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DEVELOPMENT AND CHARACTERIZATION OF INSECT (*ALPHITOBIUS DIAPERINUS*) PROTEIN HYDROLYSATES

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DESENVOLVIMENTO E CARACTERIZAÇÃO DE HIDROLISADOS DE PROTEÍNA DE INSETO (*ALPHTOBIUS DIAPERINUS*)

Thesis presented to Escola Superior de Biotecnologia da Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Biotechnology and Innovation

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November 2018

RESUMO

Atualmente, o consumo de insetos é feito mundialmente por cerca de 2 mil milhões de pessoas, com mais de 1900 espécies de insetos comestíveis descritas na literatura. Presentemente, os insetos comestíveis são uma promissora fonte proteica para a geração futura, uma vez que constituem uma fonte sustentável e com um reduzido impacto ambiental, comparado com outras fontes de proteína como gado bovino, suíno ou de aves. Os insetos comestíveis são nutricionalmente ricos em proteína, com vários estudos a demonstrar a sua digestibilidade, pelo que alguns estudos recentes começam a demonstrar o seu potencial como uma base proteica para obter péptidos com propriedades bioativas e funcionais. Assim, o objetivo deste estudo foi obter potenciais péptidos bioativos, analisando a sua atividade antioxidante e antimicrobiana e atividade inibitória das enzimas conversora da angiotensina (ECA) e α-Glucosidase, através de uma hidrólise enzimática da farinha comercial de inseto "Buffalo's", proveniente do inseto comestível *Alphitobius diaperinus*, de forma a desenvolver uma farinha de inseto melhorada com propriedades bioativas, rica em proteínas e aminoácidos livres. Primeiramente, foi necessário analisar várias condições para o processo de hidrólise enzimática, usando as enzimas comerciais Alcalase™ 2.5L e Corolase PP, na proporção enzima:substrato (E/S) de 0,5%, 1,5% e 3,0% e ao longo de 24 horas, de modo a selecionar a melhor condição para cada enzima. Para tal, dois parâmetros foram analisados para esta escolha, o grau de hidrólise das proteínas (GH) e capacidade antioxidante dos hidrolisados (métodos ABTS e ORAC). As melhores condições, que apresentaram um GH relevante e com capacidade antioxidante máxima, foram obtidas com a utilização da enzima Alcalase™ 2.5L numa proporção de 1,5% (E/S) durante uma hidrólise de 4 horas, com um valor de GH de 19,5%, e com a Corolase PP numa proporção de 3,0% (E/S) durante uma hidrólise de 6 horas, com um valor de GH de 36,0%. A capacidade antioxidante dos hidrolisados de ambas as condições foi também avaliada, observando-se resultados bastante similares entre as duas condições de hidrólise (95,0 e 95,7 µmol de Trolox Equivalente/g de farinha de inseto pelo método de ABTS). Na validação final pelo método ORAC, os hidrolisados obtidos pela Corolase PP obtiveram o resultado mais elevado (944,8 comparado com 825,6 µmol de Trolox Equivalente/g de farinha de inseto obtido com a Alcalase™ 2.5L). Os hidrolisados obtidos por ambas condições foram também capazes de inibir a enzima conversora da angiotensina, demonstrando possuir potencial atividade anti-hipertensiva, no entanto, os hidrolisados obtidos pela Alcalase™ 2.5L obtiveram o melhor resultado com um IC_{50} de 55,5 comparando com 107,4 µg de proteína/mL obtidos com Corolase PP, estes valores representam a quantidade de proteína necessária para inibir a atividade da ECA em 50%. Em nenhuma das condições foi possível observar capacidade inibidora da α-Glucosidase ou atividade antimicrobiana. A hidrólise enzimática foi também responsável por um aumento da quantidade total de aminoácidos livres, quando comparado com a farinha de inseto original. Em suma, o inseto comestível *A. diaperinus* demonstrou ser uma excelente fonte de proteínas, e a sua hidrólise enzimática permitiu a obtenção de péptidos com potencial bioativo, capacidade antioxidante e atividade inibitória da ECA, que podem ser usados na alimentação humana ou animal como ingrediente funcional.

Palavras-chave – Inseto, Proteína, Hidrolisados, Bioatividade

ABSTRACT

About 2 billion people already consume insects worldwide and there are more than 1900 species of edible insects already described in literature. At this moment, edible insects are a promising protein source for the future generation, due to its sustainability and low environment impact, when compared with other protein sources used in livestock, cattle or poultry. Edible insects have a high quantity of protein with a reported digestibility; therefore, some recent studies are demonstrating their potential as a protein base to obtain bioactive and functional peptides with applicability in the food industry. So the main aim of this study was to hydrolyze and analyze the potential of the Buffalo's insect powder (i.e. the edible insect *Alphitobius diaperinus*), to develop an improved insect powder with bioactive properties, properties such as antioxidant and antimicrobial activity and inhibitory capacity of the enzymes angiotensin-converting (ACE) and α-Glucosidase, rich in protein and free amino acids. Firstly, an enzymatic hydrolysis was performed in order to choose the best condition for this process using two commercial enzymes, Alcalase™ 2.5L and Corolase PP, at an enzyme:substrate ratio (E/S) of 0.5%, 1.5% and 3.0% during 24 hours. Two parameters were selected to choose the best condition for each enzyme, the protein degree of hydrolysis (DH) and the hydrolysates antioxidant activity (ABTS and ORAC scavenging assay). The best conditions that showed a relevant DH and maximized antioxidant activity were for Alcalase™ 2.5L a ratio of 1.5% (E/S) during 4 hours of hydrolysis, with a DH value of 19.5%, and for Corolase PP a ratio of 3.0% (E/S) during 6 hours oh hydrolysis, with a DH value of 36.0%. The antioxidant capacity of hydrolysates was also evaluated, and based on the ABTS scavenging assay both conditions showed a very similar result (95.0 and 95.7 µmol Trolox Equivalent/g of insect powder). The validation by the ORAC scavenging assay showed that the Corolase PP hydrolysates obtained the highest value (944.8 compared to 825.6 µmol Trolox Equivalent/g of insect powder for Alcalase™ 2.5L). Alcalase™ 2.5L and Corolase PP hydrolysates were able to inhibit the ACE demonstrating a relevant antihypertensive activity, although, Alcalase™ 2.5L hydrolysates showed the best result with an IC_{50} of 55.5 compared to 107.4 µg of protein/mL, these values represent the amount of protein necessary to inhibit the ACE activity in half. None of the hydrolysates showed α-Glucosidase inhibitory capacity or antimicrobial properties. The enzymatic hydrolysis also increased the total quantity of free amino acids, compared with the original insect powder. In conclusion, *A. diaperinus* demonstrated to be an excellent source of protein and the enzymatic hydrolysis of insect protein can be a feasible process to obtain peptides with bioactive properties, such as antioxidant and ACE inhibitory capacity, which can be used as functional ingredient for the food and feed industries.

Keywords – Insect, Protein, Hydrolysates, Bioactivity

ACKNOWLEDGMENTS

I would like to thank to Professor Manuela Pintado for all the support and help given for the realization of my thesis and a great thanks to Sandra Borges for all the effort, ideas given, patience and dedication made during this course.

I would also like to acknowledge the role that Escola Superior de Biotecnologia – Universidade Católica Portuguesa as well CBQF- Centro de Biotecnologia e Química Fina had in mine professional education.

To the researchers Sara S. and Eduardo C. who became big friends, for their support and help inside and outside of the laboratory. Also, to the researcher Manuela A. for all the help given and willingness to help me whenever I needed.

To my friends and laboratory partners David M., Mariana V., Maria J., Célia C. and João T. for all the funny moments and hours working together.

A huge thanks to my girlfriend and best friend Margarida F. for all the help, kind words, love, friendship and motivation given in the difficult moments.

To my family, parents and grandparents, for all the sacrifices made, motivation and support words given to me along my academic course.

Finally, a special thanks to my grandfather, because without him none of this would have been possible, for all the motivation, trust, advices and support given.

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LIST OF ABBREVIATIONS

AA	Amino acids
AAPH	2,2’-Azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACE	Angiotensin-converting enzyme
AH	Alcalase™ 2.5L hydrolysates
AMP	Antimicrobial peptide/protein
ATP	Adenosine triphosphate
BAP	Bioactive peptide
CFU	Colony-forming unit
CH	Corolase PP hydrolysates
DGGE	Denaturation gradient gel electrophoresis
DH	Degree of hydrolysis
DNQ	Detected but not quantified
DON	Deoxynivalenol
DPPH	1,1Diphenyl-2-picrylhydrazyl
DPP-IV	Dipeptidyl-peptidase IV
E/S	Ratio enzyme:substrate
EAA	Essential amino acid
EFSA	European food safety authority
ET	Electron transference
FAO	Food and agriculture organization of the United States
FCR	Feeding conversion rate
FD-AH	Freeze-dried Alcalase™ 2.5L hydrolysate
FD-CH	Freeze-dried Corolase PP hydrolysate
FPLC	Fast protein liquid chromatography
FRAP	Ferric reducing antioxidant power
GHG	Greenhouse gas
GLP-1	Glucagon-like peptide-1
HAT	Hydrogen atom transference
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IgE	Immunoglobulin E

kDa	kiloDalton
MRSA	Methicillin-resistant Staphylococcus aureus
ND	Not detected
PCR	Polymerase chain reaction
QSAR	Quantitative structure-activity relationship
ROS	Reactive oxygen species
rpm	Rotations per minute
SGID	Simulated gastrointestinal digestion
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TNBS	2,4,6-Trinitrobenzenesulfonic acid solution
Trolox	6-Hydroxy-2,5,7,8-tetramethylchromane-2-caboxylic acid
UV-VIS	Ultraviolet-visible spectrophotometry
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

1. INTRODUCTION

Entomophagy, the practice of eating insects, is currently an important subject and has been done for centuries around the world, although the major challenge for the growth of this practice is the western culture, since they view insects as dirty and disgusting (Looy, Dunkel, & Wood, 2014; Yen, 2009). The consumption of insects is already done worldwide, mainly consumed from the order Coleoptera and Lepidoptera (Table 1), by at least 2 billion people and there are more than 1900 species of edible insects described in literature (Huis et al., 2013). The Food and Agriculture Organization (2013) summarized some factors that may promote entomophagy, such as: (i) insects are a healthy and nutritive alternative source to chicken or beef (ii) insects production is easier, more sustainable and less polluting than most livestock; (iii) insects are efficient in converting feed into protein (iv) and it is not necessary an abundant financial investment to start their production, allowing the participation of the poorest section of society.

Table 1 - Main insects eaten worldwide (adapted from Huis, 2016)

Order	**Insect(s)**	**Consumption (%)**
Coleoptera	Beetles	31
Lepidoptera	Caterpillars	18
Hymenoptera	Bees Wasps Ants	15
Orthoptera	Locusts Grasshoppers	13
Hemiptera	True bugs	11
Isoptera	Termites	12
Odonata	Dragonflies	
Diptera	Flies	
Others		

The increase of world population will become a huge problem since the actual protein production is not enough to cover future demand, opening new ways to develop sustainable sources of protein. Insects have a good Feed Conversion Ratio (FCR), a value used to represent an animal's efficiency to convert feed mass into body weight, the FCR (kilogram feed:kilogram liveweight) for the cricket is about 1.7, less than half of the value of the FCR of pork (5.0) and even less when compared with the FCR of beef (10.0) (Huis, 2013). Another important reason to use insects as a protein source is associated with the fact that he livestock production of insects emits much less Greenhouse gas (GHG) and ammonia than conventional livestock production, as demonstrated by Oonincx et al. (2010) comparing five species of insects (*Pachnoda marginata*, *Tenebrio molitor*, *Blaptica dubia*, *Acheta domesticus*, *Locusta migratoria*) with pigs and beef cattle, showing that all the insects had a lower emission of ammonia when compared with conventional livestock and four of the five insects analysed demonstrated a much lower GHG emission per kg of mass gained.

The production of insects has the same characteristics that can be found in other animal productions, insects need a substrate and water to grow, but substrate can be waste products like forest biomass waste (Varelas & Langton, 2017), manure from swine, poultry, dairy or organic waste (Huis, 2013). Insects can be grown in a closed environment where the substrate, water, and atmosphere can be controlled, generally this type of production is performed without the need to use antibiotics, hormones or chemicals, although, in an intensive mass production, antibiotics can be used to treat diseases and biocides to disinfect the environment between batches (EFSA Scientific Committee, 2015).

1.1 Food safety of edible insects

Since insects can be a great source of protein for the future generation, food safety issues like chemical hazard (e.g. heavy metals), potential pathogens (e.g. *Bacillus cereus*) and allergens (e.g. tropomyosin) require systematic screening to ensure safe edible insects (Belluco et al., 2013; Huis, 2015)

Food allergy is an adverse immune-mediated response which occurs when a person interacts with a certain food, and can be mediated or non-mediated by Immunoglobulin-E, or can combine both (Ig-E mediation and non-Ig-E mediation) (Turnbull, Adams, & Gorard, 2015). Many foods can cause allergic reactions, the most common in Europe are cow's milk, egg, wheat, soy, peanut, tree nuts, fish and shellfish (Nwaru et al., 2014). In order to evaluate the allergic potential of insects, a study was made with 15 shellfish allergic patients and it was tested their reaction to protein from *T. molitor.* A positive reaction was observed and the authors concluded that patients with allergy to shrimp can have allergic reaction if they consume mealworm proteins (Broekman et al., 2015).This result is corroborated with other study where patients with allergies to crustaceans had an adverse reaction to *T. molitor* (Verhoeckx et al., 2014). There are some allergens already identified in shellfish, such as Tropomyosin and Arginine Kinase, and since crustaceans and insects share the same phylum (Arthropoda), it's expected to observe cross-reactivity between shellfish and insects (Pedrosa, Boyano-Martínez, García-Ara, & Quirce, 2015). A recent study evaluated the proteins allergenicity from *Gryllodes sigillatus* and it was observed a reaction between the proteins and the shrimp-allergic sera. However, after an enzymatic hydrolysis (around 60-85% of hydrolysis), the hydrolysates didn't demonstrate any reaction (Hall, Johnson, & Liceaga, 2018), providing new information about how allergenicity can be attenuated by enzymatic hydrolysis.

The presence of microorganisms in edible insects is still under study and more research needs to be done to fully understand the composition of edible insects' microbiota. Bacteria species belonging to *Bacillus*, *Staphyloccocus*, *Campylobacter*, *Pseudomonas*, *Micrococcus*, *Acinetobacter*, *Proteus*, *Escherichia* and Enterobacteriaceae were previously found in insects (reviewed by van der Spiegel, Noordam, & van der Fels-Klerx, 2013). An analysis made by Stoops et al. (2016) in living mealworm larvae (*T. molitor*) and grasshopper (*Locusta migratoria*) sold for human consumption in Belgium, showed a considerable contamination in both insects with around 8 log CFU/g of total viable aerobic bacteria, 7 log CFU/g of bacteria from Enterobacteriaceae and 3 log CFU/g of spore forming bacteria. Enterobacteriaceae is usually associated with faecal contamination, spoilage capacity and besides that, many microorganisms of this family are known pathogens such as *Salmonella* spp. (Motarjemi, Moy, Jooste, & Anelich, 2014). Similar results were observed for the mealworm larvae (*T. molitor*), small cricket (*A. domesticus*) and the large cricket (*Brachytrupus* spp.), since the amount of microorganisms were 7 log CFU/g for total viable aerobic bacteria, 4-6 log CFU/g for Enterobacteriaceae and 2-4 log CFU/g for sporeforming bacteria (Klunder, Wolkers-Rooijackers, Korpela, & Nout, 2012). The microbiological load in insects from different rearing companies at an industrial scale from Belgium and Netherlands was also investigated, and as previously presented by other authors, a similar quantity of Enterobacteriaceae was detected (ca. 7-8 log CFU/g) (Vandeweyer, Lievens, & Van Campenhout, 2015). A more extensive study carried out with sixteen samples of marketed processed (boiled, grounded or dried) edible insects bought from companies based on Netherland and Belgium, determined the microbiological profile by direct and indirect methods of cricket powder (*A. domesticus*), whole dried small crickets, whole dried locusts (*L. migratoria*) and whole dried mealworm larvae (*T. molitor*). Pathogens such as *Salmonella* spp. and *Listeria monocytogenes* were absent in 25 grams of sample, the presence of viable counts of *Staphylococcus aureus* was observed in all samples (less than 1 log CFU/g) and *Bacillus cereus*, a very well-known pathogen responsible for food poisoning (Granum & Lund, 2006), was detected in quantities of 5.1 log CFU/g and 3.6 log CFU/g for cricket powder and small crickets, respectively. Among the different samples tested, the cricket powder exhibited the major contamination with a significant quantity of all microorganisms analysed, this contamination may be explained by the considerable processing (grinding process) of cricket powder (Osimani et al., 2017). The same analysis and type of samples (cricket powder, whole small dried crickets, whole dried locusts and whole dried mealworm larvae) were used in other study. As previously observed, *L. monocytogenes* and *Salmonella* spp. were absent in all samples; lactic acid bacteria and total mesophilic aerobes were found in a higher quantity in cricket powder and whole small dried crickets when compared with all the samples tested (Garofalo et al., 2017).

Culture-independent methods were also used to study the microbiological profile of insects and it was possible verify a dominance of the spore-forming bacteria from the genera *Bacillus* and *Clostridium* using a Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis (Osimani 2017 and Osimani 2018). Garofalo et al. (2017) used a 16S rRNA gene pyrosequencing and observed a dominance of the genus *Spiroplasma* in the mealworm larvae, which was also reported by Vandeweyer et al. (2017). This genus is associated with plants and insects since this bacteria use them as a host (Gasparich, 2010). The genus *Weisella* was dominant in the locusts, which is normally found in fermented food and considered as safe (Abriouel et al., 2015). The family *Ruminococcaceae* was dominant on the powdered crickets, but this family is usually found in the gut and is responsible for cellulose digestion derived from plant ingestion by the host (Biddle, Stewart, Blanchard, & Leschine, 2013).

The storage of insects at 5-7 ºC with a previously bleaching process (5-10 minutes) has proved to be an efficient solution to completely eliminate Enterobacteriaceae, reduce the mesophilic aerobes and reduce the bacterial spores to half of the initial amount. The main problem with insects (raw and after processing) is the presence of bacterial spores, which is explained by the contact of insects with soil (Klunder et al., 2012).The well-known examples of spore-forming bacteria from soil are the pathogenic microorganisms *Clostridium botulinum*, usually found in vacuum packaged products, because this bacterium is anaerobe, and *Bacillus cereus*, usually found in dairy and ready-to-eat products (Heyndrickx, 2011).

In addition to microbiological hazard and food allergy, several chemicals are present in insects, some of them are naturally produced or can be acquired by ingestion of contaminated food (van der Spiegel et al., 2013). Compounds like heavy metals and residues of pesticides can be found in edible insects collected in fields caused by the use of pesticides in agriculture (Huis et al., 2013). According with Poma et al. (2017), insects can be a source of hazard chemicals but in a very low concentration, unlike common food sources such as meat or egg. However, a variability of chemical contaminants was observed between samples from different companies, because the type of contaminants can be influenced by the conditions of rearing, soil and substrate used. More studies are necessary to understand how these conditions can affect the presence of chemical compounds in insects.

The diets of insects can be contaminated with mycotoxins, for example wheat flour, maize and barley are substrates commonly contaminated by deoxynivalenol (DON), which is a mycotoxin produced by the fungi *Fusarium* (Pestka, 2007). In a recent study, *T. molitor* larvae were grown using (i) a non-contaminated wheat flour, (ii) a naturally contaminated wheat flour (4.9 mg/kg of DON) and (iii) a wheat flour incorporated with a controlled dose of DON (8.0 mg/kg). After harvest of the larvae, the presence of this toxin was analysed in the larvae and in the faeces. The mycotoxin was not detected in larvae, around 14.0% of the total amount of ingested mycotoxin was detected in faeces of larvae grown on the naturally contaminated substrate and around 41.0% on larvae grown on wheat flour contaminated with a controlled dose of DON. So, the larvae may degrade this toxin and it may be possible to grow this insect in contaminated substrate. Although, it’s not known if the metabolites produced by the degradation of this toxin cause harm to humans (Van Broekhoven, Gutierrez, De Rijk, De Nijs, & Van Loon, 2017).

1.2 *Alphitobius diaperinus*

Alphitobius diaperinus, commonly known as Lesser Mealworm or Buffalo worms, is a specie that belongs to the Coleoptera order, the most consumed order of edible insects worldwide (Huis, 2016). This insect is usually found in poultry houses (Axtell & Arends, 1990) and it’s the most abundant beetle present in the litter of broiler houses in Brazil (A Chernaki, Almeida, Sosa-Gómez, Anjos, & Vogado, 2007).

The life cycle of this insect, as can be observed in the Figure 1.2, is divided in four stages, the Egg stage, Larvae stage, Pupae stage and the Adult stage. Temperature is the key factor for insect development, with temperatures of 17 ºC no eggs hatch or larvae development is observed, at temperatures of 20, 25, 30, 35 and 38 ºC in a relative humidity of 50-60% the median development duration is respectively, 13.4, 6.0, 4.4, 2.6 and 2.6 days for the egg stage, 133.0, 46.0, 26.2, 22.4 and 23.9 days for the larvae stage, 17.0, 8.0, 5.5, 4.0 and 4.1 for the pupae stage and 164.4, 60.3, 37.9, 29.0 and 30.8 from oviposition to adult form (Rueda & Axtell, 1996). Similar results were reported by Chernaki and others (2001). Thus, temperatures around 30-35 ºC and a high relative humidity are the ideal conditions for the insect development.

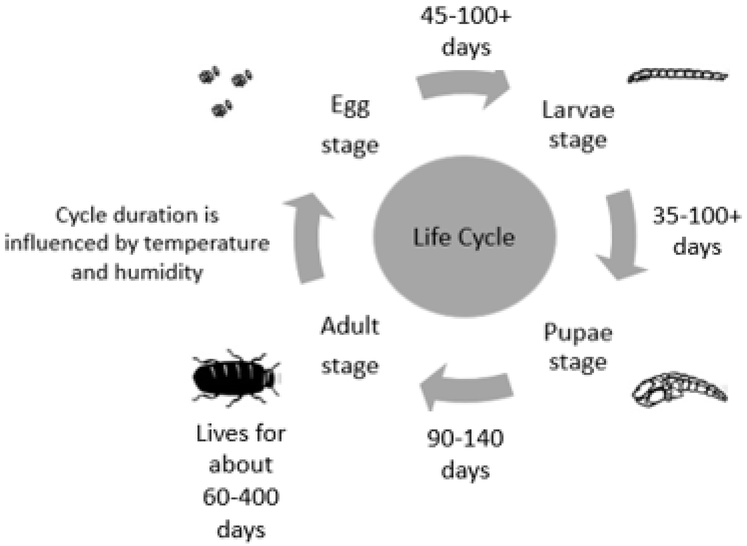

Figure 1.2 - Life cycle of the insect *A. diaperinus* (adapted from Dam & Taylor, 2016).

Nutritionally, this insect has a very similar amount of essential amino acids (EAA) compared with soybean and a bit less total EAA than bovine casein, therefore this insect is very analogous to conventional food (L. Yi et al., 2013). The amino acid composition of *A. diaperinus*, soybean and bovine casein is demonstrated in Table 1.2.1.

Table 1.2.1 - Amino acid composition of *A. diaperinus*, soybean, bovine casein and the recommended levels of amino acids for adult consumption (adapted from L. Yi et al., 2013).

Essential Amino acid	***A. diaperinus* (mg/g of protein)**	**Soybean (mg/g of protein)**	**Bovine casein (mg/g protein)**	**Recommended levels of amino acids for adults (mg/g of protein) (FAO/WHO/UNU, 1985)**
Total EAA	459	439	531	277
Histidine (H)	34	25	32	15
Isoleucine (I)	43	47	54	30

Table 1.2.1 - Amino acid composition of *A. diaperinus*, soybean, bovine casein and the recommended levels of amino acids for adult consumption (adapted from L. Yi et al., 2013) (Continued).

Leucine (L)	66	85	95	59
Lysine (K)	61	63	85	45
Methionine (M) + **Cysteine (C)**	26	24	35	22
Phenylalanine (F) + **Tyrosine (Y)**	120	97	111	38
Threonine (T)	39	38	42	23
Tryptophan (W)	12	11	14	6
Valine (V)	58	49	63	39

Other study suggested that this insect presents a greater percentage of protein (49.6%), based in the total amino acid present in the sample comparing with *T. molitor* (44.7%) and *Hermetia illucens* (36.0%) (Janssen, Vincken, Van Den Broek, Fogliano, & Lakemond, 2017). These results were also corroborated by Bosch et al. (2014), they analysed the nutritional composition of various insects and *A. diaperinus* (64.8%) had a higher protein percentage than *T. molitor* (52.0%) and *H. illucens* (56.1%).

1.3 Insect proteins

The main characteristic of using insects as food is their protein content, which can be a solution for the future demand of animal protein. Recently, various studies demonstrated the nutritional value of insects namely the high content of protein, fat, and other important nutrients (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Kouřimská & Adámková, 2016). Different insects have different nutritional compositions, even in the same order. The most consumed order insects has approximately 40.7% of protein (Table 1.3), however, protein percentages vary between 8.9 and 71.1% inside this order. This variation suggests that the stage of growth of the insect, type of feed, and also the methods used to determinate insects composition may influence the results obtained (Rumpold & Schlüter, 2013).

Bosh and co-workers (2014) evaluated the nutritional composition of different insects, such as housefly larvae (*Musca domestica*), adult house cricket (*A. domesticus*), yellow mealworm larvae (*T. molitor*), lesser mealworm larvae (*A. diaperinus*), morio worm larvae (*Zophobas morio*), black soldier fly larvae and pupae (*H. illucens*), adult six spot roach (*E. distanti*), adult death's head cockroach (*B. craniife*) and adult female Argentinian cockroach (*Blaptica dubia*) and compared with conventional products such as Soybean meal, Fish meal, and Poultry meat meal. They concluded that the percentage of protein among those insects were between approximately 47 and 71%, and in general most of insects demonstrate a content of protein around 60% depending of the species (Huis, 2016). These values when compared with the conventional meals revealed that the most of insects have more protein than Soybean meal, and most of them have similar percentages compared with Fish meal and Poultry meal. This study showed that the insects with a higher protein content belong to the species *A. domesticus* (70.6% of protein), *E. distanti* (66.3%), *B. craniife* (65%) and *A. diaperinus* (64.8%) (Bosch, Zhang, Oonincx, & Hendriks, 2014).

Table 1.3 - Nutritional composition of the most consumed orders of insects. (adapted from Rumpold & Schlüter, 2013).

Order	Protein (%)	Fat (%)	Fiber (%)	Carbohydrates (%)	Ash (%)
Coleoptera	40.7	33.4	10.7	13.2	5.1
Lepidoptera	45.4	27.7	6.6	18.8	4.5
Himenoptera	46.5	25.1	5.7	20.3	3.5
Orthoptera	61.3	13.4	9.6	12.9	3.8
Hemiptera	48.3	30.3	12.4	6.1	5.0
Isoptera	35.3	32.7	5.1	22.8	5.9
Odonata	55.2	19.8	11.8	4.6	8.5
Diptera	49.5	22.8	13.6	6.0	10.3

Some studies assessed the digestibility of these proteins through the simulation of the gastrointestinal digestion (SGID). A study made in Mexico suggested that the insect proteins have digestibility percentages around 77 and 98% (Ramos-Elorduy et al., 1997). Digestibility of insect proteins can be influenced by the way they are processed, for example *T. molitor*. *Schistocerca gregaria* and *G. sigillatus* digestibility are different when they are raw, baked or boiled. Significant differences were observed between the raw sample and the heat treated (boiled or baked) samples. *T. molitor* had the biggest difference, since the digestibility improved from ca. 15 to 31% with this treatment (Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015).

In addition, insects are a great source of polyunsaturated fatty acids and monounsaturated fatty acids. Furthermore, insects are also important source of various micronutrients like copper, zinc, iron and magnesium (Zielińska et al., 2015).

1.4 Bioactive peptides (BAPs)

Bioactive peptides (BAPs) are peptides with a beneficial biological activity composed by approximately 3-20 amino acids. They are obtained through the enzymatic hydrolysis. This process is defined to cleave peptide bonds with the addition of water molecules by hydrolytic enzymes (Szekely & Didaskalou, 2015), this cleavage can be done by digestive enzymes or microbial enzymes (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008), or by exogenous enzymes like Alcalase™ or Flavourzyme™, which are usually chosen over endogenous enzymes due to their ability to obtain consistent peptide profile and composition (Samaranayaka & Li-Chan, 2011).

Due the high protein content of insects, many studies have been done to develop and characterize hydrolysates from insect proteins, in order to increase protein digestibility and obtain bioactive properties. Some bioactive properties of hydrolysates have been already demonstrated, such as antioxidant activity (Hall et al., 2018; Tang et al., 2018; Zielińska, Karaś, & Jakubczyk, 2017), antihypertensive activity (Dai, Ma, Luo, & Yin, 2013; Hall et al., 2018; Pan, Wang, Jing, & Yao, 2016; Tao et al., 2017; Vercruysse, Smagghe, Beckers, & Camp, 2009; Vercruysse, Smagghe, Herregods, & Van Camp, 2005; Wei Wang et al., 2011; Wei Wang, Wang, & Zhang, 2014; Wu, Jia, Yan, Du, & Gui, 2015), anti-inflammatory activity (Zielińska, Baraniak, & Karaś, 2017) and antidiabetic activity (Hall et al., 2018; Lacroix, Dávalos Terán, Fogliano, & Wichers, 2018; Nongonierma, Lamoureux, & FitzGerald, 2018) (Table 1.4).

Table 1.4 Summary of some protein hydrolysates derived from insects and enzymes used in the process and the bioactive properties obtained.

Insect	Enzymes used	Bioactive properties	Reference
Alphitobius diaperinus	• Alcalase™ • Flavourzyme™ • Thermolysin • Papain • SGID (Pepsin and pancreatin)	• Antidiabetic activity	(Lacroix et al., 2018)
Amphiacusta annulipes	• SGID (α-amylase, Pepsin, pancreatin and bile extract)	• Antioxidant activity	(Zielińska, Karaś, et al., 2017)
Bombyx mori	• SGID (pepsin, trypsin and α-chymotrypsin) • Alcalase™ • Thermolysin	• Antihypertensive activity	(Vercruysse et al., 2005)
	• Alcalase™ 2.4L	• Antioxidant activity	(Yang et al., 2013)
	• Alcalase™	• Antihypertensive activity	(Jia, Wu, Yan, & Gui, 2015)
	• SGID (pepsin, trypsin and α-chymotrypsin)	• Antihypertensive activity	(Wu et al., 2015)
	• Neutral protease	• Antihypertensive activity	(Tao et al., 2017)
	• Alkaline proteases	• Antihypertensive activity	(Zhou, Ren, Yu, Jia, & Gui, 2017)
Enteromorpha clathrata	• Alcalase™	• Antihypertensive activity	(Pan et al., 2016)
Gryllodes sigillatus	• SGID (α-amylase, pepsin, pancreatin and bile extract)	• Anti-inflammatory activity • Antioxidant activity	(Zielińska, Baraniak, et al., 2017)
	• Protamex™ • SGID (pepsin and Corolase PP)	• Antidiabetic activity	(Nongonierma et al., 2018)
	• Alcalase™ • SGID (pepsin, pancreatin and bile extract)	• Antihypertensive activity • Antidiabetic activity • Antioxidant activity	(Hall et al., 2018)

Table 1.4 Summary of some protein hydrolysates derived from insects and enzymes used in the process and the bioactive properties obtained (Continued).

Locusta migratoria	• SGID (α-amylase, Pepsin, pancreatin and bile extract)	• Antioxidant activity	(Zielińska, Karaś, et al., 2017)
Spodoptera littoralis	• Alcalase™	• Antihypertensive activity	(Jia et al., 2015)
Tenebrio molitor	• Alcalase™	• Antihypertensive activity	(Dai et al., 2013)
	• SGID (α-amylase, pepsin, pancreatin and bile extract)	• Anti-inflammatory activity • Antioxidant activity	(Zielińska, Baraniak, et al., 2017)
	• Alcalase™ • Flavourzyme™	• Antioxidant activity	(Tang et al., 2018)
Zophobas morio	• SGID (α-amylase, pepsin, pancreatin and bile extract)	• Antioxidant activity	(Zielińska, Karaś, et al., 2017)

1.4.1 Antioxidant activity

One of the most important bioactive properties obtained from hydrolysed protein is the antioxidant activity, an antioxidant compound is essential for being able to inactivate a radical compound or to prevent this radical development (Neužil & Stocker, 1993). This is important to prevent oxidative stress caused by reactive oxygen species (ROS), which are naturally formed by human body and they are important in some physiological functions, like Adenosine triphosphate (ATP) generation. In normal conditions, the human body has capacity to protect itself from ROS, maintaining a redox homeostasis with the participation of enzyme systems in our body. However, a disproportionate generation of these radicals can occurs with an alteration in the redox homeostasis (Trachootham, Lu, Ogasawara, Valle, & Huang, 2008). This disturbance can mainly be caused by endogenous or exogenous factors like pollution, radiation, chemical exposure, smoking, sunlight (Rahal et al., 2014). These free radicals can injury human body by killing cells, damaging proteins, membranes and genes, this creates a chain reaction and causes the production of even more free radicals (Rahal et al., 2014). Several studies were already done using insects, protein hydrolysates from *B. mori* demonstrated to have an improved antioxidant activity compared with the unhydrolyzed proteins. It was reported by Zhou and colleagues (2017) that the protein structure is affected by enzymatic hydrolysis, ultrasound and micronization. They observed a notable improvement of antioxidant activity among all conditions tested; the enzymatic hydrolysis demonstrated the highest antioxidant activity, followed by ultrasound and lastly by micronization (Zhou et al., 2017).

The antioxidant activity of *T. molitor* (larvae), *S. gregaria* (adult) and *G. sigillatus* (adult) hydrolysates, obtained from 3 different initial conditions (raw, boiled and baked), revealed to be a good way to obtain peptides with excellent antioxidant activity. The highest activity against the radical ABTS was observed in raw *T. molitor* hydrolysates (IC_{50}value of 5.3 μg/mL) and using DPPH method the most promising results were for baked *G. sigillatus* hydrolysates (IC_{50}value of 28.5 μg/mL). After passage through a simulated saliva solution and then gastrointestinal enzymes at 37 ºC, peptides fractions from *G. sigillatus* also showed the best results for the antioxidant activity after an absorption process (dialysis) (Zielińska, Baraniak, et al., 2017). Other study accomplished with hydrolysates from *B. dubia* (adult), *Gromphadorhina portentosa* (adult), *L. migratoria* (adult), *Z. morio* (larvae) and *Amphiacusta annulipes* (adult) obtained through a simulated gastrointestinal digestion, verified a relevant potential of insects to be source of peptides with antiradical activity. Among all the insects used in this research, *A. annulipes* and *Z. morio* demonstrated the best results against the DPPH radical and the ABTS radical (Zielińska, Karaś, et al., 2017). Protein hydrolysates obtained with enzymatic digestion from *Spodoptera littoralis* (larvae) also achieved good outcomes for antioxidant activity (Vercruysse et al., 2009). More recent studies also reported a great antioxidant potential of bioactive peptides from the edible insects *T. molitor* (larvae) obtained by enzymatic process using Alcalase™ and Flavourzyme™ (Tang et al., 2018) and from *G. sigillatus* (adult) hydrolysates obtained with Alcalase™ (Hall et al., 2018), and the antioxidant potential was evaluated through a variety of methods such as ORAC, FRAP and others.

1.4.2 Antihypertensive activity

Hypertension is a recognized worldwide problem and is the major risk factor for cardiovascular diseases, when hypertension is not treated can cause numerous health problems like disability, stroke, kidney disability, coronary heart disease or even death (Balti, Nedjar-Arroume, Bougatef, Guillochon, & Nasri, 2010). There is an intense demand for novel compounds with antihypertensive activity, i.e. compounds capable of decreasing blood pressure inhibiting the Angiotensin I-Converting Enzyme (ACE), which is responsible for the increasing of blood pressure in the human body (Mondorf et al., 1998). This extreme need resulted in various studies, pointing hydrolysates from insect proteins to be a great source of peptides with antihypertensive properties. This property was already detected in numerous species of insects. Hydrolysates from *S. littoralis*, *B. mori*, *S. gregaria*, *B. terrestris* and *T. molitor* with antihypertensive activity were already documented, being originated through enzymatic hydrolysis with Alcalase™, Thermolysin, mucosal peptidases and SGID (Dai et al., 2013; Vercruysse et al., 2009, 2005). Different methods to obtain peptides such as micronization, enzymolysis and ultrasound were evaluated by Zhou et al. (2017), they observed a significant increase in the bioactivity of peptides in all methods used. The enzymatic hydrolysis proved to be the best approach to obtain peptides with antihypertensive activity instead of micronization and ultrasound. Isolated peptides with antihypertensive activity were also reported, a tripeptide of *B. mori* (Ala-Ser-Leu) with antihypertensive property was achieved by Wu et al. (2015). This peptide was obtained in the end of the hydrolysis of *B. mori* protein after passing through gastrointestinal proteases. An isolated and purified peptide from *Enteromorpha clatharata* (Pro-Ala-Phe-Gly) was also obtained after an enzymatic hydrolysis using Alcalase™ (Pan et al., 2016).

Antihypertensive properties from insect's hydrolysates were also evaluated *in vivo*, using hydrolysates originated from *B. mori* (pupae) proteins after an enzymatic hydrolysis with an acid protease, and the originated peptides exhibited antihypertensive properties in spontaneous hypertensive rats and it was not verified negative effects in mice after 7 days of treatment with different doses of peptides (Wei Wang et al., 2011).

1.4.3 Antidiabetic activity

Diabetes mellitus, a disease characterized for a congenital (type 1) or acquired (type 2) incapacity to transport glucose from the blood stream to cells (Gunawan-Puteri & Kawabata, 2010), is a well-known worldwide disease and without an adequate and prompt treatment, diabetes can cause hyperglycaemia, diabetic ketoacidosis or non-ketotic hyperosmolar coma, in a long-term can appear complications such as cardiovascular disease, renal failure or retinal damage (Yu et al., 2011). For the treatment of diabetes, are usually used drugs like insulin, a hormone responsible to maintain the glucose level in the blood stream and facilitates cellular glucose uptake by regulating the metabolism (Wilcox, 2005). Other compounds with inhibitory effect on α-amylase and α-glucosidase (e.g. acarbose) may delay the digestion of carbohydrates along the digestive tract and the rate of absorption of glucose is negatively affected (Bhandari, Jong-Anurakkun, Hong, & Kawabata, 2008; Rabasa-Lhoret & Chiasson, 2004). In addition, compounds with inhibitory effect on dipeptidyl-peptidase IV (DPP-IV) (e.g. sitagliptin) are also used, glucagon-like peptide-1 (GLP-1) is an important hormone responsible for lowering the sugar levels in the blood through diverse mechanisms. The circulating levels of GLP-1 rise after a meal but are rapidly inactivated by DPP-IV, so to prevent this step, inhibitors for this enzyme are used to slow the degradation of this hormone (Pathak & Bridgeman, 2010). Only a few studies made with hydrolysates obtained from enzymatic hydrolysis demonstrated capacity to inhibit α-glucosidase, for example Silk cocoons hydrolysates using Protease N (Lee et al., 2011) and Silk fibroin hydrolysates using Elastase (Hu, Cui, Ren, & Peng, 2008). Zhang et al. (2016), recently used a quantitative structure-activity relationship (QSAR) method and a database of protein sequences from *B. mori* to create a simulated computational digestion using a software called PeptideCutter, they analysed only peptides with less than 10 amino acids. This method helps to identify peptides with inhibitory activity of α-glucosidase, comparing docking characteristics of various peptides with this property and peptides obtained through the simulation. This method predicted four peptides with this capacity. After testing these peptides (chemically synthetized and purified through HPLC), the best peptide obtained was Ser-Gln-Ser-Pro-Ala (IC_{50}value of 20.0 μmol/L), followed by the peptides Gln-Pro-Gly-Arg (IC_{50}value of 65.9 μmol/L), Asn-Ser-Pro-Arg (IC_{50}value of 205.0 μmol/L) and Gln-Pro-Pro-Thr (IC_{50}value of 560.0 μmol/L). More recent studies also demonstrate that hydrolysates obtained from *G. sigillatus* protein using the enzymes Alcalase™ (Hall et al., 2018) and Protamex™ (Nongonierma et al., 2018) and also hydrolysates from *A. diaperinus* protein, using Alcalase™, Flavourzyme™, Papain and Thermolysin were capable of inhibiting the DPPV-IV enzyme, responsible to regulate glucose homeostasis and the inhibition of this enzyme is usually an approach used for diabetes type 2 (Dsouza & Lakshmidevi, 2015).

1.4.4 Antimicrobial activity

Insects are a natural source of antimicrobial peptides and they have been used for a long time for their potential to treat wounds, infections or diseases (Józefiak & Engberg, 2017). Insects produce some antimicrobial peptides/proteins (AMPs), more than 150 are already documented, and they are divided in four groups according their particular structures: α-helical peptides (cecropin and moricin), cysteine-rich peptides (insect defensin and drosomycin), proline-rich peptides (apidaecin, drosocin and lebocin) and glycine-rich peptides (attacin and gloverin). AMPs are usually small peptides or large proteins (gloverins and attacins) and they have activity against specific bacteria, fungi, parasites and some viruses. Their production is induced when the insect is injured and in response to an infection these AMPs can be produced by different mechanisms, such as the fat body cells of the insect or a specific local like the gastrointestinal tract for a prompt localized response (Józefiak & Engberg, 2017; Y. Yi, Chowdhury, Huang, & Yu, 2014). It is possible to observe that insects are a great source of antimicrobial compounds, however is still unknown if peptides with antimicrobial properties can be obtained through enzymatic hydrolysis with a specific enzyme or by digestion.

1.5 Insects as food and feed

A recent report prepared by Global Market Insight (2018) indicates that the Edible Insects Market size in 2017 was over $50 Billion and they expect until 2024 a growth of more than 40%. Insects producers like Enviroflight® (USA), a producer of black soldier fly larvae for animal feed, Entomo farms (USA), with a vast variety of products available for human and animal consumption, Haocheng Mealworm Inc. (China), a huge producer of edible insects for animal consumption, and Agriprotein (Africa), a producer of insects fed with organic waste for animal consumption, are recognized as major players in this industry. Some European companies are also taking big steps in insects production and development for animal feed, with companies like Protix (Netherlands) and Ynsect (France) receiving an investment of more than $50 millions by public and private investors (Cosgrove, 2017).

In Europe Union (EU), under the novel food regulation (No 2015/2283) in force since 1 January 2018, whole insects and their parts are considered as Novel Foods. Countries like Netherlands and Belgium considered whole insects as an exception under the past regulation for novel foods (No 258/97), since insects weren't specifically mentioned as novel foods and some countries didn't agree to put whole insects in the category e) - "foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use". So, they concluded that whole insects weren't regulated by Europe Union and decided to apply national rules, however under the new regulation these products can't be legally sold without an approval (Belluco, Halloran, & Ricci, 2017). If a European company has an ingredient considered as Novel Food, for example an insect, and wants to sell it in Europe, according with the regulation, the company needs to submit an application to the European Comission. After the validation of the documents, they will become available for the EU members and a scientific assessment will be made by EFSA within 9 months (European Food Safety Authority, 2018).

Initially, processed proteins from animals were prohibited for animal feed because of the transmissible spongiform encephalopathy regulation (No 999/2001), later this prohibition was adjusted by the regulation No 56/2013, allowing processed animal proteins (e.g. insects) for feed, excluding ruminants in aquaculture (Lähteenmäki-Uutela et al., 2017). According with the literature, some researchers determined the impact of replacing part of the fish meal for insect meal as an alternative protein source in the diet of african catfish (*Clarias gariepinus*) (Ng, Liew, Ang, & Wong, 2001), blackspot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017), channel catfish (*Ictalurus punctatus*), blue tilapia (*Oreochromis aureus*) (Bondari & Sheppard, 1987), Atlantic salmon (*Salmo solar*) (Belghit et al., 2018), European seabass (*Dicentrarchus labrax*) (Lock, Arsiwalla, & Waagbø, 2016; Magalhães et al., 2017), Jian carp (*Cyprinus carpio*) (Li, Ji, Zhang, Zhou, & Yu, 2017), among others. It was possible to conclude that is plausible to replace part of the fish meal by insect meal without affecting the fish growth, although, some authors reported that the fish meal can't be completely substituted by insect protein due to dietary needs, stress and growth issues (Bondari & Sheppard, 1987; Li et al., 2017; Ng et al., 2001).

The main reasons that lead consumers to buy insects and insect-based products are the curiosity and interest that these type of food products arouse, the expectation that insects are more environment-friendly and sustainable than conventional meat, and the desire to introduce new products into diets. The repeat consumption is influenced by the price, taste and accessibility of these products in the market (House, 2016). Insects can be consumed in three ways: as a whole, as a powder or as a protein isolate (Klunder et al., 2012). The inclusion of insects as an invisible ingredient (e.g. supplement) can probably increase the willingness to try an insect-based product, although, this may not be enough because without a different appearance, taste or any other characteristic, the product has less chance to be selected between others (Le Heron, 2016). A study verified that the participants were more predisposed to accept and eat insects if they were mixed with other ingredients. They were also more likely to eat them if they were unrecognizable, in this way, the authors suggested to mix insects with other products to reduce the impact caused of eating the whole insect (Lensvelt & Steenbekkers, 2014). Many companies have already manufactured products with insects incorporated: cereal bars made of *T. molitor* powder (Portugalbugs, Portugal), muesli bar with mealworms (Essento, Switzerland), peanut butter incorporated with *A. diaperinus* powder (Delibugs, Netherlands) and hamburguers made with soy and *A. diaperinus* (Bugfoundation, Germany) are some of the many developed products using insects as an ingredient.

Instead of using insects as whole or their parts, they can be added to common products as an additive to provide technological properties, researchers are suggesting insects' proteins as a viable alternative to conventional additives. Functional properties of insects' proteins have recently been studied by Zielińska and others (2018), proteins extracted from *T. molitor* (larvae), *S. gregaria* (adult) and *G. sigillatus* (adult) were analysed regarding their water and oil holding capacity, solubility, foaming and emulsifying properties. In conclusion, it was observed a great protein solubility in a wide range of pH values (with a very low solubility when the pH value was around 5 and 6), a remarkable water and oil holding capacity, a relevant emulsion activity and stability. Proteins solubility can be improved with an enzymatic hydrolysis as shown by Hall et al. (2017), hydrolysed proteins from *G. sigillatus* had their solubility improved at a pH values of 3, 7, 8 and 10 when compared with the non-hydrolysed proteins. Therefore, protein hydrolysis of insects can be a feasible way to obtain extracts with a wide functionality and application for food and feed industries.

1.6 Objectives

Considering all the information above, insects are a great source of protein and demonstrated a great potential to be used to obtain peptides with improved bioactivity and functionality, through an enzymatic hydrolysis process. Peptides obtained with this process already demonstrated to have bioactivity properties such as antioxidant, antihypertensive, antidiabetic and anti-inflammatory activity. This work purposes the development of insect protein hydrolysates, using an insect powder obtained from the edible insect *A. diaperinus* (larvae), and a characterization of the final hydrolysates. The work can be summarized by the following steps: (i) Analysis of the enzymatic hydrolysis using two commercial enzymes (Alcalase™ 2.5L and Corolase PP) (ii) Perform a final enzymatic hydrolysis, using the best condition for each commercial enzyme used, followed by a freeze-drying process (iii) Analysis of the bioactivity of the insect protein hydrolysates (Antioxidant activity, ACE and α-Glucosidase enzyme inhibition and antimicrobial activity) (iv) Characterization of the freeze-dried hydrolysates at the best chosen conditions (protein, ash, moisture and total carbohydrates), analysis of the free amino acids composition of the freeze-dried hydrolysates and analysis of the peptide profile after the enzymatic hydrolysis.

2. MATERIALS AND METHODS

2.1 Enzymatic hydrolysis analysis

2.1.1 Enzymatic Hydrolysis

Enzymatic hydrolysis of Buffalo's insect powder (Kreca®, Netherlands) was performed according to Coscueta et al. (2016) and Hall et al. (2017), with some modifications. During this assay, it was used Alcalase™ 2.5L (Aquitex, Portugal), a serine endopeptidase from *Bacillus licheniformis*, at ratios of enzyme:substrate (E/S) of 0.5, 1.5 and 3.0 % (v/w) and it was also tested the enzyme Corolase PP (AB Enzymes GmbH, Germany), an enzymatic mix from porcine pancreas composed by endo and exopeptidases, at ratios of E/S of 0.5, 1.5 and 3.0% (w/w). The optimal conditions for both enzymes, temperature of 50 ºC and a pH of 8.0, was ensured (Hall et al., 2017; O'Loughlin, Murray, Kelly, Fitzgerald, & Brodkorb, 2012). For the control, no enzyme was added. Briefly, a solution of phosphate buffer (pH 8.0) with 1% (w/v) of Buffalo's insect powder was used to perform the enzymatic hydrolysis with Alcalase 2.5L or Corolase PP, the solution was placed at a temperature of 50 ºC for an optimal enzymatic activity and the enzymes were added. Aliquots (5 mL) were taken initially before the enzymes were added, after 30 min, 1, 2, 4, 6, 8 and 24 h. After hydrolysis, the enzymes were inactivated at 100 ºC during 15 min. Then, the samples were cooled and centrifuged at 8000 rpm for 10 min at 4 ºC (Universal 320R, Germany). The supernatant was collected and stored at -80 ºC for future analysis. Hydrolysis was done in duplicate for each condition.

2.1.2 Determination of the degree of hydrolysis

The determination of the degree of hydrolysis (DH) was obtained according to Hsu (2010) with some modifications. The DH of hydrolysate was determined as the ratio of the quantity of α-amino groups released during the hydrolysis to the maximum amount of α-amino groups in Buffalo's insect powder. The maximum amount of amino acids (Lmax) present in the sample was obtained by hydrolysis of the sample with HCl (6 M) during 24 h at 105 ºC and then it was neutralized with NaOH (6 M) before α-amino acid quantification. Briefly, 50 µL of sample (or water) was added to 125 µL of 200 mM sodium phosphate buffer (pH 8.2) and 50 µL of TNBS (2,4,6-Trinitrobenzenesulfonic acid solution) (Sigma-Aldrich, USA) at 0.025% in a 96-well microplate (Sarstedt, Germany). Then, the microplate was incubated at 45 ºC during 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 (Sigma-Aldrich, USA) was used to express the results.

The DH was calculated with the following expression:

$$\mathrm{DH}\ (\%) = \left(\frac{\mathrm{Lt\text{-}L0}}{\mathrm{Lmax\text{-}L0}}\right)\times 100 \tag{2.1}$$

Lt = Amount of amino groups released during the hydrolysis time.
L0 = Amount of amino groups in the sample at initial hydrolysis time.
Lmax = Maximum amount amino groups present in the sample.

The DH was analysed in duplicate for each condition.

2.1.3 Total Antioxidant activity

2.1.3.1 ABTS scavenging assay

The total antioxidant activity of hydrolysates was determinated by ABTS (2,20-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (Sigma-Aldrich, USA) radical scavenging activity according to Re et al. (1999). Briefly, the production of the ABTS radical cation was made by the reaction of ABTS (7 mM) and 2.45 mM of potassium persulfate (Sigma-Aldrich, USA), during 16 h at room temperature in dark. The absorbance of ABTS solution was ajusted with deionised water until an absorbance of 0.700±0.020 at 734 nm. After, 1 mL of ABTS solution reacted with 10 µL of sample during 6 min in the dark and the absorbance was read in the UV-VIS Spectrophotometer (Shimadzu, Brasil). The % of inhibition was then calculated comparing with a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-caboxylic acid) (Sigma-Aldrich, USA) [0.15-0.50 mg/mL], results are expressed as µmol of Trolox Equivalent (TE)/mL of sample. All determinations were performed in triplicate.

2.1.3.2 ORAC-FL scavenging assay

The oxygen radical absorbance capacity (ORAC-FL) assay was made according the metholody used by Contreras et al. (2011). The reaction ocurred at 40 ºC in 75 mM of phosphate buffer (pH 7.4), the final assay mixture (200 µL) was composed by 120 µL of 70 nM Flurescein sodium salt (Sigma-Aldrich, USA), 60 µL of 14 mM AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) (Sigma-Aldrich, USA), and 20 µL of Trolox [$9.98\mathrm{x}10^{-4}$-$7.99\mathrm{x}10^{-3}$ µmol/mL] or 20 µL of sample at different concentrations. The fluorescense was recorded during 137 min, with a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) with 485 nm excitation filter and 520 nm emission filter. The program used during this assay was the FLUOstart Control software (version 1.32 R2) and it was used a Black polystyrene 96-well microplate (Nunc, Denmark). AAPH and Trolox solutions were made daily and fluorescein was prepared daily from a stock solution previously elaborated (1.17 mM) in 75 mM of phosphate buffer (pH 7.4). The final results were expressed in µmol of TE/mL of sample. All reactions were performed in duplicate and three independent runs were done.

2.2 Enzymatic hydrolysis under the best conditions

Considering the results obtained after the enzymatic hydrolysis of Buffalo's insect powder (DH and antioxidant activity), only the best condition for each enzyme was chosen to proceed with the characterization of the insect protein hydrolysates. For this, an enzymatic hydrolysis was made as previously explained in 2.1.1, with some modifications. Instead of using a phosphate buffer solution, the hydrolysis was performed with water, in order to remove the interference of the buffer solution salts in the freeze-dried hydrolysates, and the pH value was adjusted before and during the time of hydrolysis with 2 M NaOH. Two hydrolysis conditions were selected, in the case of Corolase PP a ratio of E/S of 3.0% (w/w) during 6 h were chosen and in the case of Alcalase™ 2.5L a ratio of E/S of 1.5% (v/w) during 4 h were chosen. When the solution, composed by Buffalo's insect powder in water at 1% (w/v), reached 50 ºC, the enzymes were added into the conditions tested. For the control, no enzymes were added. After inactivating the enzymes during 15 min in boiling water, the samples were centrifuged at 8000 rpm during 10 min at 4 ºC. Then, the supernatants were filtered and the soluble content was frozen at -80 ºC. Hydrolysis was done in duplicate for each condition.

2.2.1 Freeze drying of hydrolysates

After the enzymatic hydrolysis, the frozen samples were freeze dried for 3 days (Armfield SB4 model, UK) and stored in a desiccator for further experiments.

2.3 Determination of the degree of hydrolysis and yield

The DH was performed as described in 2.1.2. The yield was calculated in order to understand the efficiency of the enzymatic hydrolysis process. The yield for each chosen condition (Alcalase™ 2.5L hydrolysis, Corolase PP hydrolysis and control) was done according to the following formula:

$$\text{Yield (\%)} = 100 \times \frac{\text{Weight of Insect powder used for the enzymatic hydrolysis}}{\text{Weight of freeze-dried hydrolysate obtained}}$$

(2.2)

2.4 Bioactive properties of hydrolysates obtained under the best conditions

2.4.1 Total Antioxidant activity

The total antioxidant activity of hydrolysates was performed as described in 2.1.3. The samples used were composed by 10 mg of freeze dried hydrolysate diluted in 1 mL of deionised water (10 mg/mL). Results were expressed in µmol TE/g of insect powder.

2.4.2 Angiotensin-converting enzyme inhibition

The inhibitory effect of Angiotensin-converting enzyme (ACE) was measured by the fluorimetric assay proposed by Sentandreu and Toldrá (2006), with modifications made by Quirós et al. (2009). Briefly, ACE (peptidyl-dipeptidase A, EC 3.4.15.1) (Sigma-Aldrich, USA) working solution was diluted in 0.15 mM Tris buffer (pH 8.3), the final reaction solution contained 0.04 U/mL of enzyme and 0.1 mM of $ZnCl_2$. After, it was used a Black polystyrene 96-well microplate (Nunc, Denmark), containing in each well 40 µL of water (blank) or enzyme (control and samples), 40 µL of water (blank and control) or sample and 160 µL (blank, control and sample) of o-Abz-Gly-p-Phe(NO_2)-Pro-OH (Bachem, Switzerland) (0.45 mM), dissolved in 150 mM Tris buffer (pH 8.3) with 1.125 M of NaCl, to start the enzymatic reaction. The mixture was incubated at 37 ºC. The fluorescence was measured after 45 min using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany). The wavelengths used were 350 nm (excitation) and 420 nm (emission). The software used during the assay was FLUOstar control (version 1.32 R2). The activity of each sample was tested in duplicate. A sample of 10 mg/mL of freeze dried hydrolysate was used. The half maximal inhibitory concentration (IC_{50}) used to express the results obtained is the total protein content needed to inhibit the ACE activity in 50%. The formula applied to calculate the percentage of inhibition was:

$$\text{ACE inhibiton (\%)} = 100 \times \frac{\text{(Control-Sample)}}{\text{(Control-Blank)}}$$

(2.3)

Non linear fitting to the data was determined to obtain the IC_{50} values as previously done by Quirós et al. (2007). The amount of total protein present in the samples was estimated by Kjeldahl method. The ACE inhibitory activity was analysed in duplicate for each condition.

2.4.3 α-Glucosidase inhibition

The α-Glucosidase inhibitory activity was determined in 96-well microplates according to Kwon et al. (2008). Briefly, 40 µL of sample (10 mg/mL of freeze dried hydrolysate) were mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-Glucosidase (1.0 U/mL) (Sigma-Aldrich, USA). The microplate was pre-incubated at 25 ºC for 10 min. After, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (Sigma-Aldrich, USA) solution in 0.1 M phosphate buffer (pH 6.9) was added to each well. The reaction was performed at 25 ºC for 5 min, before and after the reaction, the absorbance readings were recorded at 405 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany). Acarbose (Sigma-Aldrich, USA) was used as positive control at the concentration of 10 mg/mL, phosphate buffer was used as negative control. The α-Glucosidase inhibitory activity was calculated according with the following equation:

$$\alpha\text{-Glucosidase inhibiton (\%)} = 100 \times \frac{(\Delta \text{Abs}_{\text{CONTROL}} - \Delta \text{Abs}_{\text{SAMPLE}})}{(\Delta \text{Abs}_{\text{CONTROL}})} \tag{2.4}$$

$\Delta \text{Abs}_{\text{CONTROL}}$ = Variation of absorbance of the control.

$\Delta \text{Abs}_{\text{SAMPLE}}$ = Variation of absorbance of the samples.

The α-Glucosidase inhibitory activity was analysed in triplicate.

2.4.4 Antimicrobial Activity

To evaluate the antimicrobial activity of the protein hydrolysates, a well diffusion assay was performed according to Silva et al. (2013). Firstly, the bacterial isolates of *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *L. monocytogenes* ESB 3562 and Methicillin-resistant *S. aureus* (MRSA) CCUG 60578 were grown overnight in Tryptic soy broth (Biokar Diagnostics, France). After, plates with 20 mL of Mueller-Hinton Agar (Biokar Diagnostics, France) were seeded with a bacterial suspension equivalent to a 0.5 McFarland standard (10^8 CFU/mL) using a swab. Then, 40 µL of filtered (0.22 µm) sample (10 mg/mL of freeze dried hydrolysate) or sterile water (negative control) were added to wells with 4 mm of diameter. The plates were incubated during 24 h at 37 ºC. The presence of a translucent halo around the wells indicates inhibition of the microorganisms, the absence of a translucent halo zone indicates that no antimicrobial activity occurs. Assays were done in triplicate.

2.5 Characterization of hydrolysates obtained under the best conditions

2.5.1 Total protein content

The total protein content of the samples was determined using the Kjeldahl method. The freeze-dried samples were digested with concentrated sulfuric acid in the presence of catalysts to convert the organic nitrogen into ammonium sulfate. After cooling the samples, an excess of 10 M NaOH was added to release the ammonia. Then, the ammonia previously released was distilled into an excess of boric acid solution and then titrated with HCl (0.1-0.5 M). The crude protein content is calculated from the quantity of ammonia produced (ISO 1871:2009E). This analysis was made in duplicate. The conversion factor used to calculate the protein content was 5.6, as proposed by Janssen et al. (2017) for protein extracts derived from insects.

2.5.2 Moisture and Ash content

To evaluate the moisture, the freeze-dried hydrolysates were placed at 100 ºC during 24 h. To determine the ash content, samples were placed at 550 ºC in a muffle during 5 h for incineration (ISO 2171:1980). This analysis was made in duplicate.

2.5.3 Total carbohydrates

The quantification of the total amount of carbohydrates was made as described by Dubois et al. (1956) with modifications. Briefly, into a test tube were added 80 µL of sample (10 mg/mL of freeze-dried hydrolysate), 150 µL of a aqueous phenol solution at 5% (w/v) and 1 mL of concentrated sulphuric acid. After the reaction, the samples were let to cool during a few minutes. The absorbance was then measured at 490 nm using the UV-VIS Spectrophotometer (Shimadzu, Brasil). A calibration curve of glucose at different concentrations was used to calculate the amount of carbohydrates present in the sample. All reactions were made in duplicate.

2.5.4 Free amino acids determination by High Performance Liquid Chromatography (HPLC)

The free amino acid determination was performed using a Liquid chromatography aparatus (HPLC Gold 128 Solvent module, Beckman Coulter, USA) with a High-Resolution Fluorescence Detector (Waters 474, USA) and an autosampler (model 410 Varian prostar, Agilent technologies, USA). Firstly, two eluents (A and B) and three reagents (A, B and C) were prepared. The eluent A (pH 6.9) composed by disodium phosphate (Sigma-Aldrich, USA), acetonitrile (Fisher Chemical, USA) and ultrapure water. The eluent B constituted by methanol (Fisher Chemical, USA), dimethyl sulfoxide, acetonitrile and ultrapure water. Reagent A (pH 9.5) composed by a standard solution, with homoserine and norvaline (Sigma-Aldrich, USA), mercaptoetanol (Fluka Analytical, USA), sodium tetraphenylborate (Merck, Germany) and borate buffer. Reagent B contained iodoacetic acid (Sigma-Aldrich, USA) and borate buffer, the pH was then adjusted to a value of 9.5 with 4 M NaOH. Reagent C consisted of o-Phtaldialdehyde (Sigma-Aldrich, USA), methanol, borate buffer and mercaptoethanol. Finally, the reagent A, B and C were mixed with 100 µL of filtered sample (10 mg/mL of freeze-dried hydrolysate) and then 10 µL of the mix were injected into the HPLC. The system was connected to a Chromolith® Performance RP18 (4.6 × 100 mm) (Merck, Germany) column. The flow gradient was 0.8 mL/min. A calibration curve of 17 different amino acids was made in various concentrations (0.8-100 mg/L). The measurements were analysed in duplicate and the results are expressed in amino acid (mg) per gram of insect powder.

2.5.5 Peptide profile analysis by Fast Protein Liquid Chromatography (FPLC)

To analyse the peptide profile of the hydrolysates, 100 µL of filtered sample was injected in a FPLC system (AKTA pure, GE Healthcare Life Sciences). A Superdex™ Peptide 200 Increase 10/300 GL column (GE Healthcare Life Sciences) was eluted with a solution composed by 0.025 M phosphate buffer, 0.2 g/L of sodium azide (Sigma-Aldrich, USA) and 8% NaCl. The flow gradient was set at 0.5 mL/min. To establish a molecular weight standard curve, standard proteins with known molecular weights were used, such as 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). All the analysis were performed in duplicate and the results are expressed in milli Absorbance Units (mAU) per eluted volume (mL).

2.6 Statistical analysis

Data are expressed as mean values ± standard error of the mean. The normality of the distributions was evaluated through the Shapiro Wilk’s Test and the differences were evaluated using One-way ANOVA test. Means were considered to be different at a significant 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 analysis

3.1.1 Degree of hydrolysis

An initial analysis of the enzymatic hydrolysis of Buffalo's insect powder was done to choose the best conditions to obtain peptides with bioactivity. As reported by various authors, different hydrolysis processes, using simulated gastrointestinal digestion or commercial enzymes, can affect the bioactive properties of the resulting hydrolysates (Vercruysse et al., 2009; Yang et al., 2013). With the same enzyme, differences in functional properties were also demonstrated with different ratios of E/S (Hall et al., 2017), furthermore, different times of hydrolysis were also demonstrated to affect the bioactive properties of the obtained hydrolysates (Hall et al., 2018). In this study, two commercial enzymes (Alcalase™ 2.5L and Corolase PP) were used to hydrolyse Buffalo's insect powder. Various E/S ratios and different times of hydrolysis were tested and two parameters were analysed to select the best hydrolysis condition for each enzyme, the determination of the degree of hydrolysis (DH) to observe how hydrolysis was evolving over time, and the antioxidant activity of hydrolysates to observe how this bioactive property was affected in each condition used.

The choice of the E/S ratio, hydrolysis time, pH value and temperature, can highly influence the DH and the resulting peptide functionality (Hall et al., 2017). A higher DH in insect proteins appears to have a positive effect on bioactive properties (Yang et al., 2013). In order to obtain peptides with potential bioactivity it was used Alcalase™ 2.5L and Corolase PP, as described above. Alcalase™, a protease from *Bacillus licheniformis*, is an useful resource and an enzyme largely used in insects' proteins with the purpose of getting bioactive hydrolysates (e.g. antihypertensive activity) (Nongonierma & FitzGerald, 2017). Corolase PP, an enzymatic preparation derived from porcine pancreas, was used by various authors in order to obtain peptides with bioactive properties (e.g. antioxidant activity) in other food matrices like soy protein (Coscueta et al., 2016; Guan, Diao, Jiang, Han, & Kong, 2018), whey protein (Contreras et al., 2011) and fish protein (Slizyte et al., 2016) but was used for the first time in protein insect in this study.

The DH obtained by Alcalase™ 2.5L and Corolase PP hydrolysis are presented, respectively, in Figure 3.1.1 and Figure 3.1.2. 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 to hydrolyse the insect proteins. The increasing of the DH between 8 and 24 h in the control, can be explained by protein degradation due to the thermal impact over the time and the inactivation process for the enzymes (Chi, Krishnan, Randolph, & Carpenter, 2003).

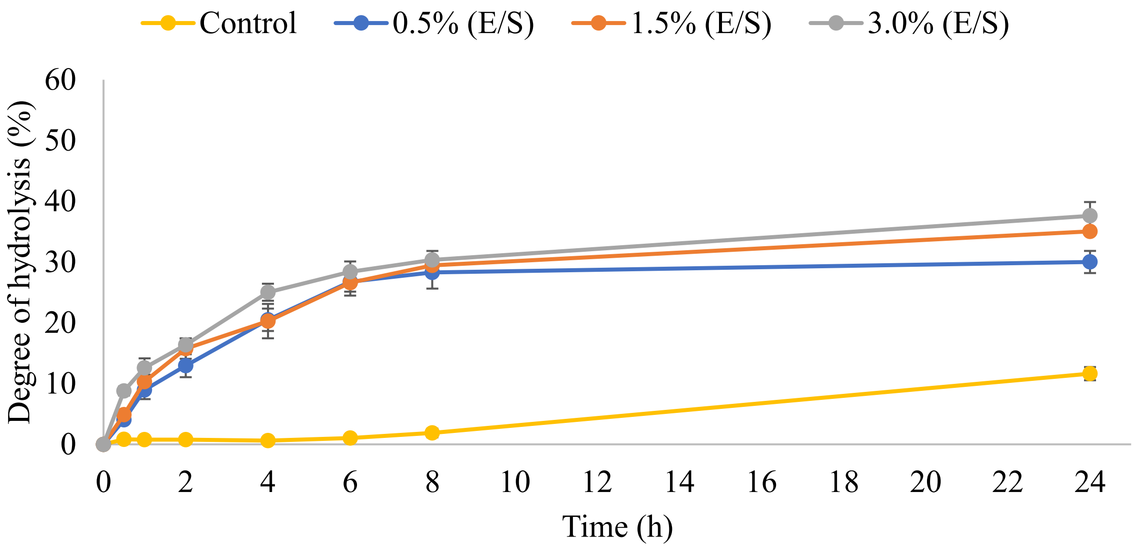

Figure 3.1.1 - Degree of hydrolysis obtained using the enzyme Alcalase™ 2.5L as a function of hydrolysis time expressed in hours. Enzymatic hydrolysis was done at pH of 8.0 and 50 ºC. The ratio of enzyme:substrate (E/S) used was 0.5% (—●—), 1.5% (—●—) and 3.0% (—●—). For the Control (—●—), no enzyme was added.

According to Figure 3.1.1, the conditions 0.5, 1.5 and 3.0% (E/S), demonstrated a very similar behaviour along the hydrolysis. During the hydrolysis, it was possible to observe that the condition 0.5% and 1.5% weren't significantly different ($p > 0.05$) during the first 8 h of hydrolysis. It was also noted that at 6 and 8 h of hydrolysis, no significant differences ($p > 0.05$) were observed in the three conditions studied for Alcalase™ 2.5L.

The DH results obtained for Alcalase™ 2.5L hydrolysates (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 are very similar to the results reported by Dai et al. (2013), after using Alcalase™ for an enzymatic hydrolysis of *T. molitor* larvae defatted flour, they estimated a DH of around 20.0% after approximately 4 h of hydrolysis with Alcalase™ at 1.0% (E/S). In the present study, using Alcalase™ 2.5L at 3% (E/S) during 6 ha DH of 28.4% was reached and a proximate value (ca. 32%) was published by Yang et al. (2013) after performing a hydrolysis of a 10% (w/v) solution of *B. mori* protein with Alcalase™ 2.4L at 4.0% (E/S) during 5 h of hydrolysis. Although the DH achieved after 1 h of hydrolysis time were 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 in a study by Hall et al. (2017), after an enzymatic hydrolysis of a *G. sigillatus* solution at 50% (w/v) with Alcalase™

and testing various ratios of E/S, DH 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 other study of Hall et al. (2018), using the same enzyme and the same insect. The DH obtained in both studies may have been influenced due to a prior pasteurization of the sample at 90 ºC during 15 min made by the authors, since a thermal process can increase proteins degradation (Jood, 1989) and it was already demonstrated that a previous heat treatment can influence the DH (O'Loughlin et al., 2012).

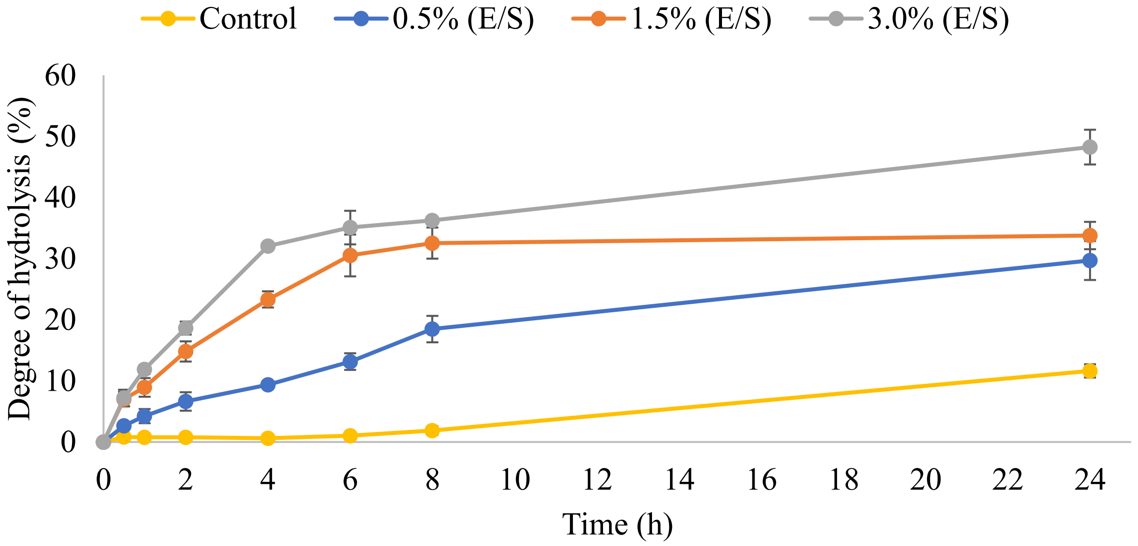

Figure 3.1.2 - Degree of hydrolysis obtained using the enzyme Corolase PP as a function of hydrolysis time expressed in hours. Enzymatic hydrolysis was done at pH of 8.0 and 50 ºC. The ratio of enzyme:substrate (E/S) used was 0.5% (—●—), 1.5% (—●—) and 3.0% (—●—). For the Control (—●—), no enzyme was added.

According to Figure 3.1.2, the conditions 0.5% (E/S), 1.5% (E/S) and 3.0% (E/S), demonstrated different behaviour of hydrolysis, it was verified that DH increased with time and E/S increment. For the hydrolysis using Corolase PP, no significant differences ($p > 0.05$) were observed in the first half hour between the condition 1.5% (E/S) and 3.0% (E/S), and at 24 h between the condition 0.5% (E/S) and 1.5% (E/S). Considering the others hydrolysis times, a significant difference ($p < 0.05$) among all the conditions could be observed. Figure 3.1.2 also shown that the condition 3.0% (E/S) had a DH value higher than 30% at 4 h of hydrolysis. As reported by Silvestre and colleagues (2013) using this same enzyme at 8% (E/S) on a protein equivalent basis to hydrolyse whey protein, they obtained a DH of 35.6% after 1 h of hydrolysis, with a small decrease to 34.3% after 4 h of hydrolysis; in the recent study after 1 h of hydrolysis was observed a DH of 11.9% with only 3.0% (E/S), although, the amount of enzyme used by the authors was significantly higher. Jaiswal et al. (2015), also evaluated the hydrolysis of buffalo α_S-casein with an enzyme ratio of 1%. After hydrolysis with Corolase PP, the authors reported the variation of DH values between 2 and 24 h of hydrolysis and could be concluded that the DH of Corolase PP ranged from 16.6% to 19.4% in 24 h of hydrolysis. Better results were obtained in this study, with Corolase PP at 0.5% (E/S), DH values ranging from 6.6% (2 h) to 29.7% (24 h). The DH in a whey protein isolate with Corolase PP at 1.0% (E/S) on a protein equivalent basis was assessed by O'Loughlin et al. (2012), they observed that after 1 h of hydrolysis a DH of ca. 3.5% was attained, and very similar result was obtained with Corolase PP at 0.5% (E/S) in this assay with a DH of 4.2%.

To our knowledge, insect hydrolysates obtained through Corolase PP hydrolysis, hasn't been tested yet and no study was published. So, the enzymatic hydrolysis of Buffalo's insect powder using this enzyme, points to the further exploitation of these insect protein hydrolysates and to their potential use.

3.1.2 Antioxidant activity of the hydrolysates

The hydrolysis efficiency was evaluated by the DH, however to define the optimal conditions (E/S ratio and hydrolysis time) for each enzyme, the bioactivity of the hydrolysates obtained throughout all process was also evaluated. To further understand how the DH is influencing the antioxidant activity of the resulting hydrolysates, it was analysed the protective capacity of the hydrolysates against the ABTS radical cation. This method is usually considered as an Electron Transfer (ET) based assay, the antioxidant donates an electron to reduce the target compound, and Hydrogen Atom Transfer (HAT), the antioxidant is able to reduce the target compound through a hydrogen donation mechanism (Huang, Boxin, & Prior, 2005; Prior, Wu, & Schaich, 2005; Schaich, Tian, & Xie, 2015). This method evaluate the capacity of the antioxidant compound to reduce the ABTS radical cation, when in contact with the antioxidant the radical color decreases when reduced, from blue-green color (oxidized) until no color (reduced) (Re et al., 1999). Antioxidant activity of peptides is associated with their amino acids composition (Korhonen & Pihlanto, 2006) and this composition is influenced by the enzyme used in order to obtain them (Coscueta et al., 2016).

According to the results obtained by Alcalase™ 2.5L hydrolysates (AH) and Corolase PP hydrolysates (CH), respectively in the Figure 3.1.3 and Figure 3.1.4, it was possible to observe that the AH and the CH of the condition 3.0% (E/S) at 24 h had the highest Trolox Equivalent Antioxidant Capacity (TEAC), with 5.8 μmol TE/mL and 6.6 μmol TE/mL, proving a clear increase of the protective capacity over time. In control, no significant differences were observed between the 0 and 8 h of hydrolysis time, and the protective capacity increased only after 24 h of hydrolysis, probably related with the increase of the DH at that time and, as above explained, may have resulted due to protein denaturation by the thermal impact during enzymatic hydrolysis and the inactivation process made at 100 ºC during 15 min.

Comparing the three hydrolysis conditions using Alcalase™ 2.5L (Figure 3.1.3), the hydrolysates obtained from the conditions 1.5% (E/S) and 3.0% (E/S), had very similar TEAC values over time. Due to this similiarity and since these two conditions are significantly different ($p < 0.05$) than the condition 0.5% (E/S), it is possible to concluded that the condition 1.5% (E/S), due to the fact of using less enzyme to obtain the same protective activity of the condition 3.0% (E/S), seems the best condition among the AH obtained.

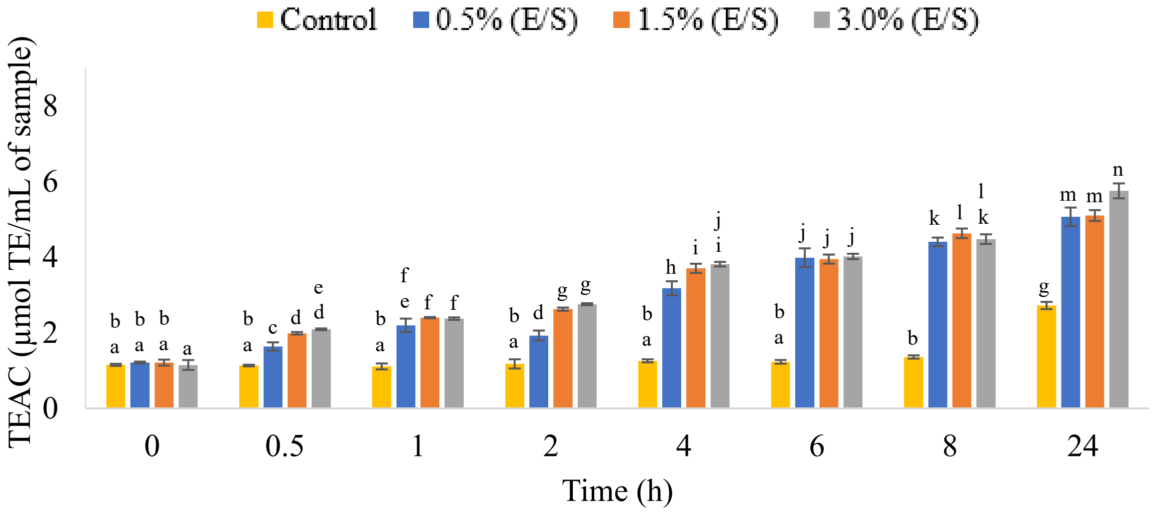

Figure 3.1.3 - Antioxidant activities of Alcalase™ 2.5L hydrolysates against radical ABTS as a function of hydrolysis time expressed in hours. The ratio of enzyme:substrate (E/S) used was 0.5% (🟦), 1.5% (🟧) and 3.0% (◼). For the Control (🟨), no enzyme was added. Same letters mean no significant difference between them ($p > 0.05$).

Evaluating the relation between the DH and the ABTS radical cation reduction, it's possible to observe that a higher DH resulted in a higher antioxidant capacity of the hydrolysates. The same relation was reported by Sbroggio et al. (2016) using okara protein hydrolysates obtained with Alcalase™ 2.4L at a ratio of 10% (E/S), the hydrolysates had their antioxidant activity potentiated with a higher hydrolysis time.

After analysing the Figure 3.1.4, the condition 0.5% (E/S) had similar TEAC values to condition 1.5% (E/S) in the first hour of hydrolysis ($p > 0.05$). After 2 h and until final hydrolysis time, the TEAC values obtained in condition 0.5% were significantly lower ($p <$ 0.05) from conditions 1.5% (E/S) and 3.0% (E/S). In conclusion, the condition 0.5% (E/S) demonstrated a poor increase of antioxidant activity over time, contrary to the conditions 1.5% (E/S) and 3.0% (E/S), and both exhibited a great increase along the hydrolysis time. However, the condition 3.0% (E/S) achieved higher TEAC values, proving to be the best condition in this study. A relation between the DH obtained on the hydrolysis of Buffalo's insect powder with Corolase PP and the TEAC values attained was notable, since the CH originated with higher DH value revealed a higher antioxidant activity, corroborating with the results described by Mann et al. (2015). They evaluated the antioxidant activity of the whey protein hydrolysates obtained using three different enzymes (Alcalase™, Flavourzyme™ and Corolase PP), concluding that a higher DH value, obtained by Corolase PP at 2% (E/S) during 7 h of hydrolysis, had also the higher TEAC value.

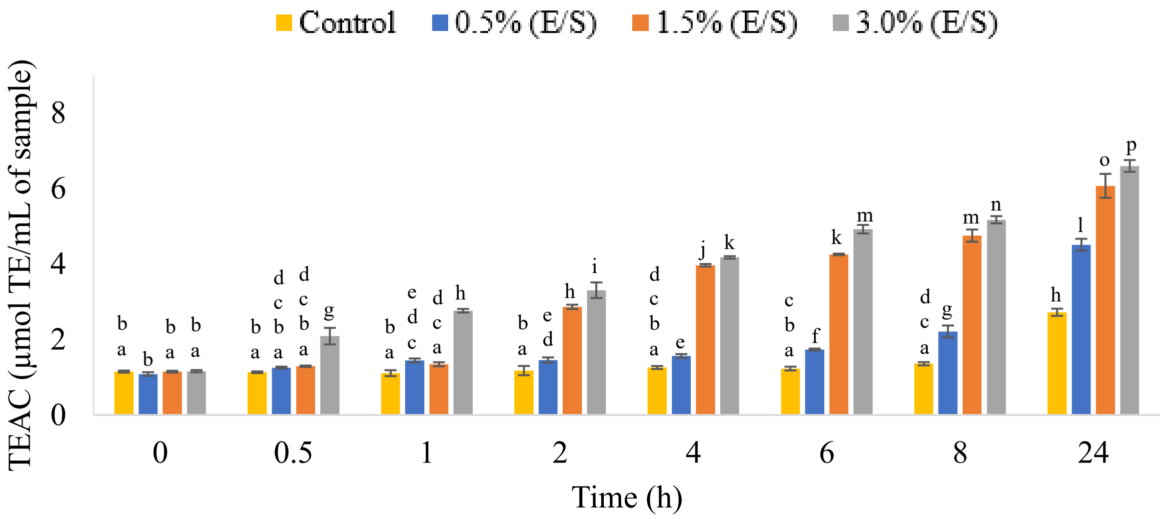

Figure 3.1.4 - Antioxidant activity of Corolase PP hydrolysates against radical ABTS as a function of hydrolysis time expressed in hours. The ratio of enzyme:substrate (E/S) used was 0.5% (🟦), 1.5% (🟧) and 3.0% (◼). For the Control (🟨), no enzyme was added. Same letters mean no significant difference between them ($p > 0.05$).

Analysing the results obtained by the two parameters, DH and ABTS radical scavenging activity, for each enzymatic hydrolysis there is an increase of the DH along the time with the values stabilizing after 6 h of hydrolysis. The TEAC values obtained with both enzymes also increased over time, demonstrating the best antioxidant capacity at the highest hydrolysis times, 8 and 24 h. Even though the antioxidant capacity is higher in a higher hydrolysis time, using extended hydrolysis involves several risks that may affect a future industrial application. Risks such as microbial contamination of the batch and high costs to maintain the ideal conditions for the enzymes (pH and temperature). So, it's appropriate to consider that 6 h of hydrolysis should be the hydrolysis time limit. In this sense, for the higher TEAC values obtained until 6 h of hydrolysis was done other antioxidant complementary method, the Oxygen Radical Absorbance Capacity method (ORAC). According with the two parameters discussed above (DH and TEAC), two-hydrolysis time were chosen for further analysis, 4 and 6 h.

To analyse the antioxidant capacity of the hydrolysates in these two-hydrolysis time, the ABTS scavenging assay was complemented with the ORAC scavenging assay. ORAC assay is categorized as a HAT based assay and evaluates the capacity of the hydrolysates to reduce the peroxyl radical, generated by the thermal decomposition of AAPH, through a hydrogen atom transfer mechanism. This method evaluates the fluorescence decay over time caused by the fluorescent probe (fluorescein) degradation caused by the peroxyl radical, resulting in a nonfluorescent compound, and the protection capacity of an antioxidant compound to reduce this peroxyl radical and delay the fluorescein degradation. On opposite to the TEAC method, which uses a radical source not found in the mammalian biology, the ORAC method is considered as biologically relevant since the mechanism evaluated usually reflects what occurs *in vivo*, since peroxyl radicals are responsible for the lipid oxidation in food and in biological systems. This method is also largely used in food industry as a reference method to measure antioxidant capacity (Huang et al., 2005; Prior et al., 2005; Schaich et al., 2015).

In the Figure 3.1.5 and 3.1.6 are represented the results obtained by the ORAC method for the AH and the CH, respectively. As can be observed in the results, the antioxidant activity of control, at 4 and 6 h of hydrolysis, is significantly lower than the hydrolysates and constant in the both hydrolysis times analysed ($p > 0.05$). For AH (Fig. 3.1.5), 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) had values of antioxidant activity significantly higher than the condition 0.5% (E/S). No significant differences ($p > 0.05$) were observed between the ORAC values obtained by the conditions 1.5% and 3.0% at 4 and 6 h of hydrolysis, being approximately 9.3 μmol TE/mL. In general, the values obtained by ORAC doesn't seem to have a relation with the DH, since a higher DH, due to higher hydrolysis time and enzyme concentration, didn't demonstrate a correspondent high antioxidant activity in the case of AH. A similar pattern was accessed by Bernardi et al. (2016), analysing the antioxidant activity of hydrolysates obtained from processed Nile tilapia residues with Alcalase™ at 0.2% (w/w) for different hydrolysis times, and the hydrolysates obtained showed very similar TE values among the different hydrolysis times.

In conclusion, the best chosen condition for the final hydrolysis of Buffalo's insect powder with Alcalase™ 2.5L was the one with lower enzyme concentration and shorter hydrolysis time that have high TE values, in the condition 1.5% (E/S) during 4 h of hydrolysis.

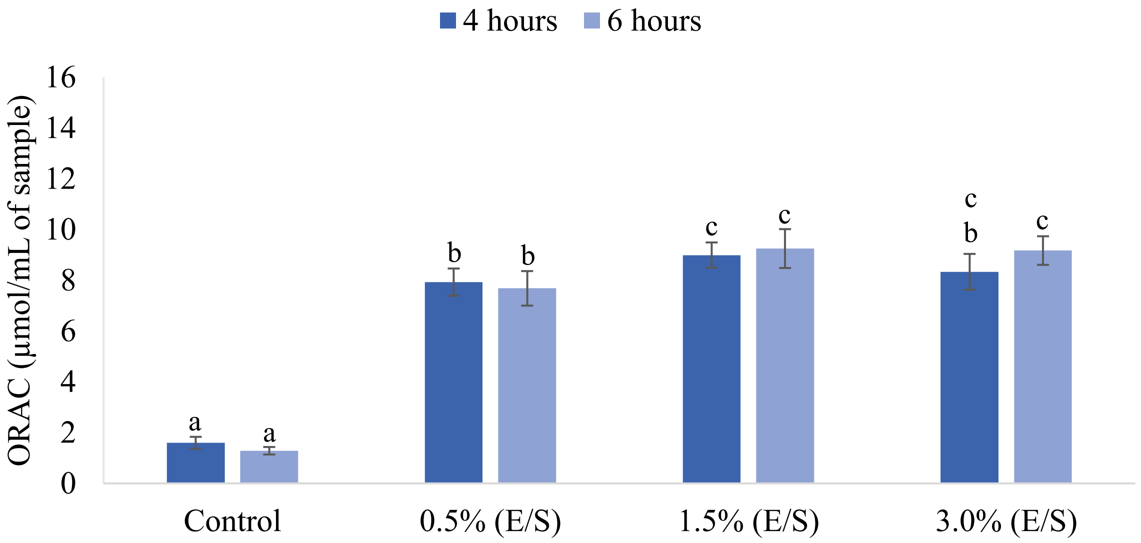

Figure 3.1.5 - Antioxidant activity of Alcalase™ 2.5L hydrolysates at 4 and 6 h of hydrolysis by ORAC. Same letters mean no significant difference between them ($p > 0.05$).

According with the results obtained by CH at 4 and 6 h of hydrolysis (Fig. 3.1.6), a significant difference ($p < 0.05$) of antioxidant activity between the different ratios of enzyme was detected. The highest ORAC values obtained for CH were the condition 3.0% (E/S) at 4 h (9.5 µmol TE/mL), 1.5% (E/S) at 6 h (9.7 µmol TE/mL) and 3.0% at 6 h (12.3 µmol TE/mL). Therefore, the condition 3.0% (E/S) at 6 h presented an antioxidant activity significantly higher than the others conditions.

Analysing the results obtained for CH, the hydrolysis time and enzyme concentration seem to have an effect on the antioxidant activity by ORAC, potentiating it. Along the hydrolysis, it's possible to observe a clear growth in the Trolox Equivalent (TE) values over time. Hydrolysates with higher ORAC values obtained from longer hydrolysis were verified by Nongonierma et al. (2017), casein hydrolysates obtained by the enzyme Protamex™ were influenced by the hydrolysis time (1-5 hours), corroborating the results obtained in the present study.

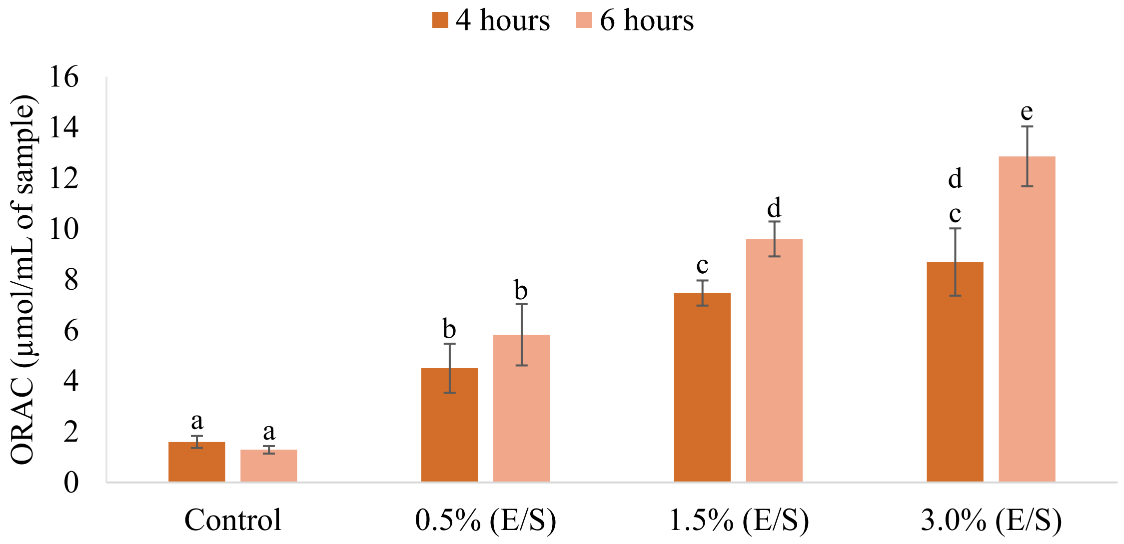

Figure 3.1.6 - Antioxidant activity of Corolase PP hydrolysates at 4 and 6 hours of hydrolysis by ORAC. Same letters mean no significant difference between them (*p* > 0.05).

Although, peptides obtained from deffated soy protein isolates through hydrolysis with Corolase PP at 1% (E/S), the DH didn't influence the values obtained by ORAC, since hydrolysates obtained from lower hydrolysis time had higher TE values when compared with longer hydrolysis times and subsequently with higher DH values (Coscueta et al., 2016).

In conclusion, since the hydrolysis time and enzyme concentration influence the ORAC capacity of the CH, the best chosen condition for the final hydrolysis of Buffalo's insect powder with Corolase PP was 3.0% (E/S) during 6 h of hydrolysis.

3.2 Bioactive properties of hydrolysates obtained under the best conditions

After hydrolysing the Buffalo's insect powder and freeze drying the hydrolysates obtained by the best conditions for each enzyme, i.e, Alcalase™ 2.5L at 1.5% (E/S) for 4 h

Table 3.2 - Yield and DH value of the final hydrolysates performed for each condition.

Condition	**DH (%)**	**Yield (%)**
Alcalase™ 2.5L 1.5% (4 h)	19.5 ± 1.6	63.3 ± 1.2
Corolase PP 3.0% (6 h)	36.0 ± 1.0	67.4 ± 0.3
Control	-	24.0 ± 1.0

(FD-AH) and Corolase PP at 3.0% (E/S) during 6 h (FD-CH), the bioactive properties of the hydrolysates were tested. For the control, no enzyme was added and the soluble part, was filtered and freeze-dried for further analysis, as performed in enzymatic hydrolysis. In Table 3.2 is shown the DH value and the yield of the process for each condition studied.

The bioactive properties analysed in the FD-AH and FD-CH were the antioxidant property, antihypertensive activity (ACE inhibition), antimicrobial and antidiabetic (α-Glucosidase inhibition) activities.

3.2.1 Total antioxidant activity

The total antioxidant activity was analysed using the two complementary methods applied during the initial enzymatic hydrolysis phase, ABTS and ORAC scavenging assay, and according with the results obtained for the antioxidant activity of the hydrolysates (Table 3.2.1), it was verified very similar TEAC values between the FD-AH and the FD-CH ($p > 0.05$). When comparing the ORAC values obtained by both conditions, it was observed a significant difference ($p < 0.05$) between the FD-CH and FD-AH, and the peptides obtained through Corolase PP demonstrated the best values.

Table 3.2.1 - Antioxidant activity of the hydrolysates obtained by Alcalase™ 2.5L and Corolase PP. Same letters mean no significant difference between them ($p > 0.05$).

Condition	**TEAC (µmol TE/g of insect powder)**	**ORAC (µmol TE/g of insect powder)**
Alcalase™ 2.5L 1.5% (4 h)	95.0 ± 0.8^a	825.6 ± 85.5^c
Corolase PP 3.0% (6 h)	95.7 ± 1.0^a	944.8 ± 68.1^d
Control	24.3 ± 0.4^b	230.7 ± 15.5^e

According with the literature, one study performed an enzymatic hydrolysis with Alcalase™ to obtain antioxidant peptides using various ratios of E/S in a 50% (w/v) solution of *G. sigillatus* protein (Hall et al., 2018). The results of the present study were lower when compared with the TEAC values reported by Hall and others (2018), the authors reported TEAC values of 403.2 µmol TE/mg of sample using a ratio of 0.5% (E/S) and values of 512.0 µmol TE/mg of 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%). The pasteurization process made previously to the enzymatic hydrolysis could had positively affected the protein digestibility and the antioxidant activity. Tang and colleagues (2018) reported results for 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), IC_{50} value of 2.7 µmol TE/mg of sample was achieved. Even though only two studies reported results using Trolox as an equivalent, 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 (Zielińska et al., 2018), alkaline protease (Zhou et al., 2017) and Alcalase 2.4L (Yang et al., 2013). The values obtained by both antioxidant methods also indicate that the peptides generated by both enzymes during the enzymatic hydrolysis act mainly against the peroxyl radical, using the HAT mechanism instead of the ET mechanism, the same outcome was also demonstrated in defatted soy hydrolysates using Corolase PP, where the ORAC assay values were higher when compared with the ABTS assay values (Coscueta et al., 2016).

There is a lack of studies analysing the TEAC and ORAC values for insect protein hydrolysates and no studies evaluating the antioxidant capacity in the insect *A. diaperinus*.

3.2.2 Angiotensin-converting enzyme inhibitory activity

Peptides capable of inhibit ACE have been already extracted from various common products such as soy protein (Coscueta et al., 2016), milk (Jäkälä & Vapaatalo, 2010), fish protein (Fujita & Yoshikawa, 1999) and egg white protein (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). Various peptides with ACE inhibitory capacity are already in the composition of some patented products such as Calpis® and Evolus®, two fermented products (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Since 2005, various authors reported that hydrolysates derived from insects proteins can be a great source of peptides with ACE inhibitory activity (Nongonierma & FitzGerald, 2017; Vercruysse et al., 2005). Various insects were studied and demonstrated this property, consequently it was also analysed this capacity in the FD-AH and FD-CH.

The results obtained for the ACE inhibition of FD-AH and FD-CH (Table 3.2.2), revealed that the FD-AH had a better ACE inhibitory activity, because the FD-AH obtaining 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 literature, similar results to those obtained by FD-AH were reported for other insects. Hall et al. (2018) described a slightly lower IC_{50} value of 40.0 µg/mL for *G. sigillatus* hydrolysates obtained through an enzymatic hydrolysis using Alcalase™ at 3.0% during 20 min of hydrolysis time.

Jia et al. (2015) studied *B. mori* hydrolysates using the same enzyme, although the process used to obtain these hydrolysates had a previous ultrasonic pre-treatment for 32 min (410W) and 50 minutes of hydrolysis time. The authors observed an IC_{50} value of 91.3 µg/mL, but better results were obtained in the present study for FD-AH (IC_{50} of 55.5 µg/mL).

Table 3.2.2 - Angiotensin-converting enzyme (ACE) inhibitory activity of the hydrolysates obtained by Corolase PP and Alcalase™ 2.5L. Same letters mean no significant difference between them ($p > 0.05$).

Condition	**ACE (IC_{50} µg of protein/mL)**
Alcalase™ 2.5L 1.5% (4 h)	55.5 ± 6.2^{a}
Corolase PP 3.0% (6 h)	107.4 ± 9.7^{b}
Control	171.6 ± 38.0^{c}

Dai and others (2013) showed an IC_{50} value of 390 µg/mL for the *T. molitor* hydrolysates obtained from deffated flour of this insect using Alcalase™ at 1% (E/S) during 4 h of hydrolysis time and Vercruysse et al. (2009) reported an IC_{50} value of 827 µg/mL for *S. littoralis* hydrolysates also obtained by an enzymatic hydrolysis using Alcalase™ at a ratio of 48 U/kg for 3 h. These values are much higher than the results presented in Table 3.2.2, which highlights the high antihypertensive property of those peptides.

Since no studies were published using Corolase PP to perform an enzymatic hydrolysis of insect protein, it was only possible to compare the results obtained by FD-CH with hydrolysates obtained from other food sources. Similar results for FD-CH were reported by Silvestre et al. (2012), after an enzymatic hydrolysis of whey protein using Corolase PP at 3.0% (E/S) during 5 h, they observed an IC_{50} value of 153 µg/mL. Coscueta et al. (2016) had also performed an enzymatic hydrolysis using this enzyme at 1% (E/S) in defatted soy protein isolates and the ACE inhibitory activity revealed an IC_{50} value of 177.7 µg/mL for hydrolysates after 10 h of hydrolysis time.

These results confirm the important potential antihypertensive activity of the hydrolysates of Buffalo's insect powder, being much better than other food hydrolysates.

3.2.3 Antimicrobial activity and α-Glucosidase inhibitory activity

The antimicrobial activity assay against the 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 with the literature, only one study reported the potential capacity of *B. mori* pupae hydrolysates, using the QSAR method, to inhibit the α-Glucosidase enzyme (Zhang et al., 2016). Hydrolysates obtained by an enzymatic hydrolysis of silk cocoons and silk fibroin also reported to have this property (Hu et al., 2008; Lee et al., 2011). Despite the fact that are known more than 150 AMPs derived from insects (Y. Yi et al., 2014), no studies were found with antimicrobial capacity for hydrolysates obtained by an enzymatic hydrolysis of insect proteins.

3.3 Characterization of the hydrolysates obtained under the best conditions

The composition of the Buffalo's insect powder hydrolysates was performed and the following parameters were quantified: total protein, carbohydrates, moisture and ash content of the freeze-dried samples (Table 3.3.1). The total free amino acid composition of the free-dried hydrolysates and insect powder were also analysed (Table 3.3.2). The peptide profile was analysed for the hydrolysates obtained under the best conditions and control, in order to perceive how the enzymatic hydrolysis is affecting the peptide profile (Figure 3.3.3).

Table 3.3.1 - Characterization of FD-AH, FD-CH and control.

Condition	**Alcalase™ 2.5L 1.5% (4 h)**	**Corolase PP 3.0% (6 h)**	**Control**
Total protein (%)	58.9 ± 7.9	66.7 ± 3.8	53.5 ± 3.6
Total Carbohydrates (%)	0.4 ± 0.1	0.3 ± 0.1	1.2 ± 0.2
Moisture (%)	5.6 ± 2.5	7.3 ± 3.3	11.1 ± 2.5
Ash (%)	6.3 ± 0.2	7.3 ± 0.3	12.6 ± 0.4
Others (%)	28.8	18.4	21.6

According with the results showed in the Table 3.3.1, it was verified that the final hydrolysates had at least half of their composition in protein, constituting an important source of protein. It's also possible to observe that at least 28.8%, 18.4% and 21.6% of the content is not described for the FD-AH, FD-CH and control, respectively, but is expected to be mostly fat, since insects have a high quantity of protein followed of a reasonable amount of fat in their composition, fibres and minerals. Also, the initial Buffalo's insect powder is composed by around 60% protein content, around 25% of fat and around 5% of fibres, according with the nutritional values provided by the company in their label.

Table 3.3.2 - Free amino acid composition of the FD-AH, FD-CH, control and insect powder.

Amino acid	**Alcalase™ 2.5L 1.5% (4 h) (mg/g of Insect powder)**	**Corolase PP 3.0% (6 h) (mg/g of Insect powder)**	**Control (mg/g of Insect powder)**	**Insect Powder (mg/g 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

Table 3.3.2 - Free amino acid composition of the FD-AH, FD-CH, control and insect powder (Continued).

Amino acid	**Alcalase™ 2.5L 1.5% (4 h) (mg/g of Insect powder)**	**Corolase PP 3.0% (6 h) (mg/g of Insect powder)**	**Control (mg/g of Insect powder)**	**Insect Powder (mg/g of Insect powder)**
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

In Table 3.3.2 is shown the free amino acid composition of the samples (FD-AH, FD-CH and control) and the original matrix (Buffalo's insect powder). It was expected that the hydrolysates would obtain a higher amount of free amino acids when compared to the original Buffalo's insect powder, because they were subjected to an enzymatic hydrolysis and the breakdown of native proteins into amino acids and small peptides should be potentiated (Sbroggio et al., 2016) and also because the DH value affects the amount of free amino acids released in hydrolysis (Ge et al., 1996).

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. The FD-AH (DH of 19.5%) had a total amount of 28.8 mg of amino acids.

As observed in the Table 3.3.2, tyrosine, included in a group of amino acids (methionine, histidine, lysine and tryptophan) known for antioxidant properties (Wenyi Wang & Gonzalez De Mejia, 2005) and one the most active against the ABTS radical cation as reported by Coscueta and others (2016), was the one found in bigger 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 (Vasdev & Gill, 2008) and a supplementation with this amino acid can potentially be used to prevent endothelial dysfunction in patients with diabetes (Kohli et al., 2004). Leucine is also an amino acid detected in remarkable amounts in FD-CH, which can be used as a potential obesity and metabolic syndrome treatment (Layman & Walker, 2006), and Phenylalanine, when metabolized into Phenylethylamine is presumed to have antidepressant properties (Kapalka, 2010). Other amino acids like cysteine and methionine were found in very low quantities, being that methionine was not detected in Buffalo's insect powder. Taking in consideration the amount of amino acids found in each sample, the best condition to obtain free amino acids is indubitably the enzymatic hydrolysis using Corolase PP.

In Figure 3.3.3 is presented the peptide profile of the hydrolysates obtained under the best chosen conditions. Fours peaks represented by the letter 1-4 are shown, corresponding to small peptides, smaller than the whey peptide used as standard, with less than 1 kDa and amino acids In general, the Corolase PP hydrolysate obtained the highest peaks among the other samples, followed by the peaks obtained by Alcalase™ 2.5L hydrolysate and then the control. This result can be explained by the DH value obtained by both enzymes, since the Corolase PP hydrolysate obtained a DH of 36.0% and the Alcalase™ 2.5L hydrolysate obtained a DH of 19.5%, and since it's expected to observe more peptides and amino acid generation at a higher DH value (Ge et al., 1996). So, the peptide profile demonstrated a clear hydrolysis in the both enzymatic conditions. The FPLC result also corroborates the results obtained by the free amino acids analysis.

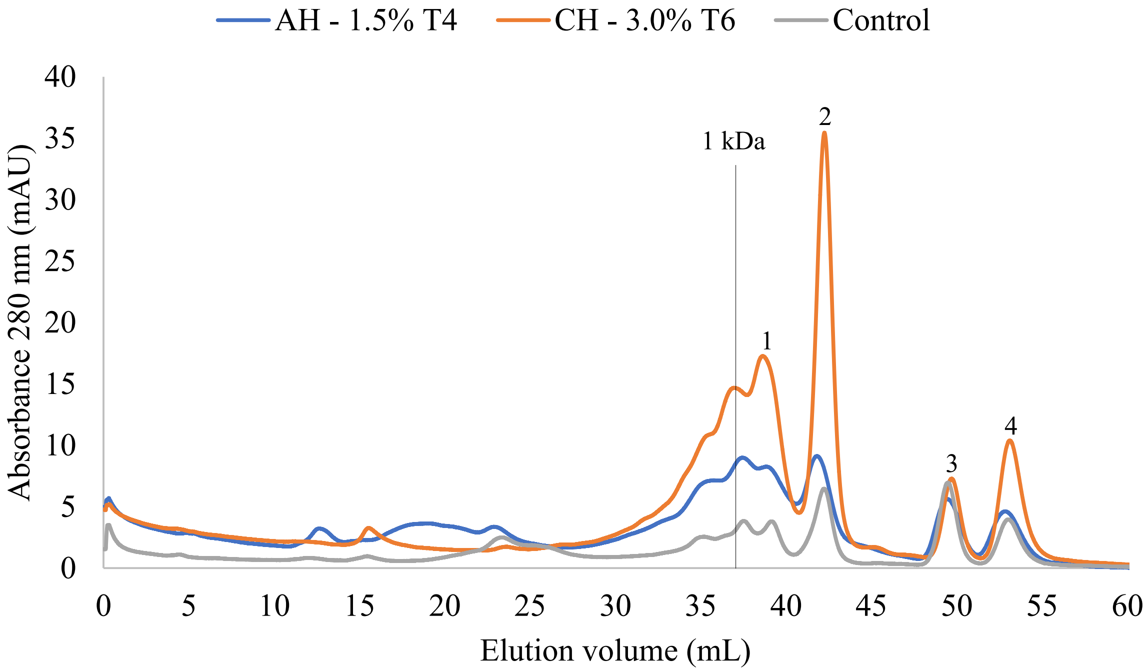

Figure 3.3.3 - Peptide profile analysis of the Alcalase™ 2.5L hydrolysate, at 1.5% (E/S) during 4 hours of hydrolysis (AH - 1.5% T4), Corolase PP hydrolysates, at 3.0% (E/S) during 6 hours of hydrolysis (CH - 3.0% T6) and Control.

The Corolase PP hydrolysate showed a big intensity in the peaks 1, 2 and which are related to small peptides and amino acids originated during the enzymatic hydrolysis. The Alcalase™ 2.5L hydrolysate presents a higher intensity of peaks between 35 and 40 mL of elution volume comparatively to control, which confirms the generation of peptides with low molecular weight, caused by enzymatic hydrolysis.

CONCLUSION

In conclusion, this work demonstrated that is possible to improve the bioactive properties and nutritional value (increase of free amino acids and small peptides) of the insect powder through an enzymatic hydrolysis process. This type of process, usually using commercial enzymes to hydrolyse proteins, is widely used as a controlled way to obtain bioactive peptides. In this study, an analysis of the enzymatic hydrolysis was done in order to find the best relation between enzyme:substrate ratio and hydrolysis time. Various parameters were used to guarantee the better conditions to obtain insect protein hydrolysates with the enzymes Alcalase™ 2.5L and Corolase PP. After defined the best conditions, based on the use of the least amount of enzyme and the shortest hydrolysis time without injury the antioxidant activity, a new batch was made to produce insect hydrolysates and it was performed their characterization and more bioactive properties were analysed. Considering that consumers are looking for foods with benefits to health, various properties were studied such as antihypertensive (ACE inhibitory activity), important to control blood pressure in antihypertensive patients, antioxidant activity, essential to inactivate free radicals in the human body, antidiabetic (α-Glucosidase inhibitory activity), reducing the sugar released by the carbohydrates degradation during the digestion process and, antimicrobial property that allow the inhibition of pathogenic microorganisms. The insect protein hydrolysates had promising results for the antioxidant and antihypertensive activities. The enzymatic process also reinforced the amount of small peptides and free amino acids, being that the FD-CH demonstrated a considerable increase.

Therefore, the Buffalo's insect powder demonstrated to have potential to be used as a source of protein to of generating bioactive peptides through enzymatic hydrolysis with commercial enzymes. The insect hydrolysates had proven *in vitro* bioactive properties, such as antioxidant and antihypertensive, which are validated properties with impact on chronic diseases, namely cardiovascular disorders. The potential of these modified insect powder's 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.

FUTURE WORK

The present study demonstrated the nutritional and biological relevance of protein hydrolysates obtained from the edible insect *A. diaperinus*.

For the future work, it's important to evaluate the antioxidant and antihypertensive activities of the hydrolysates after simulation of gastrointestinal digestion. Other bioactive properties such as anti-inflammatory and Dipeptidyl peptidase-IV inhibitory capacity, could also be analysed.

A further characterization of the hydrolysates such fat content, fibres, mineral content and fatty acids profile is also necessary. The microbiological profile, presence of chemical contaminants and allergenicity must also be analysed to ensure the safety of consumption of these insect protein hydrolysates.

The functional properties such as emulsion activity, foamability, gel formation and solubility at various pH values should also be studied in the future, because the enzymatic hydrolysis could improve some functional properties of proteins.

Finally, it is also relevant to develop a food product (i.e cereal bar) using these hydrolysates as an ingredient. Firstly, it will be important to understand if the bioactive properties are affected (potentiated/reduced) by a food matrix, and secondly it should be carried out a proof of concept through a sensorial analysis of the food product, evaluating the acceptability of the consumer for this type of insect-based products.

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