrointestinal digestion of proteins leads to the formation of peptides in the human tract due to the action of digestive proteases, and these have beneficial effects. However, the key in this topic is that different food-grade proteases might have different specificities (McDonald, 1985), and from the same substrate, the pool of peptides produced would be different, and would show different properties.

A protein hydrolysate is the mixture of peptides that originally formed the protein, after its hydrolysis. The complex structure of proteins in the native state hides the functionality of the peptides, preventing them from exerting their bioactivity by association with some other molecules.

The hydrolysis reaction consists of breaking the peptidic bonds and releasing different-sized peptide chains, whose properties may well vary depending on the properties of the amino acids included in each peptide (Adler-Nissen, 1986). The released peptides usually present improved technological and biological properties that allow the utilization of these products to improve food formula properties. Protein hydrolysis leads to an improvement in different technological aspects: solubility, emulsifying and foaming capacity, water holding capacity, oil binding capacity and lipid oxidation prevention. The technological property modification of protein hydrolysates, compared to the intact protein, is an advantage related to their use in functional feeding, because it mainly involves the facilitation of the formulation of the food. This improvement is due to the exposition of the residues of different amino acids. In this context, both the molecular weights of peptides and amino acid sequences are important.

The solubility of proteins depends on the interactions between the macromolecules forming the protein (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). Protein hydrolysis leads to polar group exposition when small peptides are released, and consequently, a high degree of hydrolysis is correlated with the higher solubility of protein hydrolysates (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Kristinsson & Rasco, 2000). This increase in solubility is important in the production of food and beverages intended for parenteral or gastric administration.

Proteins are amphipathic molecules due to the different polarities of the amino acids that compose them (Damodaran & Parkin, 2017), which are absorbed into the interface formed during the emulsification process (C. Genot, 2013), reducing the interfacial tension and stabilizing the emulsion. Notably, limited hydrolysis leads to this interfacial activity, increasing the emulsifying activity (Lam & Nickerson, 2013). Foaming property improvement also depends on the surface activity of the proteins (Klompong et al., 2007), and low degrees of hydrolysis are adequate for an increase in foaming capacity (F. G. Hall, Jones, O'Haire, & Liceaga, 2017). Protein hydrolysis also enhances the water holding capacity and the oil binding capacity, depending on the amino acids composing the peptides and the lengths of them. Hydrophilic groups would retain water more effectively (Zayas, 1997), whereas for oil binding capacity, the hydrophobic residues of proteins are important due to the lipophilic character of oils, thus allowing the interaction with their hydrocarbon chains (Zielińska, Karaś, & Baraniak, 2018). These are important parameters to consider when formulating a fortified food product, because they would affect its structure.

Proteins and peptides show antioxidant activity (Aluko, 2015; Elias, Kellerby, & Decker, 2008). Numerous protein hydrolysates have been reported as antioxidants, coming from different protein sources. Concerning technological improvement, it is important to remark that these peptides with antioxidant activities and emulsifying properties can be used in complex food matrices as an emulsion, those peptides being a double-action agent (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2016; Padial-Domínguez, Espejo-Carpio, García-Moreno, Jacobsen, & Guadix, 2020). As such, these peptides would prevent the lipid oxidation process in food formulation (Lin & Liang, 2002). Another improvement provoked by hydrolysis is the increase in digestibility and the loss of antigenicity of the proteins. This is an important statement in terms of nutrition; for example, for specific groups of people, such as infants or elder. The digestibility of

proteins is increased because the available N-terminal sites are increased after enzymatic hydrolysis (Acquah, Di Stefano, & Udenigwe, 2018; Hannu & Pihlanto, 2006), and consequently, the peptidases action is enhanced. The antigenicity of proteins is caused by epitopes, specific sequences in the allergen proteins that are potentially recognizable by the immune system and would potentially activate an allergenic response. Enzymatic hydrolysis leads to allergenic epitopes degradation, and therefore helps to reduce the immunoreactivity of the native protein (García-Moreno et al., 2017). Enzymatic hydrolysis, due to its reaction conditions, does not destroy amino acids, which is desirable for food formulation because the nutritional profile of the proteins is conserved.

Nonetheless, one disadvantage that enzymatic hydrolysis can produce in terms of nutrition is the bitter taste of peptides, related to the release of hydrophobic residues. To overcome this limitation, the encapsulation of peptides inside different matrices (i.e., chitosan, glucose syrup) or the addition of flavor-developing proteases (i.e., flavourzyme) are adequate solutions. These techniques would avoid the disadvantages of traditional techniques, such as the adsorption of bitter peptides onto activated carbon, chromatographic removal, or selective extraction with alcohols (R.J. FitzGerald & O'Cuinn, 2006).

On the other hand, the similarity of food-derived peptides to the structure of human regulatory peptides also makes them suitable for interacting with some enzymes and receptors involved in human metabolism. In this way, the most important improvement of proteins after hydrolysis, concerning functional food, is the bioactivity development. Lately, the proportion of the world population suffering an illness has increased, and prevention and pre-treatment are considered good options for most of them. At the economical level, the cost savings, compared to those associated with the treatment of the disease, are high (R. Li, Zhang, Barker, Chowdhury, & Zhang, 2010). Bioactive peptides are considered to be regulator molecules operating at different levels in the organism. As was previously mentioned, protein hydrolysis during digestion releases peptides that exert bioactivity in humans, but the intake of peptides with improved bioactivity, compared to those obtained naturally, is seen as a good option for humans (Hannu & Pihlanto, 2006). This is due to the specificity of food-grade proteases employed in the industry, which are able to release peptides that digestive proteases cannot.

The bioactivity of peptides is considered to be related to the hydrophobicity characteristics of the residues, and more precisely, to the amino acid functional groups of

their sequences (Acquah et al., 2018). Focusing on the bioactivities those peptides can exert, their antioxidant, antihypertensive and antidiabetic (glycemic index regulation) activities are the most remarkable because of the diseases they would prevent, which are some of the most prevalent worldwide nowadays (cardiovascular disease, diabetes, hypertension). Recent studies, both in vitro and in vivo, show the functionality of these protein hydrolysates. In vitro analyses allow comparisons of the biological potentials of different products, by evaluating, for example, the inhibitory capacity of different enzymes involved in metabolic processes.

Bioactive peptides can exert physiological effects at a cardiovascular, digestive, endocrine, immune and/or nervous level (Patil, Mandal, Tomar, & Anand, 2015). The most studied historically are antihypertensive and antioxidant, whereas there is less information regarding the antidiabetic properties of peptides coming from food proteins. Peptides are considered to be bioactive in different metabolic pathways, depending on how they interact with the human body. When it comes to inhibiting an enzyme, peptides can interact at the active site and/or outside the catalytic site of it, preventing the enzyme from interacting with the substrate.

Peptides are defined as antihypertensive when they are able to inhibit the angiotensin-converting enzyme (ACE). This enzyme plays a key role in blood pressure regulation (Skeggs, Kahn, & Shumway, 1956), and its inhibition has a positive effect on hypertensive patients (Mizuno et al., 2005). In the antioxidant case, a wide variety of mechanisms and implications are involved. Beyond their ability to slow down lipid oxidation in food systems, these peptides would also prevent oxidative stress related to several diseases such as hypertension and ageing (Hajieva & Behl, 2006). As such, peptides with both antihypertensive and antioxidant activities can be considered anti-ageing peptides. Beyond these two bioactivities, anticholesterolemic, antithrombotic and anti-inflammatory peptides have also been described (Meram & Wu, 2017; Suleria et al., 2017). Furthermore, peptides might have diverse bioactivities, and might consequently exert synergistic effects on the human body. For example, a correlation has been proposed between diabetic and hypertensive patients, so a treatment for both problems would be ideal. Ketnawa et al. (2018) obtained both ACE- and dipeptidyl peptidase IV (DPP-IV)-inhibitory peptides from rainbow trout. In this review, we will focus on glycemic index-regulating peptides, that is, antidiabetic peptides.

## 1.2 Carbohydrates Digestion Process and Diabetes

The metabolism of carbohydrates is the process of transforming the carbohydrates ingested from food into glucose molecules, the most efficient source of energy. The carbohydrates in foods generally appear as polysaccharides, such as starch or cellulose, or as disaccharides, such as lactose or sucrose. Carbohydrate digestion involves different enzymes and a complex series of metabolic processes. A graphical simplification is depicted in Figure 1. Initially, when the bolus is ingested, digestive enzymes would hydrolyze these complex polysaccharides.

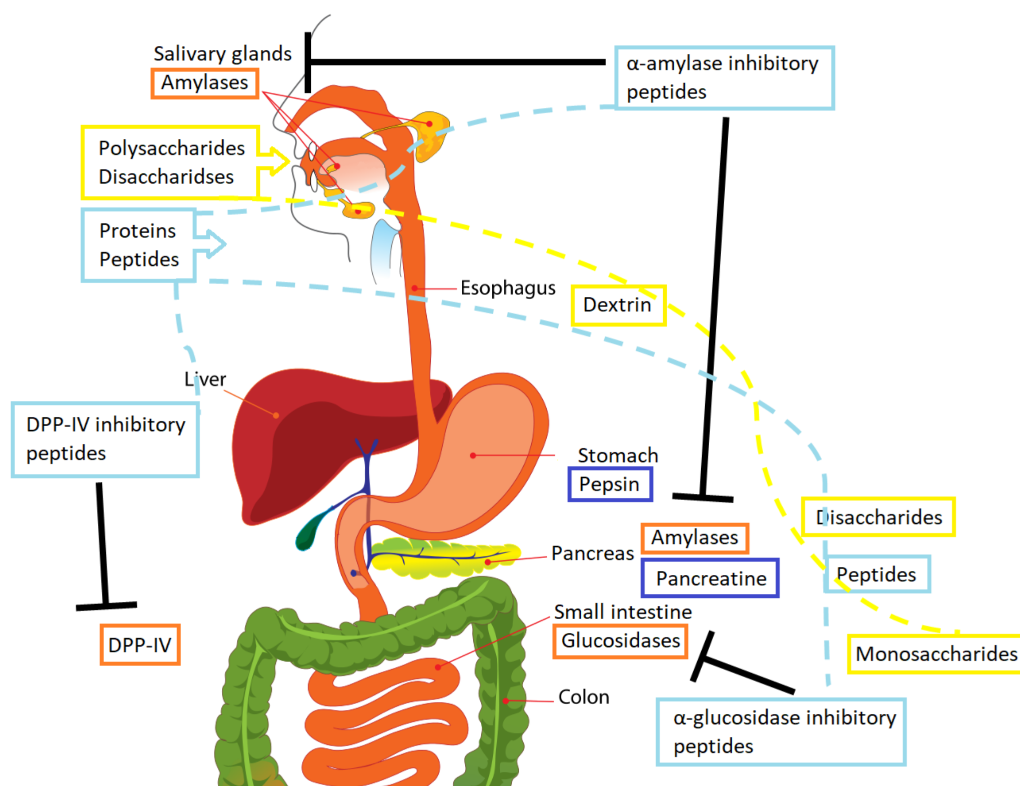


**Figure 1.** Mechanisms involved in peptides and carbohydrate digestion. The broken lines recreate the digestion process of the different molecules. Color reference: Yellow—Molecular state of carbohydrate during digestion; Orange—Main digestive enzymes involved in the carbohydrate digestion; Light blue—Molecular state of proteins during digestion; Dark blue—Digestive proteases. Permission: The original picture was released into the public domain by its author (*LadyofHats*) and modified by authors to depict the information detailed in the document.

α-Amylase (EC 3.2.1.1) hydrolyzes complex carbohydrates such as starch into oligosaccharides, which would be further hydrolyzed by α-glucosidase. This enzyme is secreted from the salivary and pancreatic glands.

α-Glucosidase (EC 3.2.1.20) is a membrane-bound enzyme found in the epithelial mucosa of the small intestine (brush border of the enterocytes). It releases free glucose molecules from terminal, non-reducing (1-4)-linked α-glucose residues.

Furthermore, food intake causes the release of intestinal hormones called incretins (gastric inhibitory polypeptide, GIP, and glucagon-like peptide-1, GLP-1). These two would affect numerous target tissues in the body, acting as endocrine signals to the pancreas, leading to insulin production in the β-cells and the suppression of the release of glucagon in the α-cells. These two incretins are responsible for ~70% of the insulin secretion after meal intake (Nauck, Baller, & Meier, 2004). This results in the uptake of glucose by the muscles, as well as a lower production of glucose in the liver. The final consequence is therefore the decrease in blood glucose after ingestion, which allows the adequate regulation of postprandial blood glucose levels. At this level, the enzyme dipeptidyl peptidase IV (DPP-IV) regulates the degradation of incretins according to physiological needs. GLP-1 and GIP have half-lives of approximately 2 min and 5–7, min respectively, before they are degraded by DPP-IV (Tahrani, Bailey, Del Prato, & Barnett, 2011; Yan, Zhao, Yang, & Zhao, 2019). DPP-IV is a cell surface (EC 3.4.14.5) that cleaves dipeptides from the N-terminus of polypeptides, in which proline is at the penultimate position (Lammi et al., 2018). DPP-IV can largely be found on the luminal surface of enterocytes; therefore, it can interact with any of the molecules from food intake before their absorption, that can be further metabolized before the molecules' interaction with soluble and vascular endothelial DPP-IV (the one affecting GIP and GLP-1 levels). Gut hormones released from the enteroendocrine cells play an important role in food intake regulation (Caron, Domenger, Dhulster, Ravallec, & Cudennec, 2017a).

Diabetes mellitus type 2 is one of the most prevalent diseases, affecting more than 400 million people and with estimations of 700 million people becoming affected by 2045 ("IDF Diabetes Atlas," 2017). This metabolic disorder is characterized by insulin resistance, that is, the inability of the organism to react to the insulin action, or an insufficient production of this hormone. It is especially important to control the postprandial glucose level, because the long-term consequences of high glucose levels in

the bloodstream are diverse, from renal failure to neurological damage and cardiovascular disorders (L. Li & Hölscher, 2007; Patil et al., 2015). Regarding the causes, both genetic and environmental factors take part in diabetes development. It is believed that the main cause is obesity, which operates through several pathways including an imbalance in the concentration of hormones, cytokines and other inflammatory signals (Tahrani et al., 2011).

## 1.3 Diabetes Prevention Strategies

Numerous strategies to manage postprandial hyperglycemia, and consequently prevent the development of type 2 diabetes, have been described (Patil et al., 2015). Insulin injection (Howard-Thompson, Khan, Jones, & George, 2018) is the direct treatment for this disease, positively regulating the functioning of the organism. The main disadvantage is that insulin cannot be orally ingested. In addition to this, medications involved in the metabolic pathway of digestion are also options as regards preventing and treating the disease. A graphical simplification of the most important mechanisms involved in diabetes prevention is depicted in Figure 1.

Food-derived peptides from food proteins play a crucial role in the regulation of glucose homeostasis, due to their implication at different levels (e.g., glucagon-like peptide 1 regulation) and due to their capacity to inhibit digestion-related enzymes. Furthermore, some authors have described peptides as being able to enhance cholecystokinin levels, a gut hormone regulating food intake (Nishi, Hara, Asano, & Tomita, 2003; Sufian et al., 2006). Peptides and amino acids would have an effect on body fat loss, insulin secretion and glycaemia reduction, but further research is needed in order to unravel these mechanisms. Further information regarding the peripheral regulation of food intake can be found in the following references (Caron et al., 2017a; Santos-Hernández, Miralles, Amigo, & Recio, 2018), including how protein digestion products act as signaling molecules in enteroendocrine cells.

Regarding the digestion process and the enzymes involved in carbohydrate metabolism, the first approach to preventing an increase in glucose blood level is to avoid the degradation of polysaccharides into glucose. Therefore, the inhibition of digestive hydrolases (amylases, glucosidases) would avoid complex polysaccharides from becoming hydrolyzed, and thus absorbed in the bloodstream. Amylases inhibition can be exerted in the saliva and in the gastrointestinal tract, lowering the blood glucose level

(Yan et al., 2019). For its part, glucosidase inhibition would essentially preclude the uptake of glucose into the blood circulation, effectively decreasing postprandial hyperglycemia (Ibrahim, Bester, Neitz, & Gaspar, 2017; Konrad et al., 2014; Patil et al., 2015). Delayed carbohydrate absorption is considered an adequate contributing factor in stimulating GLP-1 secretion, which would ultimately lead to the incretin effect.

Among the α-glucosidase and α-amylase main inhibitors, we find acarbone, miglitol and voglibose. However, numerous side-effects, such as gastrointestinal disturbances, stomach pain and flatulence, have been described for these drugs, and consequently this have limited their use as inhibitors (Patil et al., 2015). The obtaining of inhibitors for these enzymes with no side effects is consequently an interesting research topic.

On the other hand, if the body suffers from insulin resistance, considering that DPP-IV acts by degrading incretins (Kshirsagar, Aggarwal, Harle, & Deshpande, 2011), one of the oral antidiabetic drugs used today is the group of DPP-IV enzyme inhibitors called gliptins (Marya, Khan, Nabavi, & Habtemariam, 2018). The discovery that the enzyme DPP-IV inactivates more than 95% of GLP-1 has put it in the spotlight as a type 2 diabetes mellitus (T2DM) management therapy (Thoma et al., 2003). When DPP-IV is inhibited, the inhibitory action it has on incretins is suppressed, and the half-life of these incretins is increased. Protein intake can also elevate plasmatic GLP-1 levels (Hutchison et al., 2015). This causes insulin secretion to be stimulated, in addition to inhibiting glucagon release (Lammi et al., 2018), and the blood glucose level is adequately regulated. The first gliptin approved by the Food and Drug Administration (FDA) was sitagliptin, in 2006, and since then, more synthetic DPP-IV inhibitors have been approved, in spite of the adverse effects they may have. Among these, we can find nasopharyngitis, nausea, hypersensitivity, headache, skin irritations and the risk of acute pancreatitis (Juillerat-Jeanneret, 2014; R. Liu, Cheng, & Wu, 2019). Furthermore, their long-term safety remains unclear.

Although the previous strategies described are the most important in terms of health and the research related to bioactive peptides, some other ways to prevent diabetes have been described. There are different kinds of molecules acting in different organs, which also have antidiabetic effects via different mechanisms, such as insulin sensitizers, insulin secretagogues, GLP-1 mimetics or glizofins (Hsia, Grove, & Cefalu, 2017; Tahrani et al., 2011; Yan et al., 2019). There is a need for further research since some peptides are able to stimulate incretin secretion, this effect being related (or not) to the DPP-IV inhibition.

Peptides are able to interact at many physiological levels in the human body (Caron et al., 2016).

Recent studies show the importance of diabetes pretreatment in minimizing the economic impact of the disease treatment (Hewage, Wu, Neelakantan, & Yoong, 2020; Mata-Cases et al., 2020), beyond the health consequences it has on the patient. Bioactive peptides appear to be a good alternative for employment in functional foods as health-promoting ingredients. In the literature, the discovery of peptides coming from food proteins able to inhibit amylases, glucosidases and DPP-IV has been reported. These kinds of enzyme-inhibitory peptides are still in the basic research stage, and none have been approved by the FDA (Yan et al., 2019). Bioactive peptides can also regulate glucose homeostasis due to their ability to regulate gut hormones (Caron et al., 2017a). As such, bioactive peptides for preventing the development of diseases are an important field of research, the interest in which is increasing, and which could have positive effects on the human health and economic levels.

# 2. PRODUCTION OF GLYCEMIC INDEX-REGULATING PROTEIN HYDROLYSATES

## 2.1 Enzymatic Hydrolysis Reaction

Obtaining bioactive peptides from food proteins is preferably carried out by enzymatic hydrolysis rather than chemically, or via microbial fermentation. Chemical hydrolysis requires high temperatures and an extremely acidic or basic environment in order to destabilize the bond, and consequently, some amino acids are modified or even destroyed, meaning a loss in the nutritional value of the peptides. Microbial fermentation, to produce peptides, is not a reproducible technique, since there are some uncontrollable factors (i.e., enzyme levels, metabolism of microbes, etc.). However, genetic recombinant strains could help palliate these limitations (Daliri, Oh, & Lee, 2017). In this review, we will focus on obtaining bioactive peptides via enzymatic hydrolysis.

Enzymatic hydrolysis requires mild reaction conditions, and is specific and controllable. The reaction itself is simple, needing the substrate (protein) and the enzyme(s) (protease(s)). The reaction conditions (pH and temperature) are determined by the protease, and many factors, such as enzyme/substrate ratio or substrate concentration, must be taken into consideration too. The optimal conditions for obtaining highly

bioactive hydrolysates are usually achieved via different kinds of experimental designs (Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2018; Nongonierma et al., 2019; Pérez-Gálvez, Morales-Medina, Espejo-Carpio, Guadix, & Guadix, 2016). The enzymatic hydrolysis is generally carried out in a jacketed reactor, under stirring, in order to ensure the homogeneity and constant temperature of the reaction.

In terms of large-scale production, some authors have produced protein hydrolysates at a pilot or semi-pilot plant scale. In this context, different hydrolysates have been produced from such sources as fish discards (Vázquez et al., 2020) for their valorization, and trials have confirmed the results of production obtained at lab scale, or from boarfish (Harnedy-Rothwell et al., 2020), which show strong DPP-IV inhibitory activities.

Lately, scientists have been testing enzymatic hydrolysis carried out after or during the application of non-thermal techniques, such as high pressure, ultrasound or microwave. The global conclusions are that high hydrostatic pressure and ultrasound pre-treatment improve the efficacy of enzymatic hydrolysis and the consequent release of bioactive peptides (Knezevic-Jugovic et al., 2012; Perreault, Hénaux, Bazinet, & Doyen, 2017; Wali et al., 2017; H.-C. Yu & Tan, 2017). Regarding the protein structure, the tertiary and quaternary structures are generally affected by high-pressure treatment, while the secondary structures tend to be maintained. Nonetheless, we must consider the possibility that pressure treatment may lead to the denaturation of proteins, but also to aggregation or precipitation (Galazka, Dickinson, & Ledward, 2000). With respect to the primary structure, the application of pressure does not affect the covalent bonds, and so the sequences of amino acids are not lost (Marciniak, Suwal, Naderi, Pouliot, & Doyen, 2018; Muntean et al., 2016).

The main parameter for characterizing protein hydrolysates is the degree of hydrolysis. This is defined as the proportion of cleaved peptide bonds compared to the original protein. The higher the degree of hydrolysis, the smaller the peptides size would be in the product obtained after the hydrolysis reaction. It is generally reported that bioactive peptides have a length of 2 to 20 amino acids.

## 2.2 Proteases

The enzymes responsible for protein hydrolysis are called proteases (EC 3.4.X.X), and they can be classified via where they catalyze the hydrolysis of bonds. They can be considered (a) endopeptidases, if the cleavage site is inside the protein, or (b)

exopeptidases, if the cleavage sites are located at or near the ends of chains. The active site of the protease determines its substrate specificity, that is, the position where the hydrolysis will take place (Turk, 2006). Then, the choice of the protease employed is essential, since it will define the degree of hydrolysis and the profile of released peptides (Tavano, 2013).

Endopeptidases can be classified depending on their catalytic mechanism and their tertiary structure, considering the amino acid or metal present in the active site, such as aspartate, cysteine, metallo or serine-proteases. Exopeptidases can be classified as aminopeptidases, carboxypeptidases or dipeptidases (McDonald, 1985; Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018). Some examples of proteases widely employed in the industry are Subtilisin, a non-specific endo-peptidase, exerting its proteolytic activity over hydrophobic amino acids (Adamson & Reynolds, 1996), Trypsin, a specific endo-peptidase, exerting its proteolytic activity over arginine and lysine residues (Olsen, Ong, & Mann, 2004), and Flavourzyme, a complex mixture of endo- and exo-peptidases, exerting its proteolytic activity over lineal chains, releasing small peptides and free amino acids (Segura Campos, Chel Guerrero, & Betancur Ancona, 2010). Depending on the type of bioactive peptide desired, certain enzymes have been tested and considered as adequate proteases for obtaining these molecules. For example, a combination of Alcalase and Flavourzyme has been reported as a good enzymatic treatment for obtaining DPP-IV inhibitory peptides (Harnedy et al., 2018a; Rivero-Pino, Pérez Gálvez, Espejo-Carpio, & Guadix, 2020). For α-glucosidase inhibitory peptides, trypsin has been reported as an adequate protease (Connolly, Piggott, & FitzGerald, 2014), and so has Alcalase (Arise et al., 2019). Further research should be carried out, since different proteins might lead to different bioactive protein hydrolysates.

## 2.3 Protein Source

The substrates usually employed for protein hydrolysis are of natural origin, usually with a high protein percentage. The most-studied protein substrates for obtaining antidiabetic peptides to date are milk (Horner, Drummond, & Brennan, 2016; Konrad et al., 2014; Miralles, Hernández-Ledesma, Fernández-Tomé, Amigo, & Recio, 2017) and soy proteins (Lammi et al., 2018; Montesano, Gallo, Blasi, & Cossignani, 2020), due to their high biological value compared to other proteins. One such example is seen in Lacroix

and Li-Chan (2012a), who described the formation of DPP-IV inhibitors from dairy proteins, using 11 enzymes and different substrates.

Another valuable protein source is marine species. The literature reporting fish peptides with antidiabetic activity has been recently stated (Harnedy-Rothwell et al., 2020; Harnedy et al., 2018b; Huang, Jao, Ho, & Hsu, 2012; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). In this context, the use of fishing discards as protein sources for value-added products is important (Harnedy-Rothwell et al., 2020; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017; Pérez-Gálvez et al., 2016; Rivero-Pino, Espejo-Carpio, & Guadix, 2020b). The scientific community considers enzymatic hydrolysis as a helpful option for revaluing these low-quality products and increasing their potential, as they have no side effects on the patient's health. In addition, by-products of the food industry, such as whey or gluten, with adequate protein content are also possible options for generating value-added products. Recently, vegetable protein sources such as peas or lupine have also been used, given the lower ecological impact they have (Tilman & Clark, 2014). Similarly, insect or algae proteins are being used today in the food industry for the production of food products (Admassu, Gasmalla, Yang, & Zhao, 2018a; Nongonierma & FitzGerald, 2017b). These sources of proteins are reported to be sustainable sources with great potential for use in the food industry. There is already literature concerning the production of DPP-IV inhibitory peptides from these kinds of sources, such as Palmaria Palmata and brewers' spent grain (Connolly et al., 2014; Harnedy & FitzGerald, 2013). The main differences among substrates are their protein structure complexities and their amino acid sequences. Table 1 shows the amino acid profiles of different substrates considered as novel protein sources, such as insects. It can be observed that in some cases, the difference in the amount of a certain amino acid (g/100 g of substrate) is remarkable, and consequently the bioactivity related to the peptides released after hydrolysis is expected to be considerable. This statement is based on the fact that the bioactivity of peptides is mainly related to their amino acid sequence characteristics, that is, their hydrophobicity and/or length. The enormous diversity of substrates that are currently being used to obtain peptides with antidiabetic capacity is summarized in Table 2. It can be seen that marine, vegetable, insect or dairy sources are used, among others. The choice of the protein source used for the production of peptides must consider, in addition to the resulting bioactivity of the peptides, the environmental and economic factors during its production, via a life cycle assessment.

**Table 1.** Amino acid content of some vegetable, insect and fish proteins (g/100 g of substrate).

| Amino Acid | **Quinoa** | **Lentil Protein Isolate** | **Brewer Spent Grain** | **Mealworm Larvae Meal** | **Silkworm Pupae Meal** | **Mussel Meal** | **Herring** |
|---|---|---|---|---|---|---|---|
| **Essential** | | | | | | | |
| H | 2.2 | 2.0 | 3.6 | 2.9 | 2.6 | 1.9 | 2.1 |
| I | 0.8 | 3.1 | 4.2 | 4.7 | 5.1 | 4.5 | 3.3 |
| L | 2.5 | 6.9 | 7.2 | 8.0 | 7.5 | 7.2 | 7.9 |
| K | 2.3 | 5.6 | 3.1 | 6.3 | 7 | 8.3 | 10.1 |
| M | 0.3 | 0.6 | 1.4 | 1.4 | 3.5 | 2.6 | 3.3 |
| F + Y | 2.8 | 7.1 | 9.7 | 9.5 | 11.1 | 8.7 | 5.9 |
| T | 5.7 | 3.0 | 3.2 | 4.3 | 5.1 | 5.3 | 4.0 |
| W | 1.0 | | - | 1.2 | 0.9 | 1.0 | - |
| V | 1.0 | 3.5 | 6.0 | 8.5 | 5.5 | 4.6 | 4.6 |
| C | 0.1 | 0.5 | 1.4 | 0.8 | 1 | 1.0 | 1.1 |
| **Non-essential** | | | | | | | |
| R | 3.0 | 7.4 | 5.9 | 5.4 | 5.6 | 7.6 | 7.5 |
| G | 3.0 | 3.1 | 3.8 | 5.5 | 4.8 | 6.6 | 7.6 |
| E | 8.7 | 15.5 | 24.8 | 10.6 | 13.9 | 14.0 | 17.1 |
| D | 3.7 | 10.5 | 6.6 | 7.8 | 10.4 | 11.3 | 9.3 |
| P | 1.8 | 2.9 | 9.7 | 6.0 | 5.2 | 4.2 | 4.7 |
| S | 1.7 | 5.2 | 4.1 | 4.6 | 5.0 | 5.4 | 4.3 |
| A | 2.2 | 3.4 | 4.3 | 8.4 | 5.8 | 5.1 | 7.1 |
| Ref. | (Escuredo, González Martín, Wells Moncada, Fischer, & Hernández Hierro, 2014) | (Alberta N.A. Aryee & Boye, 2017) | (Connolly, Piggott, & FitzGerald, 2013) | (Azagoh et al., 2016) | (Makkar, Tran, Heuzé, & Ankers, 2014) | (Breternitz, Bolini, & Hubinger, 2017) | (Sathivel et al., 2003) |

A = alanine, R = arginine, D = aspartic acid, C = cysteine, E = glutamic acid, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine, V = valine.

**Table 2.** Summary of recent publications concerning antidiabetic bioactive peptides (from Scopus, 201

| | | | | In Vitro | | | | Cellular Assay |
|---|---|---|---|---|---|---|---|---|
| **Substrate** | **Enzymatic Treatment** | **ID** | **Verification** | **B-A** | **DPP-IV** | **GIA** | **AMY** | **Cell line** |
| Rainbow trout (*Oncorhynchus mykiss*) | Alcalase | No | No | No | Yes | No | No | No |
| Camel whey protein | PTN 6.0S | Yes | Yes | Yes | Yes | No | No | No |
| Boarfish *(Capros aper)* | Alcalase 2.4 L, Flavourzyme 500 L; Simulated digestion | Yes | Yes | No | Yes | No | No | Caco-2; BRIN-BD11 |
| Blue whiting (*Micromesistius poutassou*) | Alcalase 2.4 L and Flavourzyme 500 L Simulated digestion | Yes | No | No | Yes | No | No | BRIN-BD11, GLUTa 3T3-L1 |
| Cricket (*G. sigillatus*) | Alcalase | No | No | No | Yes | No | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Simulated digestion | | | | | | | |
| Soybean (*Glycine max*) | Simulated digestion | Yes | No | No | Yes | Yes | Yes | No |
| *Luffa cylindrical* seed | Alcalase, trypsin | No | No | No | No | Yes | Yes | No |
| Salmon *(Salmo salar)* | Alcalase 2.4 L, Alcalase 2.4 L and Flavourzyme 500 L, and Promod 144 MG | Yes | No | No | Yes | No | No | BRIN-BD11; GLUTag |
| Boarfish *(Capros aper)* | Alcalase 2.4 L, Flavourzyme 500 L | No | No | No | Yes | No | No | Caco-2; BRIN-BD11; |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Simulated digestion | | | | | | | GLUTag 3T3-L1 |
| Mealworm *(Tenebrio molitor)* | Alcalase, trypsin, ficin, flavourzyme | No | No | No | Yes | No | No | No |
| Tropical banded crickets *(Gryllodes sigillatus)* | Protamex Simulated digestion | No | No | No | Yes | No | No | No |
| Hempseed (*Cannabis sativa*) | Pepsin, trypsin | No | No | No | Yes | No | No | Yes |
| Bovine whey | Trypsin | Yes | Yes | No | Yes | No | No | No |
| Sea cucumber (*Stichopus japonicus*) | Simulated gastrointestinal digestion | Yes | No | Yes | Yes | No | No | 3T3-L1, HepG2 |
| Casein | PROTIN SD-NY10 | No | No | No | No | Yes | Yes | No |
| Walnut (*Juglans mandshurica)* | Alcalase 2.4 L | Yes | Yes | Yes | No | Yes | Yes | HepG2 cells |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mealworm (*T. molitor*), locust (*Schistocerca gregaria*), cricket (*G. sigilatus*) | Simulated digestion | Yes | Yes | No | No | Yes | No | No |
| Corn germ | Alcalase, flavourzyme, trypsin | No | No | No | Yes | Yes | Yes | No |
| Millet grains (*Panicum miliaceum*) | Simulated digestion | Yes | No | No | No | Yes | Yes | No |
| Cowpea (*Vigna unguiculata*) | Alcalase + Flavourzyme | No | No | No | Yes | Yes | Yes | No |
| Beans (*Phaseolus vulgaris*) | Simulated digestion | No | No | No | No | Yes | Yes | No |
| Basil seeds (*Ocimum tenuriflorum*) | Pepsin | No | No | No | No | No | Yes | No |
| Brewers' spent grain | Alcalase + Flavourzme | Yes | Yes | No | Yes | No | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Simulated digestion | | | | | | | |
| *Porphyra dioica* extracted protein | Alcalase + Flavourzyme | Yes | Yes | No | Yes | No | No | No |
| Red Seaweed (*Porphyra spp*) | Alcalase, neutrase, pepsin, and trypsin | Yes | Yes | No | No | No | Yes | No |
| Soybean (*Glycine max*) | Trypsin | Yes | Yes | Yes | No | Yes | No | No |
| Rice albumin *(Oryza sativa japonica*) | Trypsin | No | No | No | No | No | No | STC-1 |
| Tuber storage proteins | Simulated digestion (*In silico*) | Yes | No | Yes | No | No | No | No |
| Rambutan (*Nephelium lappaceum*), pulasan (*N. mutabile*) | Simulated digestion | Yes | No | Yes | No | No | Yes | No |
| Pinto beans *(P. vulgaris*) | Protamex | Yes | Yes | Yes | No | No | Yes | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Egg white ovoalbumin | Simulated digestion (In silico) | Yes | Yes | Yes | Yes | No | No | No |
| Salmon skin collagen (*Salmo salar)* | Pepsin, trypsin, papain,Alcalase 2.4 L | Yes | Yes | Yes | Yes | No | No | No |
| Quinoa *(Chenopodium quinoa*) | Papain, ficin, bromelain (In silico*)* | Yes | Yes | Yes | Yes | No | No | No |
| Spirulina (*Arthrospira platensis*) | Tryspin | Yes | No | No | Yes | No | No | Caco-2 |
| Tomato seed proteins (*Solanum lycopersicum*) | 15 enzymes (In silico) | Yes | No | No | No | No | No | No |
| Egg | Pepsin, trypsin (in silico) | Yes | Yes | Yes | Yes | No | No | No |
| Common carp (*Cyprinus carpio*) | Papain, neutrase, trypsin, pepsin; Simulated digestion | Yes | Yes | Yes | Yes | No | No | Caco-2 HepG2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Spirulina (*Spirulina platensis)* | Trypsin, pepsin | Yes | No | No | Yes | No | No | Caco-2 |
| Pea (*Pisum sativum)* | Alcalase, neutrase | Yes | No | No | No | No | No | No |
| Buffalo colostrum (*Bubalus bubalis*) | Simulated digestion | Yes | Yes | Yes | Yes | No | No | No |
| Chicken feet (*Gallus gallus domesticus*) | Neutrase, Protamex | No | No | No | Yes | No | No | STC-1 |
| Portuguese Oyster (*Crassostrea angulata*) | Pepsin, bromelain, papain | Yes | No | Yes | Yes | No | No | No |
| Casein | Alcalase, protamex, neutrase, bromelain, and papain | Yes | Yes | Yes | Yes | No | No | No |
| Whey | Corolase 2TS, Protamex | No | No | No | Yes | No | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Soy (*Glycine max)* | Alkaline proteinase, papain, trypsin; Simulated digestion | Yes | Yes | No | Yes | Yes | No | No |
| Egg | Simulated digestion | Yes | Yes | Yes | Yes | No | No | Caco-2 |
| Rapeseed (*Brassica napus*) | Alcalase, trypsin pepsin, flavourzyme, papain | Yes | Yes | Yes | Yes | No | No | No |
| Lesser mealworm (*A. diaperinus*) | Simulated digestion; alcalase, Flavourzyme, papain, and thermolysin | Yes | No | No | Yes | No | No | No |
| Camel skin gelatin (*Camelius dromedarius*) | Alcalase, protease from S. *Griseus* | No | No | No | Yes | No | Yes | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Chicken (*Gallus gallus)* | Corolase, Flavourzyme | Yes | No | No | Yes | No | No | Skeletal muscle |
| Kiwicha (*Amaranthus caudatus*) | Simulated digestion | Yes | No | No | Yes | No | Yes | Caco-2 |
| Silver carp (*Hypophthalmichthys molitrix*) | Alcalase 2.4 L, neutrase, pepsin, trypsin, Flavourzyme | Yes | Yes | Yes | Yes | No | No | No |
| Flaxseed (*Linum usitatissimum*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), sesame (*Sesamum indicum*), soybean (*Glycine max*) | Subtilisin, pepsin, pepsin (In silico) | Yes | No | Yes | No | No | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Bambara bean (*Vigna subterranean*) | Alcalase, thermolysin, trypsin Simulated digestion | Yes | No | Yes | Yes | No | No | No |
| Mealworm (*T. molitor*) | Pepsin, papain (In silico and experimental) | Yes | No | No | Yes | No | No | No |
| Yellow field pea (*Pisum sativum*) | Alcalase, chymotrypsin, pepsin, trypsin | Yes | No | No | No | Yes | Yes | No |
| Sardine (*Sardine pilchardus*) | Alcalase, Trypsin, Flavourzyme | Yes | No | Yes | Yes | No | No | No |
| Sodium caseinate | Simulated digestion | Yes | Yes | No | No | No | No | BRIN-BD11, 3T3-L1 |

ID: The reference includes the identification of bioactive peptides. Verification: The identified peptides' bioactivities were c
A: Any kind of bioinformatic analysis was carried out after identification of peptides. In vitro columns refer to inhibition ass
IV: Dipeptidil-peptidase IV; GIA: glucosidase; AMY: amylase. Cellular assay: Cell-based analyses were carried out, referr
refers to animal models studies. Note: Numerous references cited contain more analysis; only the antidiabetic properties anal

# 3. IDENTIFICATION OF BIOACTIVE PEPTIDES

## 3.1 Fractionation

The concentration and purification of bioactive fractions from hydrolysates is an important step in industrial implementation. Because protein hydrolysates are composed of a mixture of different peptides, some of them bioactive, some of them not, their profitable concentration by different technologies is crucial. Moreover, identification of the actual bioactive sequences in these fractions would enable us to verify their actual bioactivity, and their bioavailability, stability and functionality in the context of nutrition. Table 2 shows a summary of the recent antidiabetic bioactive peptide publications (from journals annexed to Scopus). This table includes which analyses were carried out (in vitro inhibition assays, identification of peptides, verification of bioactivity with synthetic peptides, bioinformatic analysis, cell-based assays and in vivo assays with animal models). These protein hydrolysates contain a pool of peptides obtained by the cleavage of different enzymes, and their respective abilities to inhibit DPP-IV or digestive enzymes will determine how bioactive they are. There are numerous studies reporting the production of DPP-IV inhibitory peptides (F. Hall et al., 2018; Lacroix & Li-Chan, 2016; Lammi et al., 2018; Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2018; Assaad Sila et al., 2016). Numerous authors have also identified α-glucosidase inhibitory peptides (Connolly et al., 2014; Lacroix & Li-Chan, 2013; Matsui, Oki, & Osajima, 1999; Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015; Ren et al., 2016) and α-amylase inhibitory peptides (Akkarachiyasit, Yibchok-Anun, Wacharasindhu, & Adisakwattana, 2011; Gonzalez-Montoya et al., 2018; Z. Yu, Yin, Zhao, Liu, & Chen, 2012).

Having obtained a bioactive protein hydrolysate, different technologies allow the separation of peptides based on different physicochemical properties (molecular weight, polarity or charge). The main technologies employed for fractionation are chromatography or membranes. The next step would therefore be identification by mass spectrometry, bioinformatics analysis to verify the functionality of the peptides, and ultimately assays with the chemically synthesized peptide, to establish the actual bioactivity of the peptides identified. The workflow is described below.

Chromatography is a laboratory technique for separating compounds. There are numerous types of chromatography, distinguished by their characteristics. In terms of peptide

purification, size exclusion chromatography (SEC) and reverse-phase chromatography (RPC) are the most widely used. These two separate peptides depending on their size and their hydrophobic characteristics, respectively. Usually, the combination of both techniques is adequate to obtain fractions that can be injected into a mass spectrometer (MS) so as to identify the peptides contained therein. For example, Rivero-Pino et al. (Rivero-Pino, Espejo-Carpio, et al., 2020b) reported a higher DPP-IV inhibition for sardine peptides ranging from 400 to 1400 Da after fractionation with a Superdex Peptide 10/300 GL column, using a fast protein liquid chromatography system.

Membrane technology allows the separation of a sample into retentate and permeate. In this case, the pore size of the membrane would make the peptides separate into different fractions, depending on their molecular weight. Different molecular weight cut-off (MWCO) membranes would separate the peptides depending on their size, enabling one to identify the most bioactive fractions (Espejo-Carpio, Pérez-Gálvez, Del Carmen Almécija, Guadix, & Guadix, 2014), which are usually the smallest ones, and to discard larger peptides, which are generally non-bioactive. Lacroix and Li-Chan (2012a) ultrafiltered dairy protein hydrolysates using an Ultracel Amicon ultrafiltration unit model 8400, with membrane MWCOs of 10 kDa, 3 kDa and 1 kDa, and reported a higher DPP-IV inhibitory activity for < 3 kDa fractions. For α-amylase inhibitory peptides, Ngoh et al. (2016) fractionated a Pinto bean hydrolysate using centrifugal ultrafiltration filters with MWCOs of 100, 50, 30, 10 and 3 kDa, reporting a higher bioactivity in the < 3 kDa fraction.

Considering a large-scale production of hydrolysates, purification by membranes would be an adequate means of obtaining different-sized fractions.

## 3.2 Peptide Sequence Identification

The identification of peptides is generally carried out by mass spectrometry (MS) analysis (R. Liu et al., 2015) of the most bioactive fractions after chromatographic purification or membrane separation. MS is an analytical technique that measures masses of atoms and molecules after their conversion to charged ions, with or without fragmentation, by an ionization process. This process allows one to identify unknown compounds, and to elucidate their structure and chemical properties. Characterization is done by their mass to charge ratios (m/z) and relative abundances (Perutka & Šebela, 2018). In this case, controlled fragmentation allows the determination of amino acid sequences in order to

identify peptides. As stated in Table 2, numerous studies have identified glycemic index-regulating peptides via their ability to inhibit different enzymes.

A protein hydrolysate is a mixture of peptides, some of them bioactive and some others not. The importance of the identification resides in the fact that the full characterization of the peptides involved in regulating the disease enables the manufacturer to claim the health-promoting property of the fortified product.

α-amylase inhibitory peptides are not as broadly studied as the α-glucosidase and DPP-IV inhibitors described. Some authors have suggested that peptides with branched chains (such as Lys, Phe, Tyr and Trp) and cationic residues are preferably bound to α-amylase (Arise et al., 2019; Y.-Y. Ngoh, Tye, & Gan, 2017). Further, Siow and Gan (2016) identified three α-amylase inhibitory peptides, ranging from 17 to 23 amino acids length, that might have low bioavailability due to their large molecular weight. Ngoh and Gan (2016) reported the importance of Gly or Phe at the N-terminal and Phe or Leu at the C-terminal. However, the α-amylase inhibitory peptides' features should be further researched, in order to establish similar statements as those concerning the DPP-IV inhibitory or α-glucosidase inhibitory peptides.

Concerning the α-glucosidase inhibitory peptides, Ibrahim et al. (2017) summarized the structural properties of α-glucosidase inhibitory peptides. What is remarkable is the importance of amino acids containing a hydroxyl or basic side chain at the N-terminal (which could be expected from trypsin hydrolysis), and of proline within the chain and alanine or methionine at the C-terminal. Nonetheless, factors such as the length of the peptide, its hydrophobicity and its isoelectric point are not extremely important. Ser-Thr-Tyr-Val (STYV) has been reported as the most potent glucosidase inhibitory peptide (Ibrahim et al., 2017).

Diverse features have been described for DPP-IV inhibitory bioactive peptides, such as the hydrophobic N-terminal (R. Liu et al., 2019) ideally tryptophan (Nongonierma & Fitzgerald, 2014), and proline or alanine as the penultimate N-terminal residue (Hsieh et al., 2016), or a low molecular mass (Harnedy et al., 2018a; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). Ketnawa et al. (2018) concluded that cationic peptides, obtained by electrodialysis with an ultrafiltration membrane, were the most bioactive fraction of the hydrolysate analyzed. Among the 222 peptides analyzed by Liu et al. (2019), over 88.4% had a molecular weight lower than 1000 Da, and more than half had one lower than 500 Da. Ile-Pro-Ile (IPI) has been reported as the most potent DPP-IV inhibitory peptide ($IC_{50}$ = 5 μM) (Harnedy-Rothwell et al., 2020).

The identification of bioactive peptides is a key point in this field of research. However, there are still limitations to this procedure due to the high number of molecules (free amino acids, small-/medium-size peptides, polypeptides, oligomers, undigested proteins, etc.) contained in a protein hydrolysate. Considering the presence of high molecular weight molecules, it is sometimes hard to identify low molecular weight peptides (<4 amino acids length), which are usually those responsible for the bioactivity (De Cicco et al., 2019). In this regard, bioinformatics analyses play an important role in the identification of bioactive molecules.

## 3.3 Bioinformatics Analysis

Bioinformatics analyses should be taken into consideration given their potential use in identifying, characterizing and producing bioactive peptides (Tu, Cheng, Lu, & Du, 2018). The most remarkable analyses described below are in silico analysis, molecular docking and the Quantitative Structure–Activity Relationship.

The first approach to identifying bioactive peptides is the employing of informatics tools that use knowledge about proteins and proteases. Thus, having the sequences of the protein and knowing the selectivity of the enzyme, one can expect to obtain the resulting peptides after the cleavage (Table 2, rows where the enzymatic treatment column includes the term in silico). This method has advantages concerning its feasibility, but it also has disadvantages regarding the numerous protein structures that a substrate can have, and the fact that, depending on the reaction conditions, the proteases can act one way or another. One application for this analysis would be in identifying in which protein we could expect to obtain a peptide that it is known to have antidiabetic properties. One database largely cited in the literature is BIOPEP (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008), a software that can, for example, detect bioactive peptides in a sequence, or simulate how proteases would act over a protein. There are more bioinformatics tools, such as ExPASy-PeptideCutter or Enzyme Predictor, that are capable of performing virtual hydrolysis, that is, in silico digestion (Tu et al., 2018).

The molecular docking technique predicts the preferred conformation of a molecule, when bound to another in order to form a stable complex. It is usually employed to see how an identified peptide can bind with the enzyme. Different crystal structures of DPP-IV, α-amylase and α-glucosidase can be found in the RCSB Protein Data Bank. It is a good approach to execute a screening of the different compounds, so as to choose the best candidates (R. Liu et al., 2019) and to discover where the peptide would interact with the

enzyme. The software widely employed for molecular docking and virtual screening includes AutoDock Vina (Trott & Olson, 2010) and pepATTRACT (De Vries, Rey, Schindler, Zacharias, & Tuffery, 2017).

Quantitative Structure Activity Relationship (QSAR) is an informatics tool that tries to predict the activity of a molecule based on its molecular features. This is based on the idea that structure and activity are related, and consequently, similar structures may well have similar activities (A.B. Nongonierma & Fitzgerald, 2016). The combination of different bioinformatics techniques is a good initial approach to confirming the bioactivity of identified peptides.

Fitzgerald et al. (Richard J. FitzGerald, Cermeño, Khalesi, Kleekayai, & Amigo-Benavent, 2020) recently published a manuscript on the application of in silico approaches for the generation of milk protein-derived bioactive peptides, including DPP-IV inhibitory peptides. On the same issue, Lacroix and Li-Chan (Lacroix & Li-Chan, 2012b) carried out an evaluation of the potential role of dietary proteins as precursors of DPP-IV inhibitors, via an in silico approach. Further, a structure–activity relationship was developed so as to theoretically predict the potential bioactivity of DPP-IV inhibitory peptides (Kęska & Stadnik, 2020).

Ibrahim et al. (Ibrahim, Bester, Neitz, & Gaspar, 2018) constructed a library of possible α-glucosidase inhibitory peptides based on the structural requirements of these kinds of biopeptides, which were subjected to in silico simulated gastrointestinal digestion and to molecular docking with glucosidase and amylase, in order to choose which peptides would be highly bioactive. Mora et al. (Mora, González-Rogel, Heres, & Toldrá, 2020) employed numerous in silico tools to characterize the bioactivity, resistance to digestion, permeability, allergenicity and toxicity of Iberian dry-cured ham peptides. These recent publications show the importance of in silico analysis in the discovery and/or characterization of bioactive peptides.

# 4. BIOAVAILABILITY *IN VITRO*

One important step in the research into bioactive peptides is to verify that the molecules would exert their activity in the human organism (Karaś, 2019). The biological functionality of a peptide depends, consequently, on its bioavailability. These molecules must be resistant to peptidases present in the gastrointestinal tract, the brush border and the serum. Therefore, they must escape hepatic metabolization, which impedes them from reaching the site of action (Power, Jakeman, & Fitzgerald, 2013). Bioactive peptides

orally ingested are supposed to be exposed to the action of at least 40 different enzymes before reaching systemic circulation (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Depending on where the peptide would act, the bioactivity's resistance to the human metabolism should be maintained until they reach their target.

The first approach to evaluating the efficacy of the protein hydrolysate is to simulate gastrointestinal digestion in vitro, and analyze the remaining bioactivity. The integrity of these molecules can be modified during the digestion process, before they reach their active site. At this level, it is important to establish if, in the case of further hydrolysis, the resulting peptides after digestion are still bioactive. The digestion process of the protein involves the sequential attacking of different proteases, and pH conditions at different levels:

(a) Stomach—acid pH in presence of pepsin, which specifically cleaves aromatic and hydrophobic amino acids;

(b) Intestine—basic pH in the presence of a mixture of enzymes, pancreatin, which shows trypsin, chymotrypsin and elastase activity, that is, the cleavage of arginine and lysine, as well as aromatic and aliphatic amino acids (Hou, Wu, Dai, Wang, & Wu, 2017).

Publications reporting the effects of simulated gastrointestinal digestion can be consulted in Table 2 (rows including simulated digestion in the enzymatic treatment). The effect of simulated digestion on bioactive peptides is different depending on the substrate and the enzymatic treatment employed (Huang et al., 2012; Mune Mune et al., 2018; Nongonierma et al., 2018).

It is stated that > 3-kDa peptides are more likely to be degraded by gastrointestinal proteases than < 3-kDa peptides, but this behavior depends also on the amino acid sequence of the proteins. The terminal residues are an important factor determining their resistance (B. Wang, Xie, & Li, 2019).

After digestion, the peptides obtained would be absorbed in the enterocytes, where brush border peptidases can be found. This process depends mainly on their size and hydrophobicity. Hence, intact peptide absorption can occur via different mechanisms, from the enterocyte into the portal circulation, described below (Power et al., 2013; B. Wang et al., 2019).

- PepT1-mediated transport: Small peptides (di- and tri- peptides) resistant to hydrolysis would enter via peptide transporters located on the basolateral

membrane, regardless of their amino acid sequence (Parker, Mindell, & Newstead, 2014).

- Paracellular route: Water-soluble peptides would pass between cells through tight junctions (no energy needed) (Salamat-Miller & Johnston, 2005).
- Transcytosis: Hydrophobic peptides would require precise energy to diffuse through the brush border membrane of mucosa cells via three different procedures. Large lipophilic peptides would enter into the lymphatic system due to their inability to reach the portal system (Power et al., 2013).

Nonetheless, in vitro simulated gastrointestinal digestion assays do not offer precise results because the physiological conditions are not considered, such as cellular permeability or the intestinal and brush border enzymes. For that reason, research concerning bioavailability usually involves using cell cultures for in situ analysis. The human adenocarcinoma colon cancer cell monolayer (Caco-2) is the most widely accepted in vitro model of intestinal permeability, due to its similarities with intestinal endothelium cells (human intestinal enterocytes) (Caron, Domenger, Dhulster, Ravallec, & Cudennec, 2017b; Sambuy et al., 2005). When cultured under specific conditions, Caco-2 cells form a continuous monolayer with a structural arrangement that serves as a model of both paracellular and transcellular movement. Numerous intestinal enzymes involved in food digestion are expressed on the surface of Caco-2 cells, including DPP-IV (Lammi et al., 2018; Yoshioka, Erickson, Matsumoto, And, & Kim, 1991). Therefore, it is recognized as an adequate model for drug absorption, toxicity testing and oligopeptide transport (Caron et al., 2017b; Power et al., 2013). For instance, Zhang et al. (Chi Zhang, Liu, Chen, & Luo, 2018) reported that a percentage of the DPP-IV inhibitory peptide IADHFL was degraded while passing through a monolayer of Caco-2 cells.

It is considered that di- and tripeptides are able to reach the systemic circulation via the transport means aforementioned, but these inhibitory peptides might exert their physiological effects over DPP-IV in the proximal small intestine, not in the plasma (Horner et al., 2016).

After that, serum peptidases in the human blood could ultimately degrade the peptides before reaching their target organ. Lammi et al. (2018) developed a fast, sensitive and cost-effective ex vivo DPP-IV assay for human serum by collecting venous blood from a healthy female volunteer and analyzing how peptides would inactivate the enzyme, compared to sitagliptin as a positive control. The authors characterized the bioactive

properties of a soybean peptide and a lupine one, overcoming the use of more expensive and less ethical in vivo approaches.

Specific in situ cell-based assays and ex vivo tools narrow the gap between the in vitro assays and the in vivo studies. Beyond all these physiological factors, the food matrix containing bioactive peptides would also have an effect on the bioavailability of molecules, since digestion depends also on the enzyme's susceptibility to hydrolyzed peptides, which depends on the physical availability of it and the possible interactions occurring during digestion. This topic will be discussed in the following section.

The stability of peptides can be improved by different techniques. Gianfranceschi et al. (2018) reviewed the biochemical peculiarities that can enhance the nutraceutical functionality of peptides, that is, their ability to be actually bioactive at their active site. Some techniques for the chemical modification of amino acids would prevent them from being digested by proteases, and consequently, peptide structure would be maintained, and the peptides would be expected to exert their physiological activity. Beyond that, trapping peptides inside different matrices increases their bioavailability too. For example, chitosan is a polymer able to increase the paracellular permeability of peptide drugs across mucosal epithelia (Thanou, Verhoef, & Junginger, 2001). Research on peptide absorption lacks studies regarding the influence of food matrices. It is important to investigate the influence of coexisting food components on the absorption of food-derived peptides. Harnedy-Rothwell et al. (2020) subjected DPP-IV inhibitory peptides to simulated digestion in different matrices (tomato soup and juice), and verified that bioactivity was conserved. Different food matrices influenced protein and peptide digestibility during gastrointestinal digestion and absorption, so this must be considered a major factor in characterizing the bioaccessibility and bioavailability of peptides (Karaś, 2019; Sun, Acquah, Aluko, & Udenigwe, 2020).

Unravelling the mechanisms that explain how nutrients might have physiological effects on the human body would allow to design or optimize the production of molecules with adequate molecular features for enhancing the bioactive properties of the ingredients (Santos-Hernández et al., 2018). In this sense, protein hydrolysates, sometimes poorly characterized, might have different bioactivities with synergistic effects responsible for the antidiabetic effect that they exert on humans.

Related research to be remarked upon includes the observation of a reduction in the gastrointestinal hydrolysis of a peanut protein isolate in the presence of polysaccharides, which is suggested to be due to the non-specific interactions between the polysaccharides

and the peptides (Mouécoucou, Frémont, Sanchez, Villaume, & Méjean, 2004), or the reporting that almond flour inside a chocolate mousse and a sponge cake reduces protein degradation by pepsin (Mandalari et al., 2014). In this regard, the effects of sugar-containing matrices could lead to Maillard product formation, and this would have an effect on the digestibility of proteins, since some amino acids are destroyed (Salazar-Villanea et al., 2018; Seiquer et al., 2006).

# 5. STABILITY AND FUNCTIONALITY IN FOOD MATRICES

The food processing operations currently employed in the industry include thermal treatments (sterilization, pasteurization), non-thermal treatments (high-pressure homogenization or processing, ultrasound), storage (freezing and frozen), drying (dehydration, spray drying, freeze-drying) and separation (membrane processes). Some of these processes may well affect food protein functionality, due to physical and chemical changes. Proteins and peptides are prone to interact between one another, and with other molecules. The processing of food products containing proteins and peptides could, in consequence, reduce, maintain or enhance their bioactivity (Daliri et al., 2017). The amino acid residues would interact with molecules in different ways, also depending on the location of the peptides in the food matrix, ultimately affecting their native and denatured polymeric state (Pathania, Parmar, & Tiwari, 2019; Van Lancker, Adams, & De Kimpe, 2011). It has been reported that high-fiber food matrices are adequate to carry these bioactive peptides, because chemical interactions are not likely to occur. A fiber network would avoid the aforementioned bitter taste of hydrophobic peptides, improving the sensorial acceptability of functional foods, including peptides (Sun et al., 2020; Ten Have, Van Der Pijl, Kies, & Deutz, 2015).

There are not too many studies on how food processing and/or storage modify peptide structure, and consequently their functionality and bioactive properties. Graves et al. (2016) analyzed the bioactivity of a rice bran peptide described as anti-cancer, during its 6-month storage inside an orange juice. Contreras et al. (2011) reported some antihypertensive peptides' resistance to atomization, homogenization and pasteurization, plus their retained bioactivity after incorporation into liquid yoghurt. Similar results concerning antioxidant and antihypertensive peptides' resistance to food processing techniques were reported by Rivero-Pino et al. (2020a).

As aforementioned, some authors have reported that non-thermal treatments, such as ultrasound or high pressures, enhance protein enzymatic hydrolysis. However, there is a lack of information regarding how these processing techniques would affect peptides employed as ingredients in food formulation. These techniques are nowadays seen as less aggressive in terms of nutritional loss of ingredients (X. Li & Farid, 2016), and are potentially employed for commercial sterilization or emulsifying processes.

Non-thermal processing technologies have been described to produce hypoallergenic foods due to structural epitopes changes (Chizoba Ekezie, Cheng, & Sun, 2018), same as enzymatic hydrolysis (Rahaman, Vasiljevic, & Ramchandran, 2016). The food industry can take advantage of this knowledge to fabricate hypoallergenic products without heat treatments. However, food processing may also affect amino acid composition, by forming derivatives such as lysinoalanine, d-amino acids and biogenic amines, which are usually related to undesired physiological consequences in the human body if consumed (Korhonen, Pihlanto-Leppälä, Rantamäki, & Tupasela, 1998). Hydrophobic amino acids tend to be more stable (García-Moreno et al., 2014; Ustunol, 2018).

The consequences of known high-pressure treatments mainly affect the protein structure, leading to denaturation, aggregation or precipitation (Galazka et al., 2000). The effect must be studied for each case, considering the fact that the residue characteristics of peptides would be crucial in determining the result. Ultrasound reduces the size and hydrodynamic volume of the proteins, leading to better physical-chemical and emulsifying properties (A.N.A. Aryee, Agyei, & Udenigwe, 2018). Drying processes improve the stability of products, extending the shelf-life of products by reducing water activity (Ratti, 2001).

Another important example of chemical reaction is the formation of Maillard compounds, products of the non-enzymatic glycosylation of proteins. Sugar is a widely employed ingredient in the food industry due to its sweet flavor. The combination of reduced sugar with proteins or peptides at a high temperature leads to the formation of these compounds (Fu, Zhang, Soladoye, & Aluko, 2019), affecting oxidative stability (Morales & Jiménez-Pérez, 2001) and improving the antihypertensive or antioxidant bioactivities of protein hydrolysates (Abd El-Salam & El-Shibiny, 2018; Hong, Meng, & Lu, 2015; Rivero-Pino, Espejo-Carpio, et al., 2020a; Q. Zhang, Wu, Fan, Li, & Sun, 2018; T. Zhao et al., 2018). Nonetheless, to the best of our knowledge, there is no literature reporting the increased DPP-IV inhibitory activity of Maillard reaction products coming from protein hydrolysates.

There are some techniques to avoid or slow down the effect of the digestion process, and to increase the peptides' stability when introduced into food matrices. The most widely employed technique is encapsulation (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015) with polymers or hydrogels (Lammi et al., 2019).

Many factors are also involved in the potential loss or gain of bioactivity via the modification of peptides' structure, or the aggregation of them. The state of the protein determines its properties (K. Wang, Sun, Pu, & Wei, 2017), but the primary structure is not affected by the denaturation caused by most physical processes, (Rahaman et al., 2016), whereas in a more complex aggregation can occur. It is expected that protein hydrolysates, as a mixture of defined peptides, would not suffer further modifications, since the linear sequences are affected by sequence decomposition processes, such as hydrolysis itself or fermentation. The heterogeneous chemical composition of a food, as well as its molecular structure, is related to different chemical reaction behaviors (Capuano, Oliviero, & van Boekel, 2017) and, in consequence, its functionality.

Once the product containing peptides is formulated and its bioactivity maintained, it should also be ensured that bioactivity is not lost during its life as a commercial product. Chemical reactions might occur during the storage, depending on the formulation of the product and the temperature of it. The Maillard reaction has been described to occur at high temperatures, but long periods might lead to the appearance of Maillard reaction products too. Guyomarc'h et al. (2000) reported the occurrence of the Maillard reaction within refrigerator-stored milk powder at 4 °C, whereas Albalá-Hutado et al. (1999) reported it in liquid infant's milk at room temperature. Recently, Harnedy-Rothwell et al. (Harnedy-Rothwell et al., 2020) fortified different food products (tomato-based soup and juice products) that were subjected to thermal treatments (sterilization and pasteurization) and stored at refrigerated temperature for 30 days. No modification of bioactivity was reported, indicating this treatment's potential use on foods that could contain the bioactive protein hydrolysates.

Furthermore, peptides and proteins might tend to aggregate or precipitate over time, due to some other interactions, such as the van de Waals interaction, hydrogen bonding or a hydrophobic interaction. Hence, when considering the use of a protein hydrolysate as a bioactive ingredient, its stability during the food formulation, and its stability during storage, should be established.

# 6. BIOACTIVITY ANALYSIS

## 6.1 Bioactivity Initial Approaches

Nowadays, considering the novelty of the research subject, literature concerning in vivo analysis with animals and humans is extremely highly needed, but unfortunately, also scarce. Evidently, this research point is the most important, and is the one that offers authentic evidence concerning the implementation of these bioactive peptides as nutraceutical ingredients. The formulation of foods with legal claims to being a glycemic index-regulator due to the presence of these bioactive peptides would be the final step. For this purpose, plenty of evidence and verification in humans is required. The literature currently available on protein hydrolysates and bioactive peptides focusses mainly on in vitro analysis. In this regard, for the antidiabetic analysis, different analyses can be carried out, concerning the different metabolic routes involved in the disease. The most reported bioactive peptides with antidiabetic properties are those with amylases, glucosidases and DPP-IV inhibitory properties.

Concerning cell assays, among the cell lines generally used (Table 2) for the evaluation of the functionality of antidiabetic peptides, we found:

- BRIN-BD11: insulin-secreting cells (pancreatic B cells) in response to glucose, to analyze the effect of the compounds on insulin secretion (McClenaghan et al., 1996);
- GLUTag: enteroendocrine cells that allow the secretion of GLP-1 (intestinal hormone regulated by the DPP-IV enzyme) to be measured using the ELISA technique (Drucker, Jin, Asa, Young, & Brubaker, 1994);
- 3T3-L1: adipocyte cells that allow the measurement of glucose absorption by fluorimetry (Green & Kehinde, 1975);
- STC-1: intestinal secretin tumor cell line that expresses and secretes gut hormones in response to physiological stimuli (McCarthy et al., 2015).

Different studies have employed these cellular lines in exploring DPP-IV inhibitory peptides, as can be observed in Table 2. For example, Harney et al. (Harnedy et al., 2018b) showed that a blue whiting hydrolysate mediated insulin and glucagon-like peptide-1 (GLP-1) release from BRIN-BD11 and GLUTag cells, respectively, and Li et al. (Y. Li et al., 2020) observed the inhibition by a spirulina hydrolysate of the DPP-IV activity expressed by Caco-2 cells.

Nonetheless, in vitro, in situ and ex vivo approaches are not enough for the scientific community to establish claims about the functionality of food peptides.

## 6.2 *In Vivo* Analysis

In vivo analysis should be carried out to verify effectiveness, and to establish the required dose that should be consumed for the protein hydrolysate to effectively exert its biological activity. In these analyses, different markers are evaluated that indicate the physiological influence that these hydrolysates have on the subject (Drummond et al., 2018; Nongonierma & FitzGerald, 2017a). In these investigations, model organisms, such as cell cultures or experimental animals, are used, while clinical studies in humans are less frequent. The results published so far are promising, since they show that, indeed, these protein hydrolysates have beneficial properties for the organism.

### 6.2.1 Invertebrates Models

The use of *Caenorhabdtis elegans* as the model organism (Brenner, 1974) in examining the functionality of bioactive peptides is not extensively reported in literature. Wang et al. (2016) and Zhou et al. (2018) reported a delay in senescence and stress resistance, and lifespan extension, respectively, through the antioxidant activities of bioactive peptides from *Angelica sinensis* protein and mussels (*Mytilus edulis*). Focusing on the antidiabetic activity of peptides, Zhu et al. (2016) proposed an integrated microfluidic device, that resembles the hyperglycemic condition in diabetics, using this nematode as a model, and thereby investigated the responses after exposition to continuously high glucose concentrations in a physiologically relevant manner. These first approaches suggest that this easy-to-work nematode could also be employed (Schlotterer et al., 2009).

Another model organism employed in the research is Drosophila (Brandt & Vilcinskas, 2013). In the same way, scarce information is available in the bioactive peptides field. Chen et al. (2020) reported the up-regulation of antioxidant-related genes, a prolonged lifespan and the reduction of the accumulation of peroxide products when feeding the animal with crimson snapper scale peptides. To the author’s knowledge, no studies have been published concerning the antidiabetic properties of food-derived peptides in Drosophila.

### 6.2.2 Vertebrates Models

The easiest animal models to work with in vivo are rats and mice. In these assays, different biological parameters are measured. In the case of antihypertensive peptides, blood pressure and plasma ACE and renin concentrations are measured (Suárez, Aphalo, Rinaldi, Añón, & Quiroga, 2020). Animal models of type 2 diabetes usually reflect insulin resistance and/or beta cell failure. Furthermore, many of them are obese, reflecting the human condition, wherein obesity is closely linked to type 2 diabetes development (King, 2012). This latter author summarizes numerous examples of these animal models as related to diabetes.

In regard to the literature dealing with this topic, Harnedy et al. (2018b) and Parthsarathy et al. (2018) reported a protein hydrolysate from blue whiting and boarfish with in vitro and in vivo antidiabetic properties, using cell cultures and mice. Similar research was carried out by Jung et al. (2017) with silk fibroin hydrolysate, and by Hsieh et al. (2016) with milk proteins. Mochida et al. (2010) reported that zein-derived peptides induced glycemic regulation via GLP-1 secretion, and DPP-IV inhibition in rats, whereas Ishikawa et al. (Ishikawa et al., 2015) obtained similar results by employing rice-derived peptides. Valencia-Mejía et al. (2019) studied the antihyperglycemic and hypoglycemic activity of naturally occurring peptides and protein hydrolysates from beans in male Wistar rats. D'Souza et al. (2012) introduced an α-amylase inhibitor peptide into Lactococcus lactis, a bacteria usually employed to produce a yogurt, and diabetic mice fed with it showed a reduction in blood glucose levels after 20 days. Along the same line, Wang et al. (2015) improved glycemic control in diabetic rats vis administration of fish skin gelatin hydrolysates, with a DPP-IV inhibitory capacity and a GLP-1 stimulation capacity. Drotningsvik et al. (2016) showed that fish protein hydrolysates could affect different metabolic parameters, such as postprandial glucose regulation and lipid metabolism in obese Zucker rats. Swine have been used as a model due to their similarities to human species. We share cardiovascular anatomies and functions, metabolisms, lipoprotein profiles, tendencies to obesity, etc., making swine adequate for testing the functionality of molecules altering metabolism (Bellinger, Merricks, & Nichols, 2006). The use of swine as an animal model for diabetes is stated (Baek et al., 2019; Ribel et al., 2002). However, studies wherein peptides are included in their diets do not focus on the bioactive effects on these features, but on the palatability or feed efficiency, in order to improve their nutritional status and gut function (Hou et al., 2017).

### 6.2.3 Humans

Finally, the authentic evidence that bioactive peptides are adequate for employment in the food industry as nutraceuticals must overcome the clinical analysis carried out in humans. Peptides have extensive applications in medicine nowadays. The Food and Drug Administration (FDA) has approved more than 60 peptide drugs for marketing, and thousands of preclinical studies are being carried out for numerous peptides (Fosgerau & Hoffmann, 2015). Concerning the regulatory requirements of protein hydrolysates from food proteins, different countries have developed different protocols to approve them as health-promoting ingredients (Chalamaiah, Keskin Ulug, Hong, & Wu, 2019). For instance, at the European level, the European Food Safety Authority approved some angiotensin-converting enzyme inhibitory peptide products as a functional food ingredient, but no glycemic index-regulator peptides have been approved so far.

Concerning the bioactive peptides from food protein hydrolysates, studies are mainly carried out by employing dairy or fish proteins hydrolysates, since these are the most studied ones. Focusing on dairy proteins, the large amount of proline residues in casein makes this protein exceptional for the production of DPP-IV inhibitory peptides (Patil et al., 2015). There are numerous studies reporting the efficacy of casein protein hydrolysates in humans, as a pretreatment for diabetes (Drummond et al., 2018; Geerts et al., 2011; Jonker et al., 2011; Koopman et al., 2009; R. J. F. Manders et al., 2014; R. J. Manders et al., 2006), which involve the observing of different parameters related to an adequate regulation of glucose blood level in type 2 diabetes patients. Recently, Saleh et al. (2018) studied the effect of casein protein hydrolysate (a twice-daily dose of 8.5 g) in patients with gestational diabetes, concluding a moderate reduction of plasma glucose levels, suggesting the potential functionality of protein hydrolysate in the prevention of diabetes. Along the same line, whey (Frid, Nilsson, Holst, & Björck, 2005; Goudarzi & Madadlou, 2013; Petersen et al., 2009; Sartorius et al., 2019) and egg (Plat et al., 2019) protein hydrolysates have been proven to have a positive effect on postprandial blood glucose, both in T2DM subjects and in healthy subjects. Calbet and Holst (2004) reported that milk protein hydrolysates elicited about 50% more gastric secretion than the native protein, plus higher GIP plasma levels during the first 20 min of the gastric emptying process.

On the other hand, fish proteins are also seen as an adequate protein source (C. F. Zhu et al., 2010). Along this line, Hovland et al. (2020) showed the effectiveness of milk and

different fish protein hydrolysates (2.5 g/day of proteins) in affecting glucose regulation and acting as markers of insulin sensitivity in overweight adults, in a randomized, double blind study. Fish species, such as cod (Dale et al., 2018) or boarfish, proteins (Crowe et al., 2018) have also been employed in human studies concerning diabetes prevention.
However, Curran et al. (Curran et al., 2019) showed the need for further precise nutrition analysis, after showing the ability of a casein hydrolysate to improve glycemic function only in some of the individuals analyzed.
All the aforementioned evidence shows that the enzymatic hydrolysis of food proteins is an adequate methodology for obtaining a mixture of peptides that are potentially bioactive. Historically, casein protein is the most widely studied protein, and there are currently food products including it as an ingredient. However, this ingredient in food products is not stated as a bioactive compound, but as a nutritionally improved protein. For example, Arla Foods Ingredients offers a range of whey protein hydrolysates, described as being more quickly absorbed into the blood, and Abbott declare that collagen protein hydrolysate, in their Promod® Liquid Protein, helps to improve pressure ulcer healing (Lee, Posthauer, Dorner, Redovian, & Maloney, 2006). Concerning antidiabetic hydrolysates, NutripeptinTM by Copalis Sea Solutions® is described as a glycemic index-reducing peptide extracted by enzymatic hydrolysis from fresh or fresh-frozen fillets of codfish. Protein hydrolysates' functionalities as ingredients are currently an important topic. The biggest drawback concerning the bioactive properties of protein hydrolysates would be that the in vivo results show differences among individuals. Metabotyping individuals is an important step when considering which subgroups of people could benefit from protein hydrolysates as a functional food (Hillesheim & Brennan, 2020).

# 7. CONCLUSIONS

The available literature on bioactive peptides highlights their relevance to nutrition. The potential of bioactive peptides as antidiabetic agents to be employed in food formulation is a relevant field of research. A protein hydrolysate is a source of peptides capable of modulating different physiological processes. The choice of the protein source is essential, considering not only the bioactivity of peptides but also the environmental, economic and social factors. Then, its potential use as an ingredient must include the evaluation of its stability during storage, and its sensory properties via technical studies. Furthermore, there is a need to validate the antidiabetic properties of food-derived

peptides through well-designed clinical trials with cellular assays, in animals and humans, to ensure their effectiveness and safety. These studies would describe the actual activity of protein hydrolysates with the purpose of being commercially developed in the food industry for functional feeding.

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# II. Production and identification of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from discarded *Sardine pilchardus* protein*

Production of bioactive peptides via enzymatic hydrolysis is a sustainable way to take advantage of proteinaceous by-products from food industry, such as fish discards. *Sardine pilchardus* protein was subjected to different enzymatic treatments using two endopeptidases of different selectivity and one exopeptidase in order to produce hydrolysates with antidiabetic activity. The highest dipeptidyl peptidase IV inhibitory activity was obtained by the combination of three enzymes (subtilisin, trypsin and flavourzyme) employed sequentially. This hydrolysate was subsequently purified by size exclusion chromatography to obtain fractions sorted by size (hydrodynamic volume). Peptides below 1400 Dalton had the highest activity, and these pools were analysed by mass spectrometry in order to identify the peptides responsible for that activity. Numerous peptides with adequate molecular features, it is, owning an alanine (A) as their penultimate N-terminal residue (e.g. NAPNPR, YACSVR) were identified and are proposed to be antidiabetic peptides from *Sardine pilchardus* muscle.

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# 1. INTRODUCTION

Enzymatic protein hydrolysis is an effective, economic and quick technique to obtain peptides with potential bioactivity from food proteins. Among all the bioactivities described in literature, antioxidant, antihypertensive, anticholesterolemic, etc., the antidiabetic activity of peptides by inhibiting metabolic enzymes such as dipeptidyl peptidase IV or digestive hydrolases (Harnedy et al., 2018b; Huang et al., 2012) is an important issue considering the prevalence of this disease. Diabetes mellitus type II (T2D) is a metabolic disorder characterized by high glucose levels in the bloodstream. This is a consequence of insulin resistance that might occur as a result of physical inactivity or obesity. More than 400 million people are nowadays diagnosed with diabetes, and the forecasts suggest that almost 700 million people are likely to suffer diabetes by 2045 ("IDF Diabetes Atlas," 2017).

In regular metabolism, food intake results in the liberation of insulin secretion hormones known as incretins (glucagon-like peptide and glucose-dependent insulinotropic polypeptide, GLP-1 and GIP respectively) that would affect numerous target tissues in the body acting as endocrine signal to the pancreas (MacDonald et al., 2002). Pancreatic β-cells would increase insulin concentration in the bloodstream; suitable insulin secretion is a key-factor to maintain physiological blood glucose level. Furthermore, α-pancreatic cells would reduce glucagon concentration, avoiding glucose production in the liver. Then, blood glucose level is maintained in healthy physiological levels. The enzyme dipeptidyl peptidase IV (DPP-IV) would degrade incretins in order to regulate its concentration (Duez, Cariou, & Staels, 2012).

Nonetheless, T2D patients have insufficient insulin level in the bloodstream, and they end up by developing insulin resistance, leading to an increase in glucose blood level inadequate to the organism (Xia et al., 2017). According to this physiological background, DPP-IV inhibition would allow incretins to keep exercising its insulin secretion effect, and as a result, blood glucose levels will decrease to be adequate. There are already authors that have described beneficial effects of DPP-IV inhibition either *in vitro* and *in vivo* concerning glucose tolerance and insulin secretion (Duez et al., 2012) related to the increase of circulating levels of incretins (Juillerat-Jeanneret, 2014).

Multiple molecular structures have been demonstrated to inhibit DPP-IV activity. It is possible to find anti-diabetic drugs in the market that act by inhibiting DPP-IV enzyme,

known as gliptins, such as sitagliptin or saxagliptin. Nevertheless, these drugs tend to present adverse effects, such as nasopharyngitis, cystitis, and headache (Amori, Lau, & Pittas, 2007; Duez et al., 2012). To overcome these limitations, natural compounds such as biopeptides coming from protein hydrolysis are recently seen as an adequate alternative. These bioactive peptides, beyond being a source of nitrogen and amino acids, exert potential physiological functions within the body (Harnedy & FitzGerald, 2012) and consequently, they could be used in functional food to prevent some diseases, such as diabetes (Lacroix & Li-Chan, 2013).

Bioactive peptides are encrypted in the native protein sequences, and the proteases action would cleave the structure of the proteins, releasing the peptides. The enzymatic treatment employed, considering the specificity of different proteases, is essential to optimize the biological activity of the hydrolysates. Some others factors involved are the amino acid composition and the protein structures, which may well be different and have an impact on the hydrolysis and subsequent products obtained. At this level, fish protein are a promising source for biopeptides since their protein level usually ranges between 10–23% (w/w) (Harnedy & FitzGerald, 2012). Marine fish species have been widely studied as a potential source of antihypertensive and antioxidant peptides (Neves, Harnedy, O'Keeffe, & FitzGerald, 2017; Pérez-Gálvez et al., 2016). Concerning the antidiabetic activity of peptides coming from fish proteins, substrates as salmon, blue whiting or tuna cooking juice (Harnedy et al., 2018b, 2018a; Huang et al., 2012) have been previously employed.

Rather than employing commercial fish species, it is particularly interesting to take advantage of discarded fish as substrate for the production of biopeptides (García-Moreno, Espejo-Carpio, Guadix, & Guadix, 2015; Kristinsson & Rasco, 2000). *S. pilchardus* may be discarded up to a maximum of 5 % of the total annual by-catches of those species by vessels using bottom trawls board (European Commission, 2020). However, since a considerable volume of *S. pilchardus* species without commercial interest due to its small size or the oil content (seasonally variable), is landed due to the landing obligations, these could be revalued by transformation into other compounds of greater interest in the market. In the "General Fisheries Commission for the Mediterranean" area, *S. pilchardus* catches are the second most abundant with 188431 tonnes in 2016 (FAO, 2018).

Sardine protein as source for the production of biopeptides has been extensively studied, mainly regarding their antioxidant and antihypertensive activity (García-Moreno et al., 2015; Morales-Medina et al., 2016). However, to the best of our knowledge no information has been reported about peptides from sardine muscle with DPP-IV inhibitory activity. Considering that some fish species have been used to obtain DPP-IV inhibitory peptides, we hypothesed that sardine protein would be a potent source of bioactive peptides with antidiabetic effect. Hence, the aim of this study was to obtain protein hydrolysates with inhibitory activity from sardine muscle with different enzymes and the purification and identification of the peptide sequences responsible for that capacity.

# 2. MATERIALS AND METHODS

## 2.1 Materials

Raw sardines (*S. pilchardus*) were provided by the fishing harbour of Motril (Spain). Proteases used were: Alcalase 2.4L (subtilisin, EC 3.4.21.62), PTN6.0S (trypsin 3.4.21.4) and Flavourzyme 1000L (3.4.11.1), all obtained from Sigma Aldrich (USA). Human DPP-IV enzyme, Gly-Pro-p-nitroanilide, Diprotin A, pancreatin and chromatography standards were purchased from Sigma-Aldrich (USA). The digestive enzymes employed were pepsin (Merck, Germany) and pancreatin (Sigma-Aldrich, USA).

## 2.2 Enzymatic hydrolysis

Sardine was prepared by removing bones, skin and viscera. Then, sardine muscle was minced and homogenised in a cutter (SK-3 Sammic, Spain). Protein content was analysed by Kjeldahl method. A 40 g/L of protein in distilled water was hydrolysed by subtilisin at pH 8, 50ºC. The reaction was monitored by pH-stat method (Camacho, González-Tello, Páez-Dueñas, Guadix, & Guadix, 2001) using a 902 Titrando (Metrohm AG, Herisau, Switzerland) which keeps the pH constant. The amount of 1M sodium hydroxide added is related to the degree of hydrolysis (DH), as described in equation:

$$DH = \frac{Vb \cdot Nb}{mp \cdot \alpha \cdot htot} \quad (1)$$

Where Vb is the volume (mL) of base consumed, and Nb (eq/L) its normality. α is the average degree of dissociation of the α-NH2 amino groups released during the hydrolysis, which is dependent on the temperature and the pH. mp (g) is the mass of protein in the

substrate and htot (meq/g) is the number of equivalents of peptide bonds per gram of protein. Considering the reaction conditions and substrate employed, 1/α considered was 1.13, while htot was assumed to be 8.6, as reported in literature (Adler-Nissen, 1986).

Subtilisin hydrolysates at degree of hydrolysis of 10, 15 and 20% were produced (S10, S15 and S20). Moreover, sequential hydrolysis after subtilisin treatments were evaluated by adding trypsin (S20T) or flavourzyme (S20F) to the reactor after reaching DH 20% with subtilisin. A last treatment (S20TF) evaluating the effect of the sequential hydrolysis of subtilisin (DH 20%), trypsin (2 hours) and flavourzyme (2 hours) was also carried out. Subtilisin was added at an enzyme–substrate ratio (E/S) of 0.75%, 1.25% and 2.75% for DH 10, 15 and 20 respectively. Trypsin and Flavourzyme were added at an E/S ratio of 2.75%. Considering the optimal working conditions given by the manufacturer, temperature of post-subtilisin treatments was constantly 50ºC whereas pH was maintained at pH 8 for trypsin and was reduced to pH 6 by adding 1M HCl in the case of Flavourzyme. In all cases, enzymes were deactivated by heating at 90ºC for 5 minutes in a heating plate and samples were lyophilised (Labconco Lyph-Lock 6) after being stored at -20ºC for further analysis. The frozen samples were subjected to a vacuum at 5 mmHg and then heated to 15ºC to sublimate the water.

## 2.3 *In vitro* simulated gastrointestinal digestion (SGID)

Simulated gastrointestinal digestion was carried out as described in Garrett, Failla and Sarama (1999) in order to evaluate the effect of digestive enzymes *in vitro*. Reactions in triplicate were carried out in a temperature-controlled shaker (Heidolph, Germany) at 37ºC with 300 rpm shaking. Firstly, the lyophilized samples were diluted in distilled water, at 5% (w/w, in dry weight) and pepsin was added at an E/S ratio of 4% (w/w, on protein basis), previous set pH 2 with 1 M HCl. After 1 h of reaction, pH 5.3 was achieved with a solution of 0.9 M $NaHCO_3$, then, pancreatin was added at an E/S ratio of 4% (w/w, on protein basis) and the pH was set to 7.5 with 1 M NaOH. After two hours of digestion, the enzymes were thermally deactivated (90 °C for 5 min). The samples were freeze-dried and stored until analysis.

## 2.4 Characterization

Protein content of powdered samples was analysed by Kjeldahl method, taking a nitrogen-to-protein factor of 5.58 (Mariotti, Tomé, & Mirand, 2008). Molecular weight

distribution was analysed by size exclusion chromatography (SEC) using a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) employing a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden). Aliquots of 500 µL (10 mg of protein/mL) were eluted at 0.5 mL/min with MiliQ water as mobile phase. The absorbance was measured at 280 nm. The column was calibrated with the following standards: L-tyrosine (217,7 Da), vitamin B12 (1355 Da), and ribonuclease (13700 Da).

## 2.5 *In vitro* DPP-IV inhibition assay

The DPP-IV inhibition assay was performed as previously described with slight modification (Lacroix & Li-Chan, 2012a). Briefly, 25 µL of enzyme (0,02 U/ml) were mixed with 100 µL of sample solution and incubated 10 minutes. After that, reaction was started by adding 50 µL of Gly-Pro-p-nitroanilide at 1 mM and the amount of p-nitroanilide released was monitored by measuring the absorbance at 405 nm during 2 hours, each 2 minutes employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). Each sample was analysed in triplicate. Half maximal inhibitory concentration ($IC_{50}$) value was calculated by plotting the progress of reactions compared to the blank (distilled water). Results are reported in mg of protein/mL. Diprotin A was employed as positive control (maximal inhibition).

## 2.6 Fractionation

The sample with the best DPP-IV inhibition activity was fractionated with the same equipment employed for the molecular weight distribution analysis previously described, coupling the chromatograph to a collector Frac-902. 5 runs for each analysis (protein content, mass spectrometry and DPP-IV inhibitory activity) were carried out, and pooled to obtain enough material of all fractions, and fractions were freeze-dried. DPP-IV inhibition capacity of fractions was analysed as previously described (2.5). Protein content of fractions was analysed by organic elemental analysis (Flash 2000, Thermo Scientific). To this end, lyophilised samples were oxidized with pure oxygen at high temperature (1020ºC), and the combustion products are transported by helium onto a chromatographic column to separate them and finally detected by a thermal conductivity detector (TCD) that provides a signal (mV/s) proportional to the concentration of each of the individual components of the mixture. Sulphanilamide was employed for calibration.

## 2.7 Identification of peptides

The most active fractions collected from SEC were analysed employing an ACQUITY UHPLC system (Waters, Milford, CT, USA) coupled to a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer (Waters). Samples of 10 µL were injected onto an ACQUITY BEH 300 C4 column 1.7 µm (Waters) and components were eluted using a flow rate of 0.3 mL/min of water–formic acid 0.1% (buffer A) and acetonitrile (buffer B) as described by Liu et al. (2015). The MS spectra were acquired under the positive electrospray ionization using a capillary energy of 2.5 kV and sampling cone of 30 V. The analyses were performed using the standard range from 50 to 1900 m/z at the normal scan resolution. PepSeq program from BioLynx software (Micromass UK Ltd., Manchester, United Kingdom) was employed for *de novo* peptide sequencing.

## 2.8 *In silico* prediction for activity of the identified peptides

pepATTRACT (De Vries et al., 2017) was employed to perform molecular docking of peptides identified with the DPP-IV enzyme (PDB: 2AJBA) and obtain an energy score.

## 2.9 Statistical analysis

Significant differences were analysed by means of Statgraphics 5.1 by the multiple comparison test (Least Signifcant Difference) at a p-value $\leq 0.05$. Data is shown as average ± standard deviation.

# 3. RESULTS AND DISCUSSION

## 3.1 Hydrolysates characterization

Alcalase 2.4L main activity is attributed to subtilisin, an endo-peptidase high-spectrum enzyme, this is, non-specific, that binds mainly to hydrophobic amino acids (Adamson & Reynolds, 1996). This protease is widely employed to obtain bioactive peptides, whereby this bioactivity usually related to the hydrophobicity characteristics of the residues (Acquah et al., 2018). Furthermore, the availability of N-terminal sites is increased. The use of trypsin or Flavourzyme as secondary enzymes would change the peptide profile of the hydrolysates. Trypsin is a specific endo-peptidase enzyme that cleaves near arginine and lysine residues (Olsen et al., 2004). Flavourzyme is a complex mixture of endo and

exo-peptidases that is able to release very small peptides from lineal chains (amino-peptidase) and free amino acids (Segura Campos et al., 2010).

**Table 1**: Relative molecular mass distribution (%) of *Sardine pilchardus* hydrolysates

| Fraction | MW (KDa) | S10 | S15 | S20 | S20T | S20F | S20TF |
|---|---|---|---|---|---|---|---|
| A | >10 | 27.79 ± 1.27[a] | 42.66 ± 1.42[b] | 36.24 ± 1.08[c] | 41.9 ± 3.44[b] | 10.54 ± 0.45[d] | 8.13 ± 0.26[d] |
| B | 10-1.4 | 31.75 ± 1.54[a] | 21.69 ± 0.03[b] | 24.32 ± 1.91[c] | 15.21 ± 2.23[d] | 18.08 ± 0.93[e] | 21.01 ± 0.32[b] |
| C | 1.4-0.8 | 33.13 ± 2.41[a] | 30.22 ± 1.41[b] | 31.86 ± 1.17[a,b] | 21.44 ± 0.23[c] | 14.72 ± 0.53[d] | 12.89 ± 0.28[d] |
| D | 0.8-0.4 | 7.33 ± 2.15[a] | 5.43 ± 0.02[a] | 5.40 ± 0.45[a] | 16.87 ± 2.86[b] | 37.48 ± 0.39[c] | 41.84 ± 0.27[d] |
| E | <0.4 | nd | nd | 2.18 ± 0.79[a] | 4.59 ± 1.87[b] | 19.18 ± 0.33[c] | 16.13 ± 0.07[d] |

Values are presented as the mean of three replicates ± standard deviation (p-value ≤ 0.05). Different letters in the same row indicate significant differences among hydrolysates.
nd: no detected
S10, S15 and S20 refers to subtilisin hydrolysates at degree of hydrolysis of 10, 15 and 20% respectively.
S20T, S20F, and S20TF refers to sequential hydrolysis of S20, adding trypsin (T) or flavourzyme (F), or trypsin and then flavourzyme (TF).

All samples contained a high protein content, which ranged between 54.8 and 62.1%. Molecular weight distribution as deduced from SEC chromatograms (Table 1) showed that low molecular weight fractions proportion is higher as degree of hydrolysis increases. S15 showed a larger proportion of fraction A, highly likely due to the hydrolysis of insoluble proteins, whereas S20 had already achieved full hydrolysis of these larger macromolecules. The same behaviour seems to appear with S20T, where it is observed that larger molecules were hydrolysed when the enzyme was added, after the subtilisin treatment. S20F and S20TF samples showed a significant increase of fraction E (<400 Da). This fraction corresponds to small peptides (di- and tri-peptides) and free amino acids. This is due to the exopeptidase activity of flavourzyme.

## 3.2 DPP-IV inhibitory activity from hydrolysates

Hydrolysates proved to be dose-dependent DPP-IV inhibitors, which allow calculating the half maximal inhibitory concentration ($IC_{50}$) value. $IC_{50}$ values of samples are shown in Figure 1. Diprotin A $IC_{50}$ value for the conditions employed in this DPP-IV inhibition assay was estimated as 1.62 ± 0.18 µg/mL, as reported by Lacroix & Li-Chan (2012a).

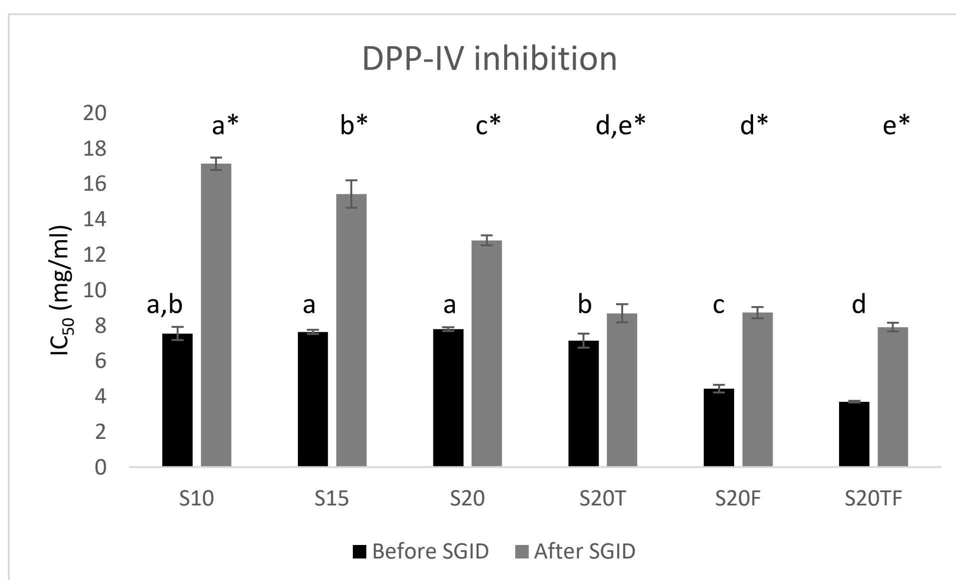


**Figure 1**: $IC_{50}$ (mg of protein/mL) for DPP-IV inhibition of samples before and after simulated gastrointestinal digestion.

Values are presented as the mean of three replicates ± standard deviation. Different letters within the same lot of samples (before and after) denotes significant differences among hydrolysates. The presence of * denotes significant difference of the same sample before and after digestion.

The substrate not subjected to enzymatic treatment did not show DPP-IV inhibitory activity even at concentrations higher than 100 mg/ml. In general terms, this behaviour is reported for bioactive peptides, since it is the releasing of peptides from native protein, the reason why these samples become bioactive. All subtilisin-treated hydrolysates (S10, S15 and S20) showed DPP-IV inhibitory capacity with no significant differences (Figure 1). Similar results were obtained from salmon protein, where hydrolysates obtained with subtilisin at different degrees of hydrolysis showed no significant differences among their DPP-IV inhibitory bioactivity (Neves, Harnedy, O'Keeffe, Alashi, et al., 2017; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). As mentioned before, low molecular weight peptides are able to inhibit this enzyme, and the degree of hydrolysis achieved in all treatments is relatively high. As observed in Table 1, the percentage of fractions C, D and E are similar, except for S20 having a ~2% of <400 Da peptides. The effect of this small amount of peptides on the overall bioactivity of the hydrolysate is negligible. This explains why all hydrolysates, regardless of the degree of hydrolysis achieved, showed a similar bioactivity.

Beyond that, specificity of enzymes is key in the release of bioactive peptides. As shown in figure 1, the $IC_{50}$ value is significantly lower in S20T than in S20. The importance of trypsin resides in its specificity (Olsen et al., 2004) which may release peptides that subtilisin is not able to obtain. Furthermore, flavourzyme treated samples confirmed the

importance of both features – low molecular weight and amino acid composition of sequences – by decreasing significantly the $IC_{50}$ value, and in the case of the most hydrolysed sample, halving the value regarding the subtilisin-treated samples.

The importance of flavourzyme specificity in the release of DPP-IV inhibitory peptides have been already described (Harnedy et al., 2018a) due to its exopeptidase activity, that enables it to release small peptides. An *in silico* analysis carried out in BIOPEP (Minkiewicz et al., 2008) with myosin and β-actin sardine proteins sequences from Uniprot identified a large number of dipeptides and tripeptides with DPP-IV inhibitory activity. These peptides would be very highly likely present in fraction E. These peptides possess structures that avoid the steric hindrance and are able to bind to different sites of the DPP-IV, inhibiting its activity. These dipeptides and tripeptides, coming from the flavourzyme cleavage, can contribute to the overall bioactivity of the hydrolysate. However, the percentage of this fraction (E) is significant lower compared to fraction ranged from 1.4 to 0.4 kDa (fraction C plus D) (Table 1).

Furthermore, Sila et al. (2015) generated protein hydrolysates from fish gelatine, obtaining $IC_{50}$ values ranging from 2.21 to 3.71 mg/ml, in the range obtained in this study for a marine species as well. Some others species, such as Atlantic salmon skin gelatine, have been reported to contain DPP-IV inhibitors peptides (Harnedy et al., 2018a). Beyond that, *in vivo* studies have been carried out showing the relevance of protein hydrolysates in their antidiabetic activity. Harnedy at al. (2018b) produced a protein hydrolysate from blue whiting with *in vitro* and *in vivo* antidiabetic properties employing cell cultures and NIH Swiss mice respectively. Hence, the peptides obtained from sardine in this study may well be adequate to exert antidiabetic activity in functional food. As it was hypothesized, employing sardine discard protein is also an adequate source to obtain DPP-IV inhibitory peptides with bioactivity values comparable to some other fish species.

## 3.3 Resistance to SGID

Digested samples showed a molecular weight distribution similar in all cases, except for S10 and S15 hydrolysates (Supplementary material). It may well be due to the fact that these samples contain larger peptides than the other ones, and even after digestive enzymes attack, they are not extremely degraded. In the case of S20, S20T, S20F and S20TF, the peptides are usually short chains that after pepsin and pancreatin activity, they may release some amino acids from terminals.

DPP-IV inhibitory capacity of digested samples was slightly reduced compared to their respective non-digested, and S20T hydrolysate was the most resistant to SGID (Figure 1). DPP-IV inhibition values are still comparable to others found in literature (Nongonierma et al., 2018). Mune Mune, Minka and Henle (2018) reported a reduced DPP-IV inhibitory activity for *Bambara bean* hydrolysates obtained with subtilisin, and an improvement of bioactivity for the trypsin hydrolysates after SGID. The difference is attributed to further hydrolysis of peptides to less-potent or non-potent fragments after SGID, since the hydrolysates contains different peptides sequences. During SGID, pepsin cleaves specifically aromatic and hydrophobic amino acids. For its part, pancreatin shows trypsin, chymotrypsin and elastase activity, which is, cleavage of arginine, lysine, aromatic and aliphatic amino acids (Hou et al., 2017). This proteolytic activity of both enzymes will eventually lead to further hydrolysis of peptides, where all hydrolysates except S20T are more prone to be affected since this latter was obtained with an intestinal protease.

Further research concerning specific peptides sequences' resistance to digestive proteases should be carried out, since some authors described some biopeptides as resistant to this SGID (Huang et al., 2012). Nonetheless, modifications of terminal sites of peptides (usually 2-20 amino acids length) may well protect it from peptidases by adopting a similar structure than the native protein. Beyond that, the effect of SGID on the bioactivity of peptides depends also if the proteinaceous material is encapsulated, for example, with glucose syrup, increasing its resistance due to the entrapment of the peptides. Another factor influencing the resistance is the food matrix where the peptide is contained, since it may interact with other molecules and their willingness to be cleaved is modified.

## 3.4 DPP-IV inhibitory activity from hydrolysate fractions

Having these key-notes reported in literature, not only the specificity of enzymes employed during hydrolysis is important in the release of biopeptides, but also the fractionation and purification methods applied. In order to purify these mixtures of different-size peptides and non-protein material, next step for better results is, in consequence, fractionation by size exclusion chromatography. The most active fractions would allow us to identify the peptides responsible for the bioactivity.

**Table 2**: Protein concentration and DPP-IV inhibitory activity of the SEC-fractions from S20TF sample.

| Fraction | Protein concentration (mg/ml) | DPP-IV Inhibitory activity ($IC_{50}$ ,mg/ml)* |
|---|---|---|
| **A** | 0.79 | >4 |
| **B** | 0.39 | >4 |
| **C** | 4.69 | 1.83 ± 0.05 |
| **D** | 3.12 | 2.89 ± 0.15 |
| **E** | 0.39 | >4 |

*Values are presented as the mean of three replicates ± standard deviation (mg of protein/mL)

The hydrolysate with the highest DPP-IV inhibitory capacity (S20TF) was subjected to fractionation. According to the fractions obtained (Table 1), five fractions were separated (from A to E) and analysed. The protein concentration and the DPP-IV inhibitory activity of the five fractions are shown in Table 2. As expected, the long duration of the hydrolysis have favoured higher concentration for <1400 Da fractions, and lower concentration for larger peptides. This concentration of shorter peptides is responsible for the increased bioactivity.

An increase in the protein content is observed for the C, D and E fraction, which means a concentration of the product compared to the original sample, that contained ~55% of protein. Concerning the bioactivity of the fractions, an increase was observed for C and D fractions compared to the original hydrolysate. The fraction containing peptides ranging from 1400 to 800 Dalton gave the highest inhibition, with an $IC_{50}$ of 1.83 ± 0.05 mg/ml, and the fraction ranging from 800 to 400 Da showed an $IC_{50}$ of 2.89 ± 0.15 mg/ml. The remaining fractions obtained did not show any bioactivity at the maximum concentration analysed ($IC_{50}$ > 4 mg /mL). In fact, the protein contained in the C and D represented 67% of the total, hence, most of the peptides range from 400 to 1400 Da, as expected for bioactive peptides. This data is important since the concentration of bioactive peptides have been optimised in terms of quantity. These results agrees with published literature, highlighting that the samples with higher <1kDa fraction percentages shows higher bioactivity (Harnedy et al., 2018a; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017).

The results obtained with sardine protein concentrated by chromatography are similar to those reported by Sila et al. (2016) employing a barbel protein hydrolysate. In that case,

after separation by SEC, the most active fractions had an $IC_{50}$ of 1.23 mg/ml and 1.83 mg/ml. The differences with the values obtained in this study can be attributed to the substrate composition and the enzymatic treatment employed. Another purification step widely used is ultrafiltration membrane. In this record, Lacroix & Li-Chan (2012a) reported higher DPP-IV inhibitory activity for <1kDa and 1-3kDa fractions, compared to the >3kDa, for sodium caseinate hydrolysate obtained with bromelain and thermolysin. Concentration with membranes is an adequate means to obtain bioactive peptides.

## 3.5 Identification of peptides with DPP-IV inhibitory activity

The most active DPP-IV inhibitory fractions obtained from size exclusion chromatography (C and D) were analysed by mass spectrometry to identify bioactive peptides (Supplementary material). Among the widely varied fish protein composition, myofibrillar ones are the most abundant. These structural proteins represent >66 wt% of the total and includes myosin – the most abundant - actin, myosin, tropomyosin, among others (Harnedy & FitzGerald, 2012; Vareltzis, 2000). The peptides identified are shown in the Table 3. Two mass spectra are shown in the Figure 2. The mass spectra and the molecular features of the sequences identified suggest that indeed these peptides are contained in the protein hydrolysate.

Some of the peptides found have features that agree with the described literature for DPP-IV inhibitory peptides. It is, owning a proline (P) or an alanine (A) as their penultimate N-terminal residue (Hsieh et al., 2016) and hydrophobic N-terminal residues (R. Liu et al., 2019). For example, for NAALGPR, CAEAGH, NAPNPR and YACSVR possess the alanine amino acid in the sequence. One such already described DPP-IV inhibitory peptide is VLGP, reported previously by Nongonierma & FitzGerald (2013) as a competitive inhibitor derived from β-casein milk proteins. The binding energy of peptides (Table 3) is in the range of results obtained by Hu, Fan, Qi and Zhang (2019), whose peptides were shown to be DPP-IV inhibitory, so it is expected that peptides identified in this research show a similar behaviour. Concerning this value, the most bioactive peptide would be CGGWLF even though molecular features do not correspond with what is expected for these kind of peptides. Verification of its bioactivity by chemical synthesis of the peptide and validation would be necessary.

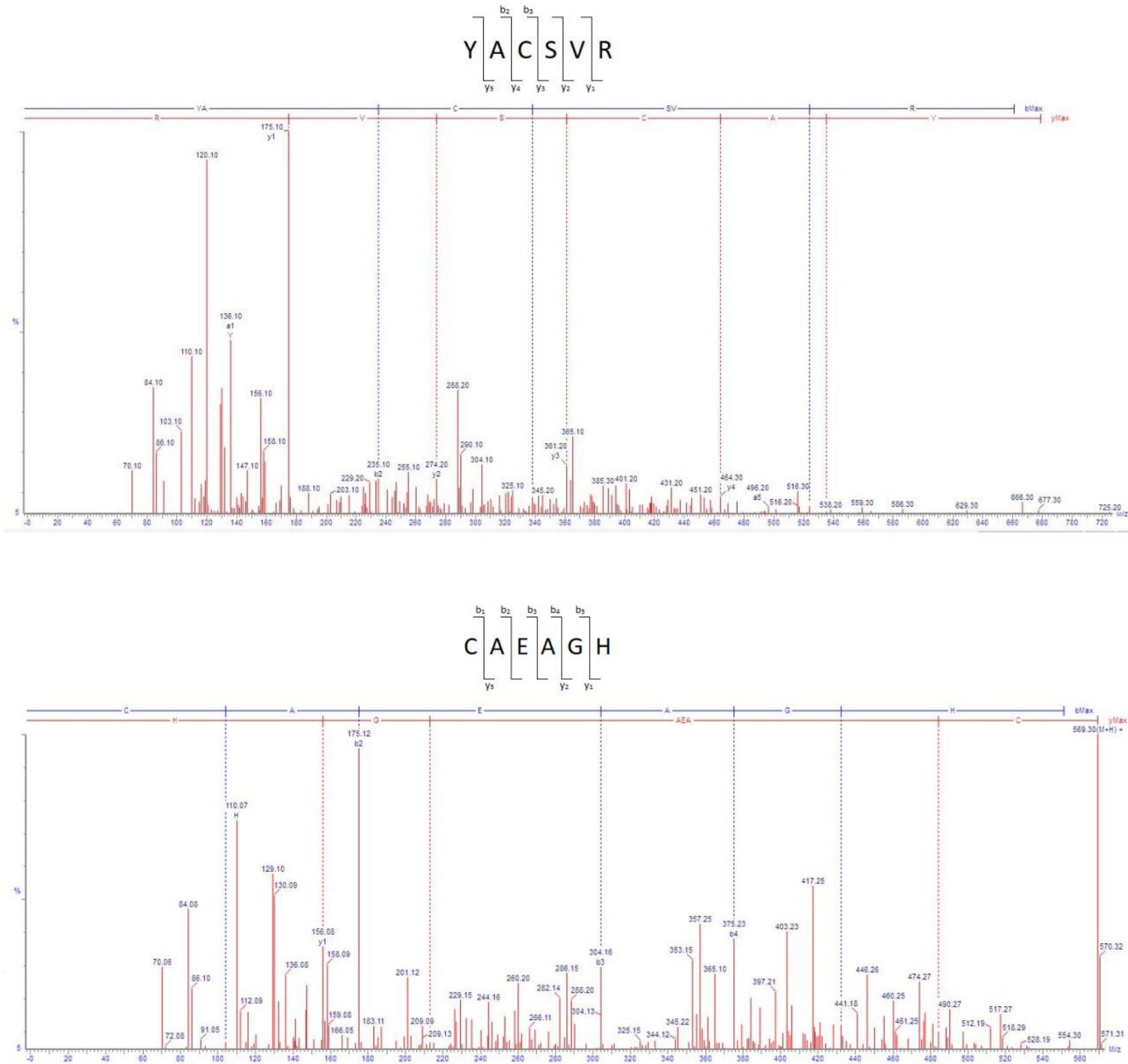


**Figure 2**: Sequence profile of two of the peptides identified from *S. pilchardus*

Effectively, these molecular features are not exclusive to characterise the peptide as DPP-IV inhibitor, but it offers an initial idea of it. The larger the peptide is, the harder it will be for it to achieve the target, and also functional groups might contribute to the steric hindrance of the interaction. DPP-IV inhibitory peptides have usually a length varying from 3-7 amino acids, the presence of proline, especially on the second N-terminus and mostly flanked by leucine, valine or phenylalanine. Neves et al. (2017) identified some peptide sequences (GGPAGPAV, GPVA, PP, GF) and two free amino acids (arginine and tirosine) from salmon hydrolysates, as capable to inhibit DPP-IV enzyme.

**Table 3**: Peptide sequences identified by UHPLC–MS/MS in the most active fractions obtained by chromatography from S20TF sample.

| Fraction | Peptide sequence | Calculated mass (Da) | Energy score* |
|---|---|---|---|
| **D** | VLGP | 384.226 | -13.304 |
| | CGSFT | 513.178 | -16.670 |
| | FNLE | 521.24 | -11.851 |
| | LLLLN | 584.38 | -14.885 |
| | CAEAGH | 586.21 | -14.829 |
| | WHSLP | 638.31 | -14.422 |
| | EVPADM | 660.27 | -14.262 |
| | NAPNPR | 667.329 | -12.699 |
| | NQGPRP | 667.329 | -12.081 |
| | DNWTF | 681.26 | -16.622 |
| | CGGWLF | 681.284 | -19.697 |
| | YACSVR | 697.311 | -14.012 |
| | TVEHVGG | 697.33 | -15.605 |
| | NAALGPR | 697.376 | -14.907 |
| **C** | DTMYDT | 744.25 | -13.999 |
| | DWSSAPP | 758.31 | -16.181 |
| | TVCLSGGGA | 763.343 | -15.261 |
| | PVNTLPLA | 823.469 | -16.462 |
| | EVYEFDR | 956.41 | -13.448 |

*Obtained with pepATTRACK

On the other hand, considering the final purpose of the hydrolysate, it is also important to obtain gastrointestinal digestion resistant peptides. Possessing an arginine at the terminal of the sequence could help these peptides to resist the attack of digestive enzymes, since digestive enzymes selectivity includes this amino acid. Among the peptides identified in this study, it is highly likely that YACSVR or NAPNPR, coming from the trypsin hydrolysis of the product, would be capable to resist the gastrointestinal digestion and could eventually achieve their final purpose.

Based on these factors (mass spectra and sequence features) authors consider NAPNPR and CAEAGH to be subjected to further analysis, that is to say, chemical synthesis of the

sequence to verify its bioactivity and its resistance to food processing, digestive enzymes attack and stability in storage conditions.

*In vitro* analysis is the first step to discover drugs with benefits to human health. Beyond the advantages of protein hydrolysates compared to drugs, concerning no consequent side effects, protein hydrolysate provide better characteristics due to their synergic effects (Lacroix & Li-Chan, 2013).

# 4. CONCLUSIONS

This is the first time that peptides stemming from sardine protein hydrolysates have been identified and reported as DPP-IV inhibitors. The amino acids composition and the proteins of the substrate, as well as the enzymatic treatment are involved in releasing these antidiabetic peptides. The enzymatic combination employed was optimised to obtain a hydrolysate with bioactive and digestive enzyme resistance peptides. The most bioactive fraction, ranging from 800-1400 Da, showed an $IC_{50}$ of 1.83 ± 0.05 mg/ml after separation by chromatography. Identified peptides were from 4 to 9 amino acids length. These results show an economic, easy, fast method to produce bioactive peptides from raw material with no initial use that could be potentially incorporated into food matrix and have health benefits in consumers.

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# III. Identification of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from vegetable protein sources*

Vegetable proteins are appearing as a sustainable source for human consumption. Food-derived peptides are an important field of research in terms of bioactive molecules. In this study, seven vegetable proteins were enzymatically hydrolysed following an optimised treatment (sequential hydrolysis with subtilisin-trypsin-flavourzyme) to obtain dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. Hydrolysates were fractionated by size exclusion chromatography and, from the most bioactive fractions (corresponding to *Glycine max, Chenopodium quinoa* and *Lupinus albus* proteins); peptides responsible for this bioactivity were identified by mass spectrometry. Peptides with adequate molecular features and based on in silico analysis were proposed as DPP-IV inhibitors from soy (EPAAV) lupine (NPLL), and quinoa (APFTVV). These vegetable protein sources are adequate to obtain protein hydrolysates for functional food.

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# 1. INTRODUCTION

Globalisation, alongside availability of natural resources and consumer trends, among others factors, has led to a revolution in food science and technology as never seen before. Currently, the most studied proteins are those coming from milks, as well as meat proteins and wheat. Nonetheless, Western dietary habits are changing progressively, giving importance to vegetal sources rather than meat (Neacsu, McBey, & Johnstone, 2017).

The consumption of vegetable proteins compared to animal protein has numerous advantages. Western diets, high in meats, refined sugars, and fats, have been proved to be worse in terms of health-promoting for humans compared to plant-based diets because the risk of developing some diseases as higher (Medawar, Huhn, Villringer, & Veronica Witte, 2019). In addition, vegetable production help to reduce agricultural greenhouse gas emissions (GHG) and to reduce land clearing and resultant species extinctions compared to animal-based foods (Tilman & Clark, 2014). Furthermore, plant production requires less resources compared to the production of the same amount of animal protein. One such example, the same land extension serves to grown 20 times the amount of soy compared to a given quantity of beef (Nadathur, Wanasundara, & Scanlin, 2017; Tilman & Clark, 2014). However, the most important limitations for plant proteins are the amino acid profile and the absorption rate, but the intake of different plant sources could solve this limitation (Nadathur et al., 2017).

Soy protein products are currently the most employed alternative to meat, as well as oat or pea, which are commonly employed in the food industry. It is remarkable to mention that pea proteins contain essential amino acids in the adequate proportion, just limited by the lack of methionine (Tulbek, Lam, Wang, Asavajaru, & Lam, 2017). Among grain legumes, lupine receives a great interest because of its high-quality protein and dietary fibre. Comparing ileal digestibility of lupine protein to milk and wheat proteins, it may be considered a very beneficial plant protein (Tomé, 2013). Recently, it has been reported that digested isolates from pea and lentil had comparable soluble nitrogen to milk proteins, employing the INFOGEST protocol (Santos-Hernández et al., 2020). Some other adequate vegetable sources are lentil and chickpea, widely ingested in Mediterranean diets. These legume sources contain also a balanced content of essential amino acids and adequate *in vitro* protein digestibility (Sánchez-Vioque, Clemente, Vioque, Bautista, & Millán, 1999). Quinoa protein is gaining popularity due to its quality,

bioavailability and adequate composition of essential amino acids, comparable to casein (Mudgil et al., 2020).

Enzymatic hydrolysis of proteins is currently employed to obtain bioactive peptides with antioxidant, antihypertensive or antidiabetic activities. Numerous vegetable proteins have been employed to obtain peptides with different bioactivities (García, Puchalska, Esteve, & Marina, 2013; Lammi et al., 2018). Particularly, some authors have identified antidiabetic peptides, related to their ability to inhibit the dipeptidyl peptidase IV (DPP-IV) enzyme, from soy, lupine or bean (Lammi et al., 2018; Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015). The role of DPP-IV is degrading insulin secretion hormones. Hence, if DPP-IV is inhibited, the half-life of these hormones is increased and the insulin level regulated in order to achieve adequate blood glucose levels (Kshirsagar, Aggarwal, Harle, & Deshpande, 2011). The inhibition of this enzyme via oral medicaments is currently one of the most employed antidiabetic methods, but some side effects have been described (Juillerat-Jeanneret, 2014). Consequently, prevention and pre-treatment of this metabolic disorder by functional food, including bioactive peptides as ingredient, seems promising.

Currently, there are not too many articles focused on the identification of DPP-IV inhibitory vegetable peptides. Based on the amino acid content, abundant in alanine and proline (Lammi et al., 2018), it could be expected that peptides from soy show high DPP-IV inhibition, whereas those coming from lentil, pea and chickpea, containing lower quantity of proline (Samaranayaka, 2017), would present lower DPP-IV inhibitory activity. Authors found no report of lentil DPP-IV inhibitory peptides. Chickpea DPP-IV inhibitory peptides have been reported (Felix, Cermeño, Romero, & FitzGerald, 2019), likely due to its high content of alanine (Sánchez-Vioque et al., 1999), an important amino acid for these kind of bioactive peptides (Hsieh et al., 2016). Potato DPP-IV inhibitors peptides have only been identified by *in silico* analysis (Ibrahim, Bester, Neitz, & Gaspar, 2019), but no experimental research has been carried out to the author's knowledge. Previously, we defined an enzymatic treatment that maximized the DPP-IV inhibitory activity of different protein hydrolysates, such as casein (data not published) and sardine (*S. pilchardus*) (Rivero-Pino, Espejo-Carpio, & Guadix, 2020) employing three different proteases. Furthermore, due to the trypsin action (whose specificity is similar to the one exerted by digestive proteases), the hydrolysate contained digestion-resistant peptides and the length of the hydrolysis allowed maximizing the quantity of bioactive peptides.

Hence, it has been considered that enzymatic treatment as adequate to obtain a hydrolysate with high bioactivity for other substrates as well (Rivero-Pino et al., 2020).

Considering that some vegetable sources have been used to obtain DPP-IV inhibitory peptides, we hypothesed that vegetable proteins subjected to an adequate enzymatic treatment would yield potent antidiabetic peptides with food industry applicability. Some of the proteins employed have not been reported as DPP-IV inhibitors to the best of our knowledge and could be potent sources of bioactive peptides with antidiabetic effect. The aim of this work was obtain protein hydrolysates from different vegetable protein sources (soy, pea, potato, quinoa, chickpea, lentil and lupine) employing an enzymatic treatment that release a high content of bioactive peptides. The sources chosen for this study were considered on different factors: amino acid content, widely studied proteins to compare results (Lammi et al., 2018; Nongonierma & FitzGerald, 2015; Vilcacundo, Martínez-Villaluenga, & Hernández-Ledesma, 2017) and new protein sources to exploit their potential as antidiabetic peptides sources. Subsequently, these hydrolysates were purified to obtain fractions with high content of bioactive peptides and in the most active ones, the peptides responsible for the bioactivity in the most active fractions obtained were identified.

# 2. MATERIALS AND METHODS

## 2.1 Materials

Vegetable flours were kindly donated by different companies. Thereby, pea (*Pisum sativum*) and potato flour (*Solanum tuberosum*) from Trades (Barcelona, Spain), lupine flour (*Lupinus albus*) from Dayelet (Barcelona, Spain), soy (*Glycine max*) from Solae LLC, (St. Louis, MO, USA), whereas chickpea (*Cicer arietinum*), lentil (*Lens culinaris*) and quinoa (*Chenopodium quinoa*) were bought in a local market. The protein content of the samples were potato 81%, pea 80%, soy 85%, lupine 32.5%, chickpea 50.9%, lentil 46.2% and quinoa 59.4%. Proteases employed were: Alcalase 2.4L (subtilisin, EC 3.4.21.62), PTN 6.0S (trypsin 3.4.21.4) and Flavourzyme$^{TM}$ 1000L (3.4.11.1). All the enzymes were provided by Nozoymes (Bagsvaerd, Denmark).

For the enzyme inhibition assay: Human DPP-IV enzyme, Gly-Pro-p-nitroanilide as substrate and Diprotin A as positive control (maximal inhibition) were purchased from Sigma-Aldrich (Spain).

## 2.2 Enzymatic hydrolysis

The chosen substrates were diluted in distilled water at 40 g/L of protein and hydrolysed for seven hours at 50 ºC. Enzyme-to-substrate ratio was fixed at 2.75%. Alcalase 2.4L was added at t = 0 h, trypsin was added at t = 3 h without modifying pH nor temperature. The pH for the first five hours was 8, maintained by the addition of 1M sodium hydroxide using a 902 Titrando (Metrohm AG, Herisau, Switzerland. Finally at t = 5 h, the pH was reduced to pH 6 with 1 M HCl and Flavourzyme was added, exerting its activity for two hours more (until t = 7 h). Then, the proteases were deactivated by heating at 90ºC for five minutes and samples were freeze-dried. This sequential hydrolysis was previously analysed by our group, leading to an adequate DPP-IV inhibitory bioactivity (Rivero-Pino et al., 2020). Hydrolysis reactions were carried out in duplicate.

## 2.3 Characterization of samples

Protein content of hydrolysates was analysed by Kjeldahl method. On the other hand, elemental analysis was carried out to measure the protein content of the fractions (Flash 2000, Thermo Scientific). In this technique, freeze-dried samples were oxidized with pure oxygen at 1020 ºC and the combustion products were transported through a chromatographic column by helium to separate them. At the end, a thermal conductivity detector (TCD) provides a signal (mV/s) proportional to the concentration of the individual components of the mixture. The nitrogen content was transformed into protein content of each sample, considering the nitrogen-to-protein factor for each one: 5.36 for pea, 5.5 for soy and lupine, 5.93 for quinoa (Fujihara, Sasaki, Aoyagi, & Sugahara, 2008) 5.6 for potato and 5.4 for chickpea and lentil (Mariotti, Tomé, & Mirand, 2008). Calibration sample was sulphanilamide.

Molecular weight profile was analysed in duplicate by size exclusion chromatography (SEC) employing a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden). 500 µL of samples flowed at 0.5 ml/min with MilliQ as mobile phase. The absorbance was measured at 280 nm. Standards for calibration were: L-tyrosine (217.7 Da), vitamin B12 (1355 Da), aprotinin (6512 Da), cytochrome C (12384 Da) and ribonuclease (13700 Da).

## 2.4 Purification by SEC fractionation

The samples were fractionated coupling the equipment employed for the molecular weight distribution analysis described in the section 2.3, to a collector Frac-902. In order to obtain enough mass for analysing DPP-IV inhibitory activity and protein content of each fraction, five runs were carried out and the fractions collected were freeze-dried to be analyzed Samples chosen for mass spectrometry analysis were fractionated once again. DPP-IV inhibition capacity of fractions was analysed as described below (section 2.5).

## 2.5 DPP-IV inhibitory activity

The *in vitro* DPP-IV inhibition assay for the hydrolysates and the fractions obtained by size exclusion chromatography was performed as previously described (Lacroix & Li-Chan, 2012) with slight modification. Briefly, 25 µL of the enzyme (DPP-IV) at 0.02 U/ml were incubated with 100 µL of hydrolysate at different concentration for 10 minutes. Then, reaction was initiated by the addition of 50 µL of 1 mM Gly-Pro-p-nitroanilide. The amount of reaction product (p-nitroanilide) released was tracked by measuring the absorbance at 405 nm during 2 hours, each 2 minutes, employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). Samples were analysed in triplicate. Half maximal inhibitory concentration ($IC_{50}$) value for each sample was calculated by plotting the progress of reactions compared to the blank (distilled water). Results were expressed in mg of protein/mL, as mean ± standard deviation.

## 2.6 Identification of peptides and *in silico* analysis

The most active fractions from different sources collected from the chromatography (section 2.4) were analysed using an ACQUITY UHPLC system (Waters, Milford, CT, USA) coupled to a Synapt Mass Quadrupole Time- of-Flight Mass Spectrometer. A volume of 10 µL of the selected samples was injected in an ACQUITY BEH 300 C4 column 1.7 µm (Waters) and components were eluted employing water–formic acid 0.1% as buffer A, and acetonitrile as buffer B, at a flow rate of 0.3 ml/min (Liu et al., 2015). The MS spectra were acquired in positive electrospray ionization mode using a capillary energy of 2.5 kV and sampling cone of 30 V. The analyses were performed using an m/z range from 50 to 1900 at the normal scan resolution. PepSeq program from BioLynx software (Micromass UK Ltd., Manchester, United Kingdom) was employed for sequencing peptides *de novo*.

BLASTp (Basic Local Alignment Search Tool) was used to compare the sequences identified in the mass spectra with corresponding proteome, to verify that the peptides are encrypted in a protein sequence reported. *Glycine max* (Taxon identifier: 3847) as soybean. *Lupinus albus* (Taxon identifier: 3870) as lupine. *Chenopodium quinoa* (Taxon identifier: 63459) as quinoa.

The likelihood of the identified peptides to being bioactive was predicted with the Peptide Ranker software and the likelihood of being cell penetrating was analysed with CPPpred tool (http://distilldeep.ucd.ie). The iDPPIV-SCM tool, a sequence-based predictor for identifying DPP-IV inhibitory peptides was employed to obtain a score to determine if the peptide would inhibit the key enzyme (Charoenkwan, Kanthawong, Nantasenamat, Hasan, & Shoombuatong, 2020).

## 2.7 Statistical analysis

Statgraphics 5.1 was employed to calculate significant differences by the multiple comparison test (Least Signifcant Difference) at a p-value $\leq 0.05$. Data is shown as average ± standard deviation.

# 3. RESULTS AND DISCUSSION

## 3.1 Hydrolysates characterization

In this research, differences in the amino acid content and the proteins structure are responsible for the molecular weight profile and bioactivity differences among substrates. The non-hydrolyzed proteins did not show DPP-IV inhibitory activity even at 50 mg/ml. Diprotin A $IC_{50}$ value was 1.62 ± 0.18 µg/mL, considered the positive control for this assay, reporting the maximal inhibition of the DPP-IV enzyme. The $IC_{50}$ values for *in vitro* DPP-IV inhibition of the hydrolysates analysed are reported in table 1. Soy hydrolysate gave the highest inhibition among all the samples ($IC_{50}$ value of 2.39 ± 0.19 mg/ml), followed by chickpea ($IC_{50}$ value of 2.68 ± 0.18 mg/ml). Soybean amino acid composition present higher content of alanine and proline (Samaranayaka, 2017), whose residues are proved to be determinant in DPP-IV inhibition, compared to chickpea, lentil and pea. This might be one reason why soy protein hydrolysate showed better bioactivity than the other sources. In the same line, Gonzalez-Montoya et al. (2018) reported an $IC_{50}$ value of 1.49 ± 0.14 mg/mL for a soybean protein digested with porcine pepsin and pancreatin. Vegetable protein hydrolysates potential DPP-IV inhibitory activity has been

previously reported for different enzymatic treatment. Pea and soy hydrolysates with similar $IC_{50}$ values were reported for different enzymatic treatment, employing Protamex and Promod 144MG, whereas Corolase L10 hydrolysates did not show inhibition at the concentration analysed for both substrates (Nongonierma & FitzGerald, 2015). Some authors have also reported DPP-IV inhibitory peptides from quinoa by *in vitro* simulated gastrointestinal digestion and fractionation by ultrafiltration (Vilcacundo et al., 2017). Recently, Mudgil et al (2020) reported $IC_{50}$ values ranging from 0.72–1.12 mg/ml for quinoa protein hydrolysates obtained with bromelain, chymotrypsin and Pronase E, highlighting the importance of the specificity of the protease in releasing bioactive peptides.

**Table 1**: *In vitro* DPP-IV inhibitory activity (as $IC_{50}$ value) of the hydrolysates and their fractions.

| Protein | Pea | Soy | Lupin | Chickpea | Lentil | Quinoa | Potato |
|---|---|---|---|---|---|---|---|
| **Hydrolysate** | $2.93 \pm 0.22^{b,c}$ | $2.39 \pm 0.19^{a}$ | $3.42 \pm 0.09^{d}$ | $2.68 \pm 0.18^{a,b}$ | $2.92 \pm 0.10^{b,c}$ | $3.25 \pm 0.23^{c,d}$ | $5.41 \pm 0.39^{e}$ |
| **A (>10kDa)** | > 3.5 | >2.5 | >2.5 | >2.5 | >2.5 | >2.5 | >2.5 |
| **B(10-3kDa)** | $4.91 \pm 0.04^{a}$ | $3.92 \pm 0.03^{b}$ | >2.5 | >3.5 | >3.5 | >2.5 | >4 |
| **C(3-0.8kDa)** | $2.09 \pm 0.04^{a}$ | $1.16 \pm 0.07^{b}$ | $1.73 \pm 0.02^{c}$ | $3.45 \pm 0.05^{d}$ | $1.81 \pm 0.02^{e}$ | $1.47 \pm 0.06^{f}$ | $1.84 \pm 0.02^{e}$ |
| **D(800-400Da)** | $2.84 \pm 0.08^{a}$ | $1.74 \pm 0.07^{b}$ | $2.16 \pm 0.12^{c}$ | $2.32 \pm 0.05^{d}$ | $3.22 \pm 0.06^{e}$ | $1.52 \pm 0.05^{f}$ | $2.35 \pm 0.01^{d}$ |
| **E(<400 Da)** | >3 | >3.5 | >2.5 | >2.5 | >2.5 | >2 | >2 |

$IC_{50}$ refers to half maximal inhibitory concentration value of DPP-IV in mg of protein/mL. Different letters in the same row indicate significant differences among substrates (n=3)

Recently, bioinformatics analysis are gaining importance because it is possible to predict the bioactive peptides sequences derived from an hydrolysis by *in silico* analysis, for example, simulating the gastrointestinal digestion of tuber storage proteins (Ibrahim et al., 2019). However, further research should be carried out to exploit the vegetable potential as source of DPP-IV inhibitory peptides. Lentil DPP-IV inhibitory peptides have not been reported in literature and this study shows its potential as good source for antidiabetic peptides, showing an $IC_{50}$ value of 2.92 mg/mL, significantly lower than potato and lupine ($IC_{50}$ values of 5.41 and 3.42 mg/ml respectively). Lupine has been described as an adequate source for this kind of bioactive peptides (Lammi et al., 2018). The values obtained for these vegetable protein hydrolysates are also comparable to DPP-IV inhibitory values from animal protein hydrolysates. For instance, camel skin gelatin hydrolysates showed an $IC_{50}$ value ranging from 0.70–2.4 mg/mL (Mudgil et al., 2019), or from bovine lung, with $IC_{50}$ value ranging from 1.43–6 mg/mL (Lafarga & Hayes, 2017). Regarding marine sources, silver carp hydrolysates exerted up to 35% of DPP-IV inhibition at 5 mg/ml, considering different proteases and reaction times (Zhang, Liu,

Hong, & Luo, 2019) and DPP-IV inhibitory peptides from sardine showed an $IC_{50}$ value ranging from 3.7-7.8 mg/ml (Rivero-Pino et al., 2020). Similarly, camel milk protein hydrolysates have been reported to have $IC_{50}$ values ranging from 0.52 to 1.26 mg/ml, comparable to the activity reported for these vegetable protein hydrolysates (Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2017). These results suggest that, in addition to being more sustainable protein sources and health-promoters that those from animal, their hydrolysis into bioactive peptides show adequate and comparable results to these traditional sources. The differences among the samples are due to the peptides sequences released after the enzymatic treatment. The native protein structure, including the sequences of the proteins, would determine the peptides released for each protein source. In order to concentrate the bioactive peptides depending on their size, the hydrolysates were fractionated by means of size exclusion chromatography and the obtained fractions analysed.

## 3.2 Fractionation

The *in vitro* bioactivity of the different fractions from all the protein sources analysed is shown in Table 1. In general terms, the most bioactive fractions are C and D, containing peptides ranging from 3 to 0.4 kDa, and the fractions A (>10 kDa) showed little or no DPP-IV inhibitory activity and consequently, in none of them the $IC_{50}$ values could be determined. Fractions of similar size to those obtained in this work were also described as the most bioactive for sardine muscle (Rivero-Pino et al., 2020). Concerning the other fractions, some differences in terms of inhibition were reported. As described above, soy protein hydrolysate gave the highest inhibition at minimal concentration among all the protein sources analysed ($IC_{50}$ value of 2.39 mg/ml). Then, fraction C (ranging from 3 kDa to 800 Da) was the most bioactive fraction in soy, and among all the fractions from the hydrolysates analysed, with an $IC_{50}$ value of 1.16 ± 0.07 mg/mL. Due to limitation of the technique, $IC_{50}$ values could not be determined for the less active fractions.

**Table 2**: Protein concentration of the fractions obtained for each hydrolysate by size exclusion chromatography (mg/mL).

| Protein | Pea | Soy | Lupin | Chickpea | Lentil | Quinoa | Potato |
|---|---|---|---|---|---|---|---|
| **A (>10kDa)** | 0.65 | 1.59 | 0.36 | 0.44 | 0.87 | 0.28 | 0.19 |
| **B(10-3kDa)** | 0.87 | 2.18 | 1.51 | 1.52 | 1.54 | 0.53 | 1.28 |
| **C(3-0.8kDa)** | 1.92 | 2.77 | 2.18 | 1.30 | 0.71 | 1.50 | 1.06 |
| **D(800-400)** | 2.28 | 3.50 | 2.51 | 2.44 | 2.22 | 2.80 | 2.66 |
| **E(<400 Da)** | 0.27 | 0.47 | 0.26 | 0.22 | 0.41 | 0.55 | 0.44 |

In table 2, it is depicted the protein concentration of fractions, where it is observed that fraction D is the most concentrated in peptides, followed by C and B, depending on the protein employed. In general terms, fraction A (larger molecules) and fraction E (di and tri peptides) are the less concentrated fractions. This is an optimistic result since the majority of bioactive peptides (ranging from 3 kDa to 400 Da) are present in the most concentrated fractions. These results are important when considering scaling-up at industrial level, where concentration by membranes with molecular weight cut-offs of 3 kDa would be adequate to easily purify the hydrolysates.

In general, low molecular weight peptides, ranging from 3 kDa to 400 Da seems to be responsible for the bioactivity. This behaviour have been reported in some other protein sources, for example, Lacroix & Li-Chan (2012) described that <3kDa peptides exerted higher bioactivity than those from the >3kDa fraction in a sodium caseinate hydrolysate and Vilcacundo et al. (2017) reported higher bioactivity for <5kDa fractions in a quinoa protein hydrolysate, compared to the >5kDa fraction. The high bioactivity of soy and lupine peptides as DPP-IV inhibitors has been previously described (Lammi et al., 2018), and was confirmed with these results.

Nonetheless, there are differences comparing the different fractions of the analysed hydrolysates (Table 1). Fraction E, containing mainly di and tri-peptides was reported slightly bioactive only in quinoa and potato hydrolysates at the concentration analysed (inhibition around 30% were reported at 2 mg/mL, not shown in table). This could be due to the predisposition of the hydrolysate after subtilisin and trypsin treatment, to be hydrolysed by flavourzyme. In this case, higher proportion of <3 aa residues peptides could be obtained, increasing the bioactivity of this fraction. Carrying out an *in silico* analysis with the BIOPEP software (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008) with the major proteins sequences of each source from Uniprot led to the identification of a large number of peptides of 2 or 3 amino acids length with DPP-IV inhibitory activity, that would encompass fraction E. However, as stated in Table 2, the protein concentration generally obtained for this fraction is lower than the value obtained for fractions C and D, and their contribution to the bioactivity might not be as important as the exerted from these two active fractions.

In quinoa, larger peptides can be responsible for the DPP-IV inhibitory activity, as some inhibition could be reported for fraction B. This is in agreement with previous report for quinoa peptides obtained by simulated gastrointestinal digestion (Vilcacundo et al.,

2017), highlighting that also high molecular weight peptides from quinoa can be DPP-IV inhibitors. Based on potato fractions inhibition percentages (Table 1), this correlation between length and DPP-IV inhibitory activity could be similar for potato peptides. In the case of lentil peptides, even when the concentration of fraction C (Table 2) is lower than fraction D, the bioactivity in fraction C is significantly higher than in D, highlighting the importance and the potential high bioactivity of these peptides.

Overall, these results agree with published literature that highlights the potential of low molecular weight peptides to inhibit DPP-IV. Furthermore, the amino acid composition and sequence of peptides is important (Gonzalez-Montoya et al., 2018; Nongonierma & Fitzgerald, 2016), and it depends on the enzymatic treatment employed. As it was hypothesized, the peptides obtained from the vegetable protein sources we have exploited in this research exert values of DPP-IV inhibition comparable to some other traditional sources.

## 3.3 Identification of peptides

The identification of the bioactive sequences would enable to characterise the peptides obtained to ensure its functionality in the scope of nutrition. The most active fractions (fraction C from soy, lupine and quinoa) were analysed in order to identify the peptides responsible for the bioactivity. These fractions were also some of the most high-protein content. The peptides identified and the scores obtained from the *in silico* analysis are shown in the Table 3. Some of the mass spectra obtained from the samples analysed are shown in the Fig 1. Based on the mass spectra and the molecular features of the sequences identified it is expected that these peptides are contained in the protein hydrolysate and would exert the bioactivity described.

Some of the peptides found in soy (TPVGM, APFL and EPAAV), lupine (NPLL, APLAVR), and quinoa (APFTVV) possess molecular features that have been described as common in peptides with DPP-IV inhibitory activity. For instance, possessing the amino acids proline or alanine in the penultimate N-terminal residue (Hsieh et al., 2016) or having hydrophobic N-terminal residues (Liu, Cheng, & Wu, 2019). However, these molecular features previously described are not exclusive to characterize a peptide as DPP-IV inhibitor, as well as the lack of these amino acids does not mean that the peptide cannot exert the bioactivity. For example, Zhang et al. (2019) identified the peptide AALEQTER as highly bioactive, but the peptide LLDLGVP also exerted inhibition over

the enzyme DPP-IV even when no alanine or proline was the penultimate N-terminal residue.

**Table 3**: Peptide sequences identified by UHPLC–MS/MS in the most active SEC fractions and the scores obtained from the *in silico* analysis.

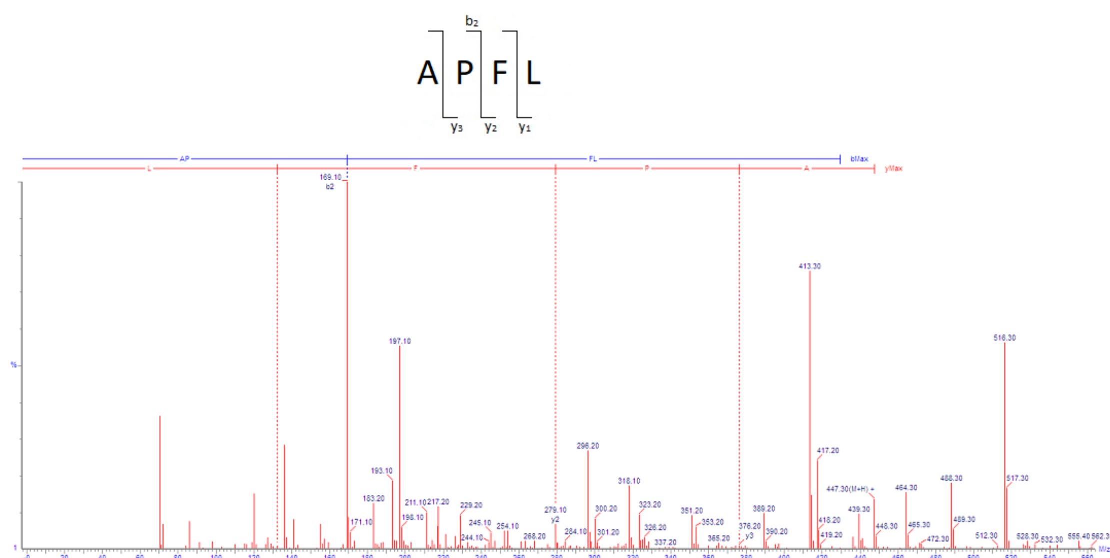
| Substrate | Peptide sequence | Probability of being bioactive* | Probability of being cell penetrating** | DPP-IVi predictor*** |
|---|---|---|---|---|
| **Soy (*Glycine max*)** | KTLDTS | 0.0435 | 0.236 | ~~219.4~~ |
| | KDVDTS | 0.049 | 0.104 | ~~205.8~~ |
| | TPVGM | 0.391 | 0.191 | 401.75 |
| | EPAAV | 0.117 | 0.162 | 369.75 |
| | APFL | 0.952 | 0.145 | 415.67 |
| | PVDTY | 0.163 | 0.082 | 382.25 |
| | VLFPT | 0.528 | 0.192 | 400.25 |
| | DLLGLW | 0.823 | 0.375 | 327.0 |
| | APEDY | 0.265 | 0.058 | 362.75 |
| **Lupin (*Lupinus albus*)** | RDDVPT | 0.180 | 0.194 | 307.2 |
| | RNTSPQ | 0.114 | 0.241 | 303.2 |
| | HDLPG | 0.487 | 0.077 | 355.0 |
| | NPLL | 0.655 | 0.393 | 446.0 |
| | APVPEM | 0.484 | 0.121 | 426.2 |
| | APLAVR | 0.311 | 0.669 | 313.6 |
| | APENPV | 0.232 | 0.122 | 407.8 |
| **Quinoa (*Chenopodium quinoa*)** | RQGMR | 0.569 | 0.740 | ~~261.25~~ |
| | RMGPL | 0.838 | 0.541 | 350.5 |
| | RSTPL | 0.393 | 0.465 | 295.25 |
| | APFTVV | 0.338 | 0.175 | 365.4 |

*Score obtained by PeptideRanker. **Score obtained by CPPpred ***Score obtained by iDPPIV-SCM (Values higher than 294 are considered as positive result, negative results are represented crossed out)

Based on BLASTp analysis, the peptide EPAAV can be found in numerous proteins (serine/threonine-protein kinase ATM; hypothetical protein GLYMA_08G316100; alpha,alpha-trehalose-phosphate synthase [UDP-forming] 10; activating signal cointegrator 1 complex subunit 2; auxin response factor 9 and

bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic) and might be one of the peptides obtained that highly contributes to the DPP-IV inhibitory activity of the hydrolysate. Furthermore, this peptide, EPAAV, shares the two C-terminal residues with the peptide DPP-IV inhibitory peptide GGPAGPAV, isolated from salmon gelatine (Neves et al., 2017). In the same way, APFL from soy could be found in kinesin-like protein KIN-12D, cellulose synthase interactive 1, putative callose synthase 8 and UDP-glucose:glycoprotein glucosyltransferase-like isoform X1. This peptide showed a score of 0.952 with the PeptideRanker software, suggesting its high potential to be bioactive.

NPLL identified in lupine fraction can be found in numerous proteins from these substrate. For example, putative DNA helicase chromatin remodeling SNF2 family , putative ABC transporter and P-loop containing nucleoside triphosphate hydrolase. This peptide showed a potential of being bioactive of 0.655 and a likelihood to be cell penetrating of 0.393, suggesting its adequate biological activity. Peptides such as AVPTGVA, YVVNPDNDEN, YVVNPDNNEN, LTFPGSAED, LILPKHSDAD, GQEQSHQDEGVIVR have been previously identified in soy and lupine as DPP-IV inhibitors *in vitro* (Lammi, Zanoni, Arnoldi, & Vistoli, 2016). Furthermore, an *in vivo* study showed that the peptide YPFVV from soy showed hypoglycemic activity in diabetic mice (Yamada et al., 2012). Hence, soy protein appear as a potent DPP-IV inhibitory peptides precursor.



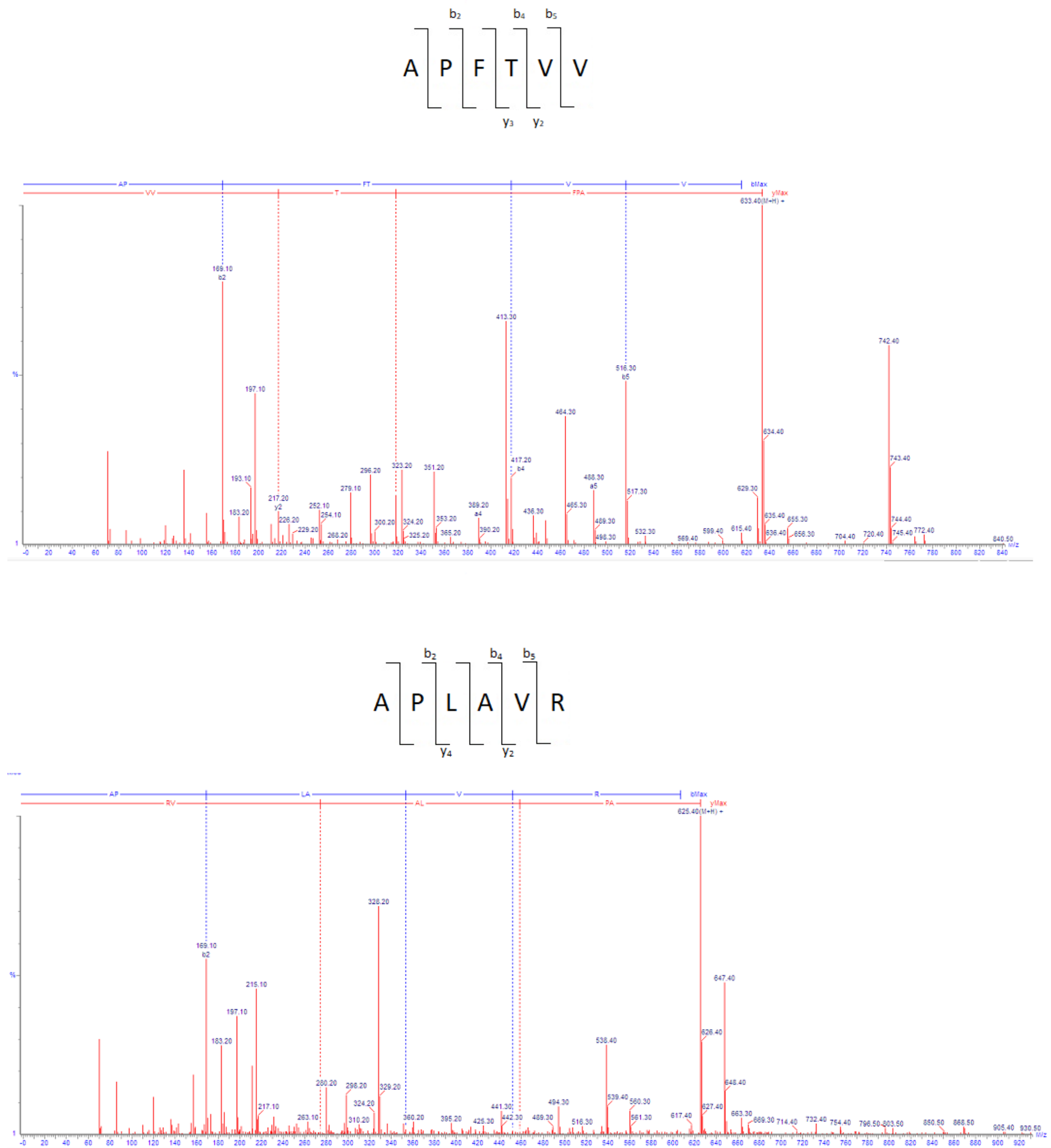



**Fig 1.** Mass spectra of peptides a) APFL, from soy b) APFTVV, from quinoa c) APLAVR, from lupine.

Concerning BLASTp analysis of quinoa peptides, the proteome of this substrate currently available on Uniprot only encompass 224 unreviewed proteins. Hence, the analysis might not be entirely accurate. However, some more peptides were identified by mass spectrometry (unpublished data), but no correspondence to quinoa protein could be attributed, likely due to the fact that the proteome is not fully characterized. In regard to the peptides, RMGPL was found in an uncharacterized protein from quinoa. This one showed a high potential to be bioactive based on the PeptideRanker software (Score = 0.838). However, RSTPL could be found in transcription factor MYB3R-5-like; ankyrin

repeat-containing protein NPR4-like; 50S ribosomal protein L3, chloroplastic and cytochrome b-c1 complex subunit Rieske-4, mitochondrial-like proteins, which enhances its importance as bioactive peptide. Based on the known features of DPP-IV inhibitory peptides, APFTVV could be a strong inhibitor, due to the presence of proline in that position, even when the score by *in silico* analysis was low (0.338). However, further analysis with synthetic peptides should be carried out to confirm its bioactivity.

Based on the iDPPIV-SCM analysis, all the identified peptides, except for KTLDTS and KDVDTS from soy and RQGMR from quinoa, would be DPP-IV inhibitory peptides. The score for the positive control, Diprotin A is 469 and the threshold to consider a peptide as inhibitor is 294. As observed in table 3, some of the peptides obtained a high score close to the positive control (NPLL, from lupine = 446; APFTVV from quinoa = 365.4 or APFL, from soy = 415.67). The *in silico* analysis are an easy and economic approach to identify bioactive peptides prior to their synthesis.

Having identified the protein containing the DPP-IV inhibitory peptides, the bioactive peptides production process could be optimized for example by extracting the proteins before the enzymatic hydrolysis based on their isoelectric point. These would avoid the presence of non-bioactive peptides. Furthermore, during hydrolysis, separation by means of membranes could be implemented to concentrate the quantity of bioactive peptides, knowing the molecular weight the peptides have.

To the best of our knowledge, these peptides stemming from protein hydrolysates have not been identified previously. However, similarity of sequences were found between some peptides found in this research and published literature. One such example is the soy peptide TPVGM, similar to TPVVVPP from β-casein (Nongonierma & Fitzgerald, 2016) or APFL and APFTVV, from soy and quinoa respectively, containing the DPP-IV inhibitory peptide APF (FitzGerald, Cermeño, Khalesi, Kleekayai, & Amigo-Benavent, 2020). Some of the peptides identified (Table 3) contain the residues AP at the N-terminal, molecular feature described for DPP-IV inhibitory peptides (Nongonierma et al., 2018), so it could be expected that this peptide has high bioactivity too. Quinoa-derived peptides have been also previously identified, whose amino acid length are in the range of those obtained in this research (700-1000 Da), such as IFQEY or SFFVFL (Vilcacundo et al., 2017) but the amino acid sequences do not share evident similar molecular features with the peptides reported in this research.

Despite the consideration of vegetable proteins as adequate protein sources, it must be taken into consideration the advantages and disadvantages among them. For example, quinoa shows low recovery and low protein content compared to soy and pea (Scanlin & Lewis, 2017). It should be also considered the production prices of each protein or if the protein source to obtain bioactive peptides is a means to revalue the product, such as lupine protein, as an agricultural product. For this reason, beyond all the experimental data carried out, a Life Cycle Assessment, to assess the environmental impacts of a product or service during all stages of its existence would be interesting for its industrial scale-up.

# 4. CONCLUSIONS

Recently, numerous factors (i.e. environmental aspects, health, production prices, etc.) are making food industry to orient its priorities towards plant ingredients more than animal sources. In this way, the improvement of different sustainable protein bioactivity was carried out by enzymatic hydrolysis. All plant proteins hydrolysates showed DPP-IV activity after enzymatic treatment with subtilisin, trypsin and Flavourzyme. The hydrolysate with the highest antidiabetic potential was soy ($IC_{50}$ value of 2.39 ± 0.19 mg/ml) but chickpea, lentil and pea hydrolysates also showed potent DPP-IV inhibitory activity ($IC_{50}$ values of 2.68 ± 0.18 mg/ml, 2.92 ± 0.10 mg/ml and 2.93 ± 0.22 mg/ml respectively). Peptides ranging from 400 to 3000 Da highly contributes to the DPP-IV inhibitory activity of the hydrolysate. The most potent fraction was obtained in soy ($IC_{50}$ value of 1.16 ± 0.07 mg/ml), followed by lupine and quinoa, where different peptides were identified by mass spectrometry. EPAAV, NPLL and APFTVV were suggested to be adequate active peptides responsible for DPP-IV inhibitory activity in soy, lupine and quinoa respectively.

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# IV. Evaluation of *Tenebrio molitor* protein as source of peptides modulating physiological processes*

The increase of world population has led to the need of searching new protein sources as insects, whose harvesting is economically and environmentally sustainable. This study explores the biological activities (angiotensin-converting enzyme inhibition, antioxidant capacity, and dipeptidyl peptidase IV inhibition) of *Tenebrio molitor* hydrolysates produced by a set of food grade proteases, namely subtilisin, trypsin, ficin and flavourzyme, and degree of hydrolysis (DH) ranging from 5% to 20%. Trypsin hydrolysates at DH 10% presented the highest ACE inhibitory activity at DH 10% ($IC_{50}$ 0.27 mg/mL) in the experimental series, which was attributed to the release of short peptides containing Arg or Lys residues in the C terminus, described as ACE inhibition feature. The levels of *in vitro* antioxidant activities were comparable to those reported for insect species. Subtilisin and trypsin hydrolysates at DH 10% displayed optimal DPPH scavenging and ferric reducing activities, which was attributed to the presence of 5 – 10 residue active peptides, as reported in literature. Iron chelating activity was significantly favoured by increasing DH, attaining a minimal $IC_{50}$ of 0.8 mg/mL at DH 20%, regardless of the enzymatic treatment. Similarly, *in vitro* antidiabetic activity was significantly improved by extensive hydrolysis, and more specifically, to the presence of di- and tripeptides. To this regard, the combined treatment subtilisin-flavourzyme at DH 20% showed the maximal DPP-IV inhibition ($IC_{50}$ 2.62 mg/mL). To the author's knowledge, this is the first study evaluating the DPP-IV activity of *Tenebrio molitor* hydrolysates obtained from the commercial proteases mentioned above.

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# 1. INTRODUCTION

Insects represent the highest percentage of biomass on the planet, and are increasingly regarded as a valuable source of protein for human consumption. Insects are particularly nutritious and even though western societies are reluctant to its consumption, entomophagy is widely expanded in other regions. Not only are they rich in protein and healthy fats (mainly monounsaturated fatty acids), but also in micronutrients and fibre (Azagoh et al., 2016). Insect farming is more economic and environmentally-friendly compared to traditional livestock. Moreover, reared insects present an average feed to protein conversion ratio higher than other species (Osimani et al., 2017; van Huis et al., 2013). Since 1st January 2018, insects are considered as novel foods at the European level, which paves the way to further research on its nutritional value and its incorporation into foodstuffs (European Council Regulations No. 2015/2283, 2015) .

Mealworms are already being raised at the industrial scale, and are considered as future good protein sources (De Marco et al., 2015). Some insects species, such as *Tenebrio molitor*, can be found in some stores in European countries for human consumption (van Huis et al., 2013). Considering the estimated increase of world population up to 9 billion people by 2050, we hypothesize entomophagy will be normalised in most of the countries in the short term (Gravel & Doyen, 2020; Makkar et al., 2014).

Enzymatic hydrolysis of proteins to obtain bioactive peptides from insects was early reported in 2005, where angiotensin-converting enzyme (ACE) inhibitory peptides from four insects were identified (Vercruysse, Smagghe, Beckers, & Camp, 2009). Recently, the biological potential of proteins from edible insects subjected to enzymatic hydrolysis have been reviewed (Nongonierma & FitzGerald, 2017b). Focusing on *T. molitor*, some bioactivities have been described for peptides released from this source by enzymatic treatments, such as antioxidant (Zielińska, Baraniak, & Karaś, 2018), antihypertensive (Dai, Ma, Luo, & Yin, 2013), or antithrombotic (F. Chen, Jiang, Gan, Chen, & Huang, 2019) .

Most of the peptides exhibiting antihypertensive activities are able to inhibit the proteolytic activity of Angiotensin I Converting Enzyme, an enzyme involved in blood pressure regulation (Mizuno et al., 2005; Skeggs et al., 1956). As for the antidiabetic peptides, the major mechanism is the inhibition of dipeptidyl-peptidase IV, an enzyme involved in the carbohydrate metabolism (Duez et al., 2012; Pospisilik et al., 2002).

Antioxidant activities may be exerted through a wide range of mechanisms, such as the scavenging of free radicals, chelation of metal ions, and the ability to reduce oxidised species (Frankel & Meyer, 2000; Hunyadi, 2019). Antioxidants are widely employed in food processing to avoid lipid oxidation and oxidative (Hajieva & Behl, 2006; Lin & Liang, 2002; Wang, Huang, Kong, & Xu, 2018).

These natural compounds released from protein hydrolysis are recently regarded as adequate biological regulators because they exert physiological functions within the body (Harnedy & FitzGerald, 2012), and consequently, they could be used in functional food to prevent some diseases without having side effects (Lacroix & Li-Chan, 2013; Perez-Gregorio & Simal-Gandara, 2017). The functionality and potential bioactivity of a given peptide depends on its length and amino acid composition, and therefore is influenced by the enzyme and operating parameters of the hydrolysis reaction. Scarce references are found to date investigating the impact of the enzymatic treatment on both antioxidative and antihypertensive bioactivities of *T. molitor* hydrolysates. To the authors' knowledge, little on *in vitro* DPP-IV inhibitory properties of *T. molitor* protein or derived hydrolysates has been reported so far (Dávalos Terán et al., 2019).

The aim of this work is to explore the *in vitro* ACE inhibitory, antioxidant capacity and DPP-IV inhibitory activities *Tenebrio molitor* hydrolysates obtained by a set of conventional and combined enzymatic treatments employing commercial food grade proteases (i.e. Subtilisin, Tripsin, Ficin and Flavourzyme), studying the influence of both enzymatic treatment and degree of hydrolysis on the *in vitro* activities.

# 2. MATERIALS AND METHODS

## 2.1 Tenebrio molitor meal and enzymes

*Tenebrio molitor* meal was kindly donated by MealFoodEurope (Salamanca, Spain) in April 2019. The samples contained by average 46.8% w/w of protein.

Four commercial proteases were employed for the enzymatic treatments: Subtilisin (EC 3.4.21.62) and pancreatic trypsin (EC 3.4.21.4), which act as serine endoproteases; Ficin (EC 3.4.22.3), which is cysteine-endoprotease and Flavourzyme 1000L$^{TM}$ (3.4.11.1), which is a enzymatic complex mostly comprising exoprotease (i.e. aminopeptidase and dipeptidase) fractions (Merz et al., 2015). All the enzymes were provided by Nozoymes

(Bagsvaerd, Denmark). The reagents employed for the analytical assays were purchased from Sigma Aldrich (St. Louis, US).

## 2.2 Hydrolysis procedure

The enzymatic reaction was conducted in a jacketed reactor coupled to an automatic titrator (718 Stat Titrino, Metrohm, Herisau, Switzerland) to maintain constant pH. All the hydrolysis reactions were conducted at 50ºC and pH 8. A 30 g/L of protein from *T. molitor* was diluted in distilled water. The enzyme-to-substrate ratio was set at 3% and the reaction was allowed until achieving the degree of hydrolysis desired.

Titration allows monitoring the degree of hydrolysis (DH) as a function of the base consumption (NaOH 1 M) required to maintain pH in the course of the reaction (Camacho et al., 2001). A set of protein hydrolysates were produced by six different enzymatic treatments, classified into two groups:

(i) Single enzyme reactions, employing subtilisin (noted as S); porcine trypsin (T) or ficin (F) as sole catalysts.

(ii) Combined treatments employing 1:1 w/w combinations of subtilisin-porcine trypsin (S-T), subtilisin-ficin (S-F) and subtilisin-flavourzyme (S-E). Based on previous studies on the proteolysis of the substrate, subtilisin was chosen as component in all the enzyme combinations.

For every enzymatic treatment, four levels of DH - 5%, 10%, 15% and 20% - were assayed. Under the experimental conditions in this work (pH 8, 50ºC and E/S=3%), the experimental range of DH could not be completed for some enzymatic treatments. For instance, trypsin did not allow obtaining protein hydrolysates above DH 15%. Porcine trypsin is an endo-peptidase that bonds near arginine and lysine residues (Olsen et al., 2004). This specificity restrained the number of peptide bonds prone to enzyme attack, and therefore the final DH reached. Ficin is reported to attack a broad range of peptidic bonds such as Ala, Asn, Gly, Leu, Lys, Tyr and Val (Hou et al., 2017). However, *Tenebrio* meal showed limited proteolysis by the ficin treatment, attaining a steady maximum of DH 5%. Given the broad specificity of subtilisin, it was chosen to take part in all the combined treatments (i.e. S-T, S-F and S-E). Finally, flavourzyme is an enzymatic mixture containing mostly amino and di-peptidases (Segura Campos et al., 2010). Due to its specificity towards N terminal sites, this enzyme needs the initial degradation of the

native protein by an endopeptidase (Nchienzia, Morawicki, & Gadang, 2010), so it was not employed as single catalyst but in combination with subtilisin (treatment S-E).

After completing the hydrolysis treatment, the reaction was stopped by enzyme denaturation after heating at 90ºC for 5 minutes. The hydrolysates were then stored at -20ºC prior to analysis.

## 2.3 Molecular weight distribution of the protein hydrolysates

Molecular mass distribution was estimated by gel filtration chromatography by a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Aliquots of 500 µL (10 mg protein per mL) were eluted at 0.5 mL min−1 with MiliQ water as mobile phase in a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden). The absorbance was measured at 280 nm. A molecular mass calibration curve was prepared using the following standards: L-tyrosine (217,7 Da), vitamin B12 (1355 Da), and ribonuclease (13700 Da).

## 2.4 Determination of protein solubility and protein content

Protein solubility was determined as described by Amiri-Rigi *et al.* (2012) with some modifications. To this end, 800 mg of powdered hydrolysate were diluted with 20mL of ultra-pure water and stirred at 500 rpm and 20ºC for 5 min and then centrifuged at 5000 rpm for 5 min. Supernatant, 10 mL, was recovered and freeze-dried. The protein solubility of the sample was expressed as the percentage of protein, related to the initial protein mass, recovered in the supernatant.

Protein content of samples was analysed in a Flash 2000 Organic elemental analyser (Thermo Scientific). Gases coming from combustion with oxygen at high temperature (1020ºC), are transported through a gas chromatographic column to separate them and a thermal conductivity detector (TCD) detect the signal for each element proportional to the concentration. Nitrogen-to-protein factor was considered 5.6, as reported by Janssen *et al.* (2017).

## 2.5 ACE inhibitory activity

ACE inhibitory activity was determined as described by Shalaby *et al.* (2006). The percentage of inhibition of ACE exerted by the hydrolysates was determined spectrophotmetrically by mixing 10 µL of the enzyme (0.25 U/mL), 10 µL of a wide

range of concentrations of samples and 150 μL of 0.88 mM of substrate (N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine, FAPGG) in buffer Tris–HCl 50 mM, pH 7.5 and 0.3 M of NaCl. The decrease in absorbance was recorded at 340 nm during 30 min using a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). A blank solution was prepared containing ACE and FAPGG without addition of hydrolysate. The percentage ACE inhibition was calculated by the ratio of the slopes of absorbance against time of reaction for the sample $s_i$ and the slope of the blank solution $s_0$ (i.e. sample containing FAPGG and ACE in absence of hydrolysate), as expressed by Eq. [1].

$$\% \, ACE \; Inhibition = \left(1 - \frac{s_i}{s_0}\right) \cdot 100 \qquad [1]$$

The concentration of hydrolysate inhibiting ACE activity by 50% is referred by the half maximal inhibitory activity $IC_{50}$.

## 2.6 Antioxidant activities of the hydrolysates

### 2.6.1 DPPH scavenging activity

DPPH scavenging activity was determined as described by Picot *et al.* (2010) and García-Moreno *et al.* (2014). To this end, a mixture 1:1 mixture containing hydrolysate at increasing protein concentrations (1 – 10 mg/mL) and 0.1 mM DPPH in methanol was shaken and stored for 0.5 h at 25 °C in the dark. Then, the absorbance of the reaction mixture was measured at 515 nm. The DPPH scavenging activity was calculated by Eq. [2]:

$$\text{DPPH scavenging activity, \%} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \qquad [2]$$

where the control sample was prepared by using methanol instead of DPPH and the blank solution contained 1 mL DPPH and 1 mL distilled water. The half maximal inhibitory concentration ($IC_{50}$) value was determined as the concentration of hydrolysate which reduces DPPH activity by 50%.

## 2.6.2 Ferrous ion chelating activity

Chelating activity of hydrolysates was determined as described by Decker and Welch (1990) and García-Moreno *et al.* (2014) For that purpose, 1 mL of hydrolysate at increasing protein concentration (0.5 – 4 mg/mL) was mixed with 3.7 mL of de-ionised water and 0.1 mL of 2mM ferrous chloride. After 3 minutes of incubation, 0.2 mL of 5mM ferrozine were added to stop the reaction. After 10 min of incubation, the absorbance of the mixture was measured at 562 nm. Ferrous ion chelating activity was then calculated by Eq. [3]:

$$\text{Ferrous ion chelating activity, \%} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \qquad [3]$$

where the control sample was prepared without adding ferrozine and water was added instead of hydrolysate for the blank solution. The ferrous ion chelating activity of each hydrolysate was reported as $IC_{50}$ value.

## 2.6.3 Reducing power (FRAP)

Reducing power of hydrolysates was determined as described by Oyaizu (1988). To this end, a mixture of 2 mL of hydrolysate at different protein concentration (1 – 20 mg/mL), 2 mL of 0.2 mM phosphate buffer and 2 mL of 1% potassium ferricyanide were incubated for 20 min at 50ºC. Then, 2 mL of 10% TCA solution was added, the mixture stirred and after centrifugation, 2 mL of supernatant were extract and mixed with 2 mL of water and 0.4 mL of 0.1% ferric chloride. Absorbance was measured at 700 nm after incubating 10 minutes at room temperature. Since the absorbance increase with the hydrolysate concentration, the reducing power capacity was reported by the $EC_{0.5}$ value, which is defined as the concentration of protein whose absorbance was 0.5 units.

## 2.7 DPP-IV inhibitory activity

The DPP-IV inhibition assay was performed as described by Lacroix & Li-Chan (2012a), with slight modification. Briefly, 25 µL of enzyme (0,02 U/mL) were mixed with 100 µL of hydrolysate solution –previously centrifuged- at different concentrations and incubated for 10 minutes. After that, the reaction was initiated by adding 50 µL of 1 mM of Gly-Pro-p-nitroanilide and the absorbance at 405 nm was measured during 120 minutes, each 2 minutes employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa,

Finland). Half maximal inhibitory concentration ($IC_{50}$) was calculated by plotting the progress of reactions compared to the control.

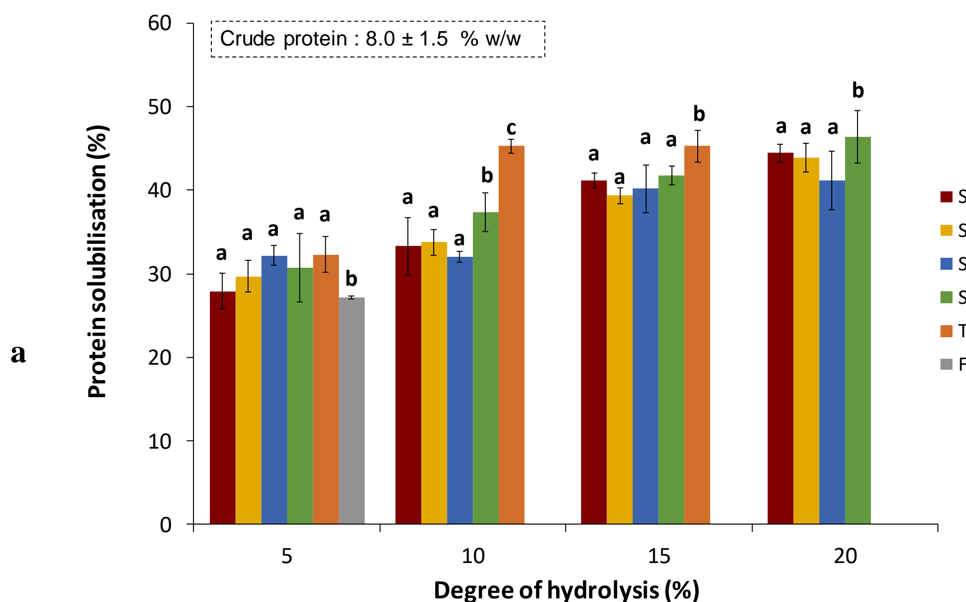
# 3. RESULTS AND DISCUSSION

## 3.1 Protein solubilisation and molecular weight profiles of the hydrolysates

The average levels of protein solubilisation showed by the raw protein and the set of hydrolysates are depicted in Fig. 1a. After the enzymatic treatments, all the resulting hydrolysates presented improved solubility compared to the undigested sample of *T. molitor*, which presented on average 8.0 ± 1.5 % w/w of protein solubility. Indeed, protein solubility of the hydrolysates ranged from 27% to 46% w/w. On average, protein solubility increased with DH, regardless of the enzymatic treatment. Indeed, most of the enzymatic treatments attained 44% w/w of protein solubilisation on average at DH 20%, except for the treatment Alcalase-Flavourzyme (SE20) which presented the maximum of the experimental series (46% w/w on average). Although single trypsin could not attain DH 20%, this treatment was the most efficient to solubilise *Tenebrio* protein, attaining 45.3 % w/w on average at DH 10%. Interestingly, this value was not improved at DH 15%.

The solubility of the protein in a medium depends on a range of parameters such as the molecular weight, hydrophobicity, amino acid charges, pH or ionic strength (Wouters et al., 2016) Increasing degree of hydrolysis is generally correlated with better solubilisation as new polar groups are exposed by enzyme attack (Klompong et al., 2007; Kristinsson & Rasco, 2000). The high solubilisation attained after trypsin treatment is explained by its specificity towards Arg or Lys residues, releasing positively charged (and therefore hydrophilic) peptides to the medium. The specificity of trypsin explains why further hydrolysis above DH 10% did not improve solubilisation, since it only cleaves accessible bonds and numerous insoluble sequences are not hydrolysed. As for the hydrolysate SE20, which presents the maximal protein solubilisation, the combined action of subtilisin and flavourzyme fragmented the original protein down to dipeptides and free amino acids, as shown by its molecular weight distribution (Fig. 1b). Indeed, this sample presented the highest percentage of peptides below 0.5 kDa (19.6% w/w). According to the molecular weight profiles, the fraction of peptides below 0.5 kDa showed little

variation with DH, except for the combined treatment S-E, which increased percentage area from 11% (DH 5%) to 19.6% (DH 20%). This treatment has been employed in the hydrolysis of other proteins of animal origin (Nchienzia et al., 2010; Saadaoui, Espejo-Carpio, Guadix, Amar, & Pérez-Gálvez, 2019), when the release of free amino acids or short chain peptides was targeted.




Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

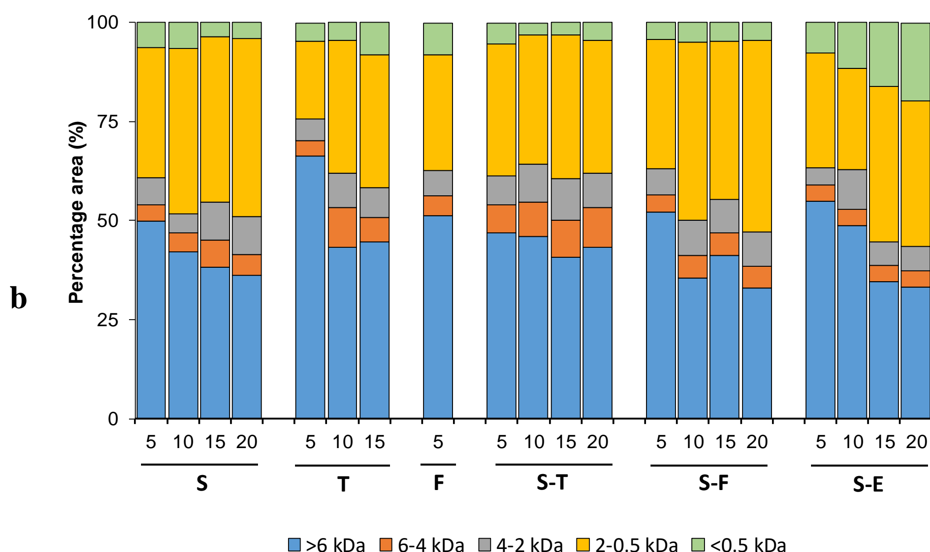



**Figure 1.** Effect of enzymatic treatment and degree of hydrolysis on (a) protein solubilisation, (b) molecular weight distribution of the hydrolysates.

## 3.2 ACE Inhibitory Activity

Figure 2 illustrates the influence of both the enzymatic treatment and the degree of hydrolysis on the ACE inhibitory activity of the hydrolysates, expressed as $IC_{50}$ (mg/mL). The raw *Tenebrio* protein exhibited low ACE inhibition ($IC_{50}$ >100 mg/mL), whereas all the enzymatic treatments led to a significant improvement in this activity. Indeed, the average $IC_{50}$ values determined for the hydrolysates ranged from 0.26 to 1.28 mg/mL, which represents a significant improvement with respect to the intact protein. The tryptic hydrolysates T10 and T15 presented the best levels of ACE inhibition of the experimental series, with an average $IC_{50}$ of 0.27 ± 0.01 mg/mL. To this regard, an average $IC_{50}$ of 0.10 mg/mL was reported for the HPLC purified fraction of *Tenebrio* larva hydrolysates produced by gastrointestinal enzymes (i.e. pepsin, trypstin, chymo-trypsin) (Cito et al., 2017). As for the subtilisin hydrolysates S5 to S20, they showed a significant improvement of ACE inhibition with increasing DH, presenting a minimal $IC_{50}$ (0.35 ± 0.02 mg/mL) at DH 20%. This value is similar to the ACE inhibition reported for subtilisin hydrolysates from *Tenebrio* larva at DH 20% (Dai et al., 2013). The combination of subtilisin with other enzymes did not improve the ACE inhibitory activity in respect to the hydrolysates produced with subtilisin as unique enzyme. As a general trend, ACE inhibition was positively correlated with DH for the hydrolysates S-T, S-F and S-E in the range from DH 5% to DH 15%. However, extensive hydrolysis seems to be detrimental to this activity (e.g. $IC_{50}$ was 0.56 mg/mL and 1.28 mg/mL for ST15 and ST20, respectively), likely due to the breakage of active peptide sequences released previously.

ACE inhibitory peptides identified so far are usually small chain peptides (2-20 amino acids) containing hydrophobic residues (e.g. Pro, Phe, Tyr) in the tripeptide sequence at the C terminal end, which facilitates the interaction with the active site of the Angiotensin I Converting Enzyme (G.-H. Li, Le, Shi, & Shrestha, 2004). The high levels of ACE inhibition attained by the subtilisin and trypsin treatments can be attributed to their specific proteolytic mechanisms. Subtilisin has been reported to produce ACE inhibitory peptides (Connolly et al., 2014), since it cleaves preferably peptide bonds with participation of hydrophobic residues. Trypsin reacts specifically those peptide bonds involving arginine and lysine residues. Tryptic peptides are reported to display strong ACE inhibition when the charged Arg and Lys residues are placed at the C terminus (G.-H. Li et al., 2004). Moreover, it is reported that trypsin presents chymotrypsin-like

behaviour at reaction temperatures above 45ºC, releasing new peptides with hydrophobic residues (Tyr, Trp, Phe) at the carboxyl side (i.e. potential ACE inhibitors) (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010). We hypothesize that this observation could explain the high levels of ACE inhibition displayed by tryptic hydrolysates, which were produced at 50ºC.

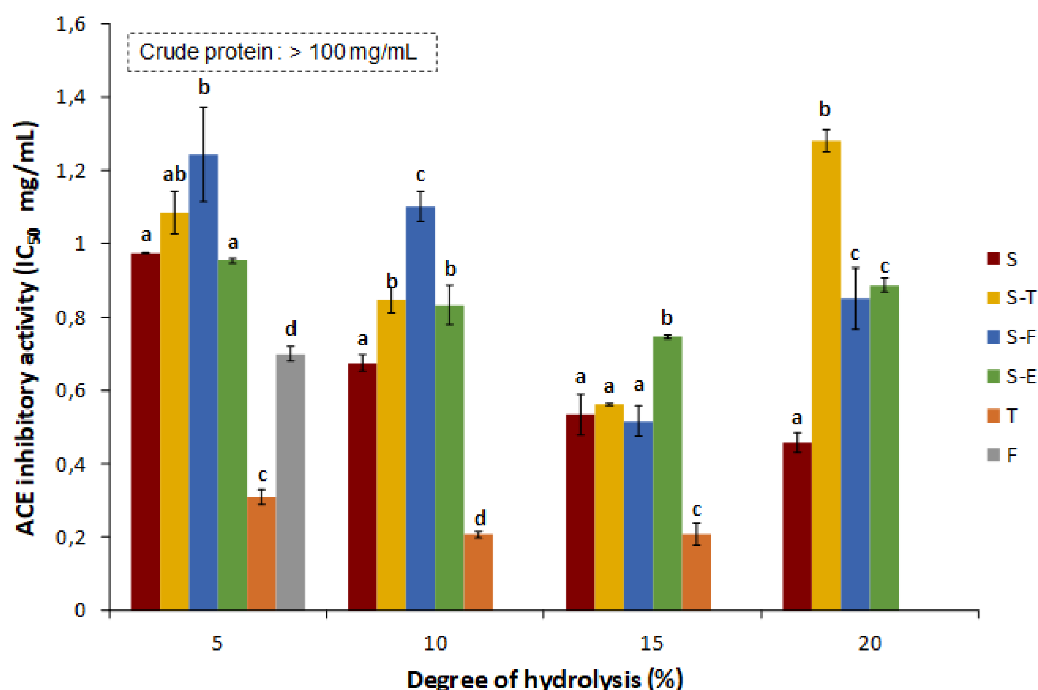



**Figure 2.** *In vitro* ACE inhibitory activity of the hydrolysates as a function of enzymatic treatment and degree of hydrolysis. Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

## 3.3 Antioxidant Activities of the *T. molitor* hydrolysates

### 3.3.1 DPPH Scavenging Activity and reducing power

Figures 3a and 3b present the observed values of DPPH scavenging activity and $Fe^{3+}$ reducing power of the hydrolysates. Although both antioxidant mechanisms act by different pathways, they are related to the electronic transfer and were influenced in a similar trend by DH and enzymatic treatment. All the enzymatic treatments assayed improved the antioxidant capacities of the hydrolysates, related to the intact *Tenebrio* protein. Indeed, the series of hydrolysates presented $IC_{50}$ values of DPPH ranging from 1.03 to 2.31 mg/mL, while the $IC_{50}$ of the undigested protein was 3.01 mg/mL. As shown in Fig. 3a, low DH hydrolysates exhibited weak radical scavenging activity, which improved with increasing DH until attaining an optimum. Above this value, further

hydrolysis up to DH 20% was detrimental to both DPPH scavenging and $Fe^{3+}$ reducing power. The enzymatic treatments S, T and S-T showed maximal DPPH scavenging activity at DH 10% (e.g. $IC_{50}$ of 0.93 ± 0.01 mg/mL for T10, minimum of the experimental series) while the treatments S-F and S-E did at DH 15%.

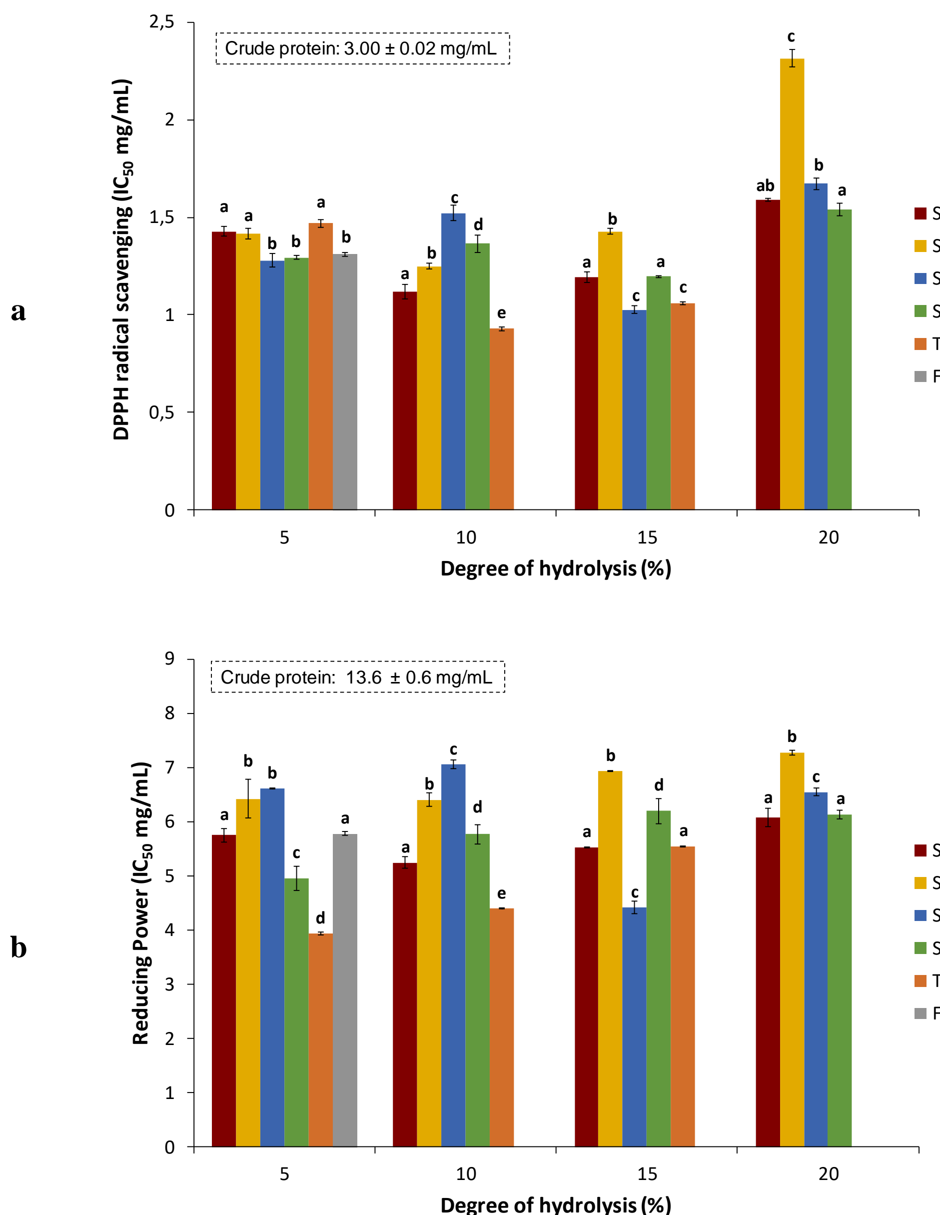



**Figure 3.** Influence of enzymatic treatment and DH on the *in vitro* DPPH scavenging activity (a) and iron reducing power (b). Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

As for the reducing power activity, hydrolysis improved this property by 46 - 67%, related to the intact protein ($EC_{0.5}$ =13.6 ± 0.6 mg/mL). As observed with radical scavenging activity, tryptic hydrolysates presented the lowest $EC_{0.5}$ value of the experimental series

(~ 4 mg/mL) at DH 5%. This value was kept at DH 10%, while higher DH was detrimental to this property.

According to the molecular weight profile (Fig. 1b), the peptide fraction [0.5 – 2 kDa] was the most abundant in the hydrolysates displaying better DPPH and $Fe^{3+}$ reducing power (i.e. S10,T10, ST10, SE15). This fraction corresponds to peptide chains between 2 – 10 amino acid residues. To this regard, the antioxidant peptides identified so far in *T. molitor* hydrolysates, such as AAAPVAVAK, YDDGSYKPH and AGDDAPR (Zielińska, Baraniak, et al., 2018), fall into the size range mentioned above.

Several authors have reported a direct relationship between the presence of hydrophobic residues in the peptide sequence and its potential radical scavenging activity (Girgih et al., 2015; Ovissipour et al., 2013), highlighting the good antioxidant properties of subtilin and trypsin hydrolysates. As for the length, of the peptide chain, it is widely accepted that the antioxidant capacity is favoured by the presence of short peptides (Ajibola, Fashakin, Fagbemi, & Aluko, 2011; Jin, Liu, Zheng, Wang, & He, 2016), due to the steric hindrance associated to larger peptides (Nwachukwu & Aluko, 2019). The trend observed in this work is in agreement to previous works, which reported that DPPH scavenging and reducing power activities presented an optimal DH with minimal $IC_{50}$. Above this value, active peptides were cleaved by further enzymatic treatment, worsening the DPPH scavenging activity of the hydrolysate (Elmalimadi et al., 2017; P. Mudgil, Omar, Kamal, Kilari, & Maqsood, 2019).

Some previous references report the improvement in antioxidant activities observed when subtilisin was employed in combination with other enzymes, such as trypsin, ficin or flavourzyme. Higher radical scavenging activity of *T. molitor* hydrolysates was obtained by combination of subilisin and flavourzyme than that obtained by the single treatments (Tang et al., 2018). Antioxidant properties of horse mackerel hydrolysates produced by combination of subtilisin and trypsin at different proportions has been studied. The authors found that the ratio of both enzymes in the combined treatment had a great impact on the antioxidant properties, suggesting a ratio 2:1 subtilisin-trypsin for optimal DPPH scavenging and $Fe^{3+}$ reducing power activities (Morales-Medina, Pérez-Gálvez, Guadix, & Guadix, 2017).

## 3.3.2 $Fe^{2+}$ Chelating Activity

The ferrous chelating activity of the hydrolysates (Fig. 4a), reported as $IC_{50}$, ranged from 0.53 – 2.12 mg/mL. Intact *Tenebrio* protein presented an $IC_{50}$ of 4.86 ± 0.34 mg/mL, concluding that the enzymatic treatment had a positive impact on this property. Unlike radical scavenging and ferric reduction capacities, ferrous chelating activity improved continuously with increasing levels of DH, attaining an optimum (average $IC_{50}$ ~ 0.8 mg/mL) at DH 20%, regardless of the enzymatic treatment. The minimal $IC_{50}$ value corresponded to the hydrolysate SF20 (0.53 mg/mL) obtained by combination of subtilisin and ficin. This value was statistically equivalent to the chelating activity observed for the samples S20, ST20 and SF20.

Overall, higher degree of hydrolysis is correlated with increased chelating bioactivity of peptides, since new sites able to bind metal ions are exposed. The metal binding capacity of protein hydrolysates depends on the presence of specific residues able to bind metals in the C terminus, such as Gly, Asp, Lys or Arg (Bougatef et al., 2009; Q. Liu, Kong, Xiong, & Xia, 2010; Ovissipour et al., 2013). Other residues such as His show high affinity to metal coordination when located at the N terminus (Dunbar, Martens, Berden, & Oomens, 2018). To this regard, the improvement of chelating activity with DH observed in T and S-T hydrolysates could be explained by the release of new peptides containing Arg or Lys at the side chain. So far, the references reporting chelating peptides from insect protein are limited to some insect species hydrolysed by gastrointestinal enzymes. To this regard, chelating activities inferior to our results ($IC_{50}$ 2.03 mg/mL) for silkworm larva protein subjected to simulated gastrointestinal digestion (i.e. pepsin, trypsin and quimotrypsin) have been reported (Q.-Y. Wu, Jia, Tan, Xu, & Gui, 2011). Similarly, antioxidant activities of the digested protein (i.e. alpha-amylase, pepsin and pancreatin) from some edible insect species have been recently reported (Zielińska, Baraniak, et al., 2018). The authors identified some peptides displaying strong $Fe^{2+}$ chelating activity ($IC_{50}$ 0.11 mg/mL on average) such as AAAPVAVAK, YDDGSYKPH and AGDDAPR from *Tenebrio molitor* or GKDAVIV and AIGVGAIER from *Schitocerca gregaria*.

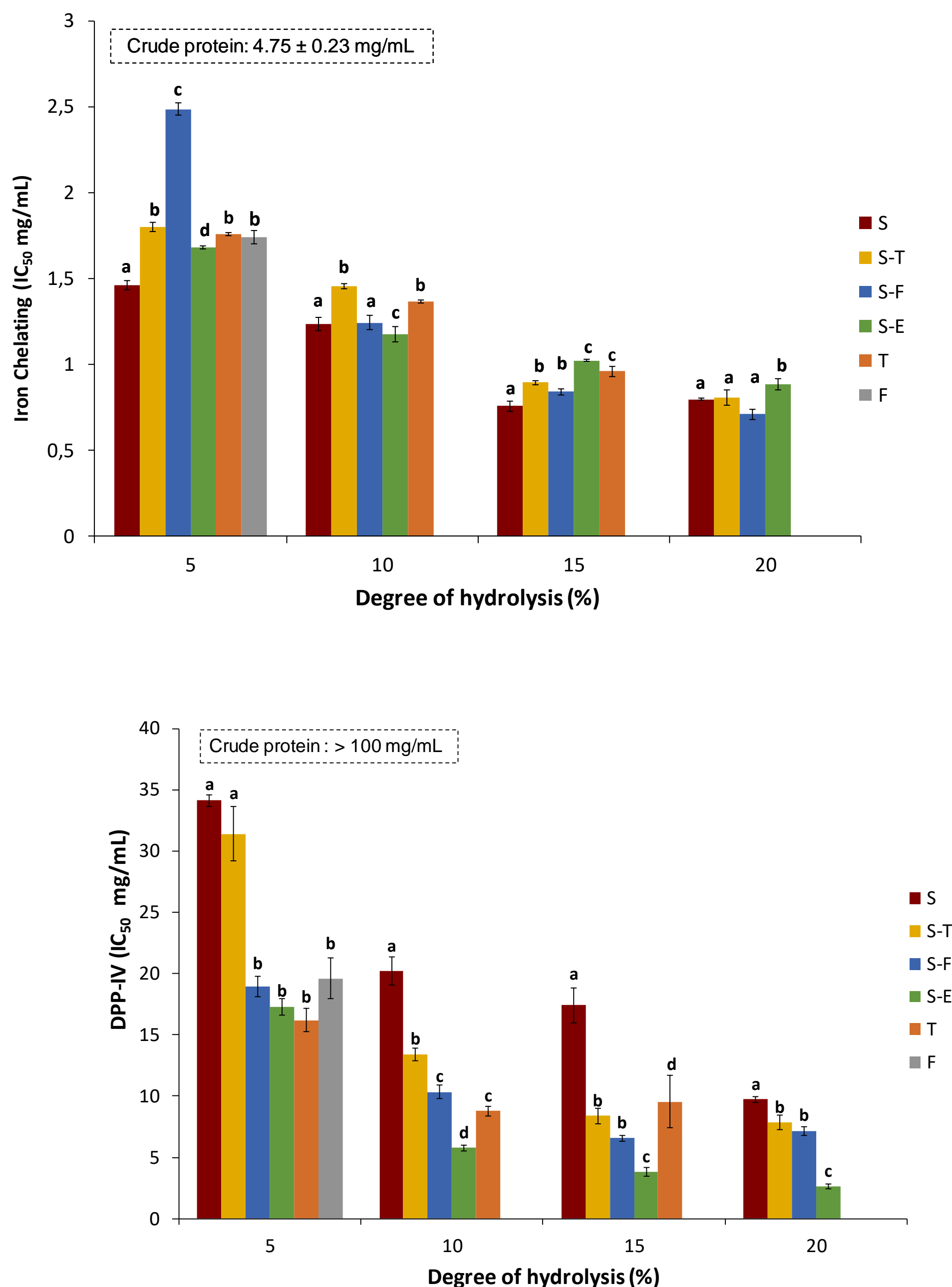



**Figure 4.** Influence of enzymatic treatment and DH on the *in vitro* Ferrous chelating activity (a) and DPP-IV inhibitory activity (b). Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

## 3.4 DPP-IV Inhibitory Activity

The DPP-IV Inhibitory Activity of the hydrolysates is presented in the Figure 4b. The $IC_{50}$ values ranged from 2.62 to 34.13 mg/mL, whereas the intact protein did not show bioactivity ($IC_{50}$ > 100 mg/mL). The results show significant differences in DPP-IV inhibitory activity among enzymatic treatments. Moreover, we observed a positive correlation between DH and DPP-IV activity of the hydrolysates, regardless of the

enzymatic treatment. Stabilisation of the antidiabetic activity above DH 10% reported for subtilisin hydrolysates has been also described for other substrates (Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). The subtilisin-flavourzyme treatment led to the best improvement in DPP-IV activity, attaining maximal inhibition at DH 20% ($IC_{50}$ 2.62 ± 0.16 mg/mL). Compared to the sole use of subtilisin, the combined treatment with flavourzyme improved significantly the levels of antidiabetic activity. The efficacy of the combination of subtilisin and flavourzyme has been already reported to produce good DPP-IV inhibitor peptides (Harnedy-Rothwell et al., 2020). This fact is related to the synergy between both enzymes, where subtilisin releases peptide fragments which are subsequently cleaved by flavourzyme peptidases near the carboxyl-terminus. In our case, this is confirmed by the molecular weight profile, where SE20 presents 19.6% of total area for the peptidic fraction below 0.5 kDa. Most of the antidiabetic peptides identified so far correspond to di- and tripeptides (Harnedy et al., 2018a; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017), and have been isolated from dairy, vegetable or marine sources (Drummond et al., 2018; Harnedy-Rothwell et al., 2020; Lammi et al., 2018). As for insect sources, the DPP-IV inhibitory activity of lesser mealworm (*A. diaperninus)* protein was studied, reporting the maximal antidiabetic activity for the thermolysin hydrolysate ($IC_{50}$ of 0.63 mg/mL) (Lacroix et al., 2019).

To the best of our knowledge, only high DPP-IV inhibitory peptides from cuticular protein from *T. molitor* hydrolysed with papain ($IC_{50}$ of 0.82 mg/mL) have been identified (Dávalos Terán et al., 2019). Hence, this is the first study describing the *in vitro* antidiabetic activity of *Tenebrio molitor* hydrolysates obtained from the set of commercial proteases assayed.

# 4. CONCLUSIONS

Insects are gaining much interest as novel sources for human nutrition, in view of recent changes in population concerns on the sustainability of food resources and environmental issues. The aim of this paper was to evaluate the *in vitro* biological activities (i.e. ACE inhibitory, antioxidant and DPP-IV inhibitory activities) of a set of protein hydrolysates from *Tenebrio molitor* meal, employing commercial food-grade proteases (i.e. subtilisin, trypsin, ficin and flavourzyme) as single catalyst or in mixtures 1:1. Both the enzymatic treatment and the degree of hydrolysis had a significant impact on the bioactivities of the hydrolysates, which was attributed to their specific peptide profile.

Most bioactive ACE inhibitory peptidic fractions were obtained with trypsin at DH 10% ($IC_{50}$ 0.27 mg/mL) and subtilisin at DH 20% ($IC_{50}$ 0.35 mg/mL), which was related to the release of peptides with hydrophobic residues in terminal position.

The highest DPPH scanvenging and $Fe^{3+}$ reducing activities were displayed by the subtilisin and trypsin hydrolysates at DH 10%. Above this value, active peptides were cleaved by further enzymatic treatment, worsening both properties. In contrast, ferrous chelating activity of the *Tenebrio molitor* hydrolysates was favoured by increasing DH, attaining a minimal $IC_{50}$ of 0.8 mg/mL at DH 20%, regardless of the enzymatic treatment.

The DPP-IV activity of the hydrolysates was significantly improved with increasing degree of hydrolysis, regardless of the enzymatic treatment. The combination of subtilisin with flavourzyme led to the most active hydrolysate ($IC_{50}$ 2.62 mg/mL) at DH 20%. According to the molecular weight profile, this hydrolysate presented the highest content in short-chain peptides below 0.5 kDa, which are likely to inhibit DPP-IV.

We conclude that the *Tenebrio molitor* hydrolysates produced with food grade proteases are a valuable source of active peptides able to take part as functional ingredients into food and nutraceutical preparations.

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# V. Effect of ultrasound pretreatment and sequential hydrolysis on the production of *Tenebrio molitor* antidiabetic peptides*

Diabetes prevalence has been rapidly increasing worldwide, and there is a need for searching natural compounds to prevent and treat it. Bioactive peptides derived from insect protein would be an interesting sustainable option. However, the potential of insects such as mealworms (*Tenebrio molitor*) as source for antidiabetic peptides has not been widely studied. In this work, the influence of ultrasound and the sequential hydrolysis with subtilisin and trypsin hydrolysis were analysed for the release α-glucosidase inhibitory peptides. Short time of ultrasound improved the following releasing of bioactive peptides but longer ultrasound treatment time limited their production. Subtilisin hydrolysis did not allow the release of α-glucosidase inhibitory peptides, but it is needed to cleave the protein before trypsin releases these peptides. These high-bioactive hydrolysates could be used as ingredient regulating the glycaemic index in food formulation.

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# 1. INTRODUCTION

Diabetes mellitus type II is one of the most prevalent diseases in the world. More than 400 million people suffer from this metabolic disorder characterized by high glucose levels in the bloodstream. and the forecasts suggest that around 700 million people would suffer it by 2045 ("IDF Diabetes Atlas," 2017). Commercial drugs to palliate type II-diabetes symptoms are classified based on their mechanism to restore physiological function.

One such example is the inhibition of alpha-glucosidase. This key-enzyme in food digestion is responsible for the degradation of polysaccharides into glucose molecules, which are required as energy source for the normal functioning of the organs (Patil et al., 2015). α-glucosidase inhibitors are a group of antidiabetic compounds acting as competitive inhibitors of the enzymes involved in polysaccharide degradation, reducing the glucose released into the bloodstream (Ibrahim, Koorbanally, & Islam, 2014). Acarbose, the reference inhibitor, reduces absorption of starch and disaccharides during postpandrial digestion (Ortiz-Andrade et al., 2007). However, several studies report the adverse side effects of synthetic α-glucosidase inhibitors, such as frequent gastrointestinal discomfort (Duez et al., 2012; T. Y. Wang et al., 2015).

To this regard, natural compounds such as bioactive peptides are appearing in recent years as alternative for glycaemic index management (Lacroix & Li-Chan, 2013; Mora et al., 2020; Zielińska et al., 2020). Most bioactive peptides are obtained by enzymatic hydrolysis of milk, vegetable or fish proteins (Masood & Khosravi-Darani, 2015; Pérez-Gálvez et al., 2016; Rizzello et al., 2016). Insects have emerged in recent years as a novel protein source (Gravel & Doyen, 2020; Alice B. Nongonierma & FitzGerald, 2017b) which presents an interesting content in protein, fats, fibre and micronutrients (Azagoh et al., 2016). Moreover, insect farming is more economic and environmentally-friendly compared to traditional livestock (Grau, Vilcinskas, & Joop, 2017; Oonincx & de Boer, 2012; Osimani et al., 2017). Indeed, some insect species such as mealworms (*Tenebrio molitor*) are already raised on an industrial scale (De Marco et al., 2015). Since 1st January 2018, insects are considered as novel foods at European level, which paves the way to further research on its nutritional value and its incorporation into foodstuffs (European Council Regulations No. 2015/2283, 2015). Current social situation makes necessary a reconsideration of food systems in the future, less dependent on animal-based protein sources and focused on looking for new protein sources (Galanakis, 2020).

Enzymatic hydrolysis of proteins to obtain bioactive peptides from insect was firstly described in 2005, where ACE inhibitory peptides from four insects were analysed (Vercruysse, Smagghe, Herregods, & Van Camp, 2005). Recently, Yoon at al. (Yoon, Wong, Chae, & Auh, 2019) have reported α-glucosidase inhibitory peptides obtained by subtilisin and flavourzyme hydrolysis of different insect species. However, further research should be carried out to explore the optimization of releasing these kinds of peptides.

Furthermore, the search for novel functional ingredients takes advantage of the new processing technologies, where non-thermal treatments such as ultrasound or pulsed electric field are gaining interest (Galanakis, 2012). In the field of functional compounds, recent investigations point out the efficacy of ultrasound treatment to improve the functionality of different molecules, such as polysaccharides (Dou, Chen, & Fu, 2019) or to release bioactive peptides (Abadía-García et al., 2016; Mintah et al., 2019). In this regard, Jiang et al. (2018) employed ultrasound pre-treatment in the hydrolysis of soybean protein for the production of α-glucosidase inhibitory peptides, but the effect of ultrasounds over the protein and the final yield of bioacitve peptides was not evaluated. Ultrasound treatment might provoke different structural and physical modification over proteins, including improved solubilisation (O'Sullivan, Murray, Flynn, & Norton, 2016) that would help the later hydrolysis in the case of low-soluble proteins, such as those from insects (Rivero-Pino, Pérez Gálvez, et al., 2020).

The aim of this work is to evaluate the influence of ultrasound and enzymatic hydrolysis with different specificity proteases over the production of α-glucosidase inhibitory peptides from *Tenebrio molitor* meal. To this purpose, the substrate was subjected to ultrasound pre-treatments and subsequent hydrolysis with subtilisin and trypsin during which the α-glucosidase inhibitory activity was measured.

# 2. MATERIALS AND METHODS

## 2.1 Raw material

*Tenebrio molitor* meal was kindly provided by MealFoodEurope in April 2018 (Salamanca, Spain). The *T. molitor* meal was ground to powder and stored at -16ºC until used. The protein content was determined by Kjeldahl method, assuming a nitrogen-to-protein factor of 5.6 (Janssen et al., 2017). Fat content of the samples was determined gravimetrically as previously described with slight modification (Drusch et al., 2012)

after extraction with a solvent mixture hexane/isopropanol (1:1; v:v). Protein content of the flour was estimated as 55% and fat content was estimated to be 28%, on a wet weight basis.

## 2.2 Ultrasound pretreatment

The process layout followed in this work is depicted in the Fig. 1. *T. molitor* meal was grounded and subjected to sonication as pre-treatment prior to the sequential hydrolysis employing subtilisin and trypsin as endo-proteases. The ultrasound pre-treatment aimed at increasing the exposure of *T. molitor* protein hydrophobic residues, favouring the proteolysis of the pre-treated sample.

For that purpose, the *T. molitor* grounded meal was diluted in distilled water down to 60 g of protein/L. This suspension was subjected to ultrasound pre-treatment, employing a probe-type sonicator (QSonica LLC, Newtown, Connecticut, USA). The equipment was set at 500 W and 20 KHz of frequency, with an amplitude of 75%, while the temperature was maintained with a cold bath. Ultrasound pulses were applied for 15 seconds, intersected by relaxation periods of 10 seconds. This sequence was repeated until attaining the required energy input. In our case, three different ultrasound pre-treatments were applied:

- US0. Control treatment where no ultrasound treatment was applied to the sample.
- US15. Ultrasound pre-treatment comprising 60 pulses. The sample was exposed to ultrasound pulses for a total duration of 15 min, which corresponds to an energy input of 17.3 kJ.
- US30. Ultrasound pre-treatment comprising 120 pulses (30 min, 36.1 kJ).

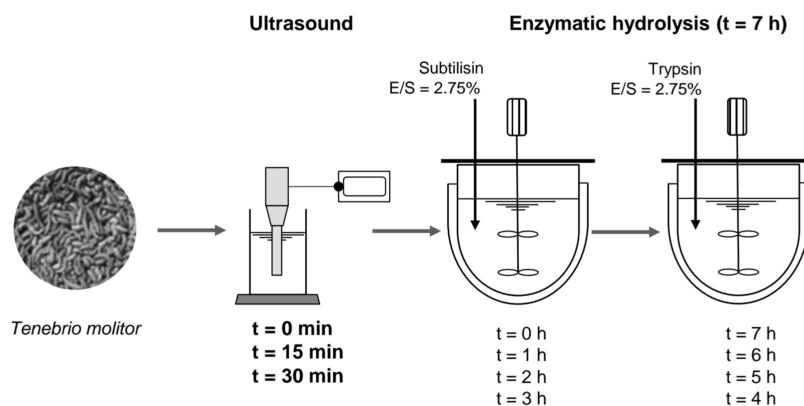


**Fig 1.** Process layout comprising the ultrasound and sequential enzymatic treatments.

## 2.3 Surface hydrophobicity of ultrasonicated samples

Surface hydrophobicity index was measured in triplicate as described by Hayakawa and Nakai (1985). This method is based on the fluorometric measurement of samples at different concentrations (from 0.4 to 0.05 mg/ml) with 1-Anilino-8-naphthalenesulfonic acid. Surface hydrophobicity was calculated as the slope of the line between the serial concentrations analysed and their corresponding fluorescence.

## 2.4 Enzymatic hydrolysis

A solution containing 15 g/L of protein in distilled water, previously sonicated (except for the control samples without ultrasound treatment) was hydrolyzed by a sequential enzymatic treatment, employing subtilisin and trypsin as catalysts. The overall duration of the sequential enzymatic treatment (i.e. subtilisin plus trypsin hydrolysis) was set at 7 h. Firstly, the enzymatic reaction was initiated by addition of Alcalase 2.4. (subtilisin, EC 3.4.21.62) at enzyme-to-substrate 2.75%, 50 °C and pH 8. Subtilisin acts as broad spectrum endopeptidase, which cleaves preferably hydrophobic residues (Hou et al., 2017). The duration of the subtilisin stage was tested at four levels: no subtilisin addition, 1 h, 2h and 3h of reaction. At this point, PTN 6.0S (trypsin, EC 3.4.21.4) was added to the reactor vessel at enzyme-to-substrate ratio 2.75%, equally at 50 °C and pH 8 and the hydrolysis was allowed until completing 7 h,. The sequential enzymatic treatments were coded employing S and T for the subitilisin and trypsin stages, respectively. For example S2T5 refers to the treatment involving 2 h of subtilisin plus 5 h of trypsin hydrolysis. A control treatment S0T7 was employed where the hydrolysis was carried out for 7 h employing only trypsin as catalyst. Based on previous literature, we hypothesize that trypsin would enhance the release of α-glucosidase inhibitory peptides (Ibrahim et al., 2017) due to its narrow specificity towards peptide bonds containing arginine or lysine residues (Olsen et al., 2004). Proteases were provided by Novozymes (Bagsvaerd, Denmark). The degree of hydrolysis (DH) was monitored in the course by means of an automatic titrator (902 Stat Titrando, Metrohm, AG, Herisau, Switzerland), employing 1 N NaOH as titration agent. According to the pH-stat method (Camacho et al., 2001), DH can be related to the amount of titration agent consumed during the reaction, as expressed by equation [1]:

$$\mathrm{DH},\% = \frac{\mathrm{N_b} \cdot V_b}{\alpha \cdot m_P \cdot h_{tot}} \cdot 100 \qquad [1]$$

where $V_b$ and $N_b$ are, respectively, the volume (mL) and normality (eq/L) of base, α is the average degree of dissociation of the α-$NH_2$ amino groups released during the hydrolysis, $m_P$ is the mass of protein (g) fed to the reactor and $h_{tot}$ is the number of equivalents of peptide bonds per gram of protein (meq/g). Considering the reaction conditions and substrate employed, 1/α considered was 1.13, while $h_{tot}$ was assumed to be 8 (Adler-Nissen, 1986). In order to monitor the α-glucosidase inhibition, 0.5 mL aliquots were drawn every 30 min of reaction. After completion of the enzymatic treatment, the reaction was stopped by thermal deactivation of enzyme (90 °C, 5 min).

## 2.5 Molecular weight distribution

Molecular weight distribution was analysed by size exclusion chromatography by a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) employing a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden). Aliquots of 500 µL (10 mg protein per mL) were eluted at 0.5 mL $min^{-1}$ with MilliQ water as mobile phase. The absorbance was measured at 280 nm. The column was calibrated with the following standards: L-tyrosine (217,7 Da), vitamin B12 (1355 Da), aprotinin (6512 Da), cytochrome C (12384 Da) and ribonuclease (13700 Da).

## 2.6 *In vitro* α-glucosidase inhibition assay

20 µL of α-glucosidase (EC. 3.2.1.20) at 0,4 U/ml (Sigma Aldrich, St. Louis, USA) were mixed with 120 µL of sample solution at different concentration in pH 6.94 sodium phosphate buffer 20 mM 6.7 mM NaCl. 100 µl of (4-Nitrophenyl-β-D- glucopyranoside (PNPG) at 0.5 mM were added after 15 minutes incubation to start the reaction and the amount of 4-nitrophenol (PNP) released from the substrate was monitored by measuring the absorbance at 405 nm during 60 minutes, each 30 seconds employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). Each sample was analysed in triplicate. Then, inhibitory activity of each sample can be calculated by Eq. 2

$$\alpha - glucosidase\ Inhibition\ (\%) = \left(1 - \frac{p_i}{p_0}\right) x100 \qquad [2]$$

where ρi is the slope in the presence of inhibitor (hydrolysate) and ρo the slope obtained in the absence of inhibitor (control). Results are expressed as the α-glucosidase inhibitory percentage of each sample point.

## 2.7 Statistical analysis

Significant differences were calculated by Statgraphics 5.1 (Statgraphics Technologies, Inc., VA, USA) employing the multiple comparison test (Tukey's Test). Significant differences among samples were computed at level of confidence 95%. The values are presented as the mean of three replicates ± standard deviation.

# 3. RESULTS AND DISCUSSION

## 3.1 Effect of ultrasound pre-treatment on *T. molitor* protein

Ultrasound treatment provokes acoustic cavitation due to the interaction between the ultrasonic waves and the liquid and dissolved gas from the solution (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). This treatment by probe method has been described as able to modify the native structure of proteins (Mir, Riar, & Singh, 2019) by cleaving some interactions between aminoacids. Consequently, it helps to expose the hydrophobic residues of proteins (Tao et al., 2019) and would have an effect in releasing different bioactive peptides. In this case, surface hydrophobicity was affected by the energy input applied by the ultrasound treatment (Table 1). It can be observed that the application of ultrasound pulses increased the surface hydrophobicity of the *Tenebrio* protein in 40%, regardless the intensity of the treatment. The lack of correlation between surface hydrophobicity and intensity of the ultrasound treatment was also reported for fish protein hydrolysates. To this regard, Rivero-Pino et al. (2020a) reported a significant increase of surface hydrophobicity after the application of a threshold energy, whereas further treatment did not improve hydrophobicity significantly.

**Table 1**: Surface hydrophobicity of the ultrasonicated samples related to the energy input.

| Time (min) | Energy (J) | Hydrophobicity Index |
|---|---|---|
| **0** | 0 | 22.42 ± 0.84$^{a}$ |
| **15** | 17330 | 30.61 ± 0.33$^{b}$ |
| **30** | 36102 | 31.35 ± 0.91$^{b}$ |

*Values in table are presented as the mean of three replicates ± standard deviation. Different letters within the same column denotes significant differences among samples.

It is expected that short ultrasonication pre-treatments led to cleavage of some aminoacids and solubilisation improvement of the protein whereas, further duration of the ultrasound treatment could originate the formation of protein aggregates. This behaviour was observed for quinoa protein after a 35 min treatment with the same ultrasound probe, while no aggregates were observed for sonication treatments below 25 min (Mir et al., 2019).

The duration of the ultrasound pulses exerts an important effect in the protein substrate. Therefore, it is expectable that the potential biological properties of the hydrolysates resulting from sonicated protein may well be different. The intensity of the ultrasound treatment will determine the effect on the protein, highlighting the importance and adequate choice of the treatment to obtain the desired peptides.

## 3.2 Effect of ultrasound and enzymatic treatments on the curves of hydrolysis

The figure 2 shows the evolution of the degree of hydrolysis throughout the complete 7-h enzymatic treatment. Ultrasound pre-treatment (US15 and US30) did not modify the degree of hydrolysis curves. As an example, the progression curves for S0T7 samples that were subjected to ultrasound were depicted too. It can be observed that these curves are coincident, presenting only slight deviation attributable to the experimental error. At the beginning of the subtilisin stage, the rate of proteolysis increases rapidly and then flattens progressively as more peptidic bounds are exhausted for protein attack (Adler-Nissen, 1986). The addition of trypsin had an instant activation effect on the hydrolysis curves, which soon attached a steady value around DH 30%, regardless the enzyme sequence. A control treatment employing sole trypsin led to final DH ~21%.

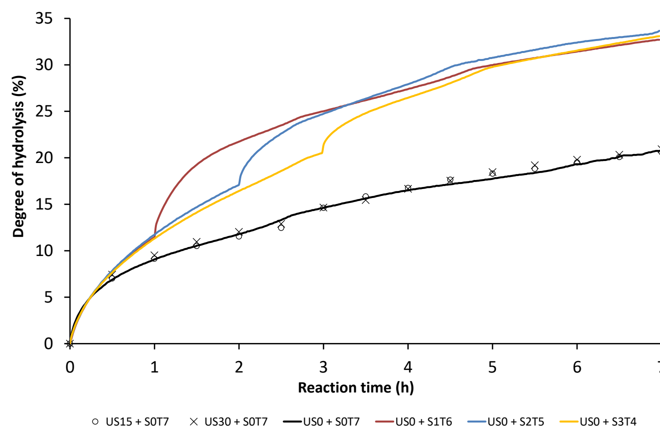


**Fig 2.** Evolution of the degree of hydrolysis during the sequential enzymatic treatment.

## 3.3 Study of the α-glucosidase inhibitory activity of the hydrolysates

### 3.3.1 Effect of the ultrasound pretreatment on α-glucosidase inhibition

Ultrasound treatment did not release α-glucosidase inhibitory peptides. The purpose of this physical treatment is to modify the structure of the native protein, which would have an effect on the later enzymatic hydrolysis to obtain bioactive peptides. Ultrasound as emerging technology is widely employed for the production of nutraceuticals because the cavitation and disrupting properties of ultrasound waves enhances bioactive compounds extraction (Galanakis, 2013).

It is observed that same-enzymatic-treatments with different ultrasound pre-treatments showed different α-glucosidase inhibition during the reaction (Figure 3), due to the differences in the starting protein structure. US15 led to protein modifications that helped to release high bioactive peptides in shorter time than US0 and US30 treatments in the S1T6 enzymatic hydrolysis.

When ultrasound treatment is applied, differences in the trypsin hydrolysis (S0T7) are observed (Figure 3). For US0+S0T7 and US15+S0T7 reactions, the peptides were not able to inhibit α-glucosidase during the course of the reaction. On the other hand, US30+S0T7 was able to inhibit the enzyme from its fifth hour (~50%), and a complete inhibition was obtained the following hour and conserved until the end of the reaction. This could be explained by the physical effect that ultrasound has on the protein structure, mainly cleaving weak bonds. The difference between US15 and US30 samples might well be due to the strength of the treatment itself, even though the increased hydrophobicity keeps the same, some key-cleavage sites could have been exposed.

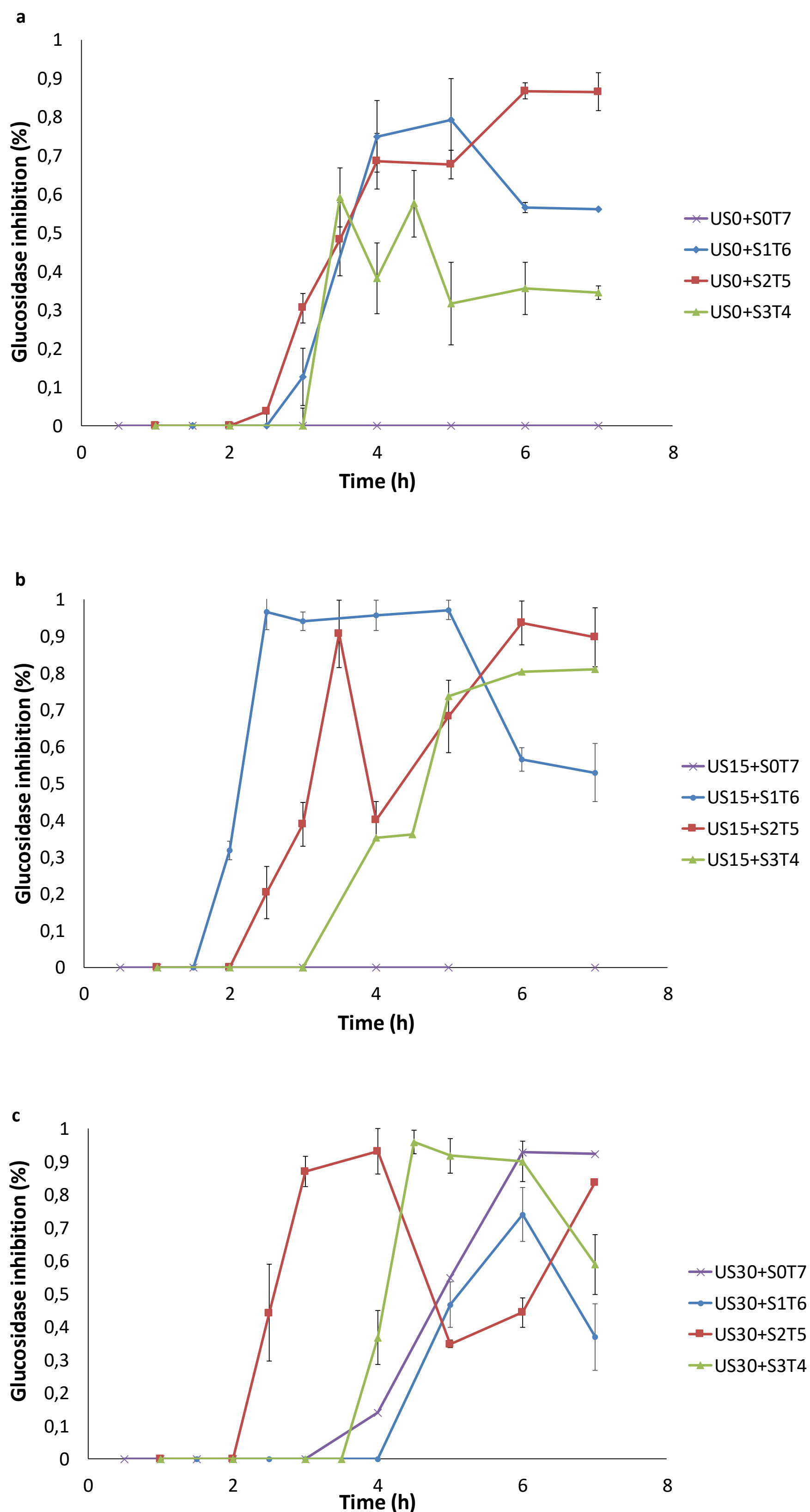


**Fig 3**. Progress curves for the α-glucosidase inhibitory activity during the enzymatic treatment after ultrasound pre-treatments US0 (a), US15 (b) and US30 (c).

Further analysis, such as circular dichroism experiments, to establish the type of bonds created should be carried out in order to unravel the mechanism involved in these reactions. However, based on literature, it is expected that secondary structure modifications occurred in the samples analysed. Different authors have reported molecular unfolding of the protein and exposure of functional groups after ultrasound, modifying the secondary structure by reducing α-helix and increasing β-sheet (Q. Wu, Zhang, Jia, Kuang, & Yang, 2018; Yang et al., 2017). Some authors have described how ultrasound pre-treatment improved the α-glucosidase (Jiang et al., 2018) or ACE inhibitory activity (J. Jia et al., 2010; Q. Wu et al., 2018) due to the change of spatial configuration and molecular structure of proteins by ultra-sonication.

## 3.3.2 Effect of the sequential subtilisin-trypsin treatment on α-glucosidase inhibition

### 3.3.2.1 Effect of subtilisin

In figure 3, the progress curves for α-glucosidase inhibition are presented. No inhibition was reported at the initial stages of reactions, where only subtilisin was employed as catalyst, even in the longest treatment (S3). During the hydrolysis of subtilisin-trypsin, a protein concentration of 15 g/L was chosen based on previous studies, in order to encompass the inhibitory activity of the hydrolysate after trypsin treatment, where significant differences could be observed at different reaction times when trypsin was included in the reaction.

The non-hydrolyzed protein is expected to have proteins whose molecular weight can vary from 14 kDa to 100 kDa (Azagoh et al., 2016), a range that cannot be encompassed by the FPLC analysis carried out for the hydrolysates. In those cases, the subtilisin samples showed an important increase of 2 kDa - 800 Da fraction as hydrolysis continues (Supplementary material). This small peptide-containing fraction seems adequate to be cleaved by trypsin in order to obtain α-glucosidase inhibitory peptides. Although when subtilisin does not release inhibitory peptides, it enhances the later releasing of α-glucosidase inhibitory peptides. The subtilisin reaction time would have an effect on the later bioactive peptide releasing, since the cleavage of the protein would be higher as the time is longer. However, it is observed that the longest subtilisin treatment S3 did not significantly enhance the bioactivity compared to the S1 and S2 in the US0 and US15 pre-treatments. The *time* parameter is important in order to optimize the energetic

resources employed during the peptides releasing. Nonetheless, different substrates, considering the amino acid content and protein sequences, and reaction conditions would have an important influence in the bioactive peptides production. For example, Ren et al. (2016) reported higher α-glucosidase inhibitory activity with subtilisin compared to trypsin for hemp seed protein hydrolysates, whereas Connolly et al. (2014) reported higher bioactivity with trypsin compared to subtilisin and other proteases for brewers' spent grain protein hydrolysates.

#### 3.3.2.2 Effect of trypsin

Overall, the α-glucosidase inhibition values were no affected by the degree of hydrolysis (Figure 2) obtained, as expected (Connolly et al., 2014; Ren et al., 2016), but did depended on the treatment employed. In the figure 3, it is observed that the α-glucosidase inhibition during trypsin hydrolysis highly depends on the previous ultrasound pre-treatment as well as the subtilisin reaction time. Trypsin has been described as an adequate protease to optimize the α-glucosidase inhibitory peptides, as previously reported by different authors (Connolly et al., 2014; Z. Yu et al., 2011). Ibrahim et al. (2017) published a review summarising the structural properties of α-glucosidase inhibitory peptides. They highlighted the importance of amino acids containing a hydroxyl or basic side chain at the N-terminal and of proline within the chain and alanine or methionine at the C-terminal, while the length of the peptide, its hydrophobicity and isoelectric point are not main factors.

Considering S1T6 samples, higher inhibition is achieved for US15 compared to US30 pretreatment. In the case of US15, a >85% of inhibition was reported after 1.5 h of trypsin, and maintained the following hours, whereas five hours of trypsin are needed in order to obtain adequate values of inhibition when US30 is employed. This could not be expected, from the idea that both pre-treatments enhance α-glucosidase inhibitory activity, but it might due to the aggregation of peptides originated that might have occurred after 30 minutes of ultrasound pre-treatment in the native protein, exposing different binding sites for the enzyme. In the US30 samples, one hour of subtilisin is not enough to break the protein before trypsin is able to release α-glucosidase inhibitory peptides faster, highly likely due to that protein aggregation. In the case of the US0 pre-treatment, one hour of subtilisin and two of trypsin were enough to obtain similar values for the US15. Consequently, increasing time of ultrasound with short subtilisin hydrolysis does not seem to enhance the α-glucosidase properties of peptides obtained.

However, when subtilisin time was increased to two hours (S2T5), α-glucosidase inhibitory activity of the hydrolysate is achieved faster when the pre-treatment with ultrasound was longer. For US0 and US15, 1.5 hours of trypsin were needed to achieve >50% inhibition of the enzyme, whereas for US30, 0.5 h of trypsin led to ~44% of inhibition, and was increased up to ~86% after extra half hour. The behaviour during the course of reaction gave different percentage of inhibition, no correlated with the degree of hydrolysis.

Finally, in the three hour subtilisin pre-treatment (S3T4), US0 sample achieved >50% after 0.5 h of trypsin, but during the course of reaction, the hydrolysate never surpasses >60% of inhibition. When ultrasound was applied, a complete inhibition of the enzyme is effectively achieved. In US15 sample, after 2 hours of trypsin, and in US30 treated, after 1.5 h.

One important factor is the reaction temperature, in this battery of reactions, constantly 50 ºC. Zhang et al. (1999) reported that trypsin proteolytic activity decreases more than its fifty percent from 47 ºC whereas subtilisin shows higher resistance against thermal degradation, since its maximal proteolytic activity is found between 50 and 60 ºC. Furthermore, trypsin is highly susceptible to loss of activity by autolysis. Similar behaviour by both enzymes was described previously (Espejo-Carpio et al., 2018; Pérez-Gálvez et al., 2016). Considering that a protein hydrolysate is composed of a mixture of different peptides, some of them bioactive, some of them not, it is expected that the hydrolysis of peptides due to subtilisin or trypsin changed the peptide profile obtained. Hence, the resulting peptides contained in the hydrolysate at different times can be further hydrolyzed, increasing or decreasing their bioactivity. This might explain why longer reaction times provoke loss of bioactivity in the hydrolysate, due to the degradation of active peptides by subtilisin. In these cases, the proportion of α-glucosidase peptides of the hydrolysate would decrease with respect to the total amount of peptides and the global bioactivity is worsened. Azagoh et al. (2016) reported a 5.4% of arginine content in *T. molitor* larvae meal, similar to the 5.9% found in pale brewers' spent grain (Connolly et al., 2013), a reported adequate source for α-glucosidase inhibitory peptides production (Connolly et al., 2014).

# 4. CONCLUSIONS

Mealworms are one of the edible insect employed nowadays in the food industry due to its protein high content and their environmentally friendly advantages. In this work, ultrasound pre-treatment was evaluated as a process to enhance the α-glucosidase inhibitory peptides during later enzymatic hydrolysis. The purpose was to achieve the shortest reaction time where the amount of bioactive peptides is high, considering industrial scale-up to produce a functional ingredient. The enzymatic hydrolysis encompassed subtilisin and trypsin as proteases with different specificity. Trypsin is crucial to obtain these bioactive peptides, but both ultrasound pre-treatment and the hydrolysis with subtilisin are important in the later releasing of α-glucosidase inhibitory peptides. Ultrasound pre-treatment modifies the native structure of the protein and subtilisin hydrolysis reduces the size of the peptide chain which favours the release of smaller active peptides. High levels of inhibition were obtained after pre-treatment with ultrasound for 15 minutes and one hour of subtilisin, and 1.5h of trypsin. These are promising results since the bioactivity of hydrolysate considering the effectiveness of different variables led to a valuable optimization of the process to obtain a bioactive hydrolysate suitable for its incorporation into foods.

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# VI. Identification of novel dipeptidyl peptidase IV and α-glucosidase inhibitory peptides from *Tenebrio molitor*[*]

The exponential increase in world population is leading to a need for new sustainable protein sources that could supply the high demands without resulting in an enormous environmental impact. Bioactive peptides from food proteins are currently seen as capable of modulating physiological processes, such as diabetes. The potential of insects as a cheap source of antidiabetic peptides is a recent research topic. In this work, fractionation and identification of dipeptidyl peptidase IV (DPP-IV) and α-glucosidase inhibitory peptides from mealworm (*Tenebrio molitor*) was carried out. Peptides from 500 to 1600 Da showed the highest level of DPP-IV inhibition ($IC_{50}$ value of 0.91 mg/ml) and peptides below 500 Da showed the highest level of α-glucosidase inhibition ($IC_{50}$ value of 2.58 mg/ml). Numerous novel peptides were identified from the most bioactive fractions, and based on the molecular features usually described for these peptides, some of them are suggested to be the bioactive peptides responsible for the inhibition observed (e.g. APVAH for DPP-IV inhibition and CSR for α-glucosidase inhibition). Hence, these insect protein hydrolysates or their purified fractions could be used as ingredients for regulation of the glycaemic index.

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# 1. INTRODUCTION

In recent years, the use of insects as ingredients in the food industry has attracted a notable interest. Insects are therefore a trending topic foodstuff in the Western society, although there is still reluctance concerning their consumption (Gravel & Doyen, 2020). They contain high levels of proteins, healthy fatty acids, micronutrients, and fibres (Azagoh et al., 2016), and their farming can be more economic and environmentally-friendly when compared to traditional livestock (Osimani et al., 2017; van Huis et al., 2013).

Based on their high protein content, insects can be employed to produce peptides with health-promoting properties (Nongonierma & FitzGerald, 2017). Bioactive peptides derived from food proteins are currently seen as potential functional ingredients due to their ability to modulate different physiological processes without side effects (Harnedy & FitzGerald, 2012). The transformation of insects into a powder after grounding, hydrolysis and drying, is a solution to include them as bioactive ingredients in human diets. In this sense, the enzymatic hydrolysis of proteins from insects as a method to obtain bioactive peptides has been reported in the literature (Chen, Jiang, Gan, Chen, & Huang, 2019; Dai, Ma, Luo, & Yin, 2013; Nongonierma, Lamoureux, & Fitzgerald, 2018; Zielińska, Karaś, Baraniak, & Jakubczyk, 2020).

The focus of this paper is on antidiabetic peptides from insects. Regarding other protein sources, the treatment of diabetes with milk and marine protein hydrolysates has been described already (Rivero-Pino, Espejo-Carpio, & Guadix, 2020a). Clinical trials have shown that the intake of milk and fish protein hydrolysate have beneficial consequences concerning different diabetes-related parameters, such as plasma and capillary glucose (Crowe et al., 2018; Saleh et al., 2018).

There are different mechanisms to modulate blood glucose level in humans. α-Glucosidase is responsible for hydrolysis of complex carbohydrates into absorbable monosaccharids in the intestine in the postprandial phase. Subsequently, blood glucose level would be regulated by the incretins, whose half-life depends on the enzyme dipeptidyl-peptidase IV (DPP-IV). It has been demonstrated that both inhibition of α-glucosidase and DPP-IV lead to an adequate regulation of the glucose level in the bloodstream. Recent literature explores the antidiabetic potential of insect peptides obtained by enzymatic hydrolysis, identifying DPP-IV and α-glucosidase inhibitory peptides (Dávalos Terán, Imai, Lacroix, Fogliano, & Udenigwe, 2019; Lacroix, Dávalos

Terán, Fogliano, & Wichers, 2019; Nongonierma et al., 2018; Rivero-Pino, Pérez Gálvez, Espejo-Carpio, & Guadix, 2020; Yoon, Wong, Chae, & Auh, 2019; Zielińska et al., 2020). However, further research concerning identification of these bioactive peptides is needed to obtain information about the potential of insects as a source for peptides to be employed in functional food for prevention of human diseases.

In this work, both DPP-IV and α-glucosidase inhibitory protein hydrolysates from *Tenebrio molitor* protein were produced and fractionated by size-exclusion chromatography. Then, the amino acid sequences of peptides were identified from the most active ones. Novel peptides with inhibitory activity against the enzymes related to development of diabetes were found.

# 2. MATERIALS AND METHODS

## 2.1 Materials

*Tenebrio molitor* meal was kindly donated by MealFood Europe (Salamanca, Spain). The *T. molitor* meal was ground to powder and stored at -16 ºC until used. The protein content was determined by the Kjeldahl method, assuming a nitrogen-to-protein factor of 5.6 (Janssen, Vincken, van den Broek, Lakemond, & Fogliano, 2017). Subtilisin, named as Alcalase 2.4L (EC 3.4.21.62), Trypsin, named as PTN 6.0 (EC 3.4.21.4) and Flavourzyme 1000L$^{TM}$ (3.4.11.1) were provided by Novozymes (Bagsvaerd, Denmark). The reagents employed for the analytical assays, including the enzymes and substrates for the enzymes inhibitory activity assays, were purchased from Sigma Aldrich (St. Louis, US).
The protein hydrolysate with high DPP-IV inhibitory activity was obtained according to the conditions reported by Rivero-Pino et al. (2020), where 20 protein hydrolysates obtained by enzymatic hydrolysis with four different food-grade proteases solely or in combination were analysed for different bioactivities, including DPP-IV inhibition. The lowest $IC_{50}$ value among all the samples was reported to be 2.62 mg/ml for the hydrolysate obtained employing Alcalase and Flavourzyme combined until reaching a degree of hydrolysis of 20%. The protein hydrolysate with high α-glucosidase inhibitory activity was obtained following the method described by Rivero-Pino et al. (2020). There, the kinetics of α-glucosidase inhibition was evaluated for the *T. molitor* protein subjected to different ultrasound pre-treatments (t = 0, 15 and 30 min) and different subtilisin and trypsin treatment times. The experimental design included 12 points in order to achieve the shortest reaction time where the amount of bioactive peptides is high. High levels of

bioactivity were obtained after 15 min of ultrasound pre-treatment followed by 1 h of subtilisin, and 1.5 h of trypsin. The inhibition reported was greater than 90% at 15 mg/mL of protein.

## 2.2 Fractionation by size-exclusion chromatography

The hydrolysates were fractionated by size exclusion chromatography using a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden) coupled to a Frac-902 collector. Aliquots of 500 µL (10 mg of protein/mL) were eluted at 0.5 mL/min with MiliQ water as mobile phase, and the absorbance was measured at 280 nm. For each analysis (protein content, mass spectrometry and enzyme inhibitory activity), 5 runs were carried out and pooled to obtain enough material of all fractions, which were freeze-dried before analysis. The mass calibration curve was prepared using the following standards: L-tyrosine (217.7 Da), vitamin B12 (1355 Da), aprotinin (6512 Da), cytochrome C (12,384 Da) and ribonuclease (13,700 Da).

## 2.3 Protein content of the fractions

Protein content of the fractions was determined using a Flash 2000 Organic elemental analyser (Thermo Scientific). Gases coming from combustion with oxygen at high temperature (1020 ºC), were transported through a gas chromatographic column to separate them and a thermal conductivity detector (TCD) detected the signal for each element, which is proportional to the concentration.

## 2.4 Enzymes inhibitory activity

The DPP-IV inhibition assay was performed as described by Lacroix and Li-Chan (2012) with slight modifications. Briefly, 25 µL of enzyme at 0.02 U/mL were mixed with 100 µL of samples at different concentrations and incubated for 10 min. Then, the reaction was started by adding 50 µL of 1 mM of Gly-Pro-p-nitroanilide and the absorbance at 405 nm was measured each 2 min for 120 min, employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland).

The α-glucosidase inhibition assay was performed mixing 20 µL of α-glucosidase at 0.4 U/ml with 120 µL of sample solution at different concentration in sodium phosphate buffer 20 mM containing 6.7 mM NaCl and adjusted to pH 6.94. The mixture was incubated 15 min at 37 ºC, then the reaction was started by adding 100 µl of 0.5 mM 4-

Nitrophenyl-β-D-glucopyranoside, and the amount of 4-nitrophenol released was monitored by measuring the absorbance at 405 nm each 30 sec for 60 min, employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland).

For both assays, each sample was analysed in triplicate. Then, the inhibitory activity of each sample was calculated by plotting the progress of reactions compared to the blank (distilled water) according to Eq. 1:

$$\text{Enzyme inhibition (\%)} = \left(1 - \frac{p_i}{p_0}\right) x100 \qquad [1]$$

where $p_i$ is the slope in the presence of inhibitor (hydrolysate) and $p_o$ is the slope obtained in the absence of inhibitor (control). Results are expressed as the half maximal inhibitory concentration ($IC_{50}$ value), as the concentration of hydrolysate which inhibits the enzyme activity by 50%.

## 2.5 Peptide identification and *in silico* analysis

The most active fractions collected from SEC were analysed employing an ACQUITY UHPLC system (Waters, Milford, CT, USA) coupled to a Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer. Samples of 10 µL were injected onto an ACQUITY BEH 300 C4 column 1.7 µm (Waters) and components were eluted using a flow rate of 0.3 mL/min of water–formic acid 0.1% (buffer A) and acetonitrile (buffer B) as described by Liu et al. (2015) The MS spectra were acquired in positive electrospray ionization mode using a capillary energy of 2.5 kV and sampling cone of 30 V. The analyses were performed using an m/z range from 50 to 1900. PepSeq program from BioLynx software (Micromass UK Ltd., Manchester, United Kingdom) was employed for *de novo* peptide sequencing. BLASTp analysis with "*Tenebrio molitor*" (Yellow mealworm beetle) [taxid 7067] was carried out in order to identify the protein where the peptides are contained.

Peptide Ranker software and CPPpred tool (http://distilldeep.ucd.ie) were employed to analyze the potential of the identified peptides to be bioactive and to be cell penetrating, respectively, by measuring a score from 0 to 1.

## 2.6 Statistical analysis

Significant differences were calculated by Statgraphics 5.1 (Statgraphics Technologies, Inc., VA, USA) employing the multiple comparison test (Tukey's Test). Significant differences among samples were computed at 95% confidence levels. The values are presented as the mean of three replicates ± standard deviation.

# 3. RESULTS AND DISCUSSION

## 3.1 DPP-IV inhibitory activity of the fractions

The protein hydrolysate obtained by simultaneous hydrolysis with subtilisin and Flavourzyme at DH of 20% had an $IC_{50}$ value of 2.62 ± 0.16 mg/mL. Subtilisin is able to cleave hydrophobic bonds (Adamson & Reynolds, 1996), breaking the protein structure in order to facilitate the action of Flavourzyme (an exopeptidase), releasing small peptides that are those usually related to inhibit the enzyme DPP-IV. Values in the same range as our result were obtained for other substrates such as seaweed *(Porphyra dioica)* protein (Cermeño et al., 2019), boarfish (*Capros aper*) protein (Parthsarathy et al., 2018) or cowpea (Castañeda-Pérez et al., 2019). The protein hydrolysate was fractionated by size-exclusion chromatography and the corresponding activity of peptides depending on their size was analysed (Figure 1). The most active fractions, C and D, showed a lower $IC_{50}$ value compared to the hydrolysate and consequently, the peptides in these fractions are the responsible for the high bioactivity of the hydrolysate.

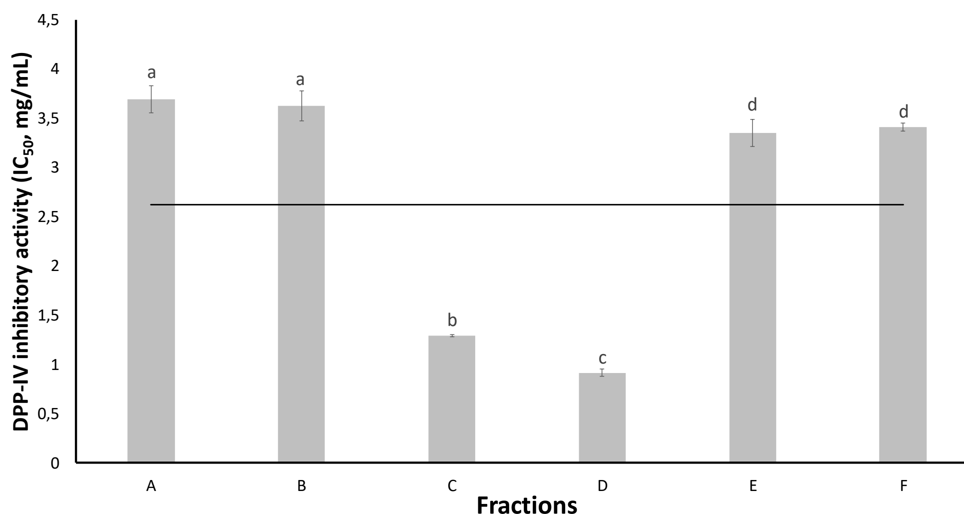



**Figure 1.** DPP-IV inhibitory activity of the SEC-fractions from the *T. molitor* protein hydrolysate obtained with Alcalase and Flavourzyme. The horizontal line represents the $IC_{50}$ value of the original hydrolysate (not fractionated). Values are presented as the mean of three replicates ± standard deviation. Different letters denotes significant differences among samples.

The high degree of hydrolysis favoured the presence of low molecular weight peptides (below 1600 Da), that are more likely to inhibit DPP-IV. In fact, the most active fraction was found to be fraction D, encompassing peptides from 500 to 1600 Da. The $IC_{50}$ value obtained was 0.91 ± 0.03 mg/mL, significantly lower than that reported for the original

hydrolysate (2.62 mg/mL). High molecular weight fractions (A and B), showed an $IC_{50}$ above the original hydrolysate.

Similar results were obtained for DPP-IV inhibitory peptides obtained from the hydrolysis of sardine muscle, where the most active ones were characterised to have a length from 4 to 9 amino acids (Rivero-Pino, Espejo-Carpio, & Guadix, 2020b). Additionally, Kang et al. (2020) reported $IC_{50}$ values of 0.79 – 0.93 mg/ml for the smallest peptide fractions of green crab protein hydrolysates obtained by different proteases solely, reaffirming the importance of the peptide size in the inhibition of the target enzyme. The highest DPP-IV inhibitory activity for low molecular weight peptides have been reported for salmon, whose fraction lower than 1 kDa showed an $IC_{50}$ value of 1.35 mg/mL (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012). This amino acid length characterisation is important in terms of scaling up the process, because membranes with the appropriate molecular weight cut-off can be selected to fractionate the bioactive peptides at large scale. In fact, as it is observed in Table 1, the highest protein concentration was found in the most active fractions (C and D). Overall, the peptides are concentrated in the range of 500-1600 Da, but it is clearly not concentrated in sole amino acids or large peptides, due to the enzymatic treatment applied.

**Table 1.** Protein concentration of the *SEC*-fractions from the DPP-IV inhibitory protein hydrolysate.

| Fraction (MW) | Protein concentration (mg/ml) |
|---|---|
| **A (>10 kDa)** | 0.09 |
| **B (10 – 3 kDa)** | 0.51 |
| **C (3 – 1.6 kDa)** | 1.21 |
| **D (1.6– 0.5 kDa)** | 1.31 |
| **E (0.5 – 0.3 kDa)** | 1.00 |
| **F (<0.3 kDa)** | 0.12 |

## 3.2 α-glucosidase inhibitory activity of fractions

The protein hydrolysate obtained by a sequential hydrolysis of subtilisin and trypsin during 1 h and 1.5 h respectively, after 15 min of ultrasound pretreatment, showed an $IC_{50}$ value of 6.52 ± 0.19 mg/mL. Ultrasound treatment increases the hydrophobicity of the sample so subtilisin is able to break more hydrophobic peptidic bonds and later, the narrow-specificity of trypsin releases highly active peptides due to their terminal residues, mainly expected to be arginine or lysine (Olsen, Ong, & Mann, 2004), that are molecular features for peptides described as α-glucosidase inhibitors (Ibrahim, Bester, Neitz, &

Gaspar, 2017). Our results are in line with those reported by Yoon at al. (2019) having a ~35% of inhibition at 2 mg/ml with an Alcalase-Flavourzyme mealworm larvae protein hydrolysate; however, our preliminary results reported that Alcalase-trypsin better increased the bioactivity of our substrate rather than the treatment with Flavourzyme.
These bioactive peptides were fractionated depending on their size, to verify the molecular weight of peptides responsible for the actual inhibitory activity. Results showed that peptides below 500 Da were the most active. This fraction E showed an $IC_{50}$ of 2.58 ± 0.09 mg/mL. The other fractions analyzed did not show any bioactivity even for the maximal concentrations analyzed (>6.5 mg/mL), showing that the bioactive peptides are those contained in the fraction E.
Our results are in line with previous literature, showing that low molecular weight peptides are those responsible for the bioactivity. In this regard, the highest α-glucosidase inhibition for the <1 kDa fraction have been also reported for a cowpea protein hydrolysate (Castañeda-Pérez et al., 2019) and for a silk cocoon protein hydrolysate (Lee et al., 2011). An $IC_{50}$ value of 2.08 mg/ml was reported for the smallest peptide fraction from almond protein extracted from an oil manufacture residue hydrolysed by Prote Ax (Gu, Gao, Hou, Li, & Fu, 2020). Regarding protein concentration, it is observed that fraction E, the most active, is not the most concentrated in protein content. This could indicate that the bioactive peptides contained, even when not highly represented in the hydrolysate, exert an enormous activity inhibiting the key enzyme.

**Table 2**. Protein concentration of the *SEC*-fractions from the α-glucosidase inhibitory protein hydrolysate.

| **Fraction (MW)** | **Protein concentration (mg/ml)** |
|---|---|
| **A (>10 kDa)** | 0.30 |
| **B (10 – 3 kDa)** | 1.15 |
| **C (3 – 1.6 kDa)** | 0.52 |
| **D (1.6 – 0.5 kDa)** | 1.34 |
| **E (< 0.5 kDa)** | 0.58 |

## 3.3 Identification of DPP-IV inhibitory peptides

The most active DPP-IV inhibitory fraction obtained from size exclusion chromatography was analysed by mass spectrometry to identify bioactive peptides (Supplementary material). The identified peptides are shown in the Table 3, with the corresponding parent proteins and the scores obtained from the *in silico* analysis. The mass spectra and the

molecular features of the sequences identified suggest that these peptides are indeed contained in the protein hydrolysate and they would exert the bioactivity reported.

**Table 3**. Peptide sequences identified by UHPLC–MS/MS in the most active fraction obtained by chromatography from the DPP-IV inhibitory hydrolysate.

| Peptide | Parent protein | Potential to be bioactive** | Potential to be cell penetrating*** |
|---|---|---|---|
| **APVAH** | Cuticular protein | 0.220 | 0.174 |
| **AVTTK** | Tenebrin | 0.042 | 0.611 |
| **AAGAPP** | Carboxylesterase CXE18 | 0.615 | 0.187 |
| **SLAPK** | Cytochrome c oxidase subunit I | 0.326 | 0.325 |
| **VHCSE** | Peptide transporter 1 | 0.097 | 0.056 |
| **PALLL** | Vitellogenin precursor* | 0.692 | 0.589 |
| **PAALST** | Larval cuticle protein A1A* | 0.186 | 0.212 |

*BLASTp analysis gave a percentage of positive coincidences higher than 80%. The lack of characterization of *T. molitor* proteins can be a reason of not being 100%. **Score obtained with PeptideRanker ***Score obtained with CPPpred

The peptide APVAH, widely found in the cuticular protein based on the BLASTp analysis (number of matches = 7, sequence ID CAA03880.1), was expected to be one of the most DPP-IV inhibitory peptides contained in the hydrolysate. Its molecular features (low molecular weight and the amino acid sequence of the peptide) make it an adequate bioactive peptide, even when the *in silico* analysis did not offer a promising result in its likelihood to be bioactive. This peptide was found in fraction D, where the highest protein concentration was reported. This information is important because the production process can be optimized by separating the proteins based on different physicochemical properties prior to the proteolysis. Dávalos Terán et al. (2019) reported higher bioactivity for cuticular proteins compared to non-cuticular proteins. Furthermore, concentration with membranes would increase the quantity of bioactive peptides contained in our sample.

Some other peptides that could be associated to a parent protein, found in the bioactive fraction, were AAGAPP, SLAPK and VHCSE. However, since the *T. molitor* proteome is not fully characterised (*Tenebrio molitor* (Yellow mealworm beetle) Uniprot proteome includes 26 reviewed but 588 non-reviewed sequences), some of the peptides found could not be completely attributed to a protein. One such example is PALLL (Figure 2a) or PAALST, that show that indeed the peptide is contained in the fraction but the BLASTp analysis found protein sequences similar but not identical to it. This remains as one of the

main challenges in the field of bioactive peptides in little studied protein sources, such as insects. In the same line, some peptides were identified in the fraction but could not be attributed to any protein, or close to one. Some peptides whose N-terminal starts with AP or AV, followed by some other amino acid have been already described, but to the authors' knowledge, not exactly the same sequences of these *T. molitor* peptides (APVAH and AVTTK) have been identified (Liu, Cheng, & Wu, 2019). Also, the peptide PAL have been described as DPP-IV inhibitor (Ji, Zhang, & Ji, 2017), but not the peptide PALLL. The score obtained with Peptide Ranker positions it as a powerful candidate (Score = 0.692 and 0.589 for being bioactive and cell-penetrating respectively). Further characterization of the substrate should be carried out, as well as verification with synthetic peptides, in order to clearly evidence the bioactivity of *T. molitor* peptides.

## 3.4 Identification of α-glucosidase inhibitory peptides

The most active α-glucosidase inhibitory fraction obtained from the size exclusion chromatography (fraction E) was analysed by mass spectrometry to identify peptides responsible for the bioactivity (Supplementary material). The peptides identified and their corresponding parent proteins are shown in Table 4, with the scores obtained from the *in silico* analysis. The mass spectra and the molecular features of the sequences identified suggest that indeed these peptides are contained in the protein hydrolysate and they would exert the bioactivity reported.

**Table 4.** Peptide sequences identified by UHPLC–MS/MS in the most active fraction obtained by chromatography from the α-glucosidase inhibitory hydrolysate

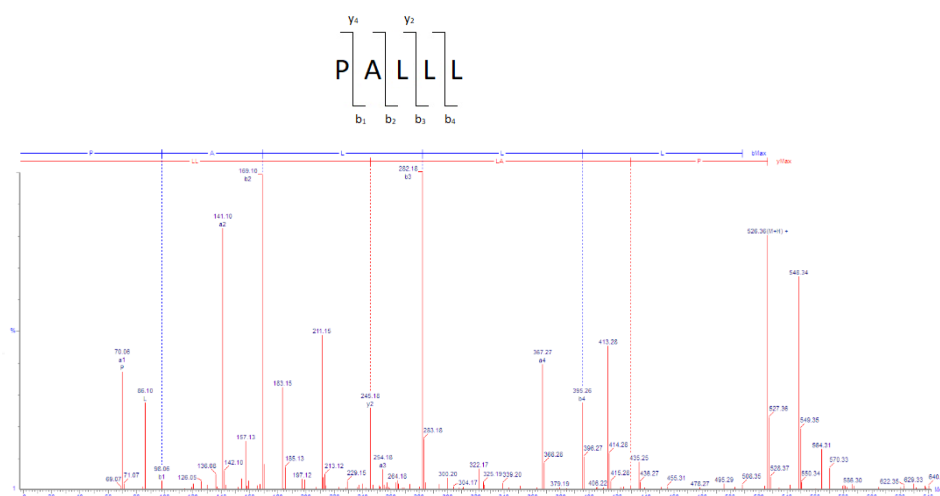
| Peptide | Parent protein | Potential to be bioactive* | Potential to be cell-penetrating** |
|---|---|---|---|
| **AR** | Numerous proteins | 0.395 | 0.860 |
| **CSR** | Aminopeptidase<br>Thermal hysteresis protein isoform<br>C1 family cathepsin L1<br>Dopa decarboxylase<br>Vitellogenin | 0.564 | 0.331 |
| **ATAL** | Tenebrin<br>Serine proteinase<br>Odorant binding protein | 0.163 | 0.480 |
| **RVGS** | Hexamerin 1 | 0.113 | 0.347 |
| **AGGP** | alpha-mannosidase | 0.786 | 0.128 |

| **APYF** | chemosensory ionotropic receptor IR1 | 0.949 | 0.060 |
|---|---|---|---|

*Score obtained with PeptideRanker **Score obtained with CPPpred

The peptide CSR (364.4 Da), coming from at least 5 different *T. molitor* proteins possess an arginine residue (Figure 2b), making it adequate to inhibit the target enzyme based on the already described peptides in the literature (Ibrahim et al., 2017). The molecular feature of this peptide is similar to the one already describe for peptides below <500 Da from silkworm pupae: QPGR, SQSPA, QPPT and NSPR (Zhang et al., 2016). The trypsin hydrolysis release peptides with arginine or lysine at the peptide terminal, which are prone to inhibit α-glucosidase, as stated for the peptide RVPSLM (Yu et al., 2011) but also the presence of N-terminal asparagine seems important to inhibit the enzyme, as stated for the peptide NVLQPS (Yu et al., 2011) and NYVADGLG (Zielińska et al., 2020). Equally, the peptide AR is found in many proteins from the substrate, including cuticle protein LPCP-23, and consequently, it is expected that this dipeptide is responsible for the high α-glucosidase inhibitory of the protein fraction.

(a)



(b)

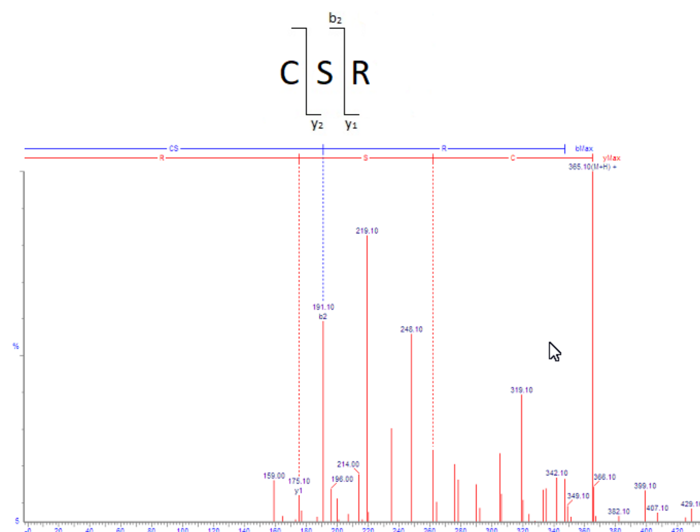



**Figure 2**. Sequence profile of two of the peptides identified from *T. molitor*. a) PALLL, as DPP-IV inhibitor b) CSR, as α-glucosidase inhibitor.

Some α-glucosidase inhibitory peptides derived from the *in vitro* digestion of *T. molitor* have been previously identified: NYVADGLG, AAAPVAVAK, YDDGSYKPH AGDDAPR (Zielińska et al., 2020). Also <5 amino acids long peptides have been described as α-glucosidase inhibitory peptides, GEY, GYG, LR and PLMLP (Lee et al., 2011; Ren et al., 2016). It is interesting to note how the peptide APYF was identified in the α-glucosidase hydrolysate, but it can be highly expected that it also possess DPP-IV inhibitory capacity, due to the presence of proline in its sequence. The score obtained in the *in silico* analysis situates this peptide as highly bioactive (Score = 0.949), but not cell penetrating. However, *in situ* analysis with cell culture would be a better approach to characterize the bioavailability. In the same line, the peptide AGGP might exert DPP-IV inhibitory activity based on the similarity with the peptide GMAGGPPLL, containing the peptide mentioned above. The identification of the peptides in the most active fractions, along with the discussed molecular features suggest that these peptides are potentially α-glucosidase inhibitors. To the authors' knowledge, these identified peptides have not been described before as bioactive in terms of regulating the glycaemic index, showing their potential to be exploited in the food industry. However, as stated for the DPP-IV inhibitory peptides identified, verification with synthetic peptides is needed in order to clearly evidence the bioactivity of *T. molitor* peptides. Furthermore, considering the scaling-up of this process, purification by membranes able to concentrate these small peptides would be adequate to produce a functional ingredient from insects.

# 4. CONCLUSIONS

Mealworms are a promising source of nutrients due to their high content in proteins. The enzymatic hydrolysis of protein from such insects, transforming them into a powder that can be used as a functional ingredient, is an alternative to overcome the reluctance that people might have to their consumption. The fractions obtained from two reported antidiabetic protein hydrolysates, with DPP-IV and α-glucosidase inhibitory activity, showed that low molecular weight peptides from *T. molitor* are adequate to regulate these carbohydrate-digestion-related enzymes. Peptides from 500 to 1600 showed a DPP-IV inhibitory $IC_{50}$ value of 0.91 ± 0.03 mg/ml, where some peptides with adequate molecular features to be bioactive were identified (APVAH and AAGAPP). Peptides below 500 Da showed a α-glucosidase inhibitory $IC_{50}$ value of of 2.58 ± 0.09 mg/mL, where some peptides with adequate molecular features to be bioactive were identified (AR, CSR). We conclude that these novel *Tenebrio molitor* peptides produced with subtilisin and trypsin or Flavourzyme, respectively, may be potential ingredients in functional foods intended for the regulation of diabetes.

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# VII. Evaluation of the antidiabetic potential of a vegetable soup fortified with *Tenebrio molitor* protein hydrolysates*

Bioactive peptides obtained from food proteins can be employed to prevent or pre-treat diabetes. These molecules can obtained from insects, a novel protein source whose interest is exponentially increasing these days, but whose potential as bioactive ingredient is scarcely studied. In this work, a commercial vegetable soup was fortified with antidiabetic (dipeptidyl peptidase IV and α-glucosidase inhibitory protein hydrolysates) Tenebrio molitor peptides and subjected to a thermal treatment (121ºC for 21 min). These sterilised samples were subsequently stored for 40 days to evaluate the influence of storage on the bioactivity. Furthermore, bioactivity after simulated gastrointestinal digestion in water and in the vegetable soup was evaluated. The inclusion of the peptides in the vegetable soup helped to maintain the bioactivity, and even increased it. Hence, these insect protein hydrolysates could be used as bioactive ingredients fortifying liquid matrices as vegetable soups for regulation of the glycaemic index.

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# 1. INTRODUCTION

The 2020s are highly likely going to put insects into human diets, overcoming little by little the reluctance concerning their consumption. They contain high levels of proteins, unsaturated fatty acids, micronutrients, and fibres (Azagoh et al., 2016), and their farming is more economic and environmentally-friendly compared to traditional food sources production (Osimani et al., 2017; van Huis et al., 2013). At European level, companies that produce insect-based foods can apply for an authorisation to produce and sell their products (European Council Regulations No. 2015/2283, 2015). In fact, the European Food Safety Authority (EFSA) has recently published that the consumption of mealworm (*Tenebrio molitor* larva) by humans either as a 'snack' or as an ingredient for other foods does not pose safety problems, although it can cause allergic reactions. The opinion issued is the first one since the regulation on novel foods in the EU came into force in January 2018.

Insect protein can be employed to obtain health-promoting peptides through enzymatic hydrolysis able to interact with endogenous human molecules without having side effects (Nongonierma and FitzGerald, 2017). These protein hydrolysates containing bioactive peptides appear as a powder, with could be used to produce fortified foods with a claimed bioactivity. Numerous articles have been recently published regarding the potential of different insects as source of bioactive peptides, specifically with antidiabetic activity (Dávalos Terán et al., 2019; Lacroix et al., 2019; Nongonierma et al., 2018; Rivero-Pino et al., 2020c; Yoon et al., 2019; Zielińska et al., 2020).

Recently, the partial replacement of meat by superworm in cooked sausages (Scholliers et al., 2020) or the effect of mealworm protein fortification in bread on different functional parameters (Roncolini et al., 2019) have been evaluated, offering results that project the use of insects as ingredients for human consumption. However, scarce information can be found about the fortification of food products with protein hydrolysates and storage, analyzing the bioactivity remaining. Antioxidant, antihypertensive (Rivero-Pino et al., 2020a), and antidiabetic (Harnedy-Rothwell et al., 2021) protein hydrolysates have been incorporated in different liquid food matrices, reporting promising results of conserved or improved bioactivity during storage and after simulated gastrointestinal digestion. Karimi et al. (2021) evaluated how a maize herm protein hydrolysate would enhance the antioxidant potential of bread before and after digestion and the inhibition of α-amylase during digestion. High-fiber food matrices seem

a suitable vehicle to carry bioactive peptides given the little possibility to chemical interactions to occur and because it would avoid the bitter taste of peptides, improving the sensorial acceptability (Sun et al., 2020; Ten Have et al., 2015). However, further research, preferably with sustainable sources such as insects, is needed in order to provide evidences to the food technology research field, to impulse their employment in food industry for human consumption to prevent the apparition of some diseases.

There are different human enzymes related to the digestive process (i.e. α-glucosidase and DPP-IV) whose inhibition has been described to lead to an adequate regulation of the glucose level in the bloodstream. Hence, a food-derived peptide is considered as bioactive with antidiabetic activity if it inhibits one or more than one enzyme related to the digestion.

The focus of this paper is on fortifying a simple liquid food matrix (vegetable soup) with antidiabetic peptides from insects. In this sense, the effect of sterilization over the peptides contained in a vegetable soup and the consequent refrigerated storage during 40 days was evaluated concerning the antidiabetic activity these peptides have proved to exert. Furthermore, the resistance of the bioactive peptides to in vitro gastrointestinal digestion in water and in the vegetable soup was evaluated to determinate the potential of employing these peptides for oral administration.

# 2. MATERIALS AND METHODS

## 2.1 Materials

The Tenebrio molitor protein hydrolysates employed for this work were reported previously by the authors as potent DPP-IV and α-glucosidase inhibitors (Rivero-Pino et al., 2021). The reagents employed for the analytical assays, were purchased from Sigma Aldrich (St. Louis, US). The vegetable soup was bought in a local market.

## 2.2 Enzymes inhibitory activity

The DPP-IV inhibition assay was performed as described by (Lacroix and Li-Chan, 2012) with slight modifications. Briefly, 25 µL of DPP-IV at 0.02 U/mL were mixed with 100 µL of the protein hydrolysate at different concentrations and incubated for 10 min. The reaction was started by adding 50 µL of 1 mM of Gly-Pro-p-nitroanilide and the absorbance at 405 nm was measured each 2 min for 120 min, employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland).

The α-glucosidase inhibition assay was performed mixing 20 µL of α-glucosidase at 0.4 U/ml with 120 µL of sample solution at different concentration in sodium phosphate buffer 20 mM containing 6.7 mM NaCl and adjusted to pH 6.94. The mixture was incubated 15 min at 37 ºC, then the reaction was started by adding 100 µl of 0.5 mM 4-Nitrophenyl-β-D-glucopyranoside, and the amount of 4-nitrophenol released was monitored by measuring the absorbance at 405 nm each 30 sec for 60 min, employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland).

For both assays, each sample was analysed in triplicate. Then, the inhibitory activity of each sample was calculated by plotting the progress of reactions compared to the blank (distilled water) according to Eq. 1:

$$\text{Enzyme inhibition (\%)} = \left(1 - \frac{p_i}{p_o}\right) x100 \qquad [1]$$

where $p_i$ is the slope in the presence of inhibitor (hydrolysate) and $p_o$ is the slope obtained in the absence of inhibitor (control). Results are expressed as the half maximal inhibitory concentration ($IC_{50}$ value), as the concentration of hydrolysate which inhibits the enzyme activity by 50%.

## 2.3 Inclusion in vegetable soup and storage study

As a commercial-like product, freeze-dried *Tenebrio molitor* protein hydrolysates were directly diluted at 30 mg/mL of protein in a commercial vegetable soup. The composition of vegetable soup as described from manufacturer is water, vegetables 10.6% (celery, carrot, spinach, leek, mushroom, onion, and tomato), salt, corn starch, spices, olive oil, natural flavours. According to the manufacturer, protein, fibre, and carbohydrates content is <0.5g/250 mL, and saturated fat content is 0.4 g/250 mL.

The percentage of solids of the vegetable soup was obtained by freeze-drying a known volume of the sample and measuring the solids obtained. The protein content was analysed by organic elemental analysis (Flash 2000, Thermo Scientific). For that purpose, the lyophilised vegetable soup was oxidized with pure oxygen at 1020 ºC, and the combustion products were transported by helium onto a chromatographic column to separate them and finally detected by a thermal conductivity detector (TCD) that provides a signal (mV/s) proportional to the concentration of each of the individual components of the mixture. Sulphanilamide was employed for calibration. Nitrogen-to-protein conversion factor was considered 4.4 (Mariotti et al., 2008). Fat content of the samples

was determined gravimetrically as previously described with slight modification (Drusch et al., 2012) after extraction with a solvent mixture hexane/isopropanol (1:1; v:v). Bioactivity of the enriched vegetable soup was evaluated before and after a stabilization process by sterilization (121 °C and 20 min) as described in section 2.3. Dilutions at 0, 25, 50 and 100% of vegetable soup were considered, being 0% the hydrolysate diluted in distilled water, as control. In order to assess the stability of bioactive properties of the peptides included in the vegetable soup during storage, sterilized samples were kept at 4 °C for 40 days and the bioactivity analysed at days 5, 10, 20, 30 and 40.

## 2.4 Simulated gastrointestinal digestion

A simplified digestion method using only proteases was employed (Garrett et al., 1999) in order to determine the remaining bioactivity of peptides after digestion process, since bioactivity would depend on protein-derived peptides. The hydrolysates were diluted in distilled water or vegetable soup at 30 mg/mL of protein and the pH was set to 2 with 1 M HCl. Gastric phase was started by adding pepsin at an E/S ratio of 4% (w/w, on protein basis) and the reaction occurred during 1 h at 37 °C at 300 rpm in a temperature-controlled shaker (Heidolph, Germany). After that, the pH was raised to 5.3 with a solution of 0.9 M NaHCO3, pancreatin was added at an E/S ratio of 4% (w/w, on protein basis) and the pH was raised to 7.5 with 1 M NaOH. This intestinal phase was maintained for 2 more hours in the shaker with the same conditions. Finally, the enzymes were thermally deactivated (90 °C for 5 min) and digested samples were freeze-dried and stored until analysis. As control, the native proteins and the lyophilized vegetable soup were also digested in the same conditions as the protein hydrolysates and the remaining bioactivity was analysed. All the analyses were carried out in triplicate.

## 2.5 Molecular weight distribution by size-exclusion chromatography

The molecular weight distributions of the processed sample were analyzed by size exclusion chromatography using a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with a Superdex Peptide 10/300GL column (GE Health- care, Uppsala, Sweden). Aliquots of 500 µL (5 mg of protein/mL) were eluted at 0.5 mL/min with a mobile phase composed of water/acetonitrile (70:30) with 0.1% trifluoroacetic acid (TFA). The absorbance was measured at 280 nm. The mass calibration line was prepared using the following standards: glycine (75 Da), L-tyrosine

(217.7 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6512 Da) and cytochrome C (12,384 Da).

## 2.6 Statistical analysis

Significant differences were calculated by Statgraphics 5.1 (Statgraphics Technologies, Inc., VA, USA) employing the multiple comparison test (Tukey's Test). Significant differences among samples were computed at 95% confidence levels. The values are presented as the mean of three replicates ± standard deviation.

# 3. RESULTS AND DISCUSSION

## 3.1 Inclusion in vegetable soup

Heat treatments are commonly employed in the food industry for different purposes, such as destroying or reducing microbial activity or modifying food structure, for example, producing Maillard compounds. The heat treatments might modify proteins and peptides, leading to denaturation or aggregation, depending on the sample treated and the treatment itself (temperature applied and time of application) (López-Sánchez et al., 2016).

The vegetable soup was chosen as a basic familiar product commonly employed in Western societies. Vegetable soup would mask the possible taste of bitter peptides, although sensory analyses reported in literature do not indicate unpleasant tastes in insect-based products. For Tenebrio molitor, different flavours have been described, such as: nutty, umami, aroma of cereal, wood, aroma of broth, flavour of vegetables and Maillard reaction products (Elhassan et al., 2019). In addition, the vegetable soup contained 0.7 g of salt / 100 mL which could help to suppress the bitterness that characterizes the protein hydrolysates by decreasing hydrophobic interactions (Xu et al., 2019).

In addition, its nutritional value in terms of lipids and sugars makes it suitable for a matrix containing antidiabetic ingredients. The pH value of the vegetable soup was 6.22 and the percentage in solids 2%. The powder obtained after freeze-drying the vegetable soup contained: 7.8% of protein and 7.6% of fat.

The protein hydrolysate obtained by simultaneous hydrolysis with subtilisin and Flavourzyme at DH of 20% had an $IC_{50}$ value of 2.62 ± 0.16 mg/ml, for DPP-IV inhibition (Rivero-Pino et al., 2021). The vegetable soup analysed directly in the DPP-IV inhibition assay had a 40% of this enzyme inhibition, showing that this food matrix is adequate for

glycaemic index management and could exert synergy with the enzymatically obtained peptides from insect protein.

Table 1 shows the influence over the bioactivity exerted by the proportion of vegetable soup in where the bioactive peptides are included, which allows to determine if the matrix itself has bioactivity, as well as the possibility of interactions to occur, after a heat treatment (121 ºC during 21 min). As control, heat treatment applied with only water decreased the DPP-IV inhibitory activity by ~48%, having an $IC_{50}$ value of 5.07 mg/ml. This loss of bioactivity, even when being statistically significant, it is still in the same order, and it is comparable to values obtained for hydrolysates from different origin without having being subjected to any heat treatment. For instance, hemp hydrolysed with Corolase for 1 h, whose $IC_{50}$ value ranged from 2.93 to 5.71 mg/ml (Nongonierma and FitzGerald, 2015). A loss of DPP-IV inhibitory activity after 1 hour of heat treatment at 120 ºC was also observed by (López-Sánchez et al., 2016) in a amaranth protein hydrolysate, in line with the results reported for our sample.

It is observed that the presence of vegetable soup helps to conserve the bioactivity. As higher proportion of soup is added, up to one, the $IC_{50}$ values decreases until being statistically equal to the control. In these cases, it is suggested that the vegetable soup is either contributing to the bioactivity and/or helping to preserve the chemical structure of the bioactive peptides exerting the bioactivity. Our results are in line with those obtained by (Harnedy-Rothwell et al., 2021) concerning the defined bioactivity of the selected matrix (tomato-based products) and the synergy with the peptides, that led to some kind of modification in the overall bioactivity reported for the product. However, these authors reported maintenance of the bioactivity after heat treatments, but those were less aggressive (90 °C during 1 min and 121 °C during 42 s) than the other exerted to our peptides (121 ºC during 21 min).

**Table 1**: Influence of vegetable soup dilution on the bioactivity of peptides after sterilisation

| Vegetable soup : Distilled water (v/v) | DPP-IV inhibition (mg/ml) | α-glucosidase inhibition (mg/ml) |
|---|---|---|
| **Control (no sterilised)** | 2.62 ± 0.16[a] | 6.52 ± 0.19[a] |
| **1:0** | 2.86 ± 0.22[a] | 12.64 ± 0.92[b] |
| **0.5 : 0.5** | 3.64 ± 0.25[b] | 12.87 ± 0.58[b] |
| **0.25 : 0.75** | 3.91 ± 0.08[b] | 12.41 ± 0.74[b] |
| **0 : 1** | 5.07 ± 0.24[c] | >15[c] |

Values are presented as the mean of three replicates ± standard deviation. Different letters in the same column denotes significant differences among samples.

The protein hydrolysate obtained by ultrasound pre-treatment, and sequential hydrolysis of Subtilisin and Trypsin had an $IC_{50}$ value of 6.52 ± 0.19 mg/mL, for glucosidase inhibition (Rivero-Pino et al., 2021). After sterilisation in distilled water, no inhibition could be reported at the highest concentration analysed (>15 mg/ml). This is highly likely due to the aggregation of peptides, not allowing them to access the key enzyme. However, when the vegetable soup, regardless of its proportion, was considered as the vehicle containing the α-glucosidase inhibitory peptides, the remaining bioactivity even when decreased, was not lost at all and was equal for all cases. These results strengthen the hypothesis that the structure of peptides is better conserved thanks to the vegetable matrix, highly likely due to the presence of fibres, since the vegetable soup showed not α-glucosidase inhibitory activity as it did for DPP-IV inhibition. To the author's knowledge, there are not available reports on literature concerning how heat treatments might modify the bioactivity of α-glucosidase inhibitory peptides. Despite the fact that these results seem promising, more studies with different food matrices should be carried out, as well as the analysis of how non-thermal treatments would affect the DPP-IV and α-glucosidase inhibitory activity of peptides because both because both physical and chemical modifications are important in determining the structure and consequently, the function of these bioactive molecules. The employment of enriched products with bioactive ingredients seems like an adequate to prevent and/or pre-treat the development of diabetes in humans. Furthermore, at economical level, the cost savings derived from pre-treat diseases with functional food compared to medical healthcare is also evident (Li et al., 2010).

## 3.2 Storage study

The maintenance of the peptides bioactivity after incorporation into food matrices and the stability during the storage as a commercial-product-like is key to declare them as functional ingredient. In consequence, a liquid matrix was considered as vehicle for these peptides, that have been proved to increase the resistance to thermal treatment, as well as being a material that could mask the bitterness that characterize these peptides.

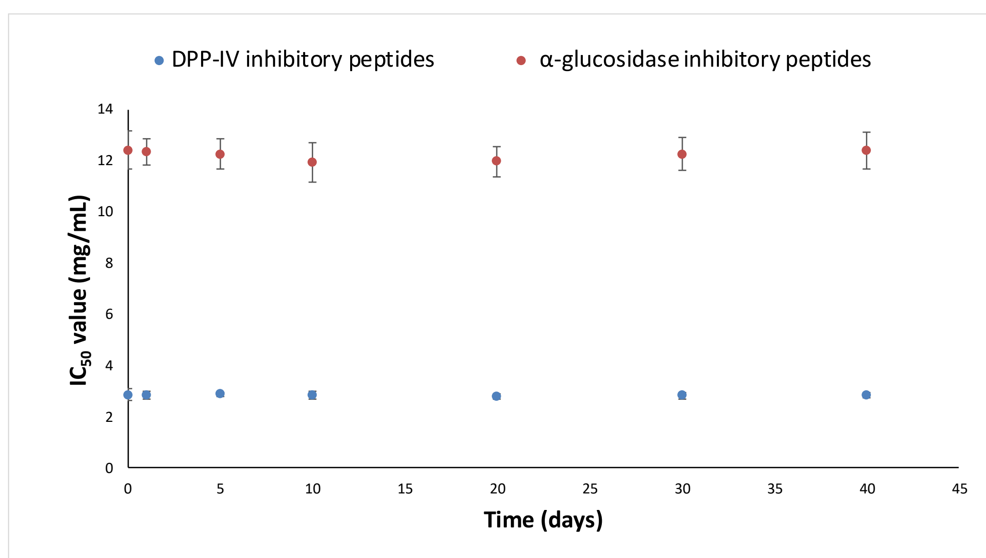


**Figure 1**: $IC_{50}$ values for enzymes inhibition in vegetable soup measured over 40 days. Red dots: for α-glucosidase inhibitory peptides. Blue dots: for DPP-IV inhibitory peptides.

In Figure 1, the evolution of the bioactivity in terms of $IC_{50}$ values of the DPP-IV inhibitory peptides is depicted (blue dots). The results showed that the bioactivity remained without modification after forty days of storage. The chemical structure of peptides contained in the food matrix seems not to change over this period. To the author's knowledge, the only report of DPP-IV inhibitory activity by food-derived peptides analysed after thermal treatment and refrigerated storage is the one from (Harnedy-Rothwell et al., 2021), employing a boarfish protein hydrolysate. These authors reported similar results than ours, confirming the adequacy of vegetable-based soup to carry antidiabetic peptides as a fortified food to be consumed by humans to prevent diabetes.

Concerning the storage of α-glucosidase inhibitory peptides, no modification of bioactivity during 40 days was observed (Figure 1 – red dots) confirming the feasibility of these hydrolysates to be employed in food industry in a commercial-like product considering a medium-term cold storage. This behaviour of maintaining bioactivity during a storage period has already been described for other types of bioactive peptides, as previously indicated with DPP-IV inhibitors, in addition to antihypertensive or antioxidant peptides (Rivero-Pino et al., 2020a). Similarly, Contreras et al. (2011) reported antihypertensive peptides' resistance to atomization, homogenization and pasteurization, plus their retained bioactivity after incorporation into liquid yoghurt. Overall, available literature supports the hypothesis that these peptides do not suffer

modifications during refrigerated storage at the short-medium term. This is the first report proving that α-glucosidase inhibitory activity of peptides is conserved during refrigerated storage. These results prove that functional beverages could be produced by fortifying these basic products with protein hydrolysates coming from sustainable sources, increasing their commercial value and health-promoting properties.

These results suggest that the resistance of the DPP-IV and α-glucosidase inhibitory activity to thermal treatment of the insect-derived peptides as hydrolysates is dependent on the vegetable soup proportion of the matrix, but not time-dependant on refrigeration at 4 °C during at least forty days, which is unquestionably promising for its application as food ingredient. However, the food matrix composition should be thoroughly investigated, since chemical interactions among molecules might lead to bioactivity changes (Capuano et al., 2017). In addition, the storage temperature and time might enhance reactions (i.e. at room temperature or longer periods of storage). Hence, further studies concerning structural compounds formed would be an issue of interest.

## 3.3 Simulated gastrointestinal digestion

Food-derived peptides are intended to be orally administrated for human consumption. In this sense, resistance of peptides to gastrointestinal digestion was evaluated in order to elucidate if peptides remain intact or their hydrolysis by pepsin and pancreatin releases more or less active peptides. Table 2 shows the bioactivity of both hydrolysates after being gastro-intestinally digested, considering water and the vegetable soup as the food matrix, and also the bioactivity that showed the substrate subjected to the SGID (digested protein).

In the DPP-IV inhibitory protein hydrolysate, the bioactivity is compromised after digestion in water, losing ~40% of the inhibition exerted. This is highly likely due to the specific cleavage exerted by pepsin and pancreatin (aromatic, aliphatic, and more precisely, arginine and lysine amino acids), since the peptides released after the hydrolysis would have molecular features not as adequate to inhibit DPP-IV as the original peptides contained in the sample.

However, when the digestion occurs in the vegetable soup, the bioactivity is not significant different from the control. Different hypothesis can be extracted from these results: i) The peptides are either resistant to digestion because digestive proteases cannot access the bioactive peptides as effectively as in water. ii) The peptides released interact with components from the food matrix, maintaining an overall equal bioactivity. iii) The

food matrix maintain the components that can inhibit DPP-IV, as seen in the section 3.1. However, this later hypothesis is not likely to occur since there is significant difference between the digested sample and the sterilised sample in vegetable soup.

The molecular weight distribution after simulated gastrointestinal digestion of the DPP-IV inhibitory peptides in water and in vegetable soup showed significant differences between them and compared to the control sample. As shown in Figure 2, the percentage of low molecular weight increased after the digestion, as expected, since the digestion reaction during three hours would undoubtedly keep on cleaving the peptides contained in the sample.

**Table 2**: $IC_{50}$ values for DPP-IV inhibition by hydrolysates and native protein subjected to simulated gastrointestinal digestion both in water and in vegetable soup.

| Sample | DPP-IV inhibition (mg/ml) | α-glucosidase inhibition (mg/ml) |
|---|---|---|
| **Control** | 2.62 ± 0.16[a] | 6.52 ± 0.19[a] |
| **H_Digested in water** | 4.54 ± 0.26[b] | 1.56 ± 0,14[b] |
| **H_Digested in vegetable soup** | 2.38 ± 0.11[a] | 1.38 ± 0.05[b] |
| **P_Digested in Water** | 7.23 ± 0,53[c] | 3.65 ± 0.12[c] |
| **P_Digested in vegetable soup** | 8.76 ± 0,62[c] | 4.48 ± 0.09[d] |

Values are presented as the mean of three replicates ± standard deviation. Different letters in the same column denotes significant differences among samples. H refers to the corresponding hydrolysate. P refers to the native protein subjected to SGID.

The effect of gastrointestinal digestion over DPP-IV inhibitory peptides activity is different depending mainly on the sample digested (the amino acid sequences of the peptides, depending on the enzymatic treatment employed). Recently, Zhang et al. (2020) reported maintained, loss and gaining of DPP-IV inhibitory activity of different hydrolysates after SGID that were originally produced by different proteases. For its part, (Harnedy-Rothwell et al., 2021) did not found bioactivity differences for the boarfish hydrolysate after digestion in the food matrices considered in their experimental work. The $IC_{50}$ values for DPP-IV inhibition exerted by the protein subjected to SGID (7.23 mg/ml in water and 8.76 mg/ml in vegetable soup) enhances the adequacy of protein hydrolysates as bioactive ingredients rather than the intake of the protein without any previous enzymatic treatment.

For its part, the α-glucosidase inhibition exerted by the hydrolysate improved up to ~300% after the simulated gastrointestinal digestion (Table 2). There are no significant differences between the sample digested in water and in the vegetable soup. Different from the DPP-IV inhibitory peptides, these kind of inhibitory peptides have usually the

molecular features of peptides that would be released from digestive enzymes. In fact, the hydrolysate was originally obtained by trypsin, whose specificity is similar to the one from pepsin and pancreatin. Hence, the product obtained after digestion is highly likely more concentrated in α-glucosidase inhibitory peptides. The original hydrolysate was the one with high bioactivity achieved in the shortest reaction time from a battery of different hydrolysates considering two parameters (ultrasound pre-treatment and reaction time of the sequential hydrolysis with two proteases) (Rivero-Pino et al., 2020b). In this regard, it is important to see how the hydrolysate kept having high bioactivity (>90%) when hydrolysing for three more hours with trypsin, results comparable with the results obtained in this research work. In the digested samples compared to control, the <250 Da peptides fraction increased around 24% its area and the 0.5-0-25 kDa increased in a 20%, and consequently the low molecular weight peptide fraction concentration is increased, enhancing the overall bioactivity of the hydrolysate. This shows how digestive enzymes kept hydrolysing the peptides into low molecular weight peptides (2-3 amino acids) and sole amino acids. Concerning both digestions, the molecular weight distribution of the peptides after digestion either in water or in vegetable soup did not show any significant difference in the percentages of the areas of the different fractions (Figure 2). This result suggest that the digestion in the sample occurs regardless the food matrix, but the bioactivity of the enriched-vegetable soup is retained due to the composition of the matrix, or the products occurring during the digestion and chemical interactions.

It is interesting the fact that the bioactivity of the native protein after the simulated gastrointestinal digestion showed an $IC_{50}$ value lower than the hydrolysate, being the $IC_{50}$ values 3.65 and 4.48 mg/ml, compared to 6.52 mg/ml of the hydrolysate (Table 2). This reported bioactivity suggests that the natural peptides released due to the action of digestive enzymes after the intake of the native protein would also exert an adequate α-glucosidase inhibitory activity. The lower bioactivity reported for the digested protein in the vegetable soup compared to the one digested in water might be a consequence of a lesser extent of hydrolysis due to the hindrance of the vegetable components to allow the hydrolysis of the protein in the matrix. However, when comparing the inhibition values reported, higher inhibition is exerted by the hydrolysate after digestion compared to the native protein after digestion. This result shows that the enzymatic hydrolysis enhances the bioactivity of the sample, due to the release of specific peptides that exert high bioactivity, and the hydrolysate would be a better option to be employed in as functional ingredient to fortify food matrices.

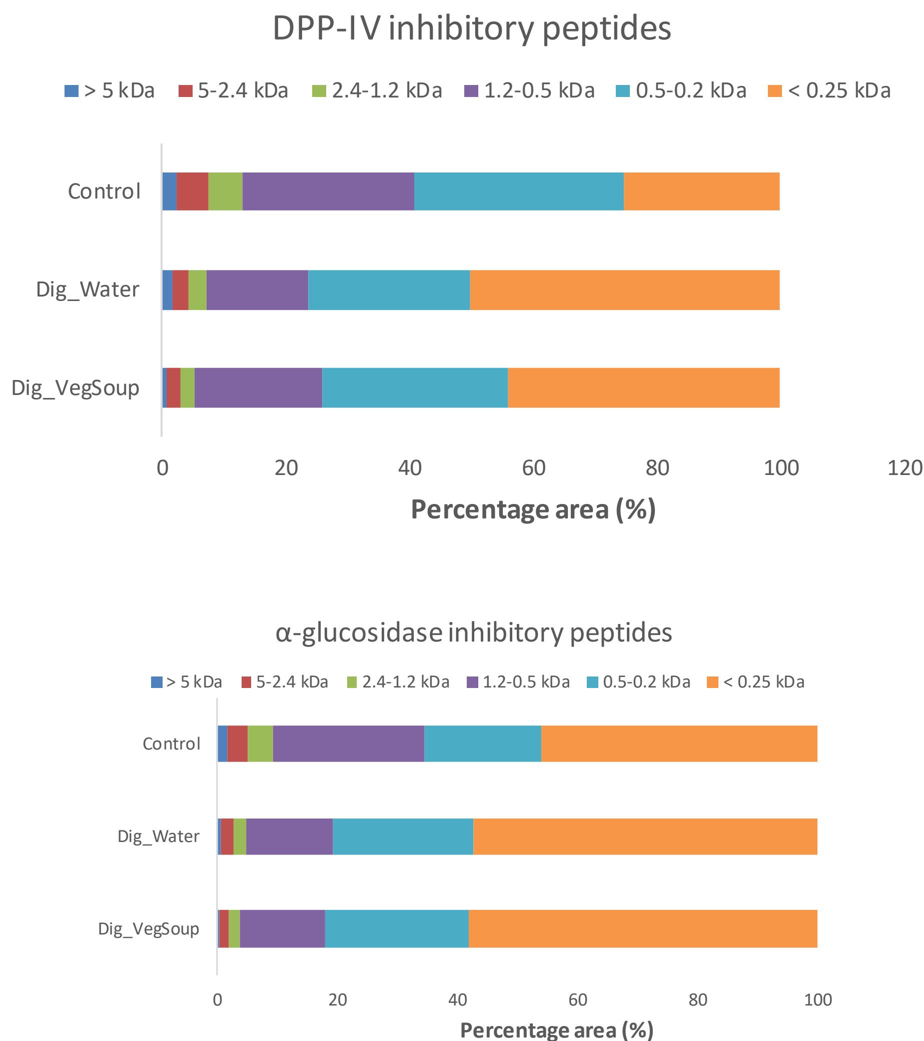


**Figure 2:** Molecular weight of distribution of the hydrolysates after digestion in water and in the vegetable soup a) DPP-IV inhibitory hydrolysate b) α-glucosidase inhibitory peptides

Zielińska et al.(2020) identified α-glucosidase inhibitory peptides obtained after in vitro gastrointestinal digestion of Tenebrio molitor, among other insect species. This supports the hypothesis that Tenebrio molitor protein is an adequate source to obtain α-glucosidase inhibitory peptides. Kang et al., (2020) reported that SGID over green crab hydrolysates would have different consequences in terms of bioactivity depending on the protease employed for obtaining the bioactive peptides. Beyond these kind of results, to the author's knowledge, there are not available reports about how gastrointestinal digestion of α-glucosidase inhibitory peptides inside a food matrix can be modified. Extensive research concerning gastrointestinal digestion of nutrients have to be carried out in order to elucidate the mechanisms of cleavage and interactions among molecules. In this regard, (Mat et al., 2016) proposed an in vitro method able to assess the behaviour of lipo-

proteinic matrices with different structures during digestion. Our results prove that peptides obtained with proteases with similar specificity to digestive enzymes' are adequate to be employed inside a liquid food matrix maintaining the bioactivity even after in vitro digestion, which is promising to consider these peptides as functional ingredient. Food matrix composition and the consequent interactions with the peptides enriching the product would affect the bioavailability of amino acids, fatty acids and other nutrients (Fardet, Dupont, Rioux, & Turgeon, 2018). Beyond that, liquid matrix behaviour would be different from a solid matrix since this latter, may well preserve the structure of the peptides longer, increasing bioaccesibility and bioavailability.

# 4. CONCLUSIONS

Mealworm proteins are a promising source of antidiabetic peptides. Their ability to inhibit enzymes related to diabetes makes them an adequate and sustainable bioactive ingredient to be employed for functional foods. Furthermore, verifying that fortification of available commercial products with these peptides do not make them lose their bioactivity is a crucial research in terms of functional food. The antidiabetic hydrolysates maintained their ability to inhibit the key enzymes after inclusion in a vegetable soup and sterilisation at 121ºC during 21 min. Subsequently, the bioactivity remained stable after 40 days of refrigeration. On the other side, the digestion of the peptides in the human digestive apparatus would not affect either the bioactivity of the peptides, and even enhance them in the case of α-glucosidase inhibitory peptides. These results proved that a vegetable soup is an adequate vehicle to carry these bioactive peptides obtained from insect protein that could potentially be used for human consumption to prevent the development of diabetes in the worldwide population.

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# CONCLUSIONES

De la investigación desarrollada se extraen las siguientes conclusiones:

1. El tratamiento enzimático con subtilisina, tripsina y Flavourzyme empleadas de manera secuencial permite obtener péptidos inhibidores de la enzima dipeptidil-peptidasa IV en proteínas de descarte de pesca (sardina) y fuentes proteicas vegetales (soja, patata, guisante, altramuz, garbanzo, lenteja y quinoa). En el caso de la proteína de Tenebrio molitor, la hidrólisis con subtilisina y Flavourzyme empleadas de manera simultánea permitió maximizar la obtención de péptidos inhibidores de la DPP-IV.
2. El tratamiento con subtilisina y tripsina de manera secuencial, empleando un pre-tratamiento con ultrasonidos, permitió obtener péptidos inhibidores de α-glucosidasa de Tenebrio molitor. Se ha comprobado que el pretratamiento con tiempos cortos de ultrasonidos de esta proteína aumenta su hidrofobicidad superficial, haciendo más accesible los enlaces peptídicos a las proteasas, convirtiendo la proteína en más susceptible a una hidrólisis que libere péptidos inhibidores de α-glucosidasa en un tiempo de reacción menor.
3. Las fracciones de péptidos con mayor capacidad inhibitoria de la enzima DPP-IV se encontraron en general, en las correspondientes a un peso molecular de 0.8 a 3 kDa, siendo el más activo en este estudio los péptidos de 0.5 a 1.6 kDa de Tenebrio molitor, con un valor $IC_{50}$ de 0.91 mg/ml. Los péptidos inhibidores de α-glucosidasa de Tenebrio molitor se concentraron en la fracción de menos de 0.5 kDa, con un valor $IC_{50}$ de 2.58 mg/ml.
4. Se han identificado *de novo* 52 péptidos reguladores del índice glucémico de las fracciones más activas: 46 inhibidores de la DPP-IV (de sardina, soja, quinoa, altramuz y Tenebrio) y 6 inhibidores de la α-glucosidasa (de Tenebrio). Como inhibidores de DPP-IV, en base a la caracterización *in silico,* destacamos NAPNPR y YACSVR en sardina, EPAAV en soja, NPLL en altramuz, APFTVV en quinoa y APVAH en Tenebrio. Respecto a la inhibición de α-glucosidasa, el más activo sería el péptido CSR, de la proteína de Tenebrio.
5. Los péptidos inhibidores de la enzima DPP-IV procedentes de sardina y Tenebrio mantuvieron su actividad o ésta disminuyó hasta un 50% su bioactividad tras ser sometidos a digestión gastrointestinal simulada. Los valores de actividad tras la digestión continúan siendo significativos en términos de alimentación funcional.

Los péptidos inhibidores de α-glucosidasa de Tenebrio incrementaron significativamente su bioactividad tras la digestión.

6. Se ha diseñado un alimento fortificado de péptidos reguladores del índice glucémico procedentes de Tenebrio molitor en una matriz líquida basada en un caldo de verduras comercial. Tras someter al alimento a un tratamiento térmico agresivo, con la finalidad de simular el procesamiento en la industria alimentaria, se ha comprobado que la capacidad reguladora de los péptidos se conserva en valores adecuados. Posiblemente este comportamiento se deba a la composición intrínseca de la matriz, que modifica las posibles interacciones y la estabilidad estructural de los péptidos.

# Lista de publicaciones

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