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

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ABSTRACT

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.

Keywords: antidiabetic, bioactive peptides, dipeptidyl peptidase IV, protein hydrolysate, sardine.

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, Jao, Ho, & Hsu, 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 (International Diabetes Federation, 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

51 background, DPP-IV inhibition would allow incretins to keep exercising its insulin
52 secretion effect, and as a result, blood glucose levels will decrease to be adequate. There
53 are already authors that have described beneficial effects of DPP-IV inhibition either *in*
54 *vitro* and *in vivo* concerning glucose tolerance and insulin secretion (Duez et al., 2012)
55 related to the increase of circulating levels of incretins (Juillerat-Jeanneret, 2014).

56 Multiple molecular structures have been demonstrated to inhibit DPP-IV activity. It is
57 possible to find anti-diabetic drugs in the market that act by inhibiting DPP-IV enzyme,
58 known as gliptins, such as sitagliptin or saxagliptin. Nevertheless, these drugs tend to
59 present adverse effects, such as nasopharyngitis, cystitis, and headache (Amori, Lau, &
60 Pittas, 2007; Duez et al., 2012). To overcome these limitations, natural compounds such
61 as biopeptides coming from protein hydrolysis are recently seen as an adequate
62 alternative. These bioactive peptides, beyond being a source of nitrogen and amino
63 acids, exert potential physiological functions within the body (Harnedy & FitzGerald,
64 2012) and consequently, they could be used in functional food to prevent some diseases,
65 such as diabetes (Lacroix & Li-Chan, 2013).

66 Bioactive peptides are encrypted in the native protein sequences, and the proteases
67 action would cleave the structure of the proteins, releasing the peptides. The enzymatic
68 treatment employed, considering the specificity of different proteases, is essential to
69 optimize the biological activity of the hydrolysates. Some others factors involved are
70 the amino acid composition and the protein structures, which may well be different and
71 have an impact on the hydrolysis and subsequent products obtained. At this level, fish
72 protein are a promising source for biopeptides since their protein level usually ranges
73 between 10–23% (w/w) (Harnedy & FitzGerald, 2012).

74 Marine fish species have been widely studied as a potential source of antihypertensive
75 and antioxidant peptides (Neves, Harnedy, O'Keeffe, & FitzGerald, 2017; Pérez-

Gálvez, Morales-Medina, Espejo-Carpio, Guadix, & Guadix, 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, Tamm, Guadix, Guadix, & Drusch, 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

126 considered was 1.13, while htot was assumed to be 8.6, as reported in literature (Adler-
127 Nissen, 1986).

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

142 2.3 *In vitro* simulated gastrointestinal digestion (SGID)

143 Simulated gastrointestinal digestion was carried out as described in Garrett, Failla and
144 Sarama (1999) in order to evaluate the effect of digestive enzymes *in vitro*. Reactions in
145 triplicate were carried out in a temperature-controlled shaker (Heidolph, Germany) at
146 37ºC with 300 rpm shaking. Firstly, the lyophilized samples were diluted in distilled
147 water, at 5% (w/w, in dry weight) and pepsin was added at an E/S ratio of 4% (w/w, on
148 protein basis), previous set pH 2 with 1 M HCl. After 1 h of reaction, pH 5.3 was
149 achieved with a solution of 0.9 M NaHCO3, then, pancreatin was added at an E/S ratio
150 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, 2012). 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.

199 2.8 *In silico* prediction for activity of the identified peptides

200 pepATTRACT (De Vries, Rey, Schindler, Zacharias, & Tuffery, 2017) was employed
201 to perform molecular docking of peptides identified with the DPP-IV enzyme (PDB:
202 2AJBA) and obtain an energy score.

203 2.9 Statistical analysis

204 Significant differences were analysed by means of Statgraphics 5.1 by the multiple
205 comparison test (Least Signifcant Difference) at a p-value ≤ 0.05. Data is shown as
206 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, Di Stefano, & Udenigwe, 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, Ong, & Mann, 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, Chel Guerrero, & Betancur Ancona, 2010).

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 (2012).

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, Dziuba, Iwaniak, Dziuba, & Darewicz, 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, Lamoureux, & Fitzgerald, 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, Wu, Dai, Wang, & Wu, 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

306 resistant to this SGID (Huang et al., 2012). Nonetheless, modifications of terminal sites
307 of peptides (usually 2-20 amino acids length) may well protect it from peptidases by
308 adopting a similar structure than the native protein. Beyond that, the effect of SGID on
309 the bioactivity of peptides depends also if the proteinaceous material is encapsulated,
310 for example, with glucose syrup, increasing its resistance due to the entrapment of the
311 peptides. Another factor influencing the resistance is the food matrix where the peptide
312 is contained, since it may interact with other molecules and their willingness to be
313 cleaved is modified.

314 3.4 DPP-IV inhibitory activity from hydrolysate fractions

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

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

328

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 (2012) 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 (Liu, Cheng, & Wu, 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.

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.

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.

CONFLICT OF INTEREST

There are no conflicts of interest among the authors.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURES AND TABLES CAPTION

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

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

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

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

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

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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}	$31.86 \pm 1.17^{a,b}$	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).

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)

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

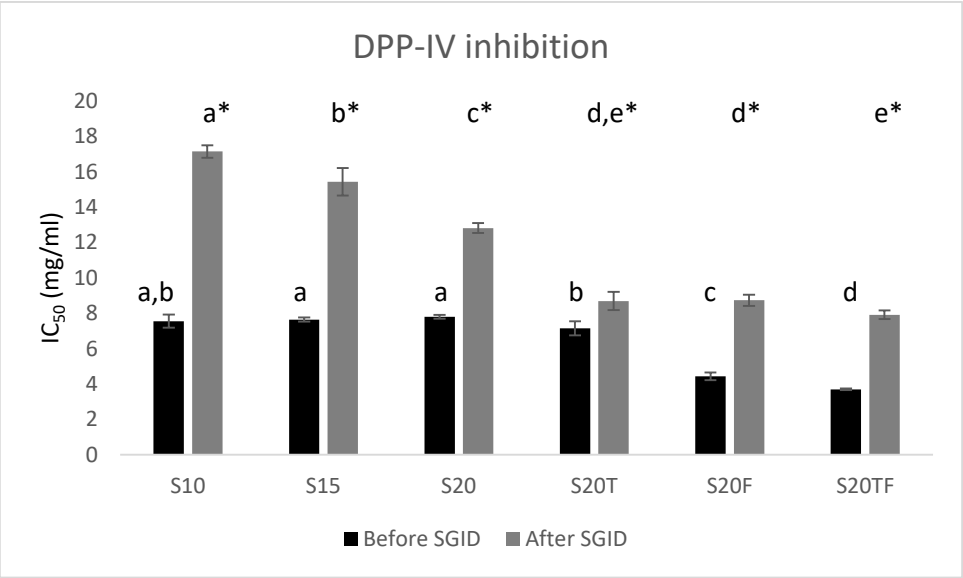

Figure 1: IC_{50} (mg of protein/mL) for DPP-IV inhibition of samples before and after simulated gastrointestinal digestion.

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.

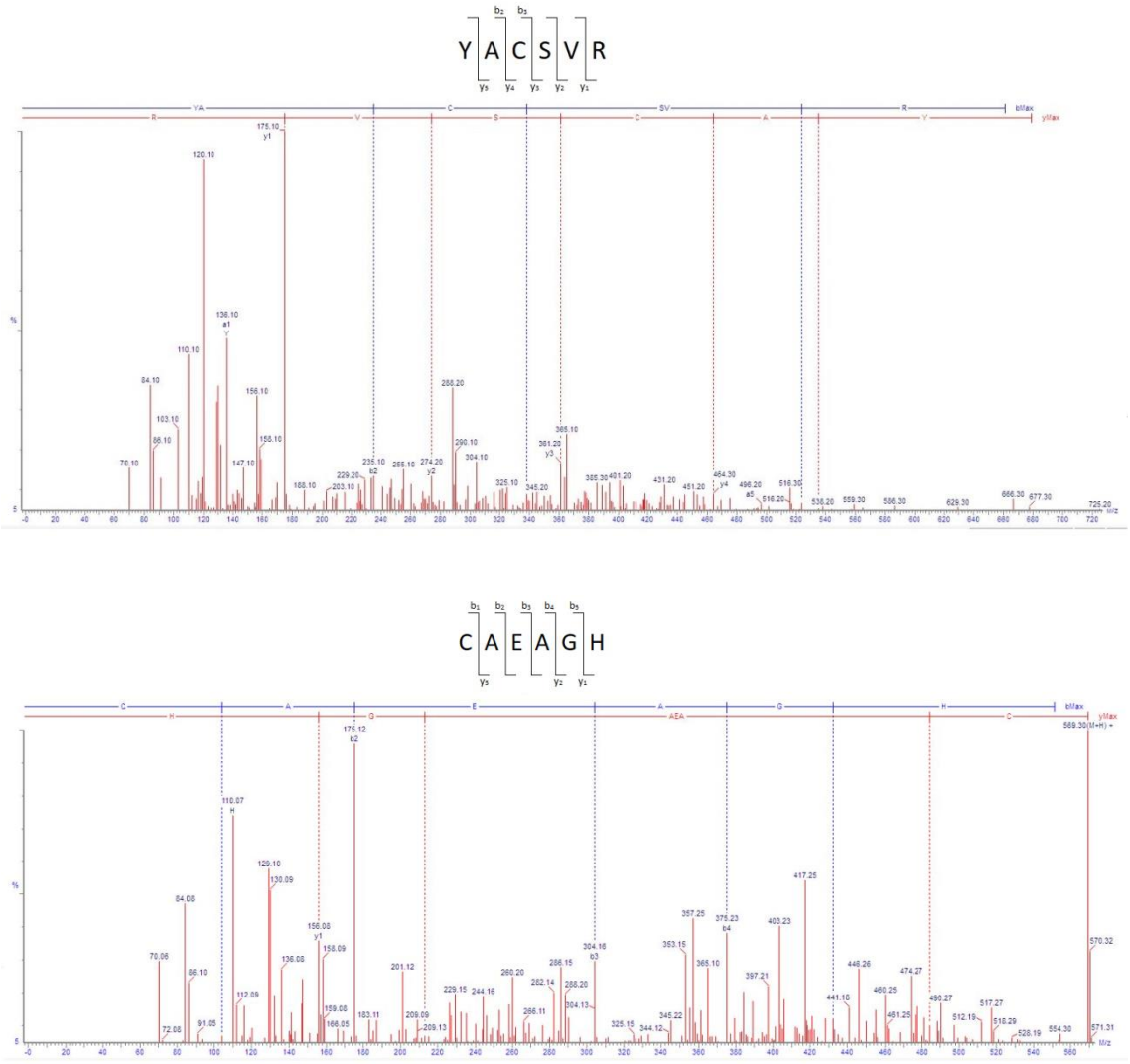

Figure 2: Sequence profile of two of the peptides identified from S. pilchardus