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Original Article

Valorisation of blood protein from livestock to produce haem iron-fortified hydrolysates with antioxidant activity

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Abstract Blood is the main by-product from slaughterhouses bearing high levels of suspended solids (18% w/w) and BOD_5 (250 000–375 000 mg O_2/L), which makes difficult its handling and disposal. This study proposes the valorisation of blood protein to produce enzymatic hydrolysates rich in haemic iron and antioxidant peptides. Haemic iron presents higher bioavailability compared to its inorganic form, but its incorporation into foodstuffs is restrained by its high tendency to oxidation. Six commercial proteases of animal, plant and bacterial origin were employed to produce the enzymatic hydrolysates. Subtilisin and trypsin treatments were able to recover 70% of haem iron as soluble peptides, in contrast with plant enzymes where the proteolysis was reduced. Moreover, some enzymatic treatments led to hydrolysates with high levels of some *in vitro* antioxidant activities such as radical scavenging (Protamex, IC_{50} 3.52 mg/mL) or metal chelating activity (trypsin, IC_{50} 0.27 mg/mL). We conclude the enzymatic valorisation of blood protein increases haemic iron bioavailability and produces antioxidant peptides. Both properties are of interest for their use as iron fortification supplements.

Keywords antioxidant activity, blood protein, enzymatic hydrolysis, haem iron.

Introduction

Blood is the most polluting by-product generated by the meat production chain in slaughterhouses. With a total nitrogen of around 30 g L^{-1}, its discharge to water streams can deplete the dissolved oxygen, induce septic conditions, and spread microbial and viral waterborne diseases (Nazifa *et al.*, 2021). An excess of blood effluents can also cause serious problems in the treatment plant, making its withdrawal necessary. Since blood is a highly perishable biomaterial, to prevent microbial spoilage it can be transformed into blood meal (Shubham *et al.*, 2020): a dehydrated powder that contains 70%–75% w/w of the total blood protein. Haemoglobin is a major protein present in blood, constituting up to 95% of the protein content in erythrocytes. It is a heterotetramer containing four non-protein haem complexes where an iron ion is coordinated to a porphyrin ring (Wang *et al.*, 2017). It is interesting from an economic and environmental point of view to revalue this blood as a source of haem iron, an affordable option for dietary enrichment.

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Iron deficiency is the most prevalent health disorder in the world, affecting at least 20% of the population. This deficiency is the root of anaemia, which is caused mainly by malnutrition or an insufficient dietary intake. In recent decades, several methods have been carried out to reduce the prevalence of iron deficiency anaemia, supplementation and fortification being the most studied (Liberal *et al.*, 2020; Shubham *et al.*, 2020),

Food fortification is considered the most cost-effective method to fight iron deficiency. Iron salts are frequently used as fortifiers; however, their present limited bioavailability and stability, as well as excessive reactivity with other food components.

Despite iron being found in a wide range of foodstuffs, its bioavailability is superior under the form of haemic iron: 15%–35%, compared to 2%–20% for the inorganic form (Banjari & Hjårtaker, 2018). Dietary supplementation of haemoglobin is not the best alternative, since the absorption of haem iron is hindered by the formation of insoluble polymers. Enzyme hydrolysis favours the solubilisation of blood proteins, releasing haem peptides which improve the assimilation of haem iron (Lichao *et al.*, 2020). Moreover, the bioavailability of haem peptides depends on the size and composition of the amino acid chain attached to the haem iron ring (Wang *et al.*, 2017). Therefore, the

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conditions of the enzymatic treatment (i.e. enzyme and degree of hydrolysis) have a great impact on the release of haemic iron peptides, as well as their ulterior functionality once introduced in food matrices (Nikhita & Sachindra, 2021).

Enzymatic hydrolysis allows releasing specific peptide sequences that may exert a range of biological activities. For instance, several studies have identified active fractions from red cell or plasma protein hydrolysates exerting antioxidant, antihypertensive or antimicrobial properties (Olagunju *et al*., 2018; Alves *et al*., 2020). Among these bioactivities, the presence of peptides exerting antioxidant activity in blood protein hydrolysates (Verma *et al*., 2019) may be advantageous when considering the dietary supplementation of haem iron. Indeed, previous studies report the role of haem iron as a catalyser of lipid peroxidation in meat and fish. Besides haem peptides, the incorporation of blood protein hydrolysates provides antioxidant species which may compensate for the pro-oxidant effect of haem iron.

The purpose of this paper was to produce a set of blood meal hydrolysates intended to be included in a food matrix (i.e. source of haem iron peptides presenting antioxidant activities). The hydrolysates were obtained employing six different commercial proteases (Alcalase, Protamex, PTN, PEM, Ficin and Papain). The enzymatic reactions were allowed for 8 h, monitoring both the degree of hydrolysis and the release of haemic iron throughout the hydrolysis. Moreover, the final hydrolysates were tested for their *in vitro* antioxidant activities (i.e. free radical scavenging, metal chelating and reducing power). These properties were related to enzyme specificity and molecular weight distribution of the hydrolysates.

Materials and Methods

Substrate and enzymes

The substrate used in this study was blood meal (Protesan, APC, Barcelona, Spain), obtained after the coagulation and drying process of porcine blood. This product has a reported content of protein above 89% wt. and 0.25% wt. of iron. The amino acid composition of the blood meal reveals an important proportion in leucine, glutamic acid, phenylalanine and lysine, all of them above 9% wt.

Six enzymes, obtained from bacterial, animal and plant sources, were used to produce the blood meal hydrolysates (BMH). Alcalase and Protamex were chosen as bacterial proteases, both purchased from Novozymes A/S (Bagsvaerd, Denmark). The former is an extract from *Bacillus subtilis*, mainly containing subtilisin (EC 3.4.21.62) as protease. Protamex is a protease complex (E.C. 3.4.21.62/3.4.24.28) from *Bacillus* spp. The animal proteases used in this work were PTN (Pancreatic Trypsin Novo, Novozymes) which is an extract of trypsin (E.C. 3.4.21.4) from bovine pancreas and PEM (Proteolytic Enzyme Mixture, Novozymes), which consists of a mixture of trypsin and chymotrypsin (E.C. 3.4.21.1) from bovine and porcine pancreas. The study was completed with two plant-derived proteases, Papain (E.C. 3.4.22.2) and Ficin (E.C. 3.4.22.3), purchased from Merk KGaA (Darmstadt, Germany).

Enzymatic treatment and determination of the degree of hydrolysis

The hydrolysis was conducted in a jacketed reactor with a 1000 mL capacity. A 718 Stat Titrino (Metrohm, Herisau, Switzerland) was used to measure the pH and temperature. It contains a dosing burette associated with a 1.5 L reservoir of 0.5 M NaOH to maintain the pH constant. The temperature was kept constant by connecting a thermostat (B3, Thermo-Haake; Karlsruhe, Germany) to the reactor jacket.

Blood meal was blended with water at a concentration of 10 g protein per 1000 mL. A file was provided as Supplementary Material, reporting the stability interval of pH and temperature, as well as the amino acid preferably cleaved by the different enzymes. The conditions of pH and temperature for maximal proteolytic activity were chosen previously, by performing a set of hydrolysis experiments on blood meal (also available as Supplementary Material). These assays allowed fixing the following conditions for the enzymatic reaction, all of them employing a 7.5% ratio of enzyme to protein:

- Bacterial proteases: Alcalase at pH 8.5 and 60 °C, Protamex at pH 7.8 and 55 °C.
- Animal-derived proteases: PTN at pH 8.5 and 50 °C, PEM at pH 9.0 and 50 °C.
- Plant-derived proteases: Papain at pH 8.0 and 55 °C, Ficin at pH 9.0 and 55 °C.

The hydrolysis reaction was allowed for 8 h, drawing samples for haem iron determination every hour of reaction. The degree of hydrolysis (DH), defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds, was correlated to the amount of base consumed during the reaction according to the pH-stat method (Adler Nissen, 1986). The reaction was stopped by heating the solution at 100 °C for 15 min, ensuring the thermal deactivation of the enzymes. The resulting hydrolysate was then vacuum filtered through an 8 μm cellulose filter and then lyophilised (Labcondo, Kansas City, MO, USA). The powdered product was kept under refrigeration (−20 °C) for further analysis.

Solubilisation of blood meal

Blood meal is a partially insoluble substrate whose solubility increases in the course of the hydrolysis

reaction. The degree of solubilisation was evaluated by the determination of the content of suspended solids in the final hydrolysate. To this end, 200 mL of the final hydrolysate were vacuum filtered through a cellulose filter paper Whatman grade 40 (8 μm of average pore size). The retained solids were dried at 110 °C for 30 min prior to being weighted.

Determination of the content of haemic iron

The release of haemic iron was monitored during the reaction by the Ockerman modified method (1985). A volume of 50 μL of acid–acetone (90% acetone, 8% distilled water and 2% HCL, v/v/w) was added to 1 mL of hydrolysate, homogenised for 30 s and kept in the dark for 1 hour. The mixture was then centrifuged at 2200 ***g*** for 10 min, and the supernatant was collected and filtered through a 0.45 μL cellulose filter. The absorbance of the filtrate was measured at 640 nm (Cary 100 Bio UV-Vis Spectrophotometer, Varian, USA). The absorbance was directly related to the concentration of haem pigment by means of a calibration curve. The haem recovery rate was expressed as the ratio between the amount of haem iron released by hydrolysis (i.e. quantified in the supernatant fraction where it is attached to soluble peptides) related to the total amount of haem iron contained in the blood meal.

Molecular weight distribution of the hydrolysates

The molecular weight distribution of the hydrolysates was determined by size exclusion chromatography (SEC) on a Superdex peptide 100/300 GL column (GE, Health care, Uppsala, Sweden), employing a mixture of ultra-pure water and acetonitrile (70:30) as mobile phase. The absorbance of the eluted samples was measured at 280 nm. A set of eight standards, namely glycine (Gly, 75 Da), alanine (Ala, 89 Da), tripeptide phenylalanine-glycine-glycine (Phe-Gly-Gly, 279 Da), hexapeptide $(Gly)_6$ (360 Da), vitamin B_{12} (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da), were employed to construct the calibration curve.

Determination of the antioxidant activities of the hydrolysates

Three antioxidant properties were investigated on the blood meal hydrolysates, namely the ability to sequester DPPH radicals, the ferrous ion chelating activity and the Fe^{3+}/Fe^{2+} reducing power. The ability of the blood meal hydrolysates to sequestrate free radicals was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method described by Picot *et al.* (2010). Ferrous ion chelating capacity was determined by the Decker & Welch (1990) method. Both properties were expressed as percentage activity, evaluated with respect to a blank solution containing the reagents and distilled water instead of hydrolysate solution. An IC_{50} value was computed as the concentration of hydrolysate yielding 50% activity.

The ferric reducing power of the samples was assayed following the Oyaizu (1988) method (FRAP assay), modified to take into consideration the proper iron content of the sample. For comparison purposes, an $EC_{0.5}$ value was defined as the concentration (mg of protein per mL) of hydrolysate leading to 0.5 absorbance units (700 nm) in the FRAP assay.

Statistical analysis

A statistical program (Statgraphics Centurion XV, VA, USA) was used for data processing and statistical analysis. The Duncan multiple range test was used to determine whether significant differences existed among enzymatic treatments and species. Differences among mean values were considered significant at a level of confidence of 95%.

Results and Discussion

Progression curves for DH and haem iron recovery

The evolution of the degree of hydrolysis (DH) and haem iron recovery against reaction time for the different enzyme treatments is shown in Fig. 1a and b. The hydrolysis curves (Fig. 1a) presented an accentuated initial slope, reaching a steady state of constant DH at large reaction times. This typical trend may be explained by a decrease in the number of peptide bonds available for enzyme attack, product inhibition or loss of enzymatic activity (Adler Nissen, 1986). The proteases employed in this work showed different proteolytic activity on the blood protein. The maximum DH (23%) was reached by Alcalase after 8 h of reaction, followed by the animal-derived enzymes, PTN and PEM which attained a steady DH above 16% after 5 h of hydrolysis. Protamex and ficin treatments hydrolysed blood meal to a lesser extent, with final DH in the range 12%–14%. Protamex is a complex presenting both endo and exoprotease activity (Cho *et al.*, 2014), while ficin is a cysteine endoprotease reported to cleave a broad range of peptidic bonds, such as alanine, glycine, leucine, lysine, tyrosine or valine (Hou *et al.*, 2017). Papain, which is also cysteine endoprotease, presented only limited proteolysis (DH 4%).

The haem iron recovery curves (Fig 1b) exhibit a similar trend to that observed in the hydrolysis curves. Trypsin (PTN) and subtilisin (Alcalase) treatments presented the highest values of haem iron recovery (79% and 73% after 8 h respectively) in the experimental series. Plant-derived enzymes displayed the

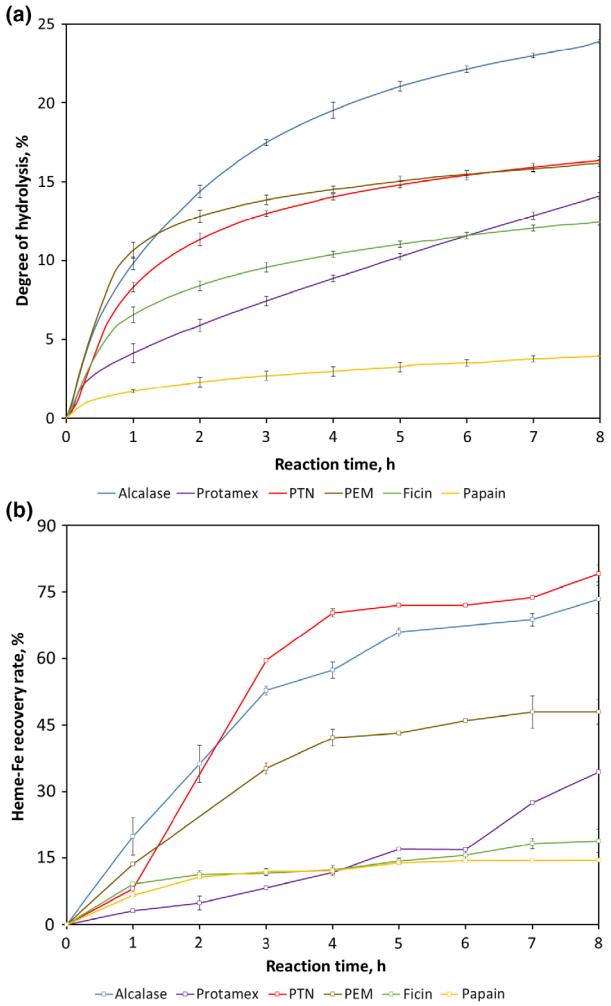

Figure 1 Time evolution of the degree of hydrolysis (a) and haemic iron recovery rate (b) for the six enzymatic treatments assayed. Mean of three replicates ± standard deviation.

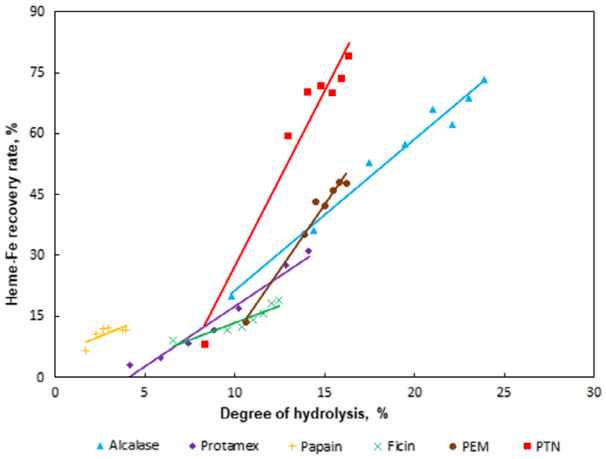

Figure 2 Linear correlation between the degree of hydrolysis and the haemic iron recovery rate for the six enzymatic treatments assayed.

lowest values of haem recovery rate, attaining only 19% recovery for ficin and 12% for papain. By comparison of Fig. 1a and b, there seems to be a direct relation between the DH reached by proteolysis and the corresponding percentages of iron recovered. This correlation is evidenced in Fig. 2, where haem recovery was plotted against DH. Except for papain, where the proteolysis was very limited, a linear correlation between DH and release of haem iron was observed, with determination coefficients R^2 ranging from 88% (Ficin) to 98% (Alcalase). The slopes of the regression lines are an indicator of the efficiency of the enzymatic treatments to release haem peptides. It was estimated that the PTN and PEM treatments released on average 2.4–2.7 milliequivalents of iron per 100 milliequivalents of peptide bonds cleaved, higher than the ratio reported for Alcalase (1.6). We concluded that PTN and PEM treatments were more efficient to release haem peptides, despite hydrolysing the blood proteins to a lesser extent. The recovery of haem iron after hydrolysis is hindered by the formation of large insoluble polymers. The latter fraction is discarded by precipitation, retaining a soluble fraction where haemic rings are bounded to short-chain peptides by hydrophobic interactions (Walters *et al.*, 2018; Wu *et al.*, 2020). The intestinal absorption of haem iron is favoured by the presence of such peptides (Vaghefi *et al.*, 2002).

Subtilisin is an endoprotease which cleaves a wide range of peptide bonds, preferably those involving aromatic and methionine residues (Adler-Nissen, 1986). Alcalase treatment hydrolysed blood meal extensively and presented the second higher haem recovery rate in the experimental series. In contrast, trypsin (PTN) presents narrow specificity, cleaving preferentially peptide bonds involving arginine and lysine residues. Previous studies reported a chymotrypsin-like behaviour of trypsin at reaction temperatures above 45 °C (Morales-Medina *et al.*, 2016; Jin *et al.*, 2020), which is our case. This causes the release of new peptides containing phenylalanine, leucine, tyrosine and tryptophan residues at the carboxyl end. This explains the overlap of PTN and PEM hydrolysis curves after 5 h of treatment (Fig. 1a) despite the lower initial reaction rate exhibited by PTN. Furthermore, the chymotrypsin activity may be responsible for the high haem recovery efficiencies of PTN and PEM treatments. Effectively, both enzyme preparations release peptides with hydrophobic residues (Phe, Leu, Trp) at the terminal end, prone to bind haemic rings.

Blood meal solubilisation and molecular weight distribution of the hydrolysates

Figure 3a shows the average values of solubilisation attained for each enzymatic treatment. The unhydrolysed blood meal only solubilised 6.57% after homogenisation at pH 8 and 50 °C for 8 h. Overall, the extent of the hydrolysis reduced the average peptide chain length and increased the exposure to polar and ionisable protein groups (Noman *et al.*, 2018), favouring the solubilisation of the blood protein. Accordingly, Alcalase, PTN and PEM treatments led to highest values of DH and protein solubilisation (above 90%) in the experimental series. Despite achieving a higher degree of hydrolysis, Protamex treatment led to 66% solubilisation, inferior to the level obtained by Ficin (75%). Neutrase, which is the main enzyme present in the composition of Protamex, is reported to produce peptides with hydrophobic residues, such as leucine and phenylalanine (Mongkonkamthorn *et al.*, 2020), which may hinder the solubilisation of blood protein. Papain treatment was the least efficient in blood meal solubilisation, in agreement with the low degree of hydrolysis observed.

Figure 3b shows the molecular weight distribution of the hydrolysates obtained after completion of the enzymatic treatment and filtration to remove the insoluble fraction. For discussion purposes, we divided the SEC profiles into four ranges of peptide size: above 10 kDa, between 5–10 kDa, between 1–5 kDa and below 1 kDa. Alcalase hydrolysates presented the highest percentage of short-chain peptides below 1 kDa (~25% wt). On the contrary, Ficin and Papain hydrolysates presented more than 60% wt. of peptide species above 10 kDa, with no significant differences between them.

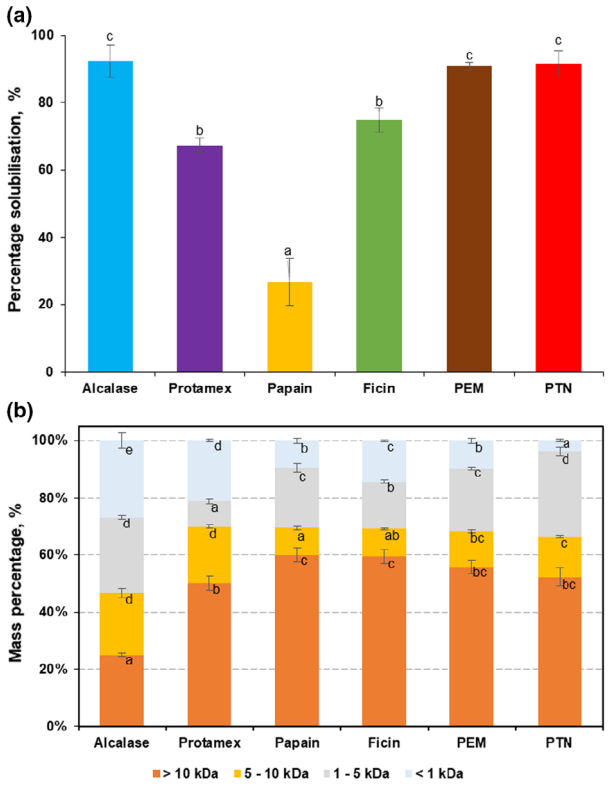

Figure 3 (a) Percentage solubilisation of the blood meal after hydrolysis and (b) molecular weight distribution for the six enzymatic treatments. Mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

Antioxidant activities of the blood meal hydrolysates

DPPH radical scavenging activity

The values of *in vitro* DPPH scavenging activity of the hydrolysates are presented in Fig. 4a. Protamex (DH 14%) and Alcalase (DH 24%) hydrolysates displayed the strongest activity, with average IC_{50} values around 3.5 mg/mL. The activity of PEM (DH 16%), PTN (DH 16%) and Papain (DH 6%) hydrolysates was significantly inferior, with IC_{50} values within the range 5–7 mg/mL. Interestingly, Ficin (DH 13%) hydrolysates, despite presenting a higher degree of proteolysis, exhibited the lowest DPPH scavenging activity in the experimental series (IC_{50} According to these results, we found no direct relationship between the degree of hydrolysis (i.e. average peptide chain length) and the radical scavenging activity of the hydrolysates. The potential ability of a peptide fraction to scavenge radicals is influenced by a number of characteristics such as molecular weight, amino acid sequence, secondary structure, presence of hydrophobic residues, among others. Previous studies concluded that the presence of low molecular weight peptides (released after large reaction times) favoured the *in vitro* levels of DPPH scavenging activity for Alcalase hydrolysates from porcine plasma (Liu *et al.*, 2010) and porcine blood (Verma *et al.*, 2019). Similarly, Mongkonkamthorn *et al.* (2020) reported maximal DPPH inhibition for the peptide fractions below 1 kDa of Alcalase and Neutrase hydrolysates from tuna blood. In our case, this could explain the high bioactivity observed for Protamex and Alcalase. According to the molecular weight distribution (Fig. 3b), both hydrolysates presented the largest content of peptides below 1 kDa, above 20% wt. Furthermore, Mongkonkamthorn *et al.* (2020) attributed the high DPPH inhibition of Neutrase (the main enzyme present in Protamex) hydrolysates to the release of peptides with hydrophobic residues, such as leucine and phenylalanine. As commented above, the chymotrypsin activity exhibited

by PEM, as well as PTN above 45 °C (Jin *et al.*, 2020) released peptides containing such residues in the terminal end. Despite its low degree of hydrolysis, the antioxidant activity of Papain hydrolysates was significantly superior to Ficin, which may be attributed to the presence of cysteine and phenylalanine-containing peptides (Zou *et al.*, 2016).

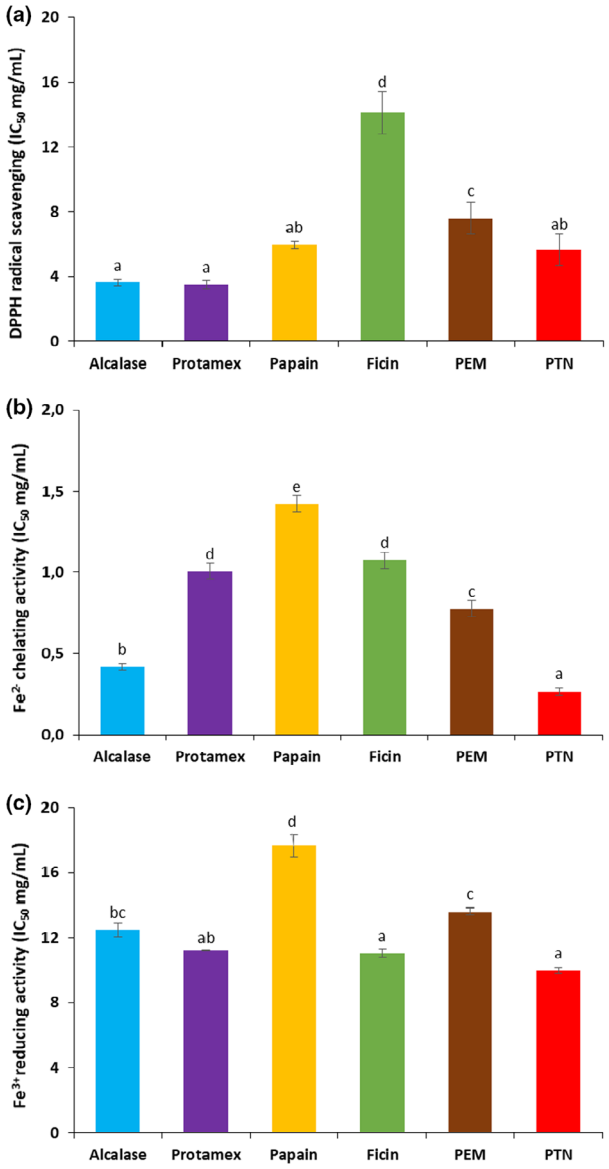

Figure 4 *In vitro* antioxidant activities of the blood meal hydrolysates produced by six enzymatic treatments (a) DPPH radical scavenging (b) Fe^{2+} chelating and (c) Fe^{3+} reducing power. Mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

Ferrous Ion-chelating activity

Figure 4b presents the average IC_{50} values of metal chelating activity, where PTN and Alcalase hydrolysates exhibited the strongest activity (IC_{50} 0.27 and 0.42 mg/mL respectively). PTN hydrolysates (DH 16%) exhibited the highest chelating activity (IC_{50} 0.27 mg/mL), followed by Alcalase hydrolysates (IC_{50} 0.42 mg/mL, DH 23%) and PEM hydrolysates (IC_{50} 0.78 mg/mL, DH 16%). The weakest antioxidant activity was found for plant-derived enzymes, which is in agreement with previous studies reporting low metal chelating activity for Papain hydrolysates of pork muscle protein (Saiga *et al.*, 2003). Some authors reported high chelating activity for peptides between 1–3 kDa (Saidi *et al.*, 2014) and even larger (~5 kDa) in fish protein hydrolysates (Slizyte *et al.*, 2016). In our work, PTN and Alcalase hydrolysates presented the largest content of peptides in the range 1–5 kDa (29.8% and 26.5% wt. respectively). Furthermore, chymotrypsin and subtilisin hydrolysates present high content of peptides with aromatic residues, which improves their ability to chelate metal ions (Zou *et al.*, 2016).

Ferric reducing antioxidant power activity

Figure 4c depicts the *in vitro* values of ferric reducing power, expressed as the concentration of hydrolysate yielding 0.5 absorbance units in the FRAP assay. The highest in vitro activity was reported for the PTN treatment, followed by Ficin and Protamex, with no significant differences among them ($EC_{0.5}$ ranging from 10.0 mg to 11.2 mg/mL). These results are in agreement with the study of Damgaard *et al.* (2015) on animal tissues, who reported an increase in ferric reduction activity for peptides containing tryptophan, methionine or tyrosine residues. This is the case for PTN at pH 8 and 50 °C, due to its chymotrypsin-like activity. Despite their higher DH and content in short-chain peptides (10–25% wt.), the bioactivity of PEM and Alcalase hydrolysates was slightly inferior, followed by Papain with $EC_{0.5}$ 17.7 mg/mL. No direct relationship was found between the molecular weight distribution and the *in vitro* ferric reduction activity of the hydrolysates. Some authors conclude that this property is influenced by other factors, such as the presence of hydrophobic or aromatic residues in terminal position (Mongkonkamthorn *et al.*, 2020; Nikhita & Sachindra, 2021).

Conclusion

The present study has been the first approach to explore the release of haem peptides through hydrolysis of blood meal, employing a wide range of commercial proteases. The highest recoveries of haem iron were obtained with PTN (79%) and Alcalase (73%), at a DH of 16.36 and 23.92%, respectively, showing a

great correlation between the degree of hydrolysis and the haem iron recovered. The inclusion of haem peptides as iron fortifiers in food matrices is restrained by the pro-oxidant tendency of haem iron. With that in mind, the blood protein hydrolysates were tested for their *in vitro* antioxidant activities. Hydrolysis with Protamex resulted in the highest DPPH scavenging activity at 11.5% DH (IC_{50} 3.51 mg/mL) while PTN treatment showed the best Fe^{2+} chelating and Fe^{3+} reducing activities at 16.36% DH. We conclude that blood meal can be used as a good substrate for the production of protein hydrolysates rich in haem iron and antioxidant peptides. Trypsin hydrolysates have proven to be the best candidate to be included in food preparations as haem iron supplements.

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Author contribution

Carmen Berraquero García: Investigation (lead); Methodology (lead); Writing – original draft (lead). **M. Carmen Almécija:** Conceptualization (equal); Methodology (equal); Supervision (equal); Writing – review & editing (equal). **Emilia María Guadix:** Conceptualization (equal); Funding acquisition (lead); Supervision (equal). **RAÚL PÉREZ-GÁLVEZ:** Conceptualization (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal).

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15616.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Material