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Protein hydrolysates from *Alphitobius diaperinus* and *Hermetia illucens* larvae treated with commercial proteases

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RESEARCH ARTICLE

Abstract

Insect proteins have been proposed as a promising alternative for feed and food formulations. In the present work protease-assisted extraction was studied as a way to separate and extract proteins from two different insect species: *Alphitobius diaperinus* (AD) and *Hermetia illucens* (HI). The proteolytic activity of seven enzymes (papain, pancreatin, dispase I, pepsin, protease from *Bacillus licheniformis*, bromelain and trypsin) was evaluated determining the protein extraction yield, the degree of hydrolysis (DH) and the released free amino acids (FAA). Both insects represent an interesting source of proteins, not only for their amount (more than 40% on dry matter) but also for the nutritional value, with essential amino acid profile exceeding the requirements proposed for human nutrition. Enzyme-assisted protein extraction, performed at laboratory scale, gave for HI an average yield of extraction of 71±8% and for AD 67±6%. Hydrolysates produced from HI gave a DH% ranging between 3 to 18%, whereas hydrolysates produced from AD yielded a DH% between 7 to 23%. The protein hydrolysates were composed by peptides and FAA (which accounted for more than 30% of the extracted protein fraction), which were released according to their abundance in initial protein. A moderate correlation between the DH% and the total amount of FAA was found, except for AD hydrolysed with trypsin and HI with papain. Based on these results, the production of hydrolysates was preliminary scaled up in a proof-of-concept experiment, focusing on the most promising insect-enzyme combination. The final product resulted to be rich in protein (60% on dry matter). This work support enzymatic hydrolysis as an effective method to extract and isolate proteins from insects, with minimal sample preparation, tailoring their composition, preserving the nutritional quality, decreasing the risk of allergic reactions and making them more accessible for their future use as feed/food supplements.

Keywords: enzymatic assisted extraction, insect, novel protein source, degree of hydrolysis, free amino acids

1. Introduction

A generalised consensus on the need to find alternative protein sources for feed and food applications is currently shared by feed and food producers, as well as researchers in the field. Increasing world population, as well as rising meat consumption per capita in developing nations, boost the necessity to find new protein sources. Novel protein sources, derived from diverse food by-products and other residual biomasses (e.g. seeds, bran, peels from plant origin) or from newly used biomasses (algae) are being recently proposed in European Union (EU) as an alternative to more commonly used proteins (FAO, 2009). Unfortunately, the diversity in protein composition and matrices represents a technological problem for a cost-competitive and a low impact processing (Russ and Meyer-Pittroff, 2004; Tuck *et al.*, 2012). Insects may constitute a possible biotechnological solution to the above problems, since some of them naturally develop on organic wastes and various biomass types. Insects incorporate the nutrients into their bodies, both reducing the amount of waste material and generating a more homogeneous and valuable biomass (Li *et al.*, 2011; Rumpold and Schlüter, 2013; Smetana *et al.*, 2016). This 'indirect' biorefinery through insects represents a way to

convert a variety of feedstocks into marketable protein end-product (Van Huis, 2013).

Insects indeed represent an optimal source of proteins: protein amount is from 39 to 64%, on dry matter basis, with a high content of essential amino acids, making them nutritionally relevant for human and animal consumption (Chen *et al.,* 2010; Sánchez-Muros *et al.,* 2016). From the legal point of view, Regulation (EC) No 999/2001 (EC, 2001) authorises the use of insect protein meals for feeding pet and fur animals, whereas forbids them for ruminants and monogastric animals. The Regulation (EC) No 2017/893 (EC, 2017) authorised the use of insect protein meals originating from seven species (including black soldier fly, *Hermetia illucens*, and lesser mealworm, *Alphitobius diaperinus*) as feed for aquaculture animals. The European Commission is currently exploring the possibility to extend their use also for feeding poultry. Regarding the food sector, from the 1st January of 2018 insects are considered novel foods, and as such, need to have EFSA safety evaluation and EU Commission approval before being placed on the market (EC, 2015).

Insect proteins meals can be obtained by different processes (e.g. chemicals, mechanical, enzymatic). Often, the protein fractionation process results in a trade-off between yield and purity (Sosa and Fogliano, 2017), thus the preparation of protein meals of acceptable purity in a cost competitive way is a challenging task. In literature, many protocols of protein extractions from insects have been proposed, often combined with a delipidating pre-step. In a previous work from our group, more than 90% of the total proteins in black soldier fly was extracted from delipidated prepupae by applying the Osborne fractionation, normally used for cereals (Caligiani *et al.*, 2018). Zhao *et al.* (2016) obtained 75% protein yield with an alkali extraction on delipidated yellow mealworm larvae. In other works, the protein extraction process was applied directly on insect flour, without any step of defatting. Purschke *et al.* (2018a) combined the action of pH with a centrifugal fractionation, recovering 58% proteins from *Tenebrio molitor*. A complete protein recovery was obtained by Yi *et al.* (2017) on *T. molitor* using high extraction pH in combination with NaCl. Soetemans *et al.* (2019) used different organic acids as adjuvants to improve lipid and protein extraction from black soldier fly.

Enzymatic hydrolysis is widely used in the food/feed sector in order to extract proteins from vegetables and meat by-products and is also exploited in order to obtain ingredients with bio- and techno-functional properties (Del Mar Contreras *et al.*, 2019; Lynch *et al.,* 2018). Enzymes are able to increase the amount of proteins extracted in forms of peptides by enhancing solubility, through a decrease in the molecular mass, and an increase in both repulsive interactions between peptides and hydrogen bond interactions with water molecules (Zhao *et al.,* 2012). The use of exogenous enzymes for a food/feed industrial process presents many advantages. First, the use of proteases could make the protein extraction process more controllable and reproducible, cheap and environmentally friendly (Ahmadifard *et al.,* 2016). The obtained hydrolysates have a high nutritional value, since hydrolysis makes the protein fraction more digestible, and essential amino acids are preserved by the mild conditions used during enzymatic hydrolysis. Moreover, the protein fractions obtained might be hypoallergenic, since allergenic proteins are also degraded to peptides. Finally, the peptides released during the hydrolysis might present bio-functional activities (Meinlschmidt *et al.*, 2015).

Among other applications, enzymatic extraction is nowadays employed in the shrimp processing industry for separating proteins from chitin wastes (Mizani *et al.*, 2005). Given the close genetic relation between crustaceans and insects, a similar process could probably be easily expanded also in the insect biorefinery. The use of proteolytic enzymes on insect biomass has been previously studied mostly in the perspective to obtain a techno functional and/or a biofunctional final protein product (Nongonierma and FitzGerald, 2017). Purschke *et al.* (2018b) compared the ability of different enzymes, used at a different concentration and for a different hydrolysis time, to solubilise proteins from a commercial locust protein flour. With the addition of enzyme, they were able to significantly increase the amount of solubilised protein from about 5 to 30%. Hall *et al.* (2017) evaluated the increasing of protein extraction during the enzymatic hydrolysis of crickets. They demonstrated that with the use of alcalase the percentage of extractable proteins increased from 3% up to about 15%, depending on the enzyme concentration and hydrolysis time. In a recent paper from our group, a thermal treatment as killing method was combined with an enzymatic hydrolysis in order to extract 97% of total proteins from *H. illucens* in form of peptides (Leni *et al.*, 2019). Despite the previous examples, the ability of different enzymes acting on the same substrate to enhance protein extraction has never been studied and compared in detail, and no data are reported on the detailed molecular composition of the obtained hydrolysates.

In the present work, the use of proteases as biotechnological adjuvant for protein extraction from raw untreated ground insects was systematically explored, as a way to boost the efficiency of the process and to obtain protein hydrolysates in an easy and efficient way. The ability of seven different commercial proteases to produce protein hydrolysates at a laboratory scale from larvae of *A. diaperinus* and *H. illucens* was deeply studied and compared, also focusing on the fine molecular composition and nutritional value of the obtained products. Furthermore, as a proof of concept,

the process was scaled up, focusing on the most promising insect-enzyme combination.

2. Material and methods

Material

Kjeldahl defoamers and catalyst were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit and AccQ-TagTM were obtained from Waters (Milford, MA, USA). DL-norleucine, amino acid standard mixture, glutamine, asparagine, tryptophan, o-phthaldialdehyde, N-acetyl-l-cysteine, DL-isoleucine and all of the enzymes tested were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other solvents, salts, acids and bases (analytical grade) were purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

Insect samples

Black soldier fly *(H. illucens*, HI) larvae were provided by Circular Organics (Turnhout, Belgium), whereas lesser mealworm (*A. diaperinus*, AD) larvae by Protifarm (Ermelo, the Netherlands). HI and AD larvae were reared in a temperature and humidity-controlled room, with standard temperature ranging between 28 and 32 °C and humidity above 60%. Larvae were fed daily *ad libitum* with the standard rearing feed. At the end of the rearing cycle (15 days for HI and 28 days for AD) the larvae were separated from the frass and transported alive in trays. Larvae were killed by packing them in vacuum sealed containers and freezing at -30 °C. After one week, dead larvae were freeze-dried and stored at -20 °C until the analysis. Before each analysis, whole larvae were ground for 2 min with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Staufen, Germany). Three different trials were performed for each insect species.

Proximate composition

Moisture, nitrogen, lipid and ash were determined using standard procedures (AOAC, 2002). Crude lipid content was determined by using an automatized Soxhlet extractor (VELP SCIENTIFICA, Usmate, Italy) with diethyl ether as solvent. Total nitrogen was determined by a Kjeldahl system (VELP SCIENTIFICA, Usmate, Italy). The nitrogen coefficient conversion for HI and AD proteins was obtained by total amino acid composition, assuming an equimolar amount of Asn/Asp and Gln/Glu (respectively, 5.50 for HI, and 5.67 for AD). The determination of chitin is described below.

Total amino acids analysis of insects

25 mg of dilapidated ground insects and 3 ml hydrolysis reagent (phenol (1 g/l)-HCl (6 M)) were mixed and heated at 110 °C for 23 h. For cysteine measurements, an additional sample was oxidised prior to hydrolysis with performic acid/phenol. After heat treatment, the samples were centrifuged for 5 min at 3,000×*g* to remove solid particles. Amino acids were analysed by high-performance anion exchange chromatography with pulsed amperometric detection in Chromeleon software. The conditions of analysis were the following: column Dionex AminoPac PA-10 (2×250 nm) and Dionex AminoPac PA-10 Guard (2×50 mm) at 30 °C; mobile phases (A) milliQ water, (B) 250 mM NaOH, (C) 1 M NaOAc, (D) 0.1 M Acetic acid; gradient 0-2 min: eluent A (76%) and B (24%), 2-11 min: eluent A (64%) and B (36%), 11-47 min eluent A (40%), B (20%), C (40%), 47.1-49.1 min: eluent D (100%), 49.2-51.2 min: eluent A (20%) and B (80%), 51.3-76 min: eluent A (76%) and B (24%) at 0.250 ml/min. Volume of injection was 10 µl.

Tryptophan (Trp) was determined following the method proposed by Delgado-Andrade *et al.* (2006) with some modifications. 0.2 g of dried insect sample were weighed into a 7 ml Pyrex glass tube and dissolved in 3 ml of 4 M NaOH. 150 µl of 5-methyl-tryptophan (0.16 mg/ml), used as internal standard, were added and mixed. Hydrolysis was then carried out at 110 °C for 18 h. After letting the tubes to cool at room temperature, the solution was carefully acidified to pH=6.5 with HCl, then diluted to 25 ml with sodium borate buffer (0.1 M, pH=9.0). Sample was centrifuged at 4,000 rpm for 5 min and supernatant filtered through 0.45 µm nylon filter membrane into UPLC vials. Trp content was calculated by dividing the area of the peak by the area of the internal standard and multiplying this value by the weight of the internal standard and the response factor of tryptophan. UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C18 column (Waters Corporation, Milford, MA, USA). The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 1.8 mins, from 100% A to 50% A by linear gradient in 11.4 mins and 0.8 mins at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.25 ml/min, injection volume 4 µl, column temperature 35 °C and sample temperature 23 °C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionisation mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, SIR acquisition mode at 188,0 and 205,0 for Trp; 202,1 and 219,1 for 5-methyl-tryptophan m/z, scan duration 1 s.

Determination of chitin

Quantification of the chitin content was performed as described by D'Hondt *et al.* (2020). Briefly, chitin was hydrolysed to glucosamine and acetate that were subsequently quantified by LC-MS and HPLC-RID, respectively. The sample was hydrolysed with 6 N HCl for 6 h at 110 °C. Glucosamine was determined by UPLC-MS/MS analysis (Waters UPLC BEH HILIC 2.1×100 mm, 1.7 µm column at 40 °C, isothermal gradient elution using water with (A) 20 mM ammonium formiate and 0.1% formic acid (pH=3) and acetonitrile (B) with 0.1% formic acid with gradient settings: 5-25% A (0-3 min), 25% A (3-4 min), 25-5% A (4-4.1 min), 5% A (4.1-7 min) at 0.4 ml/min at 40 °C). Peak detection and quantification were done using a triple quadrupole MS operated in multiple reaction monitoring mode.

Enzymatic assisted extraction

The enzymes employed were: protease from *Bacillus licheniformis* (PBL) (≥2.4 U/g; EC number 3.4.21.62), dispase I (≥10 unit/mg; EC number 255-914-4), pepsin from porcine gastric mucosa (≥250 units/mg; EC number 3.4.23.1), pancreatin from porcine pancreas (8xUSP; EC number 232-468-9), trypsin from porcine pancreas (1.000-2.000 BAEE units/mg; EC number 3.4.21.4), bromelain from pineapples (2 mAnson U/mg; EC number 232-572-4) and papain from papaya latex (1.5-10 units/mg; EC number 3.4.22.2). These enzymes were chosen among common commercial enzymes for being representative of diverse sources, being of vegetal (papain), bacterial (PBL, dispase) or animal (pepsin, trypsin, pancreatin) origin. The hydrolysis reactions were carried out on ground insects at a laboratory scale, in triplicate, in the optimal condition for each enzyme, following the indications provided by the manufacturer, which are reported in Table 1.

As general procedure for the hydrolysis, 5 g of ground insects were mixed, in a 50 ml falcon tube, with 45 ml of the specific solution above reported, and 0.05 g of enzymes were added at room temperature. The falcon tube was then placed in a heating bath at the correct temperature (Table 1) and mixed with a magnetic stirrer. The hydrolysis reaction was carried out for 18 h in order to reach the plateau phase. The enzymes were inactivated by heating at 90 °C for 5 min. The hydrolysates were then centrifuged (5810/ 5810 R; Eppendorf, Milano, Italy) at 2,683×*g* at 4 °C for 30 min. The supernatants and the pellets were separated and stored at -20 °C for subsequent analysis. As blank experiments, in order to verify protein extractions for every solution in absence of enzymes, all the above experiments were also carried out in the same conditions, but without the addition of enzymes.

Protein extraction yield

The supernatants and pellets collected after the enzymatic extraction were subjected to a Kjeldahl analysis in order to determine the nitrogen content and calculate the nitrogen mass balance. The yield of protein extraction was calculated by comparing the amount of nitrogen in the supernatant (assumed to be completely due to protein nitrogen) to the total protein nitrogen in the insects (data provided from amino acid analysis). The amount of nitrogen due to the added enzymes, being negligible (<1%), was not considered. On the other side, the contribute of salts containing nitrogen, whenever present in the extraction systems, was considered and subtracted when necessary.

Degree of hydrolysis

The degree of hydrolysis (DH), which is defined as the percentage of cleaved peptide bonds on total peptide bonds, was calculated using o-phtaldialdehyde (OPA) method described by Spellman *et al.* (2003) with some modifications (Butrè *et al.,* 2012). The hydrolysed samples were diluted in a 2% (w/v) sodium dodecyl sulfate, stirred for 20 min and stored at 4 °C overnight before the assay. The OPA/NAC (N-acetyl-cysteine) reagent (100 ml) was prepared by combining 10 ml of 50 mM OPA (in methanol) and 10 ml of NAC 50 mM, 5 ml of 20% (w/v) SDS, and 75 ml of

Table 1. Optimum condition of pH and temperature for the enzymes tested.

Enzyme	pH	Temperature	Solution buffer
PBL[1]	7.5	60 °C	Na_2HPO_4 10 mM
Dispase I	7.3	37 °C	CH_3COONa 10mM, $(CH_3COO)_2Ca$ 5 mM
Pepsin	3.0	37 °C	HCl 10 mM
Pancreatin	7.8	37 °C	NH_4HCO_3 25 mM, $CaCl_2$ 2.5 mM
Trypsin	7.8	37 °C	NH_4HCO_3 25 mM, $CaCl_2$ 2.5 mM
Bromelain	7.0	50 °C	Na_2HPO_4 10 mM
Papain	6.5	60 °C	Na_2HPO_4 10 mM, EDTA 2 mM, DL-cystin 4 mM

[1] Protease from *Bacillus licheniformis*.

borate buffer (0.1 M, pH=9.5). The reagent was covered with aluminium foil, to protect from light, and stirred for at least 1 h before use. The OPA assay was carried out by the addition of 20 µl of sample (or standard) to 2.4 ml of OPA/NAC reagent in a 5 ml of a plastic Eppendorf. Before the analysis, the samples were centrifuged at room temperature for 10 min at 280×*g*. The absorbance of the obtained solution was measured at 340 nm with Jasco B-530 UV-Vis-spectrophotometer (Jasco, Oklaoma City, OK, USA) against a control cell containing the reagent and 20 µl of the buffer used for the sample. The intrinsic absorbance of the samples was measured before OPA addition and subtracted. The standard curve was prepared using L-isoleucine (0-2 mg/ml). The DH was calculated as the ratio between the free nitrogen groups after hydrolysis and the total nitrogen groups: DH% = (Nfree / Ntotal) ×100. The former value was calculated by the OPA reactivity, whereas the total moles of nitrogen atoms involved in peptide bonds before hydrolysis were calculated by the total grams of proteins, obtained from total amino acids analysis, divided by the average of residual amino acids molecular mass (Mw 110). The average peptide chain length was obtained by the following equation: 100 / DH%, according to Adler-Nissen (1986)

Free amino acid analysis

The free amino acids (FAA) analysis was carried out on insect samples, and on the supernatants obtained after the enzymatic assisted extraction. 0.5 g of ground HI and AD were suspended in 5 ml of water and mixed with 340 µl of 5 mM norleucine (in HCl 0.1 M) for 2 h. The volume was then brought to 10 ml with the addition of deionised water and then centrifuged for 30 min at 4 °C at 2,683×*g*. As far as the analysis of FAA in supernatants, they were filtered on a 0.45 µm nylon filter membrane and collected. 100 µl of supernatants were mixed with 34 µl of 5 mM Norleucine (in HCl 0.1 M) and the volume brought to 1 ml with deionised water. Quantification was performed against a set of standard solutions. 10 µl of samples, 70 µl of borate buffer and 20 µl of reconstituted AccQ Tag reagent (Waters) were mixed and then heated at 55 °C for 10 min. The derivatised samples were diluted with 100 µl of deionised water before injecting in the UPLC/ESI-MS system. The conditions of analysis were the same described for Trp analysis.

Process scale up

Process scale up was performed on AD larvae by using protease from *B. licheniformis*, with slight modification of the protocol previously described in order to adjust for the larger scale. Specifically, 1.5 kg of freeze-dried (Christ, gamma 1-16 LSC, 36 h) ground larvae were mixed with 7.5 l of the specific buffer solution (Table 1) in a 10 l flask, at pH=7.5 and pre-heated at 60 °C. Before the addition of the enzyme, the mixture was homogenised in an incubator at 130 rpm for 30 min, while checking the pH (ProfiLine pH 3310, WTW, Xylem Analytics LLC, Weilheim in Oberbayern, Germany). The pH fluctuations were adjusted adding concentrated NaOH. Next, 0.25% (w/w) of enzyme was added and the hydrolysis was performed for 180 min. After this time, the mixture was heated at 90 °C for 5 min for the endogenous and exogenous enzymes inactivation and subsequently centrifuged at 4 °C for 30 min at 3,220×*g*. The supernatant was separated from the pellet and the lipid upper layer using a 500 µm sieve and freeze-dried for the further analysis. The hydrolysis reaction was performed in duplicate and the hydrolysates characterised in terms of proximate composition and DH% as previously described.

Statistical analysis

All experiments were carried out in triplicate. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, U.S.A.) and STATISTICA 12 of StatSoft. Significant differences were compared at a level of $P<0.05$.

3. Results and discussion

The goal of this study was to produce protein hydrolysates starting from AD and HI, by using proteolytic enzymes in order to increase the efficiency of extraction, with the final aim to obtain protein hydrolysates with potentiality for feed or food applications. Different enzymes, of microbial, vegetable ad animal origin, were employed, in order to explore the different extractant abilities, and the characteristics of the final hydrolysed mixtures obtained. Finally, the most promising insect-enzyme combination was chosen for a proof-of-concept scale up.

Insects composition

The proximate composition of HI and AD larvae, in terms of dry matter, ash, lipid, protein and chitin was first determined (Table 2).

Table 2. Proximate composition of *Hermetia illucens* and *Alphitobius diaperinus*.[1]

Composition (%)	*H. illucens*	*A. diaperinus*
Dry matter (DM)	29.5±0.3	33.6±0.3
Total nitrogen (on DM)	9.1±0.5	10.3±0.4
Proteins, from total amino acids (on DM)	41.8±2.8	53.9±5.4
Lipid (on DM)	20.7±0.2	29.6±0.4
Chitin (on DM)	7.2±0.3	4.6±0.1
Ash (on DM)	11.95±0.01	4.24±0.03

[1] Value are expressed on dry matter basis and are the results of three replicate analysis.

From the chemical composition analysis, AD resulted to have a higher amount of lipid in comparison to HI, whereas HI was characterised by a higher level of chitin and ash. In case of insect biomasses, total nitrogen is not a useful information for the calculation of protein content: indeed nitrogen originates not only from proteins and other minor sources (e.g. nucleic acids, phospholipids, and ammonia), but also (in a non-negligible way) from chitin, which is the main component of the exoskeleton. In order to precisely calculate the specific amount of protein in both insects, total amino acids analysis was then performed (Table 3). HI and AD had both a high protein content, with AD containing more proteins than HI. The protein content resulted to be higher for both insect than what reported by Caligiani *et al.* (2018) and Janssen *et al.* (2017), whereas lipid and ash were present in a comparable amount. The missing dry matter is likely represented by carbohydrates (chitin excluded), which in these insect species are between 15 and 21% on dry matter (Janssen *et al.*, 2017).

Both insects contained good quality proteins: the essential amino acid profile for both usually largely exceeded the requirement proposed for human nutrition, except in the case of sulphur AAs and tryptophan, resulting to be the limiting AAs. The samples here analysed had, to equal protein content, a slightly lower amount of essential amino acids than what reported by Caligiani *et al.* (2018), Janssen *et al.* (2017) and Leni *et al.* (2019) (about 10% less for both species). The differences in the AA profile could be related to the different substrates used to feed insects (Gligorescu *et al.*, 2018; Meneguz *et al.*, 2018; Ramos-Elorduy *et al.*, 2002).

From this AA composition it was possible to determine the conversion factor from nitrogen to protein, to be used for Kjeldahl analysis, which resulted in 5.5±0.1 for HI and 5.67±0.03 for AD. The % of proteinaceous nitrogen on total dry matter was then recalculated as 7.6±0.4% for HI (84% of the total nitrogen) and 9.5±0.9% for AD (87% of the total nitrogen). These values were used in the next experiments to compare the protein extractability with the different enzymes tested.

The % of nitrogen derived from chitin could also be back-calculated by considering the specific conversion factor, which vary from 14.5 to 11.5, assuming a fully acetylated or deacetylated glucosamine (Caligiani *et al.*, 2018). Chitin-derived nitrogen was then calculated to be between 0.5 and 0.7% in HI, and between 0.3 and 0.4% in AD. The remaining nitrogen (0.9% in HI, 10% of the total, and 0.5% in AD, 5% of the total) is to be ascribed to sources other than proteins and chitin.

Table 3. Total amino acid (AA) content for *Hermetia illucens* and *Alphitobius diaperinus* expressed as g/100 g total protein (calculated from total amino acids) and compared with the FAO/WHO standard protein (2001).[1]

Essential AA (g/100 g protein)	*H. illucens*	*A. diaperinus*	Reference protein (FAO/WHO 2001)
His	3.3±0.2	3.3±0.3	1.5
Thr	3.77±0.06	3.89±0.04	2.3
Val	5.5±0.2	5.5±0.3	3.9
Lys	5.8±0.2	6.3±0.4	4.5
Ile	3.9±0.2	4.1±0.2	3.0
Leu	6.3±0.1	6.3±0.3	5.9
Trp	0.7±0.1	0.45±0.04	0.6
Phe + Tyr	10.1±0.3	11.6±0.5	3.8
Phe	3.7±0.2	4.1±0.2	
Tyr	6.4±0.1	7.5±0.3	
Cys + Met	1.9±1.2	2.1±0.4	2.2
Cys	0.6±0.4	0.96±0.09	
Met	1.3±0.8	1.1±0.3	
Non-essential AA			
Arg	9.8±0.9	7.1±0.8	
Ala	8.12±1.02	9.96±4.07	
Gly	4.7±0.2	4.1±0.3	
Ser	3.7±0.1	3.6±0.2	
Pro	5.2±0.1	6.05±0.37	
Glu/Gln	11.8±1.1	12.4±0.6	
Asp/Asn	8.5±0.4	8.1±0.4	

[1] The results are expressed as means ± standard deviation of three replicate analysis.

Lab scale enzymatic assisted extraction and protein extraction yield

The enzymatic assisted extraction was performed, at lab scale, with seven different proteolytic enzymes: papain and bromelain (vegetal proteases), protease from PBL, dispase (bacterial proteases), pepsin, trypsin and pancreatin (animal proteases). The hydrolysis was performed in all cases overnight, with an enzyme/substrate ratio of 1:100 (w/w), at the optimal pH and temperature for each enzyme (Table 1). The long reaction time was needed in order to make sure to obtain an end point proteolysis reaction. The protein extraction yield was evaluated by Kjeldahl analysis, in order to determine the solubilised nitrogen as compared to the total protein nitrogen (see section above).

The extraction yield is reported in Figure 1, as a measure of the ability of proteolytic enzymes to extract and solubilise proteins. The yields obtained with the same solutions, but without enzymes, are also reported. Even in absence of enzymes, insect proteins were partially solubilised in the conditions applied, but almost never exceeding 50% extraction yield. These figures were higher than what was reported by Hall *et al.* (2017) for cricket (about 20%), likely due to the different insect species and the longer extraction times in our case, and the presence of different buffers at different pH. On the other side, these results are in line with our previous work, where an analogue yield (43%) was reached when extracting albumins and globulins from HI (Caligiani *et al.*, 2018). With the addition of enzymes, the amount of solubilised proteins showed an average 20% increase, supporting the enzymatic hydrolysis as an efficient way to extract and solubilise proteins from AD and HI.

Degree of hydrolysis of protein hydrolysates

The degree of hydrolysis (DH%), defined as the percentage of cleaved peptide bonds in the protein hydrolysates, was determined on the solubilised protein fractions after the enzymatic extractions. The free amino groups, i.e. the cleaved peptide bonds, were determined by the OPA assay. They were then related to the total nitrogen groups involved in peptide bond (before hydrolysis) in order to calculate the DH%. The higher the DH value, the higher the number of peptide bonds cleaved, and the shorter the peptides. The average peptide chain length can be calculated from DH% according to Adler-Niessen (1986). DH% and average peptide chain length in the protein hydrolysates are reported in Table 4. The DH% of the solutions obtained without enzymes were not measured, since in a previous paper they have already been demonstrated to be very low (Caligiani *et al.*, 2018). This also indicates a lack of a consistent endogenous protease activity.

Protein hydrolysates produced from HI had a DH% included between 3 and 18%, whereas for AD this range was between 7 to 23%. The different DH% obtained for the same species confirmed that the proteolytic activity of the different enzymes is different on the same substrates, due to the different enzyme specificities. In the case of HI, the DH% of the hydrolysate with papain presented the lowest value (3.3%), whereas trypsin yielded the hydrolysate with the highest DH (18%). For AD, the highest DH% was obtained performing the hydrolysis with bromelain (23%), whereas the lowest proteolytic activity was observed for papain (7%). In few cases, a pretty low DH% was observed, which is quite surprising, considering the overnight reaction. This might be due to enzyme specificity (which limits the maximum

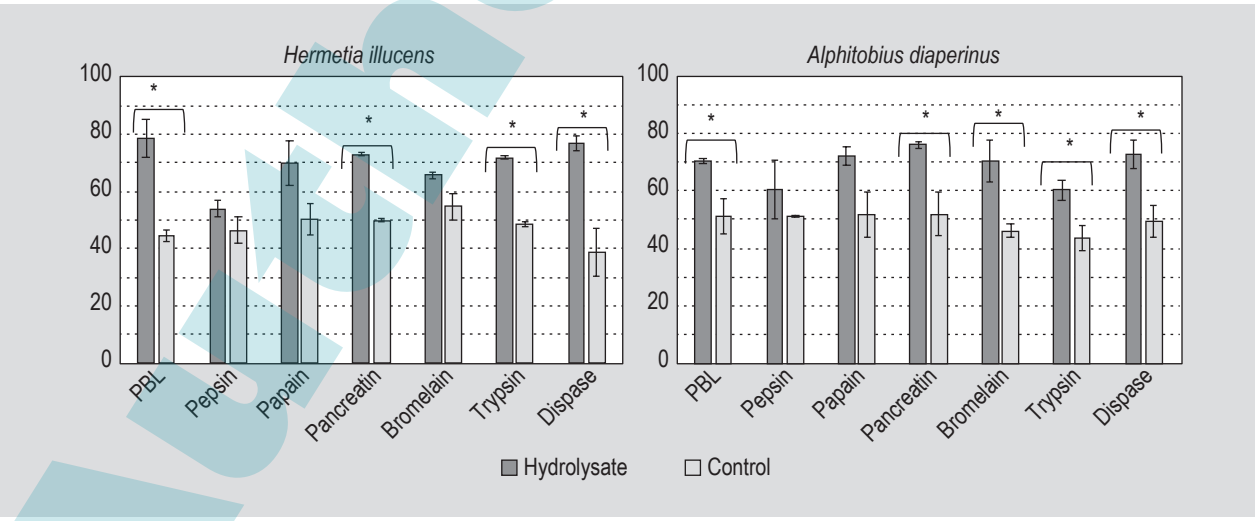

Figure 1. Protein extraction yields obtained by performing enzymatic hydrolysis with seven different enzymes to ground *Hermetia illucens* (on left) and *Alphitobius diaperinus* (on right). The protein extraction yields for the blanks (buffers with no enzymes) are also reported. The results are the mean of three separate hydrolysis experiments. Asterics indicate significant differences (P<0.05) in protein extraction yields between the enzymatic hydrolysates and the corresponding blanks.

Table 4. Degree of hydrolysis (DH, %) and average peptide chain length of protein hydrolysates obtained from *Hermetia illucens* and *Alphitobius diaperinus* larvae subjected to different enzymatic extraction.

Enzyme	*H. illucens*		*A. diaperinus*	
	DH%	Average peptide chain length (100/DH%, uncertainty included in the range)	DH%	Average peptide chain length (100/DH%, uncertainty included in the range)
PBL[1]	10.4±2.3	8-12	21.8±0.5	4-5
Pepsin	10.2±1.1	9-11	15.8±3.4	5-8
Papain	3.3±2.1	19-83	7.0±0.9	6-13
Pancreatin	8.8±4.6	7-24	17.6±3.1	5-7
Bromelain	13.3±1.5	7-8	23.1±5.8	3-6
Trypsin	18.2±0.5	5-6	21.9±5.5	4-6
Dispase I	17.3±0.2	6	15.9±1.2	6-7

[1] Protease from *Bacillus licheniformis*.

amount reachable), the cross inhibition exerted by the generated peptides, the presence of specific inhibitors. Purschke *et al.* (2018b) reported, with the same enzyme/substrate ration, a comparable DH% for the hydrolysis of migratory locust with PBL (19 to 31% from 8 to 24 h of hydrolysis), but a higher DH% for the one with papain (13% for only 8 h of hydrolysis). Hall *et al.* (2017) performed an enzymatic extraction on tropical banded crickets with PBL, reporting a higher DH (36% for 90 min of hydrolysis with 0.5% of enzyme). Thus, ours and literature data also suggests that the same enzyme can yield different degrees of hydrolysis, when applied to different insect species, even if the different ways for calculating the DH% found in literature hamper a direct comparison between experiments.

The average peptide chain length in the hydrolysate, obtained from the DH% (Adler-Nissen, 1986), can give information not only for the physicochemical and functional properties of the protein hydrolysates, but also for their potential hypo allergenicity (Adler-Nissen and Sejr Olsen, 1979). The hydrolysates from HI had an average peptide length ranging from 5-8 residues (with trypsin, dispase I and bromelain) to much larger peptides (with papain and pancreatin), whereas the same figures for AD where mostly in the range of 3-8 residues (all enzymes but papain). This is not of a secondary importance, since as reported by Nagodawithana *et al.* (2008), an average molecular weight lower than 1,500 Da (about 14 AA residues) can reduce the allergenicity property of a food product. However, these results are only to be considered as suggesting a potential hypoallergenicity, and true hypoallergenicity will have to be assessed by showing the absence of orally sensitisation after animal administration (EC, 2006).

Free amino acids profile of protein hydrolysates

The hydrolysates and the untreated larvae were also analysed in order to evaluate the FAA profile: the results are shown in Supplementary Material (Table S1 for the fractions originating from AD and Table S2 for the fractions originating from HI). The total FAA contents in untreated insect was very low (4.8 mg/g of dry HI and 5.1 mg/g of dry AD). This amount significantly increased after the enzymatic hydrolysis, ranging from 70.6 mg to 152.2 mg/g of dry HI and from 76.7 mg to 126.6 mg/g of dry AD. The predominant FAA in all the hydrolysates was Ala, for both AD and HI, ad also Leu only for AD. The amount of FAA in the supernatants, compared to the total protein (deduced from Kjeldahl) also allowed to estimate the amount of amino acids in bound form (Figure 2). The HI extracts obtained with dispase I gave the highest relative proportion of FAA (30% of total proteins were extracted as FAAs) whereas pepsin the lowest (16% of total proteins extracted as FAAs). Instead, papain acted on AD releasing the lowest relative amount of FAA (12% of total proteins extracted as FAAs), while PBL the highest amount (23%). Also this data demonstrates how the same enzymes act differently on the two different species of insects.

For both insect species a weak positive correlation emerged between DH% and FAAs (r=0.614 for HI hydrolysate and r=0.584 for AD hydrolysate). This means that, in general, high DH correlates with the release of high amount of AAs in free form. Strong outliers for this trend were the AD hydrolysate with trypsin (high DH, but low amount of free AAs) and HI hydrolysate with papain (low DH, but high amount of free AAs). In the former case, this means that the enzyme has a preference to cut on large

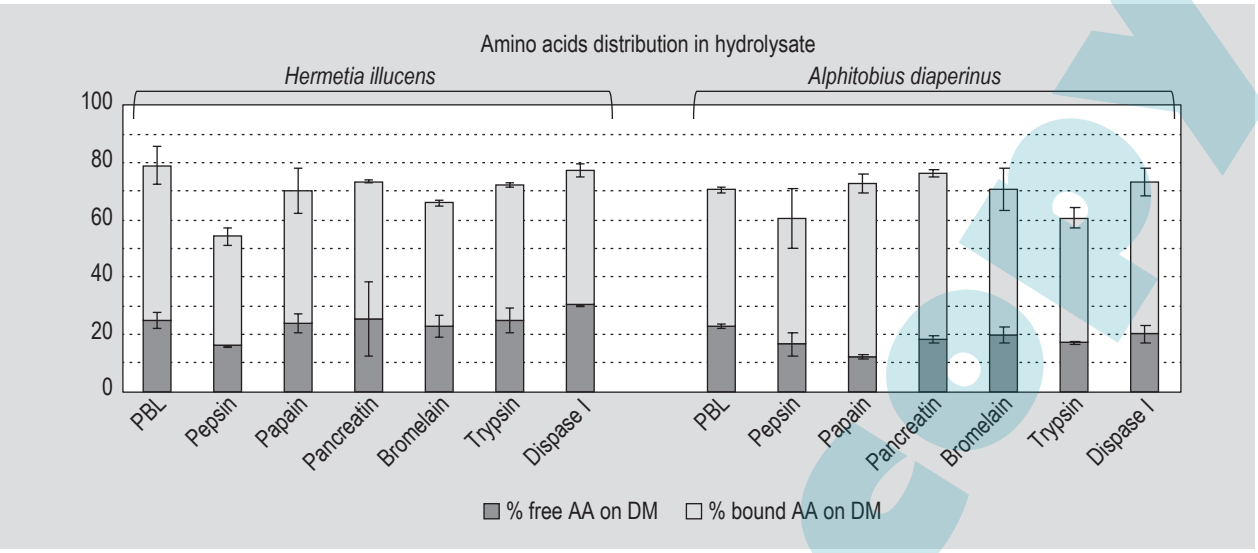

Figure 2. Distribution of bound and free amino acids in the supernatant after enzymatic hydrolysis. The full bar indicates the protein extraction yield (as reported in Figure 1) in the supernatant. The darker part of the bar indicates the amino acids present in free form and the light part of the bar the amino acids involved in peptide bond (calculated by difference).

peptides/ intact proteins, leaving small peptides relatively untouched, whereas in the latter case the opposite happens, with enzymes preferentially cleaving on small peptides rather than on large peptides/intact proteins.

The percentage distribution of FAAs essentially is consistent with the one observed for total amino acids and reported in Table 3 (with the only exception of cysteine released with trypsin). This indicates that FAA are released in the medium, in all cases, in a way which is correlated to their abundance in the proteins. The enzymes here tested are all endopeptidases, excepted for pancreatin which is a mixture of endopeptidase and exopeptidase, with a predominance of the first one. FAA release could be due either to pure chemical hydrolysis from the peptides formed, or by specific and non-specific cleavage of the endopeptidase tested. Furthermore, the proteolytic enzymes could produce reasonably high level of essential amino acids in free form (about 10% of their total amount in the insect biomass). The high levels of free essential amino acids (in theory more digestible than bound ones) enhance the value and potential of insect hydrolysates for feed and/or food formulation.

Process scale up

In order to demonstrate, as proof of concept, the scalability of the process to industrial production, the most promising insect-enzyme combination was adjusted for a reaction scale up. The reaction time was reduced as compared to the lab scale, according to energy-saving considerations, and on the assumption that, after reaching the plateau phase, most of the proteins have already been extracted in solution. For this reason, the insect-enzyme combination giving the highest DH% at lab scale was chosen. This also allowed for a reduction of the enzyme/substrate ratio, and of the volume of the buffer (also useful for reduction of environmental impact), resulting in an increased enzyme concentration as compared to the lab scale. The hydrolysis was thus performed with PBL on AD larvae. Before the enzymatic extraction, AD larvae were freeze-dried in order to improve the insect grinding. The hydrolysis was then performed on 1.5 kg of dried and ground larvae for 3 h, at optimal temperature and pH conditions. The obtained hydrolysate was freeze-dried, yielding 600 g of protein hydrolysate. This protein hydrolysate was then characterised in order to define the proximate composition (Table 5).

Table 5. Bulk composition expressed on dry matter (after freeze drying = 93%) of protein hydrolysate obtained from *Alphitobius diaperinus* (AD) hydrolysis (3 h, 60 °C, pH=7.5) with the protease from *Bacillus licheniformis* (PBL). Degree of hydrolysis (DH%) and protein extraction yield are also reported. Results are the mean of three separate analysis conducted on the two reactions performed as replicates.

Sample upscaled	Protein %	Lipid %	Ash %	DH %	Protein extraction yield %
AD+PBL	62.1±0.3	17.3±2.6	9.1±0.6	9.8±0.7	42.1±4.3

The hydrolysate resulted to be rich in protein (62.1±0.3%), with some lipid and ash. A lower extraction yield and a lower DH % was obtained as compared to the lab trials, as a result of the protocol adjustments applied (amount of sample, buffer, substrate and time). Although certainly more optimisation is needed, this experiment demonstrated the possibility of an efficient production of a protein hydrolysate from insects, which could be used as insect-based ingredients for feed and food formulations.

4. Conclusions

The enzymatic-assisted protein extraction here presented clearly represents an effective method to extract and isolate protein from two different edible insects, HI and AD. In the present work the different proteolytic activity of seven enzymes was studied, by evaluating the characteristics of protein hydrolysate produced at a laboratory scale: yields of extraction, protein integrity and FAA composition. This is the first time that several enzymes are tested and compared on these two insect species, and that the protein hydrolysates obtained underwent such a detailed assessment for their molecular composition.

Proteases were able to extract protein from insects in form of peptides and FAA, preserving their quality and making them more accessible for their future use as feed/food supplements. Protein hydrolysate, as opposed to intact protein, are more rapidly digested and absorbed, and, also important, could be potentially hypoallergenic. Furthermore, as a proof of concept, the potential scale up of the process was performed, by focusing on the most promising insect-enzyme combination. Starting from 1.5 kg of dry AD larvae, 600 g of dry hydrolysate containing more than 60% proteins, in form of peptides and FAA, were produced. Further investigations are needed in order to optimise the process for industrial production of insect-based ingredients for feed and food formulations, but the processes here presented have the potential to produce protein hydrolysates in an environmentally friendly way, even if a full LCA assessment on a perfectly optimised protocol will be needed to verify this feature.

Founding sources

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2019.0037.

Table S1. Free amino acids composition of hydrolysates from *Alphitobius diaperinus* using commercial protease under optimum conditions. Results are expressed as mg/g of dry insects employed for the hydrolysis. Results are the mean of three separate hydrolysis experiments. Different letters in the same row show significant differences (P<0.05).

Table S2. Free amino acids composition of hydrolysates from *Hermetia illucens* using commercial protease under optimum conditions. Results are expressed as mg/g of dry insects employed for the hydrolysis. Results are the mean of three separate hydrolysis experiments. Different letters in the same row show significant differences (P<0.05).

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