Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin

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Short Title: Biomolecules from black soldier fly

Abstract

Black soldier fly (BSF, *Hermetia illucens*) constitutes an economic way to convert residual biomasses into a valuable source of biomolecules, such as proteins, lipids and chitin. The present investigation was undertaken to evaluate the feasibility of applying different extraction protocols, either chemical extractions or enzymatic assisted extraction, to recover pure fat, protein and chitin fractions. First, exact proximate composition, total amino acids, fatty acids profile, and N-acetylglucosamine content of the prepupae samples were determined. BSF prepupae biomass contained, expressed on dry weight, 32% proteins, 37% lipids, 19% minerals, 9% chitin. The lipid fraction was easily recovered by organic solvents, while the most challenging issue was the separation of protein from chitin. The best separation was obtained by alkali extraction of proteins (96% of protein recovered) albeit with loss in their integrity as indicated by the measurement of the degree of hydrolysis with the O-phthaldialdehyde method. To avoid protein damage in alkali media, a stepwise protein extraction adopting milder conditions was also explored based on Osborne fractionation method, allowing the recovery of more than 85% of BSF high purity and high quality proteins, and the obtainment of chitin-enriched fraction as well. The possibility of using an enzymatic assisted extraction of proteins was also explored, obtaining a maximum nitrogen solubilisation in the best case (with *Bacillus licheniformis* protease) of about 60%. In this latter case, the chitin fraction obtained also had a significant residual protein content.

Keywords

Black soldier fly; lipids; proteins; chitin; chemical extractions; enzymatic-assisted extraction

1. Introduction

The increase in waste production because of population growth is among the major concerns in many areas around the world. One of the most innovative technology for waste management is the bioconversion of side streams by insects (Čičková, Newton, Lacy, & Kozánek, 2015). Many insects naturally feed in organic wastes, converting biomass nutrients into their own biomass and reducing the amount of waste material. *Hermetia illucens* Linnaeus 1758 (Diptera: Stratiomyidae), better known as black soldier fly (BSF), is one of the most important species proposed as a converter of organic waste. BSF larvae can develop on a wide range of substrates, including agricultural by-products and organic waste (Diener, Studt Solano, Gutiérrez, Zurbrügg, & Tockner, 2011). One of the main advantage of using BSF as waste bio converter is that adult flies do not eat, thus avoiding any disease transmission risks (Sheppard, Tomberlin, Joyce, Kiser & Sumner, 2002). BSF is reported as a good source of nutrients like proteins, lipids, minerals (Spranghers et al., 2016). For their high protein content, BSF larvae/prepupae have been proposed to be used as feed for different species as fish, chicken and pigs (Newton, Booram, Barker & Hale, 1977; Cummins et al., 2017) and as a pet food (Bosch, Zhang, Oonincx & Hendriks, 2014). Moreover, due to the large amount of fat in the prepupae, another application exploited for BSF biomass is the production of biodiesel (Li et al., 2015). Finally, yet importantly, BSF is also a source of chitin (Wasko, Bulak, Polak-Berecka, Nowak, Polakowski & Bieganowski, 2016). Chitin and its derivatives have great economical value because of their numerous applications: food, cosmetics, pharmaceuticals, textile industries etc. (Gortari & Hours, 2013). In view of a possible use of insects as foodstuff, beside regulatory issues, it should be taken into account that western consumers may be reluctant to accept whole insects for cultural reasons. Some studies showed that consumers are more willing to eat insects when they are not visible in food (Balzan, Fasolato, Maniero & Novelli, 2016). This suggests that the preferable approach would be to transform insects in meals, using protein and other fractions as food/feed ingredients. Processing of larvae into separate fractions may also address microbiological safety

issues by killing bacteria during drying and extraction steps (Lalander, Diener, Magri, Zurbrügg, Lindström & Vinnerås, 2013). In this framework, the development of protocols to fractionate biomolecules from BSF is an issue of growing importance, to obtain high added value marketable products and give further economic improvement to the organic waste recovery industry, and to agri-food by-products.

There are few protocols of extraction reported in the literature, mostly developed with the aim of separating just one constituent from the insect biomass. Del Valle et al. performed protein extraction from *Anastrepha ludens* at pH 10 and subsequent protein precipitation at pH 5 (Del Valle, Mena & Bourges, 1982). Yi et al. performed an aqueous protein extraction from five insect species (Yi, Lakemond, Sagis, Eisner-Schadler, van Huis & van Boekel, 2013). Recently Bußler et al. compared different protein extraction methods from *T. molitor* and *H. illucens* (Bußler, Rumpold, Jander, Rawel & Schlüter, 2016). Most of these studies focused on protein extraction only. However, insects and especially BSF, contain other valuable biomolecules such as lipids and chitin. Therefore, in a biorefinery approach, there is the need of established processes able to recover all the three main fractions of black soldier fly in subsequent steps along the same chain, in order to create the maximum added value from the process. While the separation of lipids is quite easy, the separation of protein from chitin is more challenging. Scarce data are available in literature dealing with this separation in insect matrix, while more data are available on crustaceans, currently the main source of commercial chitin/chitosan (Gortari et al., 2013). The most common method for recovery of chitin from crustacean shells is the chemical procedure, involving two mayor steps: demineralization and deproteination using strong acids and bases. As an alternative to the chemical process, other approaches have been investigated on crustaceans, such as fermentation and enzymatic hydrolysis (Synowiecki & Al-Khateeb, 2000). The present work explores, for the first time, several systematic approaches to separate lipid, protein and chitin from BSF prepupae into three usable fractions, with homogeneous methodologies in a biorefinery-like cascade. Extraction methods, here developed at a laboratory scale, are based on total chemical extractions or

enzymatic assisted extraction, and are designed and optimized in order to obtain the three fractions at the maximum level of purity in a subsequential homogeneous process.

2. Materials and methods

2.1. Materials

H. Illucens prepupae were purchased from Smart Bugs s.s. (Ponzano Veneto, Italy) and stored at -20°C in ziplock bags until use. Frozen prepupae were grinded with IKA A10 laboratory grinder and immediately used for the different analysis/treatments of extraction.

Chemicals. Kjeldahl defoamers and catalyst were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit and AccQ-TagTM were obtained from Waters (Milford, MA, U.S.A). Methylpentacosanoate, DL-norleucine, amino acid standard mixture, L-tryptophan, Supelco 37 component FAME mix, chitin, glucosamine, N-acetylglucosamine, beta-phenylglucoside, Pepsin from porcine gastric mucosa (250U/mg, 117K0811), Protease from *Bacillus licheniformis* (2.4U/g, SLBL2953V), pancreatin from porcine pancreas (4xUSP, SLBM4075V), papain from papaya latex (1.5–10U/g, SLBJ6115V), DL-cystine, N-acetyl-l-cysteine, DL-isoleucine, bis-trimethylsyliltrifluorocetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). o-phthaldialdehyde, D-cystein were bought from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate was purchased from Biorad (Hercules, CA, U.S.A). All the other solvents, salts, acids and bases were of analytical grade and purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

2.2 Proximate composition

Moisture, protein, lipid and ash were determined using standard procedures (AOAC, 2002). Moisture was determined in oven at 105 °C for 24 h. Crude fat content was determined using an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) using diethylether. Total ash was determined after mineralization at 550 °C for 5 h + 5 h. Total nitrogen

was determined with a Kjeldahl system (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA).

The nitrogen coefficient conversion for BSF proteins was obtained by total amino acid composition, (paragraph 2.4) assuming an equimolar amount of Asn/Asp and Gln/Glu.

2.3 Amino acids determination

2.3.1. Total amino acids profile

Five hundred milligrams of BSF prepupae were hydrolysed with 6 mL of HCl 6 N at 110 °C for 23 h, then the internal standard (7.5 mL of 5 mM Norleucine in water) was added. Cysteine and methionine were determined as cysteic acid and methionine sulphone after performic acid oxidation followed by acid hydrolysis. In this case, an amount of 0.5 g of BSF was added to performic acid freshly prepared (by mixing 9 volumes of formic acid with 1 volume of hydrogen peroxide), samples were kept in an ice bath for 16 h at 0°C. Then 0.3 mL of hydrobromidric acid was added and bromine formed was removed under nitrogen flow. Then acid hydrolysis was performed as described above. The hydrolysed samples were analysed by high performance liquid chromatography with fluorescence detector (HPLC/FLD, Waters Alliance 2695) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) according to the method described by Marseglia, Palla & Caligiani (2014). Calibration was performed with standard solutions prepared mixing 40 µL of Norleucine (2.5 mM), 40 µL of amino acids hydrolysate standard mixture, 40 µl of cysteic acid (2.5 mM) and 880 µL of deionized water.

2.3.2 Tryptophan determination by LC/ESI-MS after alkaline hydrolysis.

One hundred milligrams of sample were added to 4 ml of 4 N NaOH and hydrolysed at 100 °C for 4 h, then the solution was neutralized by adding 37 % HCl. DL-tryptophan standard solutions were treated as the sample. UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C18 column (1.7 µm, 2.1x150 mm). 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: isocratic 100 % A for 1.8 min, from 100 % A to 50 % A by linear gradient in 11.4 min and 0.8 min at 50 % A plus washing step at 0 % A (100 % B) and reconditioning. Flow rate was set at 0.25 ml/min, injection volume 2 µl, column temperature 35 °C and sample temperature 23 °C. Detection was performed by using Waters SQ mass spectrometer: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N2): 100 l/h, desolvation gas flow (N2): 650 l/h, full scan acquisition (100-2000 m/z), scan duration 1 s.

2.4 Determination of fatty acids profile by GC-MS

The Soxhlet ether extract was evaporated under vacuum at 40 °C. One hundred mg of the fatty residue were dissolved in 2 mL of 5 % HCl in methanol and heated for 1 hour at 60 °C. Five ml of hexane was added to extract the fatty acid methyl esters. Five hundred µL of the superior organic phase was added to 100 µL of methylpentacosanoate. Dilution of 1:20 of this solution with hexane was performed to match the linearity range of the GC-MS instrument. The solutions were split-injected (1µL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a Supelcowax ms capillary column (30m, i.d 25 mm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Carrier gas was helium (1 ml/min), injector and detector temperatures were kept at 250 °C, while oven temperature was programmed from 80 to 240 °C at 20 °C/min. Content of each single fatty acid was calculated in relation to the concentration of the internal standard (methylpentacosanoate), after calculating the response factors using the Supelco® 37 Component FAME Mix. Finally, results were expressed as relative percentage of each fatty acid.

2.5 Determination of chitin

Chitin was first obtained as difference from total nitrogen content after subtracting the protein contribution to total nitrogen. A specific nitrogen conversion factor of 14.5 was calculated for chitin, assuming a fully acetylated glucosamine.

The actual content of chitin was also determined by GC-MS quantification of glucosamine after chitin hydrolysis, based on the method of Flannery et al. with some modifications (Flannery, Stott, Briggs & Evershed, 2001). Briefly, one hundred mg of BSF prepupae were hydrolysed in 25 mL of 7M HCl for 4 h at 110 °C, added to beta-phenylglucoside as internal standard (0,2 mL of 5 mg/L solution in water) 1 mL of the hydrolysate was taken to dryness, dissolved in 0,8 ml of DMF and sylilated with 0,2 mL of bis-trimethylsyliltrifluorocetamide (BSTFA). The same procedure was applied to a chitin standard (Sigma Aldrich) to calculate the recovery. The solutions were split-injected (1µL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying an apolar capillary column (SLB-5, 30 m, i.d. 0,25 mm, Supelco, Bellafonte, USA). coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). GC-MS analysis was carried out in full scan mode, carrier gas was helium (1 ml/min), injector and detector temperatures were kept at 280 °C, while oven temperature was programmed from 60 to 280 °C at 20 °C/min.

The method turned out to be quite prone to yield inconsistent results in repeated analyses. In order to cross check the robustness of the obtained data, the chitin content of a commercial chitin preparation, analyzed in the same moment and with the same methodology as our samples, was routinely added as control in all our analyses, never considering any result obtained in the same batch of a standard having an accuracy level lower than 90%.

2.6 Extraction Protocol 1: chemical method with one step protein extraction

2.6.1: Lipid extraction.

Five grams of finely grinded BSF prepupae were subjected as a first step to lipid extraction. Lipids were extracted with petroleum ether (40-60 °C boiling point fraction) by a two-step method. One

part of the BSF biomass and two parts of petroleum ether (weight/volume) were stirred on a magnet stirrer for 1 h. The solvent containing fats was decanted and recovered by paper filtration. The procedure was repeated. Lipids were recovered by solvent evaporation under vacuum (40 °C) Residual solvent was removed by the defatted insect pellet by evaporation overnight.

2.6.2. One step protein extraction.

Defatted insect pellet was treated with 40 ml of 1 M NaOH in water bath at 40 °C for 1 h. The supernatant was neutralized, centrifuged (model 5810R EPPENDORF, Hamburg, Germany) for 15 min at 4000 rpm. Proteins were recovered by precipitation with 10 % TCA solution in acetone (ratio 1:1, v/v); sample was incubated overnight at -20 °C, centrifuged at 4 °C, 4000 rpm for 30 min; then, washed twice with acetone and dried in oven at 90 °C for 2h.

2.6.3. Demineralization and chitin separation

The pellet obtained from the previous step was subjected to demineralization with 40 ml of 2 N HCl for 24 h at room temperature. Then, the sample was centrifuged for 15 min at 4000 rpm; the precipitate was washed twice with water. The final residue was dried in oven at 40 °C overnight.

2.7 Extraction protocol 2: chemical method with stepwise protein extraction (Osborne fractionation)

The second protocol applied the same procedure for lipid extraction as reported in 2.6.1.

The protein extraction was performed with an alternative procedure, based on the Osborne fractionation method (Osborne, 1907) with some modifications. Two grams of defatted sample were mixed with 40 ml of a solution containing 5 mM sodium ascorbate, 2 mM EDTA and 10 mM tris-HCl. The suspension was mixed for 1 hour at 4 °C, centrifuged for 20 min at 4000 rpm at 4 °C and then the two fractions were separated. The supernatant was collected as albumin fraction. The pellet was extracted with 40 ml of a solution containing 0.5 M NaCl, 5 mM sodium ascorbate, 2 mM EDTA and 20 mM tris-HCl. The suspension was mixed for 1 hour at 4 °C, centrifuged for 20 min at 4000 rpm at 4 °C and then the two fractions were separated. The supernatant was collected as

globulin fraction. The pellet was mixed for 1 hour at 4 °C with 40 ml of 5 mM ascorbic acid in 70% EtOH. Then it was centrifuged for 20 min at 4000 rpm at 4 °C. The supernatant was collected as prolamin fraction and the pellet was extracted with 25 ml of 0.1 N NaOH and 5 mM ascorbic acid. The extraction was carried out under stirring at 4 °C for 1 h and then centrifuged for 20 min at 4 °C at 4000 rpm. The supernatant was collected as glutelin fraction and the pellet as residual. The following step of chitin demineralization was performed again as described in 2.6.3.

2.8 Extraction Protocol 3: enzymatic method

The enzymes employed for sample hydrolysis were: *Bacillus licheniformis* protease (EC 3.4.21.62), pepsin from porcine gastric mucosa (EC 3.4.23.1), papain from papaya latex (EC 3.4.22.2) and pancreatin from porcine pancreas. Whole BSF prepupae was minced and the hydrolysis reactions were performed at the optimal conditions of pH and temperature for the enzymes (Table 4). An enzyme/substrate ratio of 1:100 (w/w) was mixed with the specific solution, hydrolysed overnight, then heated at 90 °C for 10 min to inactivate the enzymes. The hydrolysed substrate was centrifuged at 4000 rpm at 4 °C for 30 min. Three fractions were obtained, from top to bottom: the lipid fraction, the supernatant, and the pellet.

2.9 Determination of the yields of extractions

The amount of fat extracted (yield) was determined by weight and compared with the total amount of fat obtained by Soxhlet (Section 2.2, Table 1). The yield of protein extractions was determined by comparing the nitrogen in the extraction systems and the protein nitrogen determined by amino acid analysis (Table 1). The contribute of salts containing nitrogen in the extraction systems was considered and subtracted when necessary. The efficiency of protein from chitin separation was determined by the nitrogen content in the different fractions determined by Kjeldahl (Section 2.2), compared with the ratio of protein and chitin nitrogen, reported in Table 1.

2.10 Determination of the integrity of protein fraction by degree of hydrolysis determination

The protein degree of hydrolysis (DH) was calculated using o‑phthaldialdehyde (OPA) method described by Spellman et al. with some modifications (Spellman, McEvoy, O'Cuinn & Fitsgerald, 2003). 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 (in methanol), 5 mL of 20 % (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil and stirred for 1 h before use. The OPA assay was carried out by the addiction of 20 μL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, OK, U.S.A) against a control cell containing the reagent and 20 μL of the specific buffer used for each sample. The intrinsic absorbance of the sample was measured before OPA addition and subtracted. A standard curve was prepared using L-isoleucine (0–2 mg/mL). The degree of hydrolysis (DH) was calculated as follows:

$$DH=A/B\times 100$$

A= moles of free nitrogen atoms from alpha amino groups after hydrolysis (determined by OPA assay)

B= total moles of nitrogen atoms in solution before hydrolysis, calculated by the ratio of total grams of proteins and the average of residual amino acids molecular mass (Mw 110).

3. Results and discussion

3.1 Black soldier fly composition

Detailed composition of BSF prepupae was important as a basis to design and evaluate the subsequent fractionation procedure, and its determination was not a straightforward task. The dry matter (DM) content of BSF prepupae was found to be 34 ± 1 %, in accordance with the results of previous studies (Sheppard, Newton, Thompson & Savage, 1994). As far as the lipid content was concerned, BSF prepupae contained 37.1 ± 0.1 % of lipids (DM basis), so they represent a good

source of energy, unlike other insect species which show a lower lipid content (Ramos-Bueno, González-Fernández, Sánchez-Muros-Lozano, García-Barroso & Guil-Guerrero, 2016). The relative fatty acid profile was determined by GC-MS on the fat extracted by Soxhlet with diethyl ether (section 2.2) after acid methylation, thus including triacylglycerol-bound fatty acids and free fatty acids. BSF fatty acid profile was dominated by lauric acid (46.7±0.6%), followed by oleic acid (15.1 ± 0.9%), miristic (8.3 ± 0.1%), palmitic (8.4 ± 0.2 %) and palmitoleic (7.6 ± 0.4 %) acids. Minor amounts of C10:0 (1.9 ± 0.2 %), C14:1 (1.3 ± 0.2 %), stearic (2.5 ± 0.1 %) and linoleic acid (1.8 ± 0.4 %) were also registered. Similar findings were obtained by Ushakova et al., using larve reared on wheat grain (Ushakova, Brodskii, Kovalenko, Bastrakov, Kozlova & Pavlov, 2016). This fatty acid profile, exceptionally rich in saturated fatty acids (72 % of the whole lipid fraction) and very poor in polyunsaturated fatty acids, suggested that the addition of antioxidants in the lipid extraction media was not essential. So this step was avoided during the development of extraction protocols.

The protein content, as determined by Kjeldhal, is generally calculated from total nitrogen using a standard nitrogen-to-protein conversion factor of 6.25. However, the total nitrogen content in BSF (in our case 6.61 ± 0.05%, Table 1), and in insects in general, includes nitrogen originating from protein and non-protein sources, and in the latter particularly chitin, a polymer of N-acetylglucosamine. Therefore, in order to obtain an accurate proximate composition of BSF, it was necessary to separate the contribution of protein and chitin nitrogen.

The nitrogen conversion factor specific to only the BSF protein can be accurately calculated from the total amino acid amount (Tkachuk, 1969). To this scope, the content of amino acids was determined (see Table 2), both in absolute (g/100g DM) as well as relative terms (mg of each amino acid/ g of crude protein). Considering the amino acid distribution as reported in Table 2, a Kjeldahl conversion factor of 5.71 ± 0.02 was calculated for BSF proteins, thus quite far from the widely used 6.25. From the total sum of amino acids, a global protein content of 32 ± 2 % (DM basis) was determined. Spranghers et al. (2016) found higher protein contents in BSF ranging from 39 to 43 %,

but they used the standard Kjeldahl conversion factor of 6.25, expected to result in an overestimation, as explained above. Our result is indeed more similar to that of Janssen et al., who calculated in a similar way the specific nitrogen conversion factor for the BSF protein (Janssen, Vincken, van den Broek, Fogliano, Lakemond, 2017). In order to estimate the contribution of nitrogen originating from proteins, as determined by Kjeldhal, the amount of proteinaceous N was recalculated from the amino acid analysis and a value of 5.59 ± 0.05% on DM was found, indicating that 84 % of all nitrogen in prepupae is located in proteins. From the amino acid composition, also the potential nutritional value of BSF proteins could be assessed. The essential amino acid contents reported in table 2 were compared with high nutritional quality animal and vegetal proteins, such as egg white and soybean. It appears that BSF proteins contain, compared to them, higher amount of tyrosine, phenylalanine and histidine, comparable amounts of lysine, threonine, valine and slightly lower amounts of leucine, isoleucine, tryptophan and sulfurated amino acids. When BSF essential amino acids were compared to the amino acids requirements for human adults calculated in the FAO/WHO reference protein, it appears that BSF proteins contain all the essential amino acids in the correct amount.

As far as chitin content is concerned, this was the most challenging determination. The protein content allowed calculating (by difference) the contribution of non-protein nitrogen to total nitrogen, consisting in 1.02 ± 0.04 % on DM. The nitrogen conversion factor for chitin can be theoretically calculated and it can vary from 14.5, assuming a fully acetylated chitin, to 11.5 for a fully deacetylated chitin. Using these factors, and assuming that all the non-protein nitrogen found was due to chitin, a chitin content ranging from a minimum of 11.7 % to a maximum of 14.6 % can be back calculated, the actual value depending on the acetylation degree. These values are indeed higher than those reported in the literature. Spranghers et al. (2016) reported a chitin content for BSF prepupe of 6-7 %, as determined by the method of Liu et al. (2012), based on the weight of the residue obtained after the total elimination of protein and mineral using strong alkaline and acidic conditions. In order to better cross check the actual chitin content in our samples, a specific GC-MS

method for the determination of glucosamine after total hydrolysis of chitin in acidic media was applied, based on the method of Flannery et al. (2001). Even if the method turned out to have low precision and low reproducibility, using suitable controls (se details in the experimental section), the chitin content by this method was reliably determined to be 9 ± 1% on DM basis. This value corresponds to a nitrogen content of 0.62 ± 0.06, indicating that 10 % of the total nitrogen of BSF prepupae is contained in chitin.

Subtracting then protein nitrogen and chitin nitrogen from the total nitrogen, a residual 0.40 % nitrogen (on DM basis) was obtained, representing 6 % of the total BSF nitrogen. This indicates that BSF prepupae contain nitrogen (see Table 1) coming from sources which are not protein and not chitin. This might include melanin. Indeed, according to Ushakova et al., melanization process can led to a melanin content ranging from 0.1 to 3 % of BSF dry matter, going from the larval to adult stage (Ushakova, Dontsov, Bastrakov, Garmash & Pavlov, 2017). The development stage considered in this work is the prepupae, so they could contain an intermediate amount of melanin between larvae and adults. Therefore, it is possible that most of the non-protein and non-chitin nitrogen in BSF was due to melanin.

Concluding on the proximate composition, the amount of the different fractions, determined with all the methods above reported, yielded a very satisfactory mass balance. According to our figures, lipids accounted for 37 % of weight, proteins for 32 % and chitin for 9 % (on DM basis). By adding 19 % of ashes, also determined in this work, the total accounted for 97 % of the dry matter weight. Since we also found clear indications that non-protein and non-chitin, nitrogen-containing compounds, possibly melanin, are also present, and considering that melanin can be present up to 3 % of the dry matter weight (and other nitrogen-containing compounds might contribute), the mass balance composition of BSF prepupae seemed to be perfectly explained by our data. This also yielded the basis for the calculation of the efficiencies of the subsequent extractions procedures.

3.2 BSF biomolecules fractionation

Extraction protocols were designed with the principal aim to recover at the highest purity level the three main biomolecules of BSF, i.e. lipids, proteins and chitin. Three different approaches were tested on the BSF prepupae: two different chemical protocols, essentially differing for the protein extraction method, and an enzymatic one. General steps in the processing of BSF biomass according to chemical protocols are schematized in Fig. 1 and Fig. 2. Enzymatic protocol steps are reported in Fig. 3. All the protocols were applied at a laboratory scale, treating grams of sample. Protocols were applied directly to whole grinded prepupae, in order to avoid the high costly freeze drying process. All the protocols were repeated three times and all the data are presented as mean and standard deviation of the different trials. The efficiency of lipid separation was determined as extraction yield related to the total fat content determined by Soxhlet. The efficiency of chitin from protein separation was evaluated measuring the crude total nitrogen content in the different fractions, considering that the relative contribute of chitin and protein to the total nitrogen was 10 ± 1% and 84 ± 1% respectively, as determined in paragraph 3.1 (Table 1). Therefore, an ideal separation process should give at the end this nitrogen distribution in the two fractions. In this approach, we decided to consider negligible the contribution of non proteic-non chitinic nitrogen, representing 6 ± 1 % of the total nitrogen.

It is to be noted that all the protocols below reported have a general validity, and thus can likely also be applied to other insects.

3.2.1 Protocol 1: total chemical extraction with one step protein extraction

The first lab-scale chemical extraction protocol is depicted in Fig. 1. Extraction was performed on five grams of whole prepupae, finely grinded. The first step was the fat extraction. Extraction of fat from *H. illucens* was tested with a two-step manual extraction: one part of the insect powder and two parts of petroleum ether were stirred on a magnet stirrer for 1 h, then the mixture was decanted and petroleum ether recovered. The procedure was repeated twice and residual solvent was removed from insect powder by evaporation overnight at room temperature. The total amount of fat extracted

with this method was 32.5 ± 0.5% on dry matter basis, corresponding to an extraction yield of 87 % respect to the total fat contained in the BSF prepupae as determined previously by ethyl ether Soxhlet extraction. Defatted BSF powder was then subjected to the deproteinization and demineralization steps in order to obtain purified protein and chitin fractions. The protein and chitin fractions were separated based on the chemical methods developed for the isolation and purification of chitin and chitosan from shrimps (Bajaj, Winter & Gallert, 2011; Zhang, Yuna, Songa, Zhanga, & Zhaob, 2017). However, these methods are generally designed and optimized for the chitin extraction and do not take care of the protein fraction; therefore, in order to isolate both protein and chitin from BSF as intact as possible, the methods were modified using the mildest conditions as possible. For the extraction of the protein fraction, a one-step total protein extraction by NaOH 1M was applied, using mild extraction condition (40 °C for 1 h). The supernatant obtained was assessed for the nitrogen content: the percentage of nitrogen accounted for 4.7 ± 0.5% on dry matter (see Fig.1), corresponding to 84 % recovery of protein fraction. A residual protein fraction (N %= 0.7 ± 0.2, 12 % of protein content, Fig.1) was further solubilized during the following acidic demineralization step. The amount of nitrogen then left in the pellet (1.1 ± 0.2 %, Fig. 1) corresponded to the chitin nitrogen, plus a small residual amount of protein and other nitrogen containing compounds, like melanin. Thus, the proposed alkali extraction of BSF defatted powder allowed a final recovery, considering together the two fractions obtained, of 96 % of protein fraction. However, it is known that alkali extraction, albeit usually quite efficient, may cause reactions in the protein backbone, such as denaturation, hydrolysis, racemization, and lysinoalanine and other cross-linked compounds formation (Schwass & Finley, 1984). These modifications might result in poor protein functionality and a reduced nutritional value. To test the protein modification in terms of hydrolysis, the OPA was explored as a rapid non specific method to evaluate protein integrity, due to its ability to detect the number of free -NH_2 group. The degree of hydrolysis was calculated as 15 % (referred to the solubilized proteins, Table 3), indicating indeed a hydrolysis induced by the alkaline extraction.

After protein separation from chitin, protein solutions were then dried using three different methods, i.e drying by rotary evaporator, freeze-drying and precipitation by trichloroacetic acid (TCA) or HCl. The first two methods resulted inconvenient, since the proteins showed significant foaming properties; therefore, some issues occurred not only in drying but also in recovering the sample. Indeed, insect proteins are known for their foaming capacity, as reported on protein hydrolysates from a tropical cricket (Hall, Jones, O'Haire & Liceaga, 2017). An additional problem is the salt resulting from neutralization, which is dried together with the sample lowering the purity in proteins. On the other hand, the precipitation by TCA resulted in a final protein yield, calculated on the starting total protein content in BSF, of 73 %. Moreover, proteins with high purity were obtained in this way, since the co-precipitation of the salt was avoided. An alternative to TCA is the use of HCl to precipitate the protein at their isoelectric point. The pH was adjusted with HCl to about 3.5-4, because this value was reported as the isoelectric point of the most insect protein (Bußler et al., 2016). This latter method indeed allowed to obtain a similar yield of precipitated proteins (67 %).

The main advantage of the above reported protocol was the rapidity and the easy up-scaling; the fraction extracted can be used for technological applications (e.g. bioplastic formulations), but also as foaming/emulsifier additives.

3.2.2 Protocol 2: total chemical extraction with stepwise protein extraction

As a variant of the previous method, during protein extraction a stepwise method, adapting the Osborne fractionation protocol (Osborne 1907), was adopted, leaving the initial fat extraction and the demineralization steps performed exactly as in the previous protocol (Fig. 2). Protein extraction was based on their different solubility in different solvents. Five fractions were obtained (Table 3). The first fraction represented the proteins extractable in water, i.e. albumins, the second fraction the globulin fraction, soluble in saline solution, the third and fourth fractions are represented by insoluble proteins (prolamins and glutelins), extractable, respectively, in alcoholic and alkali

solutions. The solid residue obtained after the four extraction steps represents the chitin fraction and the eventual residual protein fraction. From this solid it is possible to obtain a chitin fraction of higher purity applying the demineralization step as in the Protocol 1. On each protein solution, total nitrogen content was calculated in order to verify the extraction yields of proteins. The nitrogen in the extraction solutions can be assumed as protein nitrogen because chitin is practically insoluble in all the four extraction systems (Pillai, Willi & Chandra, 2009). The first fraction could have been containing also some non protein nitrogen soluble compounds, however their amount was low, as indicated by the free amino group determined by OPA method (Table 3). The main BSF protein fractions were represented by albumins and glutelins, followed by globulins and prolamines. The total nitrogen extracted in the four fractions represented 90 % of the theoretical protein nitrogen. Therefore, this method allowed to obtain a good separation of the protein from the chitin fraction. Moreover, because all of the extraction steps were made in mild conditions, the protein fractions recovered were intact. Indeed, the integrity of the protein fractions was confirmed by OPA test, which showed negligible amounts of free -NH_2 groups (Table 3). Therefore, this is the best method to recover intact proteins to be used for nutritional purposes or high added-value products (e.g. for feed/food ingredients, cosmetics, pharma). For example, soluble proteins as albumin and globulin generally present foaming and emulsifying capacities. The functional properties of each protein fraction need to be investigated as well as their specific amino acid profile and nutritional value. However, as a major disadvantage, due to the four sequential extraction steps, this method, presently developed at a laboratory scale, is likely affected by high costs when scaling it up to the industrial level.

3.2.3 Protocol 3: enzymatically-assisted fractionation

In the third protocol studied, an enzymatic hydrolysis using proteases was applied to the entire BSF prepupae. This protocol is alternative to the previous protocols and it was developed as a more sustainable and fast method, avoiding the use of organic solvents and acidic/alkali solutions. In fact,

chemical processes exploited in previous protocols, mainly the first protocol, may cause protein modification but to some extent also a possible chitin depolymerisation, affecting the polymer properties. In this method, four different proteolytic enzymes were separately tested on analytical scale directly on the grinded prepupae, without any pre-treatment, in order to find the best enzyme allowing solubilizing the most of the protein nitrogen. BSF prepupae were hydrolyzed for 16 h with one of the following commercial enzymes derived from vegetal (papain), bacterial (*B. licheniformis* protease) or animal sources (pepsin, pancreatin). Each enzyme required specific reaction conditions, chosen according to the provider specifications and from the results of a previous work (Anzani et al., 2017). The conditions used are reported in Table 4. After hydrolysis, three different fractions were collected: a floating oily fraction, a supernatant liquid under it (mainly composed by soluble intact proteins or hydrolysed proteins) and pellet (insoluble proteins and chitin). The yield in lipids, after centrifugation was only 10 %, thus much lower than that obtained with the solvent extraction method, as expected. This was also previously observed by Tzompa-Sosa et al. extracting lipids from four insect species with Soxhlet or aqueous method (Tzompa-Sosa, Yi, van Valenberg, van Boekel & Lakemond, 2016). Therefore, if a pure lipid fraction is needed, the extraction with organic solvents as a first step is certainly the most efficient method. In order to verify the protein and chitin distribution after enzymatic hydrolysis, the amount of nitrogen was calculated in both supernatant and pellet. As a blank control, the determination was done also on the samples treated in the same conditions without enzymes. Proteins extracted by this methodology are expected to be hydrolysed to peptides and free amino acids. The degree of hydrolysis (DH %) was also determined with the OPA method, calculated on the proteinaceous materials released in solution (Table 4). *B. licheniformis* protease was the enzyme with the best hydrolytic activity, with 67 ± 5 % of the total protein nitrogen released in the supernatants (solubilized), followed by pancreatin (54 ± 2 % of the total protein nitrogen solubilized), papain (51 ± 2 % of the total protein nitrogen solubilized) and pepsin, the less efficient (47 ± 3 % of the total protein nitrogen solubilized). Pancreatin and papain hydrolysis showed the highest DH (25%), followed by the hydrolysis with pepsin (17 %), and *B.*

licheniformis protease (6 %). It should be noted that this latter protease results in a higher degree of solubilisation but lower degree of hydrolysis respect to pancreatin. Both solubilisation degrees and DHs were different from those of the same enzymes acting on fleshing meat (Anzani et al., 2017), probably on the account of the different proteins being hydrolysed in the two cases. The chitin-rich residue, in this case, also contained the not hydrolysed proteins.

4. Conclusions

The present study offers the first reported systematic approach allowing to fractionate valuable biomolecules originating from BSF prepupae, and resulting into three major products (proteins, lipids, chitin) in the purest possible form. Three different fractionation approaches were pursued, all having advantages and drawbacks, and, in view of future applications, offer choices which are function of the specific end-use of the BSF biomolecules, in terms of purity, yield, integrity of the specific components. The enzymatic approach, even if less efficient than the chemical extractions, certainly deserves anyway further investigation, also in view of the possibility to tailor the hydrolysate composition obtained from BSF proteins for different purposes, such as high digestible and hypoallergenic protein supplements for feed/food, thickening agents, texturizers, foaming agents and others.

Funding

This work was done in the framework of the project "ValoriBio" co-financed by 2014-2020 ROP ERDF, Emilia-Romagna Region, Italy, DGR 774/2015 – CUP E42I15000110009.

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Figure Captions

Figure 1. Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 1 (Chemical method). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

Figure 2. Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 2 (stepwise method). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

Figure 3. Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 3 (enzymatic assisted extraction). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

Tables

Table 1. Proximate composition of BSF prepupae. Value are expressed on dry matter basis. Moisture content: 66 ± 1 %. Results from triplicate analyses.

Parameter		*Method*
Crude total Nitrogen, % DM	6.61 ± 0.05	Kjeldahl
Total amino acids, % DM	*36 ± 2*	*HPLC/FLD*
Total protein % DM	*32 ± 2*	*From total amino acids*
Protein nitrogen, % DM	*5.59 ± 0.05*	*Total aa / calculated Kjeldahl factor*
Total Non protein nitrogen, % DM	*1.02 ± 0.04*	*By difference*
Total Chitin, % DM	*9 ± 1*	*GC-MS determination of glucosamine (Flannery et al., 2001)*
Chitin nitrogen, % DM	*0.62 ± 0.06*	*Total chitin/14.5 (kjeldahl factor for chitin)*
Residual nitrogen (non protein, non chitin compounds)	*0.40 ± 0.04*	*By difference*
Crude lipid, % DM	37.1 ± 0.1	Soxhlet, ethyl ether
Ash, % DM	19 ± 1	Oven, 550 °C 5 h+5 h

Table 2. Total amino acid content of BSF prepupae (expressed as g/100 DM and for essential amino acids also as mg/g crude protein) compared with other food protein (Young & Pellet, 1991) and with the FAO/WHO standard protein. Results are the mean of triplicate analysis.

	BSF protein (g/100g DM)	**BSF protein (mg/g protein)**	**Reference protein FAO/WHO 1985 (mg/g protein)**	**Egg white (mg/g protein)**	**Soybean (mg/g protein)**
Essential Amino acids					
His	1.17 ± 0.04	33	15	22	25
Thr	1.49 ± 0.02	42	23	47	38
Val	2.4 ± 0.1	66	39	68	43
Lys	2.3 ± 0.2	65	45	70	63
Ile	1.47 ± 0.09	41	30	53	47
Leu	2.7 ± 0.1	75	59	88	85
Phe	1.3 ± 0.1	36			
Trp	0.21 ± 0.03	9	6	14	11
Cys	1.1 ± 0.1	30			
Phe+Tyr		110	38	91	97
Cys + Met		47	22	66	68
Non essential amino acids					
Asp/Asn	3.6 ± 0.4				
Ser	1.76 ± 0.08				
Glu/Gln	4.2 ± 0.5				
Gly	2.67 ± 0.01				
Arg	1.96 ± 0.03				
Ala	3.8 ± 0.3				
Pro	1.87 ± 0.09				
Tyr	2.6 ± 0.2	74			
Met	0.60 ± 0.04	17			

Table 3 Nitrogen distribution and degree of hydrolysis (DH%) in the different fractions after NaOH extraction or stepwise Osborne fractionation.

Fraction	Relative nitrogen distribution %	Free amino group (mmoles OPA/100g fresh sample)	DH%
One step extraction			
NaOH extract	71 ± 5	42 ± 3	15 % ± 1
HCl extract	10 ± 1	n.d.	
Pellet	17 ± 2	n.d.	
Stepwise Osborne fractionation			
Albumin	23 ± 2	6 ± 1	< 3 %
Globulin	14 ± 2	7 ± 2	< 3 %
Prolamin	9 ± 1	5 ± 1	< 3 %
Glutelin	31 ± 5	5 ± 1	< 3 %
Pellet	23 ± 4	n.d.	

n.d. = not determined

Table 4. Solubilisation ratio and degree of hydrolysis of BSF prepupae treated with different enzymes in the optimum conditions of pH and temperature as indicated by the enzyme suppliers. Results are the mean of three separate hydrolysis experiments.

Enzyme	Temp.	pH	Solution buffer	Solubilisation ratio (N% in solution) without enzyme[a]	Solubilisation ratio (N% in solution) with enzyme[a]	DH% with enzyme[b]
B.Licheniformis protease	60 °C	6.5	10 mM Na_2HPO_4	23 ± 3	57 ± 5	6 ± 1
Pepsin	37 °C	3.0	10 mMHCl	29 ± 2	40 ± 3	17 ± 5
Papain	60 °C	7.5	10 mM Na_2HPO_4, 2mM EDTA, 10 mM DL-cystine	29 ± 3	43 ± 2	25 ± 5
Pancreatin	37 °C	7.8	25 mM NH_4HCO_3, 2.5 mM $CaCl_2$	26 ± 1	46 ± 2	25 ± 6

a Same conditions reported applied with and without enzymes for 16 h. Results from three replicates.

b The DH was calculated taking into account only the proteins present in solution after enzymatic hydrolysis. Results from three replicates. %DH were calculated after subtracting the absorbance of the blank (samples without enzyme)

Figures

Figure 1

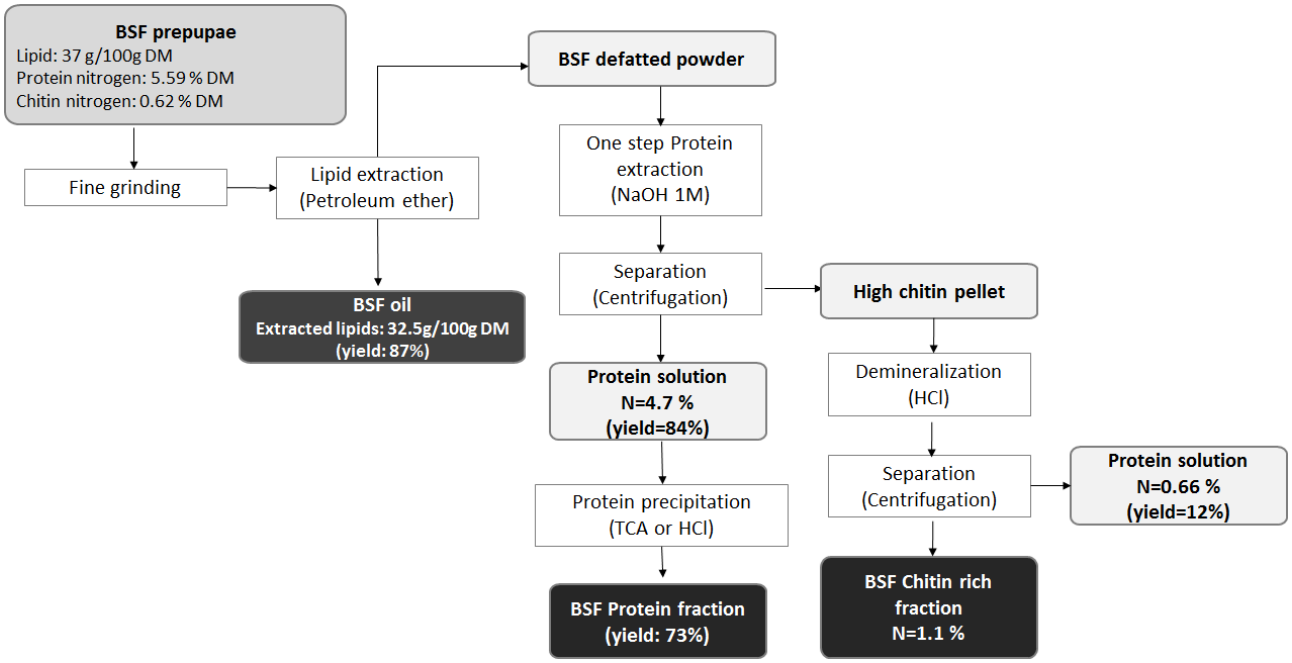


Figure 2

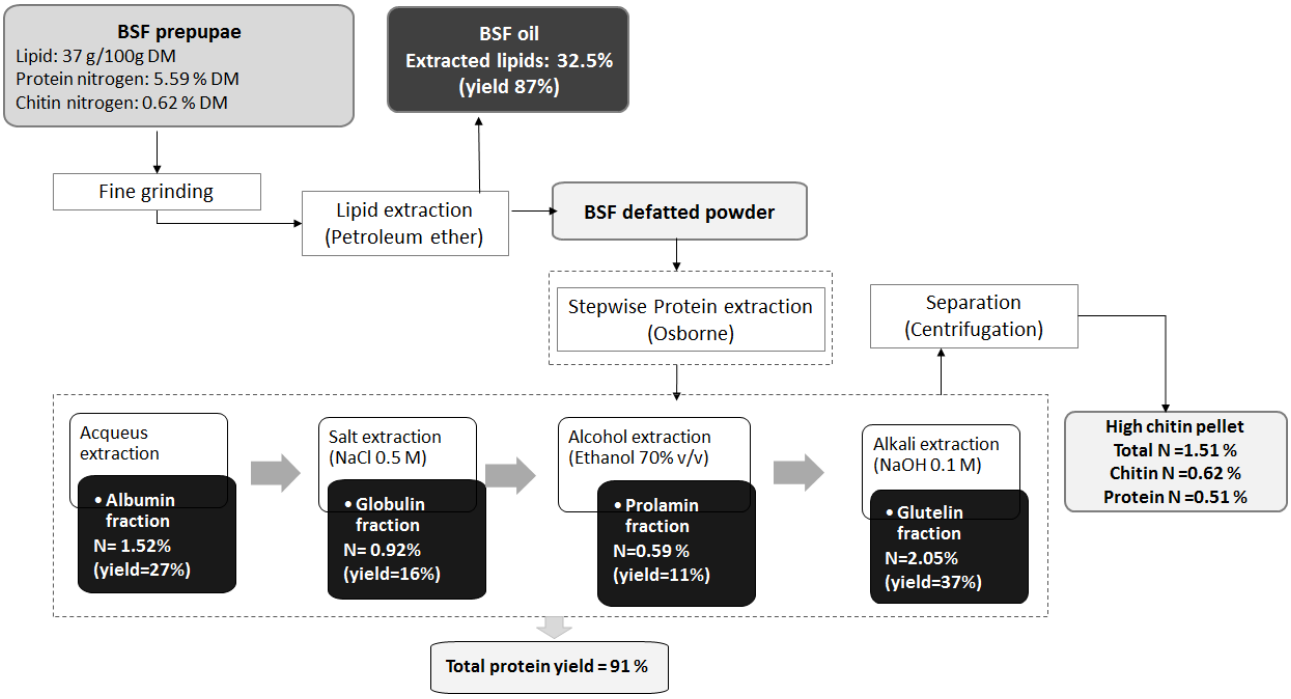
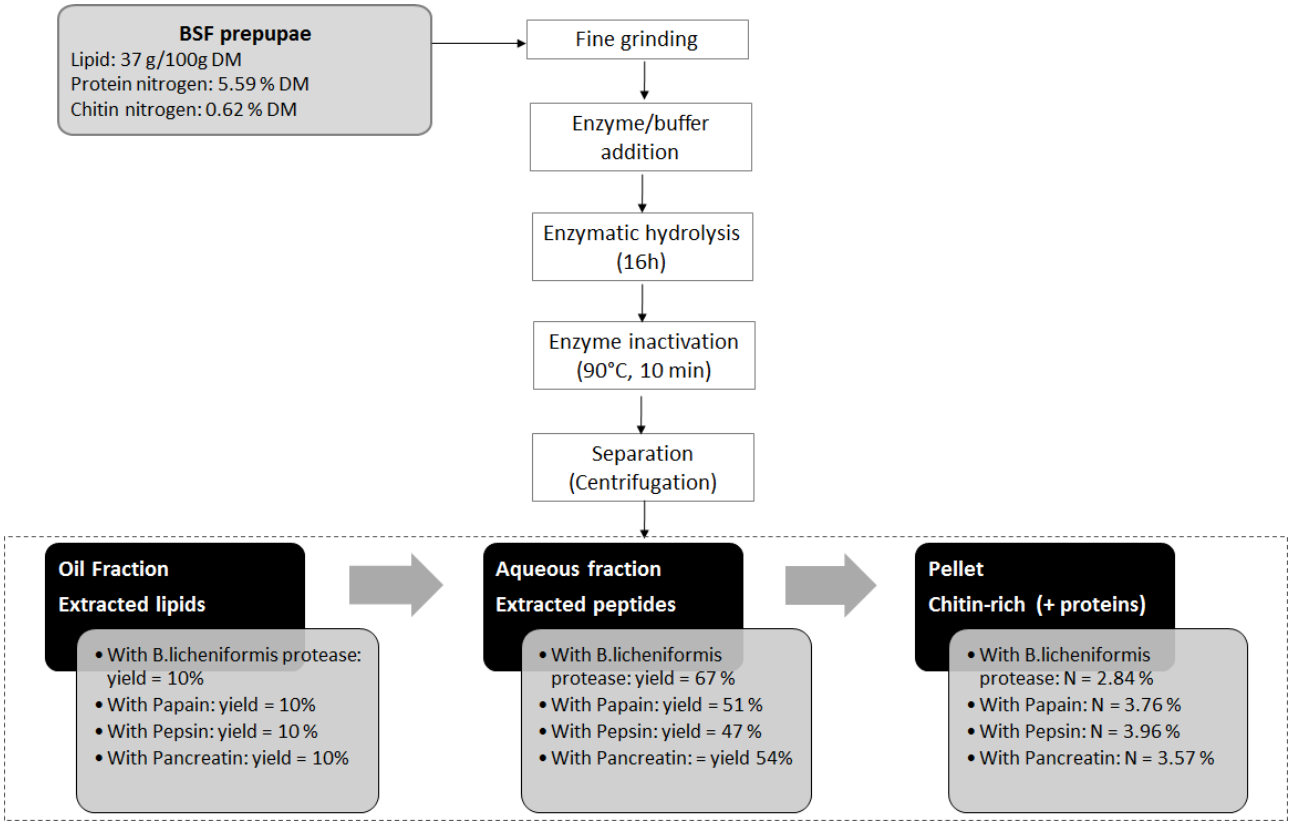

BSF prepupae
Lipid: 37 g/100g DM
Protein nitrogen: 5.59 % DM
Chitin nitrogen: 0.62 % DM
BSF oil
Extracted lipids: 32.5%
(yield 87%)
Fine grinding
Lipid extraction
(Petroleum ether)
BSF defatted powder
Stepwise Protein extraction
(Osborne)
Separation
(Centrifugation)
High chitin pellet
Total N =1.51 %
Chitin N =0.62 %
Protein N =0.51 %
Acqueus extraction
• Albumin fraction
N= 1.52%
(yield=27%)
Salt extraction
(NaCl 0.5 M)
• Globulin fraction
N= 0.92%
(yield=16%)
Alcohol extraction
(Ethanol 70% v/v)
• Prolamin fraction
N=0.59 %
(yield=11%)
Alkali extraction
(NaOH 0.1 M)
• Glutelin fraction
N=2.05%
(yield=37%)
Total protein yield = 91 %

Figure 3


BSF prepupae
Lipid: 37 g/100g DM
Protein nitrogen: 5.59 % DM
Chitin nitrogen: 0.62 % DM
Fine grinding
Enzyme/buffer addition
Enzymatic hydrolysis (16h)
Enzyme inactivation (90°C, 10 min)
Separation (Centrifugation)
Oil Fraction
Extracted lipids
• With B.licheniformis protease: yield = 10%
• With Papain: yield = 10%
• With Pepsin: yield = 10 %
• With Pancreatin: yield = 10%
Aqueous fraction
Extracted peptides
• With B.licheniformis protease: yield = 67 %
• With Papain: yield = 51 %
• With Pepsin: yield = 47 %
• With Pancreatin: = yield 54%
Pellet
Chitin-rich (+ proteins)
• With B.licheniformis protease: N = 2.84 %
• With Papain: N = 3.76 %
• With Pepsin: N = 3.96 %
• With Pancreatin: N = 3.57 %

Graphical Abstract