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Enzymatic hydrolysis of insect *Alphitobius diaperinus* towards the development of bioactive peptide hydrolysates

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Edible insects are a promising protein source for the future generation, due to their nutritional composition, sustainability and low environmental impact. Recent studies demonstrated their potential as a protein base to obtain bioactive peptides with potential applicability in the food industry. The aim of this study was to hydrolyze and analyze the potential of the edible insect *Alphitobius diaperinus* protein to develop an improved insect food ingredient with bioactive properties. After evaluating various conditions for the enzymatic hydrolysis with Alcalase 2.5L and Corolase PP, the best condition chosen for each enzyme was an enzyme : substrate ratio of 1.5% for 4 hours and a ratio of 3.0% for 6 hours, respectively. Insect protein hydrolysates are demonstrated to have relevant bioactive properties, namely antioxidant (by ABTS and ORAC methods) and antihypertensive activities (through the ability to inhibit the angiotensin converting enzyme, ACE), but no antimicrobial or antidiabetic properties were observed. Antioxidant activity values for hydrolysates obtained with Alcalase 2.5L and Corolase PP were 95.0 ± 0.8 and 95.7 ± 1.0 μmol Trolox equivalent per g insect powder by the ABTS method, 825.6 ± 85.5 and 944.8 ± 68.1 μmol Trolox equivalent per g insect powder by the ORAC method, respectively. Insect hydrolysates were able to inhibit the ACE and IC_{50} values for insect hydrolysates obtained with Alcalase 2.5L and Corolase PP were 55.5 ± 6.2 and 107.4 ± 9.7 μg of protein per mL, respectively. These insect protein hydrolysates can be used as a supplement/ingredient in the food industry with potential health benefits.

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1. Introduction

Entomophagy, the practice of eating insects, is already done worldwide by at least 2 billion people and there are more than 1900 species of edible insects described in the literature. Nowadays, this subject is becoming more relevant mostly due to population growth and future protein demand.[1] Therefore, new ways to develop sustainable sources of protein are needed and insects can be a feasible solution. Insects are a great source of protein, having approximately a content of protein of 60%[2] variable with insect and growth conditions. Comparing with conventional livestock, its production has low environmental impact and a great feed conversion ratio (FCR). The FCR is a measure of an animal's efficiency to convert feed mass into increased body mass (kilogram feed : kilogram live weight), the FCR for cricket is about 1.7, which is less than the value for pork (FCR of 5.0) and even less than the FCR of beef (10.0).[3,4] Even though entomophagy has been done for centuries around the world, the major challenge for the growth of this practice is in particular the western culture, because people view insects as dirty and disgusting.[5] Therefore, incorporating insects as an invisible ingredient can probably increase the willingness to try an insect-based product,[6] because people are more predisposed to accept and eat insects if they are unrecognizable. In this way, insects mixed with other food products can reduce the negative impact caused by eating the whole insect[7] and overcome some particular sensory properties that can make them less attractive. Furthermore, the repeated consumption of insects is also influenced by the price, taste and accessibility of these products in the market.[8] The consumption of insects can be increased by their use as a source of protein for the development of healthy ingredients such as protein hydrolysates. As reviewed by Nongonierma and colleagues (2017),[9] various studies analysed the impact of enzymatic hydrolysis on the bioactive properties of the resulting protein hydrolysates. Several bioactive peptides were obtained from insects, *i.e.*, peptides with benefits for human health and generally composed of approximately 2–20 amino acids.[10] So, enzymatic hydrolysis can potentially be used for the development of new insect-based ingredients with improved bioactive properties (*e.g.* antioxidant activity).

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Enzymatic hydrolysis can be done by using endogenous enzymes (digestive enzymes or microbial enzymes),[11] or exogenous enzymes (such as alcalase or flavourzyme), which are usually chosen over endogenous enzymes due to their ability to obtain a consistent peptide profile and composition.[12] This approach is widely used as a way to obtain bioactive peptides from various food matrices such soy protein,[13] milk protein,[14] fish protein,[15] egg white protein[16] among others. Studies using insect protein hydrolysates demonstrating bioactive properties have recently increased and they show bioactive properties such as antioxidant potential,[17–20] antihypertensive activity by the inhibition of Angiotensin converting-I enzyme (ACE),[17,19] antidiabetic activity through the dipeptidyl peptidase-IV (DPP-IV) inhibitory capacity[19,21,22] and anti-inflammatory activity.[18] Although various insects have demonstrated potential to be used as a source of bioactive peptides, there is a lack of information for some species, namely for *Alphitobius diaperinus*. This insect, commonly known as lesser mealworm, is a species that belongs to the Coleoptera order, the most consumed order of edible insects worldwide.[2] Hence, this work proposes to analyse, for the first time, the potential of this edible insect to produce antioxidant and antihypertensive peptides, through enzymatic hydrolysis using two enzymes, Alcalase™ 2.5L and Corolase PP.

2. Materials and methods

2.1. Materials

The edible insect lesser mealworm (*A. diaperinus*) powder was kindly supplied by the company Kreca® (Netherlands). The enzymes Alcalase 2.5L and Corolase PP were purchased from Aquitex (Portugal) and AB Enzymes GmbH (Germany), respectively. The other reagents were purchased from Sigma-Aldrich (USA) unless mentioned otherwise.

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of lesser mealworm insect powder was performed according to Coscueta *et al.* (2016)[13] and Hall *et al.* (2017),[23] with some modifications. During this assay, Alcalase 2.5L was used at ratios of enzyme : substrate (E/S) of 0.5, 1.5 and 3.0% (v/w) and the enzyme Corolase PP was also tested at ratios of E/S of 0.5, 1.5 and 3.0% (w/w). For the control, no enzyme was added. Briefly, 1% (w/v) of lesser mealworm insect powder was dissolved in phosphate buffer (pH 8.0) and enzymes Alcalase 2.5L or Corolase PP were added (reaction temperature 50 °C). Aliquots at different time points were taken (0, 30 min, 1, 2, 4, 6, 8 and 24 h). After hydrolysis, the enzymes were inactivated at 100 °C during 15 min. Then, the samples were cooled and centrifuged at 8000 rpm for 10 min at 4 °C (Universal 320R, Germany). The supernatant was collected and stored at −80 °C for further analysis. Hydrolysis was done in duplicate for each condition. Two analyses were performed to monitor the enzymatic hydrolysis, the degree of hydrolysis (DH) and the hydrolysate antioxidant activity (ABTS and ORAC scavenging assay).

2.2.1. Degree of hydrolysis. The DH was estimated by the determination of free amino groups by the reaction of 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) described by Hsu (2010).[24] Briefly, 50 μL of the sample (or water) was added to 125 μL of 200 mM sodium phosphate buffer (pH 8.2) and 50 μL of TNBS at 0.025% in a 96-well microplate (Sarstedt, Germany). Then, the microplate was incubated at 45 °C for 1 h and the absorbance was read at 340 nm using a Multiskan GO plate reader (Thermo Scientific, USA). A calibration curve of L-leucine was used to express the results. The DH was analysed in duplicate for each condition. The DH was calculated with the following expression:

$$\mathrm{DH}\ (\%) = 100 \times ((L_t - L_0)/(L_{\max} - L_0))$$

where L_t is the amount of amino groups released during the hydrolysis time, L_0 the amount of amino groups in the sample at initial hydrolysis time and $L_{\max}$ the maximum amount of amino groups present in the sample.

2.2.2. Total antioxidant activity. The total antioxidant activity of insect hydrolysates was determined by 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity based on Re *et al.* (1999).[25] The radical cation was produced by reacting ABTS with potassium persulfate. Then, 1 mL of ABTS solution was reacted with the sample for 6 min. The percentage of inhibition was then calculated comparing with a standard curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-caboxylic acid) [0.15–0.50 mg mL^{-1}]. All determinations were performed in triplicate. The results are expressed as μmol TE mL^{-1} of the sample.

The oxygen radical absorbance capacity (ORAC-FL) assay was performed according to the methodology used by del Mar Contreras *et al.* (2011),[26] previously described by Hernández-Ledesma *et al.* (2005).[27] The reaction occurred at 40 °C in 75 mM of phosphate buffer (pH 7.4), and the final assay mixture (200 μL) was composed of 120 μL of 70 nM Fluorescein sodium salt, 60 μL of 14 mM 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), and 20 μL of Trolox [9.98×10^{-4}–7.99×10^{-3} μmol mL^{-1}] or 20 μL of sample. The fluorescence was recorded during 137 min, with a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) with a 485 nm excitation filter and 520 nm emission filter. The program used during this assay was the FLUOstar Control software (version 1.32 R2). All reactions were performed in duplicate and three independent runs were done. Final ORAC values were expressed as μmol TE mL^{-1} of the sample.

2.3. Enzymatic hydrolysis under the best conditions

After the enzymatic hydrolysis of lesser mealworm insect powder, the best condition for each enzyme was chosen based on the degree of hydrolysis and antioxidant activity, to proceed with the characterization of the insect protein hydrolysates. For this, the enzymatic hydrolysis was performed as previously explained, with some modifications. Instead of using phosphate buffer solution, hydrolysis was performed in deionized water to remove the interference of the buffer solution salts in

the final freeze dried hydrolysates, and the pH value was adjusted before and during the time of hydrolysis with 2 M NaOH. The hydrolysis condition selected for Corolase PP was a ratio of E/S of 3.0% (w/w) for 6 h and for Alcalase™ 2.5L was a ratio of E/S of 1.5% (v/w) for 4 h. Hydrolysis was done in duplicate for each condition. After enzymatic hydrolysis, samples were freeze dried (Armfield SB4 model, UK) and stored in a desiccator for further experiments. The yield was calculated to verify the efficiency of the enzymatic hydrolysis process. The yield for each condition (Alcalase 2.5L hydrolysis, Corolase PP hydrolysis and control) was obtained according to the following formula:

$$\text{Yield}(\%) = 100 \times (\text{Weight of freeze dried hydrolysates obtained} / \text{Weight of insect powder used for the enzymatic hydrolysis}).$$

2.3.1. Peptide profile analysis. The peptide profile of insect hydrolysates was analysed after the enzymatic hydrolysis, and for that 100 μL of the filtered sample was injected in a FPLC system (AKTA pure, GE Healthcare Life Sciences, Germany) coupled with two gel filtration columns: Superdex 200 increase 10/300 GL and Superdex peptide, 10/300 GL. The eluent was composed of 0.025 M phosphate buffer, 0.2 g L^{-1} sodium azide and 8% NaCl. The flow gradient was set at 0.5 mL min^{-1}. To establish a molecular weight standard curve thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (14 kDa) and whey peptide (1 kDa) were used. All the analysis was performed in duplicate and the results are expressed in milli absorbance units (mAU) per eluted volume (mL).

2.3.2. Characterization of freeze dried hydrolysates

2.3.2.1. Chemical analysis. The protein content was measured using a Kjeltec system 1002 distilling unit (Tecator, Sweden). The crude protein content was calculated from the quantity of ammonia produced. The conversion factor used to calculate the protein content was 5.6, as proposed by Janssen *et al.* (2017)[28] for protein extracts derived from insects.

Total sugars were determined by the colorimetric method as described by Dubois *et al.* (1956),[29] using glucose as the standard.

To evaluate moisture, the freeze-dried hydrolysates were placed at 100 °C for 24 h. To determine the ash content, samples were placed at 550 °C in a muffle for 5 h for incineration.

All assays were performed in duplicate.

2.3.2.2. Free amino acid composition. The free amino acid content of each fraction was obtained by pre-column derivatization with orthophthalaldehyde (OPA) methodology. Isoindole-type fluorescent derivatives were generated in an alkaline solution (borate buffer pH 10.4) from OPA, 2-sulfanylethanol and the primary amine group of amino acid. The derivatives were separated by reverse phase-high performance liquid chromatography (HPLC) (Beckman Coulter, USA) coupled to a fluorescence detector (Waters, USA) according to Proestos *et al.* (2008).[30] Each sample (100 μL) was derivatized and the injection volume of derivatives was 20 μL. A calibration curve of 17 different amino acids was prepared in the range of 0.8–100 mg L^{-1}. The measurements were analysed in duplicate and the results are expressed in amino acid (mg) per gram of insect powder.

2.3.2.3. Total antioxidant activity. The total antioxidant activity of insect hydrolysates was evaluated by ABTS and ORAC-FL as previously described. The samples used were composed of 10 mg of freeze dried hydrolysates diluted in 1 mL of deionised water (10 mg mL^{-1}). The results are expressed in μmol TE g^{-1} of insect powder.

2.3.2.4. Angiotensin-converting enzyme inhibition. The inhibitory effect of angiotensin-converting enzyme (ACE) was measured by the fluorimetric assay proposed by Sentandreu and Toldrá (2006),[31] with modifications made by Quirós *et al.* (2009).[32] o-Abz-Gly-p-Phe(NO2)-Pro-OH (0.45 mM) (Bachem, Switzerland) was used as the substrate and the reaction was carried out in 0.04 U mL^{-1} of ACE (peptidyl-dipeptidase A, EC 3.4.15.1) pH 8.3 with 0.1 mM $ZnCl_2$. The reaction mixture was incubated at 37 °C. The fluorescence was measured after 45 min using a FLUOstar OPTIMA plate reader. The wavelengths used were 350 nm (excitation) and 420 nm (emission). The software used to process the data was FLUOstar control (version 1.32 R2). Non-linear fitting to the data was performed to calculate the IC_{50} (protein concentration needed to inhibit 50% of ACE activity).[33] The ACE inhibitory activity was analysed in duplicate for each sample.

2.3.2.5. α-Glucosidase inhibition. The α-glucosidase inhibitory activity was determined in 96-well microplates according to Kwon *et al.* (2008).[34] Briefly, 40 μL of the sample (10 mg mL^{-1} of freeze dried hydrolysate) was mixed with 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase (1.0 U mL^{-1}). The microplate was pre-incubated at 25 °C for 10 min. Then, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well. The reaction was performed at 25 °C for 5 min and, before and after the reaction, the absorbance readings were recorded at 405 nm using a FLUOstar OPTIMA plate reader. Acarbose was used as the positive control at the concentration of 10 mg mL^{-1} and phosphate buffer was used as the negative control. The α-glucosidase inhibitory activity was analysed in triplicate.

The α-glucosidase inhibitory activity was calculated according to the following equation:

$$\alpha\text{-Glucosidase inhibiton}(\%) = 100 \times (\Delta\text{Abs}_{\text{control}} - \Delta\text{Abs}_{\text{sample}})/(\Delta\text{Abs}_{\text{control}})$$

where, $\Delta\text{Abs}_{\text{control}}$ is the variation of absorbance of the negative control and the $\Delta\text{Abs}_{\text{sample}}$ is the variation of absorbance of the samples.

2.3.2.6. Antimicrobial activity. To evaluate the antimicrobial activity of the protein hydrolysates, the well diffusion assay was performed. Firstly, bacterial isolates of *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Listeria monocyto-*

genes ESB 3562 and methicillin-resistant *Staphylococcus aureus* (MRSA) CCUG 60578 were grown overnight in Tryptic Soy Broth (Biokar Diagnostics, France). Afterwards, plates with 20 mL of Mueller-Hinton Agar (Biokar Diagnostics) were seeded with a bacterial suspension equivalent to 0.5 McFarland standard (10^8 CFU mL^{-1}) using a swab. Then, 40 μL of the filtered (0.22 μm) sample (10 mg mL^{-1} of freeze dried hydrolysate) or sterile water (negative control) were added to wells with 4 mm of diameter. The plates were incubated for 24 h at 37 °C. The presence of a translucent halo around the wells indicates the inhibition of the microorganisms, and the absence of a translucent halo zone indicates that no antimicrobial activity occurs. Assays were performed in triplicate.

2.4. Statistical analysis

The results are expressed as mean ± standard deviation. The normality of the distributions was evaluated through the Shapiro Wilk test and the differences were evaluated using one-way ANOVA test. Means were considered to be different at a significance level of 0.05 and data were analysed using the Statistical Package for Social Sciences software (version 21, SPSS, USA).

3. Results and discussion

3.1. Enzymatic hydrolysis

An analysis of the enzymatic hydrolysis of lesser mealworm insect powder was done to choose the best conditions to obtain peptides with bioactivity, namely antioxidant activity. In this study, two commercial enzymes (Alcalase 2.5L and Corolase PP) were used at various E/S ratios and different times of hydrolysis were tested. Two parameters were analysed to select the best hydrolysis condition for each enzyme, the determination of DH to understand how hydrolysis was evolving over time, and the antioxidant activity of hydrolysates to perceive how this bioactive property was affected in each condition used. The DH obtained by Alcalase 2.5L (AH) and Corolase PP (CH) hydrolysates are presented in Fig. 1(A and B).

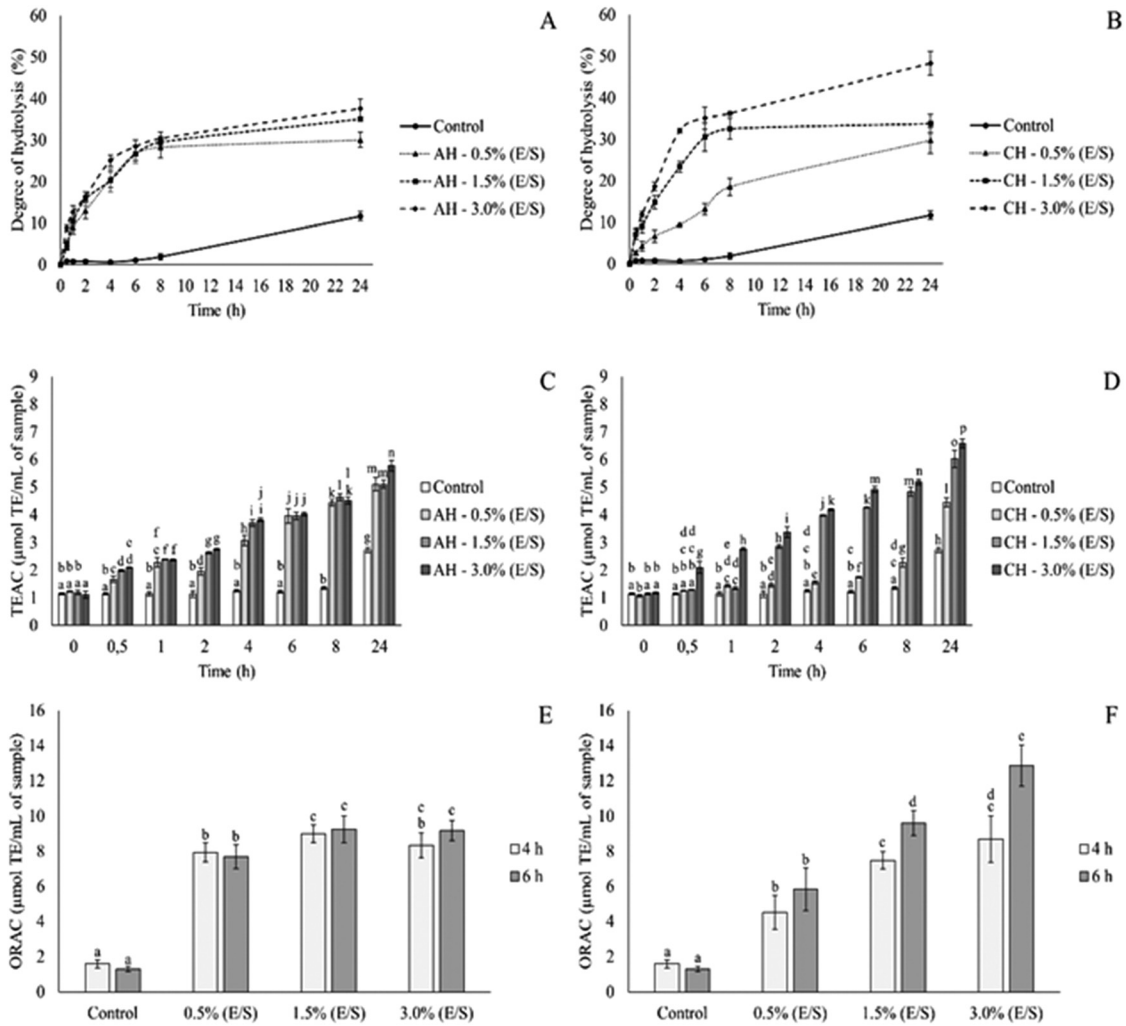

Fig. 1 Degree of hydrolysis and antioxidant activities by ABTS scavenging and ORAC-FL obtained with Alcalase 2.5L (A, C and E, respectively) and Corolase PP (B, D and F, respectively). Same letters mean no statistically significant differences ($p > 0.05$).

A significant difference ($p < 0.05$) between the control (no enzyme added) and all the other conditions studied in both enzymes was noted. This indicates that both enzymes are capable of hydrolysing the insect proteins. The main increase of DH between 8 and 24 h was observed in the control, which was also observed in other studies involving enzymatic hydrolysis of insect proteins.[19,23] According to Fig. 1(A), the conditions 0.5, 1.5 and 3.0% (E/S), demonstrated a similar behaviour for the hydrolysis with Alcalase 2.5L. The DH results obtained for AH, after 4 h of hydrolysis, were 20.5% (0.5% E/S), 20.3% (1.5% E/S) and 25.0% (3.0% E/S). These values were similar to the results reported by Dai and co-workers (2013)[35] using Alcalase at 1.0% (E/S) for the enzymatic hydrolysis of *Tenebrio molitor* larvae defatted flour, where they estimated a DH of around 20.0% after approximately 4 h of hydrolysis. In the present study, using Alcalase 2.5L at 3.0% (E/S) for 6 h a DH of 28.4% was reached, this is in agreement with a study of Yang *et al.* (2013)[36] who obtained a DH of *ca.* 32% for *Bombyx mori* protein with Alcalase™ 2.4L at 4.0% (E/S) for 5 h. The DH achieved with Alcalase 2.5L after 1 h of hydrolysis time was 8.9% (0.5% E/S), 10.3% (1.5% E/S) and 12.6% (3.0% E/S), which are considerably lower when compared with the values presented by Hall and colleagues (2017).[23] In their study the enzymatic hydrolysis of a *Gryllodes sigillatus* solution at 50% (w/v) with Alcalase and testing various ratios of E/S, DHs of 29.2% (0.5% E/S), 44.1% (1.5% E/S) and 51.2% (3.0% E/S) were obtained. Similar results were also reported by another study of Hall *et al.* (2018)[19] using the same enzyme and the same insect. The DH obtained in both studies may have been influenced by a prior pasteurization of the sample at 90 °C for 15 min, since a thermal process can increase protein degradation[37] and it was already demonstrated that a previous heat treatment can influence the DH.[38]

For the hydrolysis using Corolase PP, a significant difference ($p < 0.05$) among all the conditions could be observed (Fig. 1(B)). It was also verified that the condition 3.0% (E/S) had a DH value higher than 30% at 4 h of hydrolysis. To our knowledge, insect hydrolysates obtained through Corolase PP hydrolysis has not been tested yet and no studies have been published.

3.2. Total antioxidant activity

The hydrolysis efficiency was evaluated by the DH; however to define the optimal conditions (E/S ratio and hydrolysis time) for each enzyme, the antioxidant activity of the hydrolysates obtained throughout all processes was also evaluated. It is known that a higher DH in insect proteins appears to have a positive effect on bioactive properties.[36] To further understand how the DH affects the antioxidant activity of hydrolysates, the protective capacity was analysed through two different methods, ABTS scavenging assay and ORAC assay. The ABTS scavenging method evaluates the capacity of the antioxidant compound to reduce the ABTS radical cation, and when in contact with the antioxidant the radical color decreases when reduced, from blue-green color (oxidized) until no color (reduced).[25] The ORAC method evaluates the fluorescence decay over time caused by the fluorescent probe (fluorescein) degradation caused by the peroxyl radical, resulting in a non-fluorescent compound, and the protection capacity of an antioxidant compound to reduce this peroxyl radical and delay the fluorescein degradation. This method is also largely used in food industry as a reference method to measure antioxidant capacity.[39–41] The antioxidant activity of peptides is well known and is associated with their amino acid composition[42] and this composition is influenced by the enzyme used to obtain them.[13] According to the results obtained for AH (Fig. 1A, C and E) and CH (Fig. 1B, D and F), the condition 3.0% (E/S) at 24 h had the highest Trolox equivalent antioxidant capacity (TEAC) values, with 5.8 μmol TE mL^{-1} and 6.6 μmol TE mL^{-1}, respectively. Moreover, a higher DH resulted in higher TEAC values of the hydrolysates and the antioxidant activity increased over time for both enzymes. In the control, no significant differences were observed for 8 h of experiment and the protective capacity increased only after 24 h, probably related to the increase of the DH at that time. During enzymatic hydrolysis there is an increase of the DH along time with the values stabilizing after 6 h of hydrolysis. The TEAC values obtained with both enzymes increased over time, demonstrating the best antioxidant capacity for the highest hydrolysis times, 8 and 24 h. Even though the antioxidant activity is higher for a higher hydrolysis time, using extended hydrolysis involves several risks that may affect future industrial application, such as microbial contamination of the batch and high costs to maintain the ideal conditions for the enzymes (pH and temperature). In this sense, it was decided to further analyse the antioxidant activity through the ORAC assay for the hydrolysates obtained after 4 and 6 h of hydrolysis.

In Fig. 1(E and F), the results obtained by the ORAC method for the AH and the CH are also represented. As can be observed in the results, the antioxidant activity of the control, at 4 and 6 h of hydrolysis, is significantly lower than that of the hydrolysates ($p > 0.05$). For AH, none of the conditions demonstrated significant differences ($p < 0.05$) between 4 and 6 h of hydrolysis. However, the conditions 1.5% and 3.0% (E/S) showed values of antioxidant activity significantly higher than those of condition 0.5% (E/S). No significant differences ($p > 0.05$) were observed between the ORAC values obtained for conditions 1.5 and 3.0% at 4 and 6 h of hydrolysis, being approximately 9.3 μmol TE mL^{-1}. In accordance with the results obtained for CH at 4 and 6 h of hydrolysis, a significant difference ($p < 0.05$) of antioxidant activity between the different ratios of enzymes was detected. The highest ORAC values obtained for CH were for condition 3.0% (E/S) at 4 h (9.5 μmol TE mL^{-1}), 1.5% (E/S) at 6 h (9.7 μmol TE mL^{-1}) and 3.0% at 6 h (12.3 μmol TE mL^{-1}). Therefore, the condition 3.0% (E/S) at 6 h presented an antioxidant activity significantly higher than the other conditions.

In conclusion and considering the results obtained for the AH and CH, the best condition for the hydrolysis of the lesser mealworm insect powder with Alcalase 2.5L was the one with a lower enzyme concentration and shorter hydrolysis time that

showed the highest TE values, in this case it was the condition 1.5% (E/S) for 4 h of hydrolysis. For Corolase PP, the best selected condition for the hydrolysis was 3.0% (E/S) for 6 h of hydrolysis.

3.3. Enzymatic hydrolysis under the best condition

3.3.1. Peptide profile analysis. In Fig. 2 the peptide profile of the hydrolysates after the enzymatic hydrolysis process obtained under the best-chosen conditions is presented. The hydrolysates are composed of small peptides, smaller than the whey peptide used as the standard, with less than 1 kDa and amino acids. In general, the CH – 3.0% T6 obtained the highest peaks among the other samples, followed by the peaks obtained by AH – 1.5% T4 and then the control. This result can be explained by the DH value obtained by both enzymes, since the hydrolysis done with Corolase PP resulted in a DH of 36.0% and the hydrolysis done with Alcalase 2.5L resulted in a DH of 19.5%, and according to Ge *et al.* (1996),[43] more peptides and amino acid generation at a higher DH value are expected to be observed. In conclusion, the peptide profile demonstrated clear hydrolysis and peptide generation in both enzymatic conditions.

3.3.2. Chemical characterization of freeze dried hydrolysates. After hydrolysing the lesser mealworm insect powder and freeze drying the hydrolysates obtained using the best condition for each enzyme, Alcalase 2.5L at 1.5% (E/S) for 4 h (FD-AH) and Corolase PP at 3.0% (E/S) during 6 h (FD-CH). The chemical characterization of these hydrolysates and respective controls was performed (Table 1).

Insect hydrolysates were demonstrated to have a high amount of protein, between 59–67%, being an important source of protein. It was also verified that around 18 to 29% of hydrolysate composition is not described, but is expected to be mostly fat. In general, insects have a high quantity of protein followed by a reasonable amount of fat, fibres and minerals.

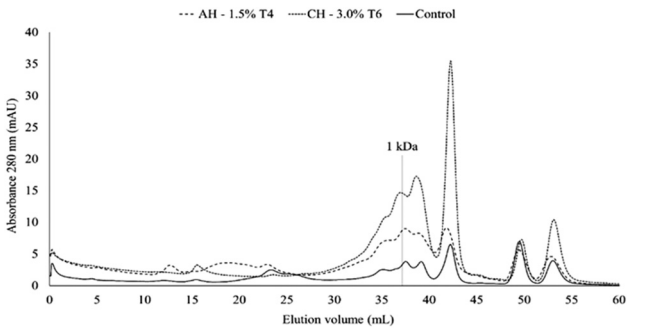

Fig. 2 Peptide profile analysis of the Alcalase 2.5L hydrolysate, at 1.5% (E/S) for 4 hours of hydrolysis (AH – 1.5% T4), Corolase PP hydrolysates, at 3.0% (E/S) for 6 hours of hydrolysis (CH – 3.0% T6) and Control.

3.3.3. Free amino acid composition of freeze dried hydrolysates. The free amino acid composition of the samples (FD-AH, FD-CH and control) and the original matrix (lesser mealworm insect powder) was analysed (Table 2).

It was expected that the hydrolysates would have a higher amount of free amino acids when compared to the original matrix, because they were subjected to enzymatic hydrolysis and the breakdown of native proteins into amino acids and small peptides should be potentiated[44] and also because the DH value affects the amount of free amino acids released in hydrolysis.[43] Considering the obtained results, a high quantity of free amino acids was observed in the sample FD-CH, the sample with a higher DH value (36.0%), with a total of 112.5 mg of free amino acids per gram of insect powder, showing that Corolase PP successfully increased by four times the amount of free amino acids when compared with all the other samples tested. This result also corroborates the peptide profile observed for the hydrolysates obtained with Corolase PP. The FD-AH (DH of 19.5%) had a total amount of 28.8 mg of amino acids. Tyrosine, included in a group of amino acids (methionine, histidine, lysine and tryptophan) is known for its antioxidant properties[45] and one the most active against the ABTS radical cation as reported by Coscueta and others (2016).[13] This amino acid was found in higher quantities in both enzymatic conditions, mainly in FD-CH. Arginine, the second amino acid found in higher quantities, is responsible for lowering the blood pressure in humans and animal models[46] and a supplementation with this amino acid can potentially be used to prevent endothelial dysfunction in patients with diabetes.[47] Leucine and phenylalanine are also amino acids detected in remarkable amounts in FD-CH. Leucine can be used as a potential obesity and metabolic syndrome treatment[48] and phenylalanine, when metabolized into phenylethylamine is presumed to have antidepressant properties.[49] The FD-AH and FD-CH were prepared under different conditions of E/S ratio and hydrolysis time, so the high amino acid composition verified in FD-CH was due to extensive hydrolysis.

3.3.4. Bioactive properties of freeze dried hydrolysates. The total antioxidant activity of the FD-AH and FD-CH was analysed using two complementary methods, ABTS and ORAC scavenging assay. According to the results obtained for the antioxidant activity of the hydrolysates (Table 3), very similar TEAC values between the FD-AH and the FD-CH ($p > 0.05$) were verified. When comparing the ORAC values obtained by

Table 1 Characterization of freeze dried hydrolysate samples (% w/w)

Condition	DH (%)	Yield (%)	Protein (%)	Ash (%)	Moisture (%)	Sugars (%)	Others (%)
FD-AH	19.5 ± 1.6	63.3 ± 1.2	58.9 ± 7.9	6.3 ± 0.2	5.6 ± 2.5	0.4 ± 0.1	28.8
FD-CH	36.0 ± 1.0	67.4 ± 0.3	66.7 ± 3.8	7.3 ± 0.3	7.3 ± 3.3	0.3 ± 0.1	18.4
Control	—	24.0 ± 1.0	53.5 ± 3.6	12.6 ± 0.4	11.1 ± 2.5	1.2 ± 0.2	21.6

Table 2 Free amino acid composition of FD-AH, FD-CH, control and insect powder

Amino acid	FD-AH (mg g^{-1} of insect powder)	FD-CH (mg g^{-1} of insect powder)	Control (mg g^{-1} of insect powder)	Insect powder (mg g^{-1} of insect powder)
Alanine (Ala)	1.8 ± 0.1	5.4 ± 0.4	1.3 ± 0.1	1.8 ± 0.4
Arginine (Arg)	4.8 ± 0.2	20.2 ± 1.6	4.9 ± 0.5	2.1 ± 0.5
Asparagine (Asn)	0.6 ± 0.1	2.8 ± 0.3	DNQ	0.3 ± 0.0
Aspartic acid (Asp)	0.7 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.1 ± 0.1
Cysteine (Cys)	DNQ	DNQ	DNQ	DNQ
Glutamine (Gln)	3.7 ± 0.4	8.7 ± 0.7	4.2 ± 0.1	2.3 ± 0.2
Glutamic acid (Glu)	2.5 ± 0.3	4.0 ± 0.3	2.5 ± 0.2	3.8 ± 0.7
Histidine (His)	2.2 ± 0.1	5.8 ± 0.5	2.0 ± 0.1	1.9 ± 0.5
Isoleucine (Ile)	0.8 ± 0.0	5.8 ± 0.6	0.7 ± 0.0	0.6 ± 0.1
Leucine (Leu)	1.6 ± 0.1	10.6 ± 0.5	0.5 ± 0.0	0.8 ± 0.2
Methionine (Met)	0.2 ± 0.0	1.6 ± 0.2	ND	ND
Phenylalanine (Phe)	1.2 ± 0.1	9.4 ± 0.2	0.5 ± 0.0	1.6 ± 0.4
Serine (Ser)	0.8 ± 0.1	2.2 ± 0.2	0.3 ± 0.0	0.7 ± 0.1
Threonine (Thr)	1.1 ± 0.0	4.0 ± 0.3	0.6 ± 0.0	0.3 ± 0.1
Tryptophan (Trp)	0.4 ± 0.0	1.3 ± 0.2	0.4 ± 0.0	0.8 ± 0.3
Tyrosine (Tyr)	4.1 ± 0.2	20.8 ± 2.6	2.6 ± 0.1	3.6 ± 0.7
Valine (Val)	2.1 ± 0.1	8.4 ± 0.4	1.8 ± 0.1	2.5 ± 0.4
Total AA	28.8 ± 1.9	112.5 ± 8.9	24.2 ± 1.5	24.3 ± 4.2

DNQ = detected but not quantified; ND = not detected.

Table 3 Antioxidant and ACE inhibition activity values for FD-AH, FD-CH and control

Condition	TEAC (μmol TE g^{-1} of insect powder)	ORAC (μmol TE g^{-1} of insect powder)	ACE inhibition (IC_{50} μg of protein per mL)
FD-AH	95.0 ± 0.8[a]	825.6 ± 85.5[c]	55.5 ± 6.2[f]
FD-CH	95.7 ± 1.0[a]	944.8 ± 68.1[d]	107.4 ± 9.7[g]
Control	24.3 ± 0.4[b]	230.7 ± 15.5[e]	171.6 ± 38.0[h]

both conditions, a significant difference ($p < 0.05$) between the FD-CH and FD-AH was observed, and the peptides obtained through Corolase PP demonstrated the highest values.

According to the literature, only one study performed enzymatic hydrolysis with Alcalase to obtain antioxidant peptides using various ratios of E/S in a 50% (w/v) solution of *G. sigillatus* protein.[19] The results of the present study were lower when compared with the TEAC values reported by Hall and others (2018);[19] the authors reported TEAC values of 403.2 μmol TE mg^{-1} of the sample using a ratio of 0.5% (E/S) and 512.0 μmol TE mg^{-1} of the sample using a ratio of 3.0% (E/S), moreover the authors reported DH values higher (31.3% and 51.8%) than our value (19.5%). Tang and colleagues (2018)[20] reported results for the ORAC method to evaluate the antioxidant capacity of *T. molitor* hydrolysates. After the hydrolysis of *T. molitor* extract powder (10% w/v) with Alcalase™ at 0.1% (E/S), the IC_{50} value of 2.7 μmol TE mg^{-1} of the sample was achieved. The antioxidant capacity of insect protein hydrolysates is well documented with various studies reporting hydrolysates obtained from edible insects through enzymatic hydrolysis with *in vitro* gastrointestinal digestion,[17,50] alkaline protease[17] and Alcalase 2.4L.[36]

Peptides capable of inhibiting ACE have been already extracted from various common food products such as soy protein,[13] milk,[14] fish protein[15] and egg white protein.[16] Various authors reported that hydrolysates derived from insect proteins can be a great source of peptides with ACE inhibitory activity,[9,51] and consequently this capacity was also analysed in the FD-AH and FD-CH. The results obtained for the ACE inhibition of FD-AH and FD-CH (Table 3), revealed that the FD-AH had a better ACE inhibitory activity, because the FD-AH resulted in half of the IC_{50} value of the FD-CH. The control, as expected, obtained the highest IC_{50} value due to the absence of enzyme to breakdown the proteins into potential bioactive peptides. Comparing the results with the reported IC_{50} values available in the literature, similar results to those obtained by FD-AH were reported for other insects. Hall *et al.* (2018)[19] described a slightly lower IC_{50} value of 40.0 μg mL^{-1} for *G. sigillatus* hydrolysates obtained through enzymatic hydrolysis using Alcalase™ at 3.0% during 20 min. Jia and coworkers (2015)[52] studied *B. mori* hydrolysates using the same enzyme, although the process used to obtain these hydrolysates used a previous ultrasonic pre-treatment for 32 min (410 W) and 50 minutes of hydrolysis time. The authors observed an IC_{50} value of 91.3 μg mL^{-1}, but better results were obtained in the present study for FD-AH (IC_{50} of 55.5 μg mL^{-1}). Dai and others (2013)[35] showed an IC_{50} value of 390 μg mL^{-1} for *T. molitor* hydrolysates obtained from defatted flour of this insect using Alcalase at 1% (E/S) for 4 h of hydrolysis time and Vercruysse *et al.* (2009)[53] reported an IC_{50} value of 827 μg mL^{-1} for *Spodoptera littoralis* hydrolysates also obtained by enzymatic hydrolysis using Alcalase at a ratio of 48 U kg^{-1} for 3 h. These results confirm the important potential antihypertensive activity of insect protein hydrolysates.

The antimicrobial activity assay against bacteria *E. coli*, *S. enteritidis*, *L. monocytogenes* and MRSA, and the assay of inhibitory activity of the enzyme α-glucosidase, demonstrated that the FD-AH and FD-CH had no antimicrobial capacity or inhibitory effect of the α-glucosidase enzyme. According to the literature, only one study reported the potential capacity of

B. mori pupae hydrolysates to inhibit the α-glucosidase enzyme, using the QSAR method.[54] Hydrolysates obtained by enzymatic hydrolysis of silk cocoons and silk fibroin were also reported to have this property.[55,56] On the other hand, antidiabetic activity through the DPP-IV inhibitory capacity has been shown for insect protein hydrolysates.[19,21,22,57] Despite the fact that there are more than 150 known AMPs derived from insects,[57] no studies were found on the antimicrobial capacity of hydrolysates obtained by enzymatic hydrolysis of insect proteins.

4. Conclusion

In conclusion, this work demonstrated that it is possible to improve the bioactive properties and nutritional value (increase of free amino acids and small peptides) of insect powder through an enzymatic hydrolysis process. The hydrolysates were demonstrated to have potential to be used as a source of bioactive peptides, with antioxidant and antihypertensive activities, which are validated properties with impact on chronic diseases, namely cardiovascular disorder. Further research is needed to identify peptide fractions for each bioactivity and to determine the bioavailability of insect hydrolysates. The potential of these modified insect powders to be used as an additive or ingredient in the food industry is huge, with the advantage of using an innovative and sustainable source of protein and with biologically active properties.

Conflicts of interest

The authors declare that they have no conflict of interest.

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