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Evaluation of *Tenebrio molitor* protein as a source of peptides for modulating physiological processes†

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The increasing world population has led to the need to search for new protein sources, such as insects, the harvesting of which can be economical and environmentally sustainable. This study explores the biological activities (angiotensin-converting enzyme (ACE) inhibition, antioxidant capacity, and dipeptidyl peptidase IV (DPP-IV) inhibition) of *Tenebrio molitor* hydrolysates produced by a set of food-grade proteases, namely subtilisin, trypsin, ficin and flavourzyme, and the degree of hydrolysis (DH), ranging from 5% to 20%. Trypsin hydrolysates exhibited the highest ACE inhibitory activity at a DH of 10% (IC_{50} 0.27 mg mL^{-1}) in the experimental series, which was attributed to the release of short peptides containing Arg or Lys residues in the C terminus, and described as the ACE-inhibition feature. The levels of *in vitro* antioxidant activities were comparable to those reported for insect species. Subtilisin and trypsin hydrolysates at a DH of 10% displayed optimal DPPH scavenging and ferric reducing activities, which was attributed to the presence of 5–10-residue active peptides, as reported in the literature. Iron chelating activity was significantly favoured by increasing the DH, attaining a minimal IC_{50} of 0.8 mg mL^{-1} at a DH of 20% regardless of the enzymatic treatment. Similarly, *in vitro* antidiabetic activity was significantly improved by extensive hydrolysis, and, more specifically, the presence of di- and tripeptides. In this regard, the combined treatment of subtilisin–flavourzyme at a DH of 20% showed maximal DPP-IV inhibition (IC_{50} 2.62 mg mL^{-1}). To our knowledge, this is the first study evaluating the DPP-IV activity of *Tenebrio molitor* hydrolysates obtained from these commercial proteases. We conclude that *Tenebrio molitor* hydrolysates produced with food-grade proteases are a valuable source of active peptides that can be used as functional ingredients in food and nutraceutical preparations.

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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 consume them, entomophagy occurs widely in many regions. Not only are insects rich in protein and healthy fats (mainly monounsaturated fatty acids), but also in micronutrients and fibre.[1] Insect farming is more economical and environmentally friendly compared with that of traditional livestock. Moreover, reared insects present an average feed-to-protein conversion ratio that is higher than for other species.[2,3] As of 2018, insects are considered as novel foods in Europe, which paves the way for further research on their nutritional value and incorporation into foodstuffs.[4]

Mealworms are already being raised on an industrial scale, and are considered as good protein sources for the future.[5] Some insect species, such as *Tenebrio molitor*, can be found in some stores in European countries for human consumption.[2] Considering the estimated increase in world population, up to 9 billion people by 2050, we hypothesize that entomophagy will be normalised in most countries in the short term.[6,7]

Enzymatic hydrolysis of proteins to obtain bioactive peptides from insects was reported in 2005, when angiotensin-converting enzyme (ACE) inhibitory peptides from four insects were identified.[8] Recently, the biological potential of proteins from edible insects subjected to enzymatic hydrolysis has been reviewed.[9] Focusing on *T. molitor*, some bioactivities have been described for peptides released from this source by enzymatic treatments, such as antioxidant,[10] antihypertensive,[11] or antithrombotic activities.[12]

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Most of the peptides exhibiting antihypertensive activities are able to inhibit the proteolytic activity of angiotensin-converting enzyme I, an enzyme involved in blood pressure regulation.[13,14] For the antidiabetic peptides, the major mechanism is the inhibition of dipeptidyl peptidase IV, an enzyme involved in carbohydrate metabolism.[15,16] Antioxidant activities may be exerted through a wide range of mechanisms, such as scavenging of free radicals, chelation of metal ions, and the ability to reduce oxidised species.[17,18] Antioxidants are widely employed in food processing to avoid lipid oxidation and oxidative damage.[19–21]

These natural compounds that are released from protein hydrolysis have recently become regarded as adequate biological regulators because they exert physiological functions within the body,[22] and, consequently, they could be used in functional food to prevent some diseases without the occurrence of side effects.[23,24] The functionality and potential bioactivity of a given peptide depends on its length and amino acid composition, and is therefore influenced by the enzyme and operating parameters of the hydrolysis reaction. To date, very few studies have been found that investigate the impact of enzymatic treatment on both the antioxidative and antihypertensive bioactivities of *T. molitor* hydrolysates. To our knowledge, little on the *in vitro* DPP-IV inhibitory properties of *T. molitor* protein or derived hydrolysates has been reported so far.[25]

The aim of this work is to explore the *in vitro* ACE inhibitory, antioxidant capacity and DPP-IV inhibitory activities of *Tenebrio molitor* hydrolysates obtained by a set of conventional and combined enzymatic treatments employing commercial food-grade proteases (*i.e.* subtilisin, trypsin, ficin and flavourzyme), by 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, on 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 a cysteine endoprotease; and flavourzyme 1000L™ (3.4.11.1), which is an enzymatic complex mostly comprising exoprotease (*i.e.* aminopeptidase and dipeptidase) fractions.[26] All the enzymes were provided by Nozoymes (Bagsvaerd, Denmark). The reagents employed for the analytical assays were purchased from SigmaAldrich (St Louis, USA).

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 50 °C and pH 8. Protein at 30 g L^{-1} from *T. molitor* was diluted in distilled water. The enzyme-to-substrate ratio was set at 3% and the reaction continued until the desired degree of hydrolysis was achieved.

Titration allowed monitoring of the degree of hydrolysis (DH) as a function of the consumption of base (1 M NaOH) required to maintain the pH in the course of the reaction.[27] A set of protein hydrolysates were produced using six different enzymatic treatments, classified into two groups as follows:

(1) Single enzyme reactions, employing subtilisin (denoted as S), porcine trypsin (T) or ficin (F) as sole catalysts.

(2) 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 a 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, 50 °C and enzyme/substrate = 3%), the full experimental range of DH could not be completed for some enzymatic treatments. For instance, trypsin did not allow protein hydrolysates above a DH of 15%. Porcine trypsin is an endopeptidase that bonds near arginine and lysine residues.[28] This specificity restrained the number of peptide bonds prone to enzyme attack and, therefore, the final level of DH reached. Ficin is reported to attack a broad range of peptidic bonds, such as Ala, Asn, Gly, Leu, Lys, Tyr and Val.[29] However, *Tenebrio* meal showed limited proteolysis with ficin treatment, attaining a steady maximum DH of 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 dipeptidases.[30] Due to its specificity towards N-terminal sites, this enzyme requires initial degradation of the native protein by an endopeptidase,[31] so it was not employed as a single catalyst but only in combination with subtilisin (treatment S–E).

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

2.3. Molecular weight distribution of the protein hydrolysates

Molecular mass distribution was estimated by gel filtration chromatography using 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 the mobile phase in a Superdex Peptide 10/300GL column (GE Healthcare, 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 (13 700 Da).

2.4. Determination of protein solubility and protein content

Protein solubility was determined as described by Amiri-Rigi *et al.*[32] with some modifications. Briefly, 800 mg of powdered

hydrolysate was diluted with 20 mL of ultrapure water and stirred at 500 rpm and 20 °C 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.

The protein content of the samples was analysed in a Flash 2000 Organic elemental analyser (Thermo Scientific). Gases from combustion with oxygen at high temperature (1020 °C) were transported through a gas chromatographic column to separate them and a thermal conductivity detector to detect the signal for each element proportional to the concentration. The nitrogen-to-protein factor was considered to be 5.6, as reported by Janssen *et al.*[33]

2.5. ACE inhibitory activity

The ACE inhibitory activity was determined as described by Shalaby *et al.*[34] The percentage of inhibition of ACE exerted by the hydrolysates was determined spectrophotometrically by mixing 10 μL of the enzyme (0.25 U mL^{-1}), 10 μL of a wide range of concentrations of the samples and 150 μL of 0.88 mM of substrate (*N*-[3-(2-furyl)acryloyl]-L-phenyl-alanyl-glycyl-glycine, 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 over 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 from the ratio of the slopes of the 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 the absence of hydrolysate), as expressed by eqn (1).

$$\%\ \text{ACE inhibition} = \left(1 - \frac{s_i}{s_0}\right) \times 100 \quad (1)$$

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

2.6. Antioxidant activities of the hydrolysates

2.6.1. DPPH scavenging activity. The DPPH scavenging activity was determined as described by Picot *et al.*[35] and García-Moreno *et al.*[36] For this purpose, a 1 : 1 mixture containing hydrolysate at increasing protein concentrations (1–10 mg mL^{-1}) 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 from eqn (2):

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

where the control sample was prepared using methanol instead of DPPH, and the blank solution contained 1 mL DPPH and 1 mL distilled water. The IC_{50} value was determined as the concentration of hydrolysate that reduces DPPH activity by 50%.

2.6.2 Ferrous ion chelating activity. The chelating activity of the hydrolysates was determined as described by Decker and Welch[37] and García-Moreno *et al.*[36] For this purpose, 1 mL of hydrolysate at increasing protein concentration (0.5–4 mg mL^{-1}) was mixed with 3.7 mL of deionised water and 0.1 mL of 2 mM ferrous chloride. After 3 minutes incubation, 0.2 mL of 5 mM ferrozine was added to stop the reaction. After 10 min of incubation, the absorbance of the mixture was measured at 562 nm. The ferrous ion chelating activity was then calculated using eqn (3):

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

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). The reducing power of the hydrolysates was determined as described by Oyaizu.[38] Briefly, a mixture of 2 mL of hydrolysate at different protein concentrations (1–20 mg mL^{-1}), 2 mL of 0.2 mM phosphate buffer and 2 mL of 1% potassium ferricyanide was incubated for 20 min at 50 °C. Then, 2 mL of 10% TCA (trichloroacetic acid) solution was added, the mixture stirred and, after centrifugation, 2 mL of supernatant was extracted and mixed with 2 mL of water and 0.4 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm after incubating for 10 minutes at room temperature. Since the absorbance increases with hydrolysate concentration, the reducing power capacity was reported using the $EC_{0.5}$ value, which is defined as the concentration of protein with an absorbance of 0.5 units.

2.7 DPP-IV inhibitory activity

The DPP-IV inhibition assay was performed as described by Lacroix and Li-Chan,[39] with slight modification. Briefly, 25 μL of enzyme (0.02 U mL^{-1}) was 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 Gly-Pro-*p*-nitroanilide and the absorbance at 405 nm was measured over 120 minutes, at 2-minute intervals employing a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). The IC_{50} was calculated by plotting the progress of the reactions compared with the control.

3. Results and discussion

3.1. Protein solubilisation and molecular weight profiles of the hydrolysates

The average levels of protein solubilisation shown by the raw protein and the set of hydrolysates are depicted in Fig. 1a. After enzymatic treatment, all the resulting hydrolysates presented improved solubility compared with the undigested

sample of *T. molitor*, which showed, on average, 8.0 ± 1.5% w/w of protein solubility. Indeed, the protein solubility of the hydrolysates ranged from 27% to 46% w/w. On average, the 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 a DH of 20%, except for treatment with subtilisin–flavourzyme (SE20), which exhibited the maximum for the experimental series (46% w/w, on average). Although single trypsin could not attain a DH of 20%, this treatment was the most efficient for solubilising *Tenebrio* protein, attaining 45.3% w/w on average at a DH of 10%. Interestingly, this value was not improved at a DH of 15%.

The solubility of protein in a medium depends on a range of parameters, such as molecular weight, hydrophobicity, amino acid charges, pH or ionic strength.[40] Increasing the degree of hydrolysis is generally correlated with better solubilisation as new polar groups are exposed by enzyme attack.[41,42] The high solubilisation attained after trypsin treatment is explained by its specificity towards Arg or Lys residues, releasing positively charged (and therefore hydrophilic) peptides into the medium. The specificity of trypsin explains why further hydrolysis above a DH of 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

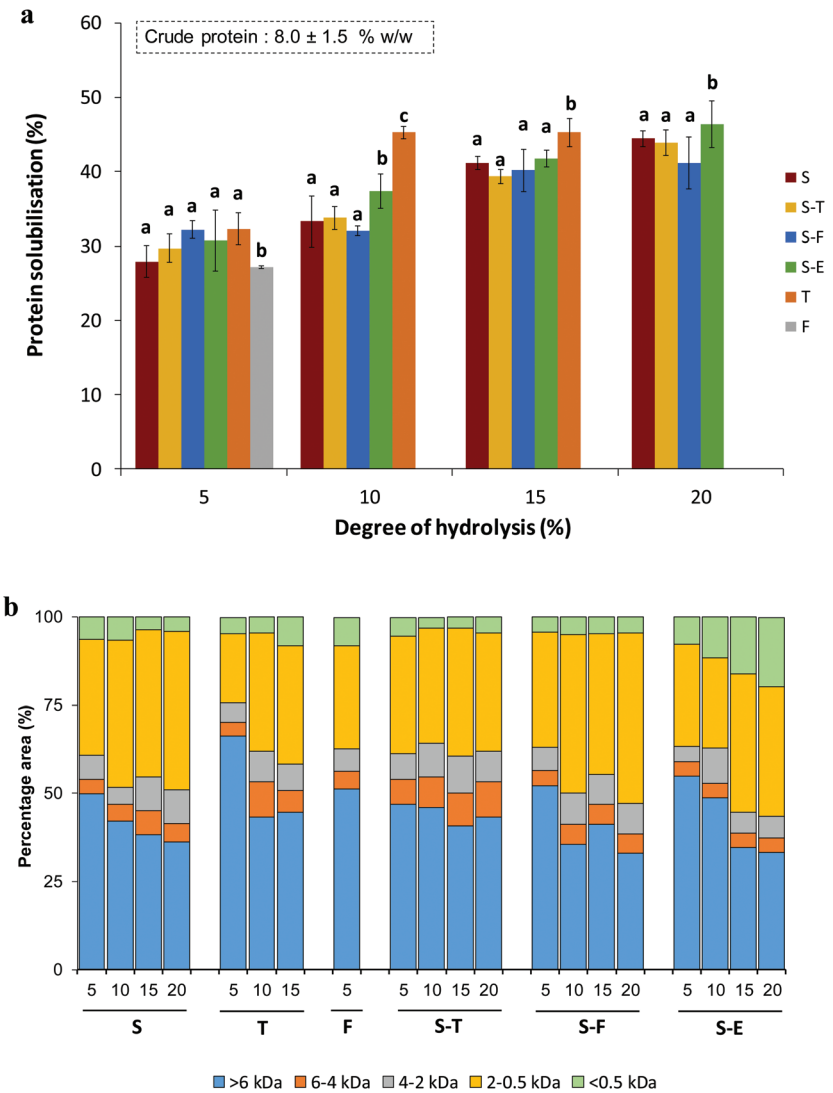

Fig. 1 Effect of enzymatic treatment and degree of hydrolysis on (a) protein solubilization and (b) molecular weight distribution of the hydrolysates. Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

variation with DH except for the combined treatment S–E, which increased the 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,[31,43] when the release of free amino acids or short-chain peptides is targeted.

3.3. ACE inhibitory activity

Fig. 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^{-1}). The raw *Tenebrio* protein exhibited low ACE inhibition (IC_{50} >100 mg mL^{-1}), 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^{-1}, 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^{-1}. In this regard, an average IC_{50} of 0.10 mg mL^{-1} was reported for the HPLC-purified fraction of *Tenebrio* larva hydrolysates produced by gastrointestinal enzymes (*i.e.* pepsin, trypsin, chymotrypsin).[44] As for the subtilisin hydrolysates S5 to S20, they showed a significant improvement in ACE inhibition with increasing DH, presenting a minimal IC_{50} (0.35 ± 0.02 mg mL^{-1}) at a DH of 20%. This value is similar to the ACE inhibition reported for subtilisin hydrolysates from *Tenebrio* larva at a DH of 20%.[11] The combination of subtilisin with other enzymes did not improve the ACE inhibitory activity with respect to the hydrolysates produced with subtilisin as the sole enzyme. As a general trend, ACE inhibition was positively correlated with DH for the hydrolysates S–T, S–F and S–E in the DH range from 5% to 15%. However, extensive hydrolysis seems to be detrimental to this activity (*e.g.* IC_{50} was 0.56 and 1.28 mg mL^{-1} for ST15 and ST20, respectively), probably due to the breaking of the active peptide sequences released previously.

The 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 interaction with the active site of the angiotensin-converting enzyme I.[45] 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,[46] since it cleaves peptide bonds preferably with participation of hydrophobic residues. Trypsin reacts specifically with 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.[45] Moreover, it is reported that trypsin presents chymotrypsin-like behaviour at reaction temperatures above 45 °C, releasing new peptides with hydrophobic residues (Tyr, Trp, Phe) at the carboxyl side (*i.e.* potential ACE inhibitors).[47] We hypothesize that this observation could explain the high levels of ACE inhibition displayed by tryptic hydrolysates that were produced at 50 °C.

3.4. Antioxidant activities of the *T. molitor* hydrolysates

3.4.1. DPPH scavenging activity and reducing power. Fig. 3a and b presents the observed values for 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 manner by the DH and enzymatic treatment. All the enzymatic treatments assayed improved the antioxidant capacities of the hydrolysates, which is related to the intact *Tenebrio* protein. Indeed, the series of hydrolysates

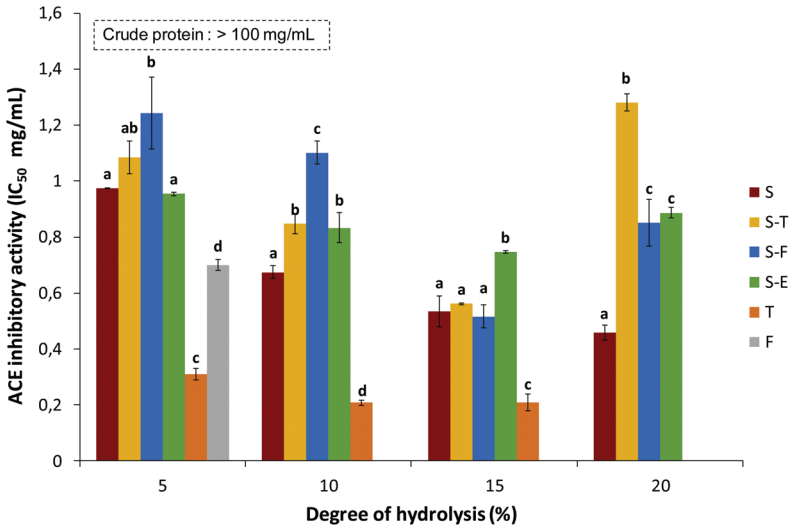

Fig. 2 *In vitro* ACE inhibitory activity of the hydrolysates as a function of enzymatic treatment and degree of hydrolysis. Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

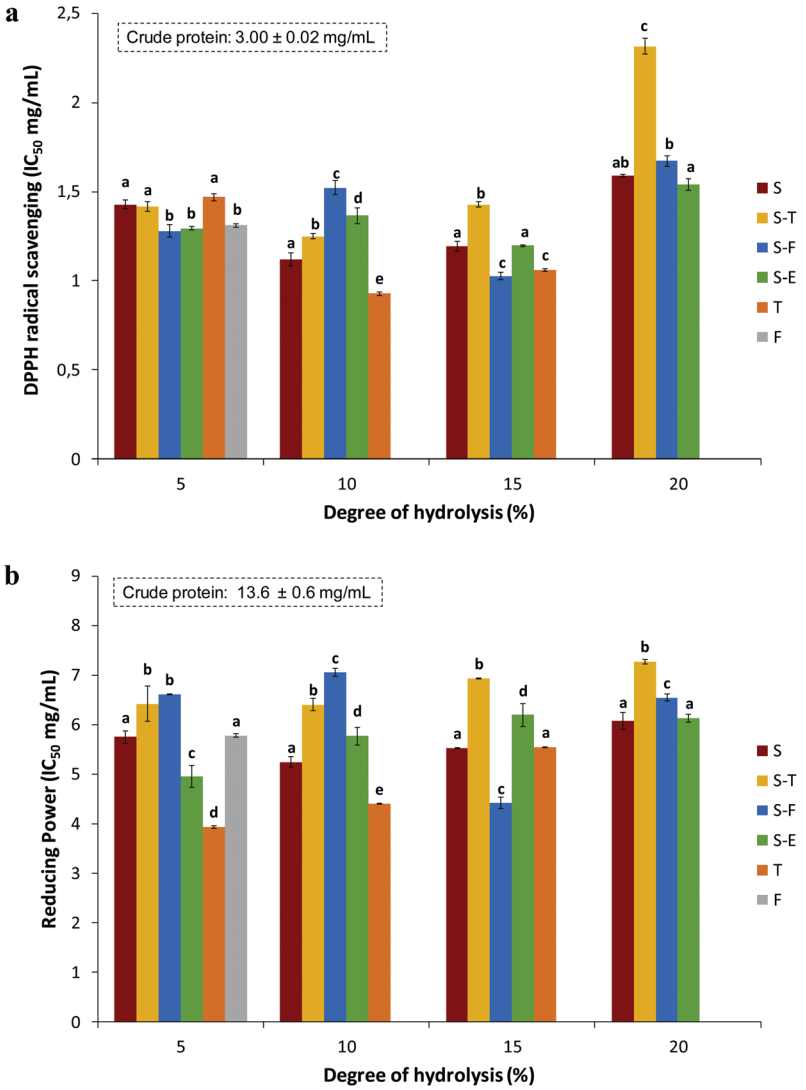

Fig. 3 Influence of enzymatic treatment and DH on (a) the *in vitro* DPPH scavenging activity and (b) the iron reducing power. Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic treatments.

presented IC_{50} values for DPPH ranging from 1.03 to 2.31 mg mL^{-1}, while the IC_{50} of the undigested protein was 3.01 mg mL^{-1}. As shown in Fig. 3a, low DH hydrolysates exhibited weak radical-scavenging activity, which improved with increasing DH until an optimum was reached. 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 a DH of 10% (*e.g.* IC_{50} of 0.93 ± 0.01 mg mL^{-1} for T10, the minimum for the experimental series) while the treatments S–F and S–E did so at a DH of 15%.

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^{-1}). As observed with the radical-scavenging activity, tryptic hydrolysates presented the lowest $EC_{0.5}$ value of the experimental series (~4 mg mL^{-1}) at a DH of 5%. This value was maintained at a DH of 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 of between 2 and 10 amino acid residues. In this respect, the antioxidant peptides identified so far in *T. molitor* hydrolysates, such as AAAPVAVAK, YDDGSYKPH and AGDDAPR,[10] fall into this size range.

Several authors have reported a direct relationship between the presence of hydrophobic residues in the peptide sequence and potential radical-scavenging activity,[48,49] highlighting the good antioxidant properties of subtilisin and trypsin hydrolysates. As for the length of the peptide chain, it is widely accepted that antioxidant capacity is favoured by the presence

of short peptides,[50,51] due to the steric hindrance associated with larger peptides.[52] The trend observed in this work is in agreement with 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.[53,54]

Some previous studies report improvement in antioxidant activities when subtilisin is used in combination with other enzymes, such as trypsin, ficin or flavourzyme. A higher radical-scavenging activity of *T. molitor* hydrolysates was obtained by combination of subtilisin and flavourzyme than was obtained with single treatments.[55] Antioxidant properties of horse mackerel hydrolysates produced by a combination of subtilisin and trypsin in different proportions have also 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 of 2 : 1 subtilisin–trypsin for optimal DPPH scavenging and Fe^{3+} reducing power activities.[56]

3.4.2. Fe^{2+} chelating activity. The ferrous chelating activity of the hydrolysates (Fig. 4a), reported as IC_{50}, ranged from 0.53 to 2.12 mg mL^{-1}. Intact *Tenebrio* protein presented an IC_{50} of 4.86 ± 0.34 mg mL^{-1}, leading to the conclusion 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^{-1}) at a DH of 20%, regardless of the enzymatic treatment. The minimal IC_{50} value corresponded to the hydrolysate SF20 (0.53 mg mL^{-1}) 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, a higher degree of hydrolysis is correlated with an increased chelating bioactivity of the peptides, since new sites

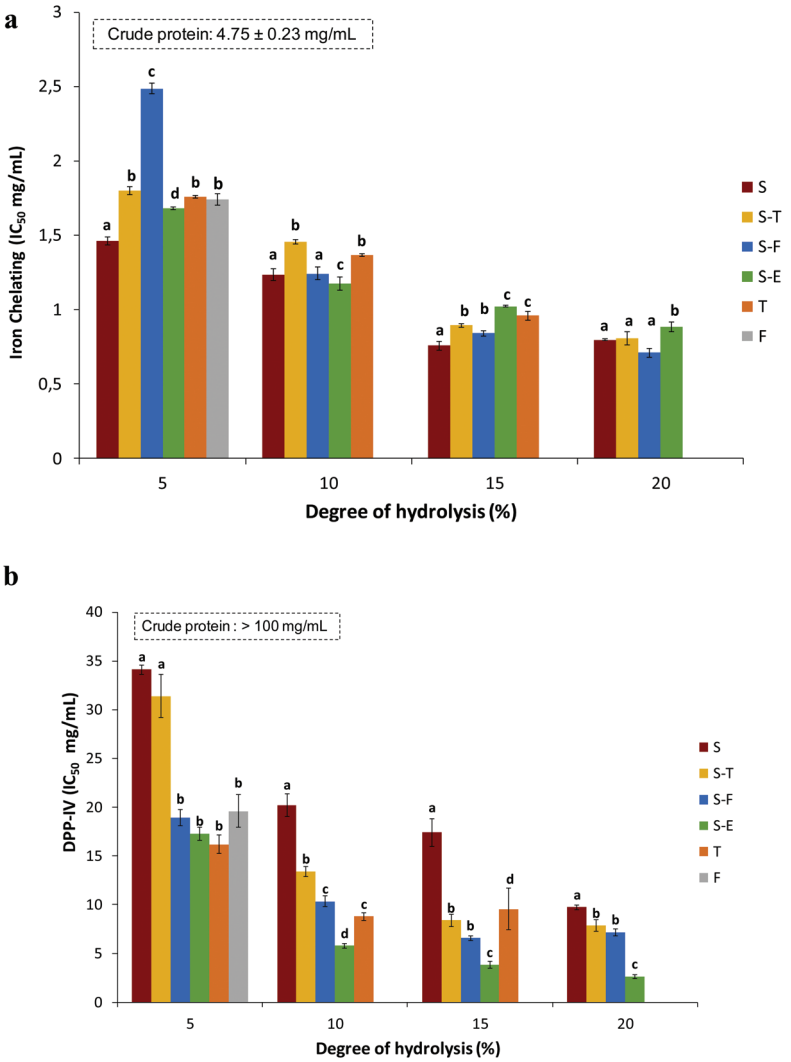

Fig. 4 Influence of enzymatic treatment and DH on (a) the *in vitro* ferrous chelating activity and (b) the DPP-IV inhibitory activity. Values are presented as the mean of three replicates ± standard deviation. Different superscript letters indicate statistically significant differences among enzymatic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

that are 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.[48,57,58] Other residues, such as His, show high affinity for metal coordination when located at the N terminus.[59] In this regard, the improvement in chelating activity with DH observed in T and S–T hydrolysates could be explained by release of new peptides containing Arg or Lys at the side chain. So far, references reporting chelating peptides from insect protein are limited to some insect species hydrolysed by gastrointestinal enzymes. In this regard, chelating activities inferior to our results (IC_{50} 2.03 mg mL^{-1}) for silkworm larva protein subjected to simulated gastrointestinal digestion (*i.e.* pepsin, trypsin and chymotrypsin) have been reported.[60] Similarly, antioxidant activities of the digested protein (*i.e.* alpha-amylase, pepsin and pancreatin) from some edible insect species have recently been reported.[10] The authors identified some peptides displaying strong Fe^{2+} chelating activity (IC_{50} 0.11 mg mL^{-1} on average), such as AAAPVAVAK, YDDGSYKPH and AGDDAPR from *Tenebrio molitor*, or GKDAVIV and AIGVGAIER from *Schitocerca gregaria*.

3.5 DPP-IV inhibitory activity

The DPP-IV inhibitory activity of the hydrolysates is presented in Fig. 4b. The IC_{50} values ranged from 2.62 to 34.13 mg mL^{-1}, whereas the intact protein did not show bioactivity (IC_{50} >100 mg mL^{-1}). The results show significant differences in DPP-IV inhibitory activity among the enzymatic treatments. Moreover, we observed a positive correlation between DH and the DPP-IV activity of the hydrolysates, regardless of the enzymatic treatment. The stabilisation of the antidiabetic activity above a DH of 10% reported for subtilisin hydrolysates has also been described for other substrates.[61] The subtilisin–flavourzyme treatment led to the best improvement in DPP-IV activity, attaining maximal inhibition at a DH of 20% (IC_{50} 2.62 ± 0.16 mg mL^{-1}). Compared with the sole use of subtilisin, the combined treatment with flavourzyme significantly improved the levels of antidiabetic activity. The efficacy of the combination of subtilisin and flavourzyme has already been reported to produce good DPP-IV inhibitor peptides.[62] This fact is related to the synergy between both enzymes, where subtilisin releases peptide fragments that 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 the total area for the peptidic fraction below 0.5 kDa. Most of the antidiabetic peptides identified so far correspond to di- and tripeptides,[61,63] and have been isolated from dairy, vegetable or marine sources.[62,64,65] As for insect sources, the DPP-IV inhibitory activity of lesser mealworm (*A. diaperninus*) protein has been studied, reporting the maximal antidiabetic activity for the thermolysin hydrolysate (IC_{50} of 0.63 mg mL^{-1}).[66]

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^{-1}) have been identified.[25] Hence, this is the first study describing the *in vitro* antidiabetic activity of *Tenebrio molitor* hydrolysates obtained from assay of a set of commercial proteases.

4. Conclusions

Insects are gaining much interest as novel sources for human nutrition, in view of recent changes in population and concerns for 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 1 : 1 mixtures. 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.

The most bioactive ACE inhibitory peptidic fractions were obtained with trypsin, at a DH of 10% (IC_{50} 0.27 mg mL^{-1}), and subtilisin, at a DH of 20% (IC_{50} 0.35 mg mL^{-1}), which was related to the release of peptides with hydrophobic residues in the terminal position.

The highest DPPH scavenging and Fe^{3+} reducing activities were displayed by the subtilisin and trypsin hydrolysates at a DH of 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^{-1} at a DH of 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^{-1}) at a DH of 20%. According to the molecular weight profile, this hydrolysate presented the highest content of 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 be used as functional ingredients in food and nutraceutical preparations.

Conflicts of interest

There are no conflicts to declare.

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References

1 C. Azagoh, F. Ducept, R. Garcia, L. Rakotozafy, M.-E. Cuvelier, S. Keller, R. Lewandowski and S. Mezdour, Extraction and physicochemical characterization of Tenebrio molitor proteins, *Food Res. Int.*, 2016, **88**, 24–31.

2 A. van Huis, J. Van Itterbeeck, H. Klunder, E. Mertens, A. Halloran, G. Muir and P. Vantomme, *Edible insects: Future prospects for food and feed security*, 2013, vol. 171.

3 A. Osimani, C. Garofalo, V. Milanović, M. Taccari, F. Cardinali, L. Aquilanti, M. Pasquini, M. Mozzon, N. Raffaelli, S. Ruschioni, P. Riolo, N. Isidoro and F. Clementi, Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union, *Eur. Food Res. Technol.*, 2017, **243**, 1157–1171.

4 European Council Regulations No. 2015/2283, Regulation (Eu) 2015/2283 on novel foods, *Off. J. Eur. Union*, 2015, **327**, 1–22.

5 M. De Marco, S. Martínez, F. Hernandez, J. Madrid, F. Gai, L. Rotolo, M. Belforti, D. Bergero, H. Katz, S. Dabbou, A. Kovitvadhi, I. Zoccarato, L. Gasco and A. Schiavone, Nutritional value of two insect larval meals (Tenebrio molitor and Hermetia illucens) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy, *Anim. Feed Sci. Technol.*, 2015, **209**, 211–218.

6 H. P. S. Makkar, G. Tran, V. Heuzé and P. Ankers, State-of-the-art on use of insects as animal feed, *Anim. Feed Sci. Technol.*, 2014, **197**, 1–33.

7 A. Gravel and A. Doyen, The use of edible insect proteins in food: Challenges and issues related to their functional properties, *Innovative Food Sci. Emerging Technol.*, 2020, **59**, 102272.

8 L. Vercruysse, G. Smagghe, T. Beckers and J. Van Camp, Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis, *Food Chem.*, 2009, **114**, 38–43.

9 A. B. Nongonierma and R. J. FitzGerald, Unlocking the biological potential of proteins from edible insects through enzymatic hydrolysis: A review, *Innovative Food Sci. Emerging Technol.*, 2017, **43**, 239–252.

10 E. Zielińska, B. Baraniak and M. Karaś, Identification of antioxidant and anti-inflammatory peptides obtained by simulated gastrointestinal digestion of three edible insects species (Gryllodes sigillatus, Tenebrio molitor, Schistocerca gragaria), *Int. J. Food Sci. Technol.*, 2018, **53**, 2542–2551.

11 C. Dai, H. Ma, L. Luo and X. Yin, Angiotensin I-converting enzyme (ACE) inhibitory peptide derived from Tenebrio molitor (L.) larva protein hydrolysate, *Eur. Food Res. Technol.*, 2013, **236**, 681–689.

12 F. Chen, H. Jiang, Y. Gan, W. Chen and G. Huang, Optimization of Hydrolysis Conditions for Obtaining Antithrombotic Peptides from Tenebrio Molitor Larvae, *Am. J. Biochem. Biotechnol.*, 2019, **15**, 52–60.

13 L. T. Skeggs, H. R. Kahn and N. P. Shumway, The preparation and function of the hypertension-converting enzyme, *J. Exp. Med.*, 1956, **103**, 295–299.

14 S. Mizuno, K. Matsuura, T. Gotou, S. Nishimura, O. Kajimoto, M. Yabune, Y. Kajimoto and N. Yamamoto, Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension, *Br. J. Nutr.*, 2005, **94**, 84.

15 H. Duez, B. Cariou and B. Staels, DPP-4 inhibitors in the treatment of type 2 diabetes, *Biochem. Pharmacol.*, 2012, **83**, 823–832.

16 J. A. Pospisilik, S. G. Stafford, H. U. Demuth, R. Brownsey, W. Parkhouse, D. T. Finegood, C. H. S. McIntosh and R. A. Pederson, Long-term treatment with the dipeptidyl peptidase IV inhibitor P32/98 causes sustained improvements in glucose tolerance, insulin sensitivity, hyperinsulinemia, and β-cell glucose responsiveness in VDF (fa/fa) Zucker rats, *Diabetes*, 2002, **51**, 943–950.

17 E. N. Frankel and A. S. Meyer, The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants, *J. Sci. Food Agric.*, 2000, **80**, 1925–1941.

18 A. Hunyadi, The mechanism(s) of action of antioxidants: From scavenging reactive oxygen/nitrogen species to redox signaling and the generation of bioactive secondary metabolites, *Med. Res. Rev.*, 2019, **39**, 2505–2533.

19 Y. Wang, Q. Huang, D. Kong and P. Xu, Production and Functionality of Food-derived Bioactive Peptides: A Review, *Mini-Reviews Med. Chem.*, 2018, **18**, 1524–1535.

20 P. Hajieva and C. Behl, Antioxidants as a potential therapy against age-related neurodegenerative diseases: amyloid Beta toxicity and Alzheimer's disease., *Curr. Pharm. Des.*, 2006, **12**, 699–704.

21 C. C. Lin and J. H. Liang, Effect of antioxidants on the oxidative stability of chicken breast meat in a dispersion system, *J. Food Sci.*, 2002, **67**, 530–533.

22 P. A. Harnedy and R. J. FitzGerald, Bioactive peptides from marine processing waste and shellfish: A review, *J. Funct. Foods*, 2012, **4**, 6–24.

23 R. Perez-Gregorio and J. Simal-Gandara, A Critical Review of Bioactive Food Components, and of their Functional Mechanisms, Biological Effects and Health Outcomes, *Curr. Pharm. Des.*, 2017, **23**, 2731–2741.

24 I. M. E. Lacroix and E. C. Y. Li-Chan, Inhibition of dipeptidyl peptidase (DPP)-IV and α-glucosidase activities by pepsin-treated whey proteins, *J. Agric. Food Chem.*, 2013, **61**, 7500–7506.

25 I. Dávalos Terán, K. Imai, I. M. E. Lacroix, V. Fogliano and C. C. Udenigwe, Bioinformatics of edible yellow mealworm (Tenebrio molitor) proteome reveal the cuticular proteins as promising precursors of dipeptidyl peptidase-IV inhibitors, *J. Food Biochem.*, 2020, **44**, e13121.

26 M. Merz, T. Eisele, P. Berends, D. Appel, S. Rabe, I. Blank, T. Stressler and L. Fischer, Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-Step Purification and Partial Characterization of Eight Enzymes, *J. Agric. Food Chem.*, 2015, **63**, 5682–5693.
27 F. Camacho, P. González-Tello, M. P. Páez-Dueñas, E. M. Guadix and A. Guadix, Correlation of base consumption with the degree of hydrolysis in enzymic protein hydrolysis, *J. Dairy Res.*, 2001, **68**, 251–265.
28 J. V. Olsen, S.-E. Ong and M. Mann, Trypsin Cleaves Exclusively C-terminal to Arginine and Lysine Residues, *Mol. Cell. Proteomics*, 2004, **3**, 608–614.
29 Y. Hou, Z. Wu, Z. Dai, G. Wang and G. Wu, Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance, *J. Anim. Sci. Biotechnol.*, 2017, **8**, 1–13.
30 M. R. S. Campos, L. A. C. Guerrero and D. A. B. Ancona, Angiotensin-I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea Vigna unguiculata hydrolysates, *J. Sci. Food Agric.*, 2010, **90**, 2512–2518.
31 H. A. Nchienzia, R. O. Morawicki and V. P. Gadang, Enzymatic hydrolysis of poultry meal with endo- and exopeptidases, *Poult. Sci.*, 2010, **89**, 2273–2280.
32 A. Amiri-Rigi, Z. Emam-Djomeh, M. A. Mohammadifar and M. Mohammadi, Spray drying of low-phenylalanine skim milk: Optimisation of process conditions for improving solubility and particle size, *Int. J. Food Sci. Technol.*, 2012, **47**, 495–503.
33 R. H. Janssen, J.-P. Vincken, L. A. M. van den Broek, C. M. M. Lakemond and V. Fogliano, Nitrogen-to-Protein Conversion Factors for Three Edible Insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens, *J. Agric. Food Chem.*, 2017, **65**, 2275–2278.
34 S. M. Shalaby, M. Zakora and J. Otte, Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates, *J. Dairy Res.*, 2006, **73**, 178–186.
35 L. Picot, R. Ravallec, F. P. Martine, L. Vandanjon, P. Jaouen, M. Chaplain-Derouiniot, F. Guérard, A. Chabeaud, Y. Legal, O. M. Alvarez, J. P. Bergé, J. M. Piot, I. Batista, C. Pires, G. Thorkelsson, C. Delannoy, G. Jakobsen, I. Johanssonm and P. Bourseau, Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties, *J. Sci. Food Agric.*, 2010, **90**, 1819–1826.
36 P. J. García-Moreno, I. Batista, C. Pires, N. M. Bandarra, F. J. Espejo-Carpio, A. Guadix and E. M. Guadix, Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species, *Food Res. Int.*, 2014, **65**, 469–476.
37 E. A. Decker and B. Welch, Role of Ferritin as a Lipid Oxidation Catalyst in Muscle Food†, *J. Agric. Food Chem.*, 1990, **38**, 674–677.
38 M. Oyaizu, *Nippon Shokuhin Kogyo Gakkaishi*, 1988, **35**, 771–775.
39 I. M. E. Lacroix and E. C. Y. Li-Chan, Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates, *Int. Dairy J.*, 2012, **25**, 97–102.
40 A. G. B. Wouters, I. Rombouts, E. Fierens, K. Brijs and J. A. Delcour, Relevance of the Functional Properties of Enzymatic Plant Protein Hydrolysates in Food Systems, *Compr. Rev. Food Sci. Food Saf.*, 2016, **15**, 786–800.
41 V. Klompong, S. Benjakul, D. Kantachote and F. Shahidi, Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type, *Food Chem.*, 2007, **102**, 1317–1327.
42 H. G. Kristinsson and B. A. Rasco, *Fish protein hydrolysates: Production, biochemical, and functional properties*, 2000, vol. **40**.
43 H. Saadaoui, F. J. Espejo-Carpio, E. M. Guadix, R. Ben Amar and R. Pérez-Gálvez, Bi-objective optimization of tuna protein hydrolysis to produce aquaculture feed ingredients, *Food Bioprod. Process.*, 2019, **115**, 26–35.
44 A. Cito, E. Dreassi, R. Frosinini, A. Zanfini, C. Pianigiani, M. Botta and V. Francardi, The potential beneficial effects of Tenebrio molitor (Coleoptera Tenebrionidae) and Galleria mellonella (Lepidoptera Pyralidae) on human health, *Redia*, 2017, **100**, 125–133.
45 G.-H. Li, G.-W. Le, Y.-H. Shi and S. Shrestha, Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, *Nutr. Res.*, 2004, **24**, 469–486.
46 A. Connolly, C. O. Piggott and R. J. FitzGerald, In vitro α-glucosidase, angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory properties of brewers' spent grain protein hydrolysates, *Food Res. Int.*, 2014, **56**, 100–107.
47 S. C. Cheison, M. Schmitt, E. Leeb, T. Letzel and U. Kulozik, Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS, *Food Chem.*, 2010, **121**, 457–467.
48 M. Ovissipour, B. Rasco, S. G. Shiroodi, M. Modanlow, S. Gholami and M. Nemati, Antioxidant activity of protein hydrolysates from whole anchovy sprat (Clupeonella engrauliformis) prepared using endogenous enzymes and commercial proteases, *J. Sci. Food Agric.*, 2013, **93**, 1718–1726.
49 A. T. Girgih, R. He, F. M. Hasan, C. C. Udenigwe, T. A. Gill and R. E. Aluko, Evaluation of the in vitro antioxidant properties of a cod (Gadus morhua) protein hydrolysate and peptide fractions, *Food Chem.*, 2015, **173**, 652–659.
50 C. F. Ajibola, J. B. Fashakin, T. N. Fagbemi and R. E. Aluko, Effect of peptide size on antioxidant properties of African yam bean seed (Sphenostylis stenocarpa) protein hydrolysate fractions, *Int. J. Mol. Sci.*, 2011, **12**, 6685–6702.
51 D. Jin, X. Liu, X. Zheng, X. Wang and J. He, Preparation of antioxidative corn protein hydrolysates, purification and evaluation of three novel corn antioxidant peptides, *Food Chem.*, 2016, **204**, 427–436.

52 I. D. Nwachukwu and R. E. Aluko, Structural and functional properties of food protein-derived antioxidant peptides, *J. Food Biochem.*, e12761, DOI: 10.1111/jfbc.12761.
53 P. Mudgil, L. S. Omar, H. Kamal, B. P. Kilari and S. Maqsood, Multi-functional bioactive properties of intact and enzymatically hydrolysed quinoa and amaranth proteins, *LWT*, 2019, **110**, 207–213.
54 M. B. Elmalimadi, J. R. Jovanović, A. B. Stefanović, S. J. Tanasković, S. B. Djurović, B. M. Bugarski and Z. D. Knežević-Jugović, Controlled enzymatic hydrolysis for improved exploitation of the antioxidant potential of wheat gluten, *Ind. Crops Prod.*, 2017, **109**, 548–557.
55 Y. Tang, T. Debnath, E. J. Choi, Y. W. Kim, J. P. Ryu, S. Jang, S. U. Chung, Y. J. Choi and E. K. Kim, Changes in the amino acid profiles and free radical scavenging activities of Tenebrio molitor larvae following enzymatic hydrolysis, *PLoS One*, 2018, **13**, 1–14.
56 R. Morales-Medina, R. Pérez-Gálvez, A. Guadix and E. M. Guadix, Multiobjective optimization of the antioxidant activities of horse mackerel hydrolysates produced with protease mixtures, *Process Biochem.*, 2017, **52**, 149–158.
57 A. Bougatef, M. Hajji, R. Balti, I. Lassoued, Y. Triki-Ellouz and M. Nasri, Antioxidant and free radical-scavenging activities of smooth hound (Mustelus mustelus) muscle protein hydrolysates obtained by gastrointestinal proteases, *Food Chem.*, 2009, **114**, 1198–1205.
58 Q. Liu, B. Kong, Y. L. Xiong and X. Xia, Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis, *Food Chem.*, 2010, **118**, 403–410.
59 R. C. Dunbar, J. Martens, G. Berden and J. Oomens, Binding of Divalent Metal Ions with Deprotonated Peptides: Do Gas-Phase Anions Parallel the Condensed Phase?, *J. Phys. Chem. A*, 2018, **122**, 5589–5596.
60 Q.-Y. Wu, J.-Q. Jia, G.-X. Tan, J.-L. Xu and Z.-Z. Gui, Physicochemical properties of silkworm larvae protein isolate and gastrointestinal hydrolysate bioactivities, *Afr. J. Biotechnol.*, 2011, **10**, 6145–6153.
61 A. C. Neves, P. A. Harnedy, M. B. O'Keeffe and R. J. FitzGerald, Bioactive peptides from Atlantic salmon (Salmo salar) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory, and antioxidant activities, *Food Chem.*, 2017, **218**, 396–405.
62 P. A. Harnedy-Rothwell, C. M. McLaughlin, M. B. O'Keeffe, A. V. Le Gouic, P. J. Allsopp, E. M. Mcsorley, S. Sharkey, J. Whooley, B. McGovern, F. P. M. O'Harte and R. J. Fitzgerald, Identification and characterisation of peptides from a boarfish (Capros aper) protein hydrolysate displaying in vitro dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulinotropic activity, *Food Res. Int.*, 2020, **131**, 108989.
63 P. A. Harnedy, V. Parthsarathy, C. M. McLaughlin, M. B. O'Keeffe, P. J. Allsopp, E. M. McSorley, F. P. M. O'Harte and R. J. FitzGerald, Atlantic salmon (Salmo salar) co-product-derived protein hydrolysates: A source of antidiabetic peptides, *Food Res. Int.*, 2018, **106**, 598–606.
64 C. Lammi, C. Bollati, S. Ferruzza, G. Ranaldi, Y. Sambuy and A. Arnoldi, Soybean-and lupin-derived peptides inhibit DPP-IV activity on in situ human intestinal Caco-2 cells and ex vivo human serum, *Nutrients*, 2018, **10**, 1–11.
65 E. Drummond, S. Flynn, H. Whelan, A. B. Nongonierma, T. A. Holton, A. Robinson, T. Egan, G. Cagney, D. C. Shields, E. R. Gibney, P. Newsholme, C. Gaudel, J. C. Jacquier, N. Noronha, R. J. Fitzgerald and L. Brennan, Casein Hydrolysate with Glycemic Control Properties: Evidence from Cells, Animal Models, and Humans, *J. Agric. Food Chem.*, 2018, **66**, 4352–4363.
66 I. M. E. Lacroix, I. Dávalos Terán, V. Fogliano and H. J. Wichers, Investigation into the potential of commercially available lesser mealworm (A. diaperinus) protein to serve as sources of peptides with DPP-IV inhibitory activity, *Int. J. Food Sci. Technol.*, 2019, **54**, 696–704.

