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Insect-based protein ingredients: extraction, molecular characterization, techno-functionality and safety aspects

Coordinatore:
Chiar.ma Prof.ssa Chiara Dall’Asta

Tutore:
Chiar.mo Prof. Stefano Sforza

Co-Tutore:
Dr. Leen Bastiaens

Dottorando:
Giulia Leni

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Abstract

In the recent years, due to the increasing in world population as well as rising in meat consumption, edible insects have been explored as a sustainable alternative source of animal proteins. In the present PhD thesis two different insect species, lesser mealworm (*Alphitobius diaperinus*) and black soldier fly (*Hermetia illucens*), have been deeply explored for their potential to provide high quality and safe proteins to the feed and food sector. Both insects have been demonstrated to be a valid source of protein material, not only for their amount, but also for the high nutritional profile, rich in essential amino acids. In order to supplement feed and food formulations with insect-based protein ingredients, different extraction protocols have been explored and compared. Besides chemical methods, based on alkali extraction and Osborne fractionation, the enzymatic assisted extraction has been demonstrated to be very promising. As a matter of fact, the use of proteases has been demonstrated to preserve the protein quality and improve digestibility. Further to that, the enzymatic hydrolysis can be tailored in order to reach the desired techno-functionalities, based on the potential end use. Insect protein quality can be hampered by endogenous enzymes. In particular they trigger enzymatic browning in insect protein fractions, with implication also on their chemical and nutritional quality. In the present thesis, blanching treatment, used as killing method for insects, has been identified as valid way to inactivate enzymes and preserve protein quality. In order to evaluate the inclusion of insect protein-based ingredients in the food chain, a safety assessment has also been performed, mostly focusing on mycotoxin presence and allergen potential. Both lesser mealworm and black soldier fly have been determined to pose an allergenic risk for future consumers. In particular, a cross-reactivity between insect proteins and known crustacean allergens has been identified, due to the close homology between taxonomically related species. Through a shotgun proteomics approach, tropomyosin has been identified as prevalent allergen in both insects and its immunoreactivity confirmed with a serum from a patient allergic to crustaceans. The previously mentioned enzymatic assisted extraction has been found to be a valid biotechnological tool in order to reduce the insect protein immunoreactivity. Besides allergens, also the potential bioaccumulation of mycotoxins in insect body was studied. Indeed, one of the main potentials in using insects is their ability to grow on a different spectrum of substrates, and potentially contaminated by-products from the agri-food chain can be used to feed them. In the present thesis, insects, grown on mycotoxin contaminated substrates, were demonstrated not to bioaccumulate mycotoxins into their body, but rather to excrete and/or metabolize them in not yet known forms.

In conclusion, the results obtained in this PhD thesis fully support edible insects as a promising and sustainable alternative source of proteins which could be introduced in both feed and food chain in order to meet the future high-quality protein demand.

CHAPTER 1

General Introduction

Novel solutions for food security for a growing population

The perspective of a growing population as presented by FAO, has focused the attention on the need to find new sources to feed the world. In 2050 the world population is expected to reach 9.7 billion people and, due to the currently increase in meat consumption in developed nations, the global livestock production has been projected to increase by 70 % between 2000 and 2030 and, as consequence, the feed production by 60 % (UN DESA, 2019; FAO, 2009).

Until half a century ago, most feed consisted of grass and by-products, cereals and other crops being too expensive to be used as feed (Westhoek et al., 2011). From the 1950s the crop production rapidly increased due to the technological innovations concerning fertilisation, irrigation, mechanisation and improved varieties (Chadd et al., 2002). Nowadays the main three types of feed ingredients can be distinguished in grass, crops and by-products (e.g. meals from oil production such as soybeans, sunflowers and rapeseed). Between these products, energy-based and protein-based feed compounds can be distinguished. Cereals and their derivates belong to the energy-based feed category, with a third of the global production currently used for this purpose. On the contrary, legumes and their by-products are considered protein-based feed, with soybean meal which has become the main feed protein source (Westhoek et al., 2011). But, the increase in non-sustainable crop production has resulted in a dramatic impact on the occupied agricultural land, water consumption and greenhouse gas-emission (Boland et al., 2013). On the other side, livestock production itself has high impact on these factors. It has been calculated that to produce 1 kg of live animal weight, 2.5 kg of feed and 2300 litres of water are required for poultry, 5 kg and 3500 litres for pork and 10 kg and 22000 litres for cattle (FAO, 2006). The agricultural land occupied by 1 kg of common livestock ranges between 5 m^2 for chicken to 420 m^2 for cattle (Flachowsky et al., 2017). Furthermore, livestock rearing is responsible for the 18 % of greenhouse gases emissions mainly due to the carbon dioxide (fertilizer production for feed crops, feed and animal transport, animal product processing), methane (fermentation in ruminants and manure) and nitrous oxide (animal manure and urine) (Kamemura et al., 2019). All these numbers are expected to significantly increase in the next decades, outlining how the availability of sustainably produced meat is a relevant challenge for the future.

In this scenario, reduce of per capita meat consumption worldwide, and finding alternative protein sources to substitute the existing high impacting ones are mandatory. Vegetable proteins could be a natural substitute for common animal proteins, since they are normally included in people's diet and have lower production associated costs. Unfortunately, they cannot represent a complete replacement of animal protein. In fact, vegetable proteins,

even when having a high protein content, are deficient in essential amino acids necessary for human and nutrition (Fasolin et al., 2019) and have to be well balanced together in order to fill the gap. Furthermore, the agriculture and crop production increasing, if carried out in a not sustainable way, lead to decrease the soil fertility, increase the pollution of water resources and improve the deforestation and desertification (Gahukar, 2016). A second solution can be the replacement of common animal protein with novel protein sources (e.g. algae, insects) (Smetana et al., 2016). Marine plants, such algae (e.g. *Spirulina*, *Chlorella* spp.), represent a promising solution. Their amino acid content is comparable to common protein sources such as eggs or soybean, but their development has been hampered by the high costs related to their production and thee technical difficulties for protein extraction and refining (Henchion et al., 2017). Among the novel sources, insects could be a cost-efficient and environmentally friendly alternative to obtain proteins.

Insects, in comparison to common livestock (e.g. cattle, pig), require significantly less land and waters for their farming and emit fewer ammonia (Sun-Waterhouse et al., 2016). The production of 1 kg of insects (mealworm, cricket and locust) generates 60 mg per day of ammonia pollution and requires 18 m^2 of land, 1.7 kg of feed and 18 L for their farming (Gahukar, 2016). This is mostly related to the fact that insects are cold-blooded and do not require feed to maintain body temperature (FAO, 2006).

Thus, insects are very efficient to biotransform a wide variety of organic materials into edible biomass (Rajendran et al.,2018). As a matter of fact, insects can be fed with a diverse spectrum of substrates, urban wastes included (Pastor et al., 2015). This is not of secondary importance, since in a circular economy perspective they could be explored as “indirect” biorefinery tool. In fact, low-value side streams, such as urban wastes and agri-food by-products, can be used as substrate of growth for insects which on turns are able to convert them into final products with high-added value, such as protein.

The attention received by insects as a novel protein source for feed and food purposes is confirmed by the exponential increase in scientific researches on edible insects (Figure 1).

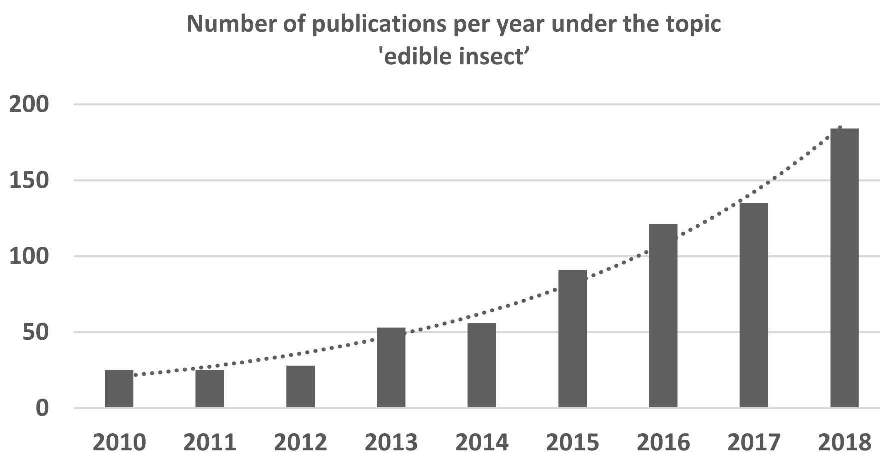

Figure 1 Number of scientific researches published from 2010 to 2018 and found with the key word "edible insect" listed in the source index of the Scopus, August 2019.

Insects as source of feed and food: a logical choice

Insects belong, just like the crustaceans (e.g. shrimp, crab, lobster), to the Arthropoda phylum and count more than one million species (Rumpold & Schlüter, 2013b). Entomophagy, the consumption of insects, is widely practised in many regions of the world (e.g. Africa, Asia, South America). More than 2000 insect species are consumed as food worldwide, but only some of them are taken into account for the breading in Europe, such as for example crickets (e.g *Acheta domesticus*), mealworms (e.g. *Tenebrio molitor*, *Alphitobius diaperinus*) and the black soldier fly (*Hermetia illucens*) (van Huis, 2013). Nevertheless, this practice is considered disgusting for European Union and north American inhabitants, who have no history of consuming insects (Sogari et al., 2017).

As a matter of fact, on the contrary, insects often represent a valid source of nutritional compounds, rich in fat, protein and micronutrients. Due to the large number of species presented in nature, these nutrients are indeed variable among the different species. For example, between the species mentioned before, *Acheta domesticus* presented the highest protein content, while *Hermetia illucens* the highest lipid amount (Figure 2).

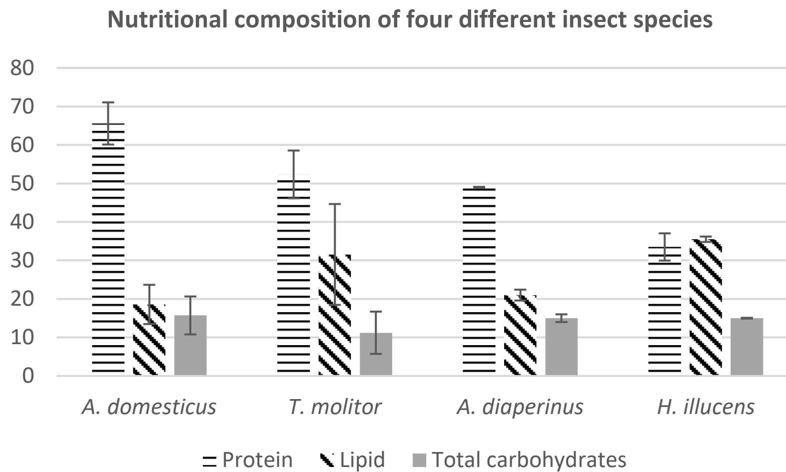

Figure 2 Nutritional composition, in terms of protein, lipid and total carbohydrates content, of four different insect species. Results are expressed as the mean of different data collected in literature (Rumpold & Schlüter, 2013; Bußler et al., 2016; Yi et al., 2013; Janssen et al., 2017)

In general, the lipid fraction of edible insects can range between the 10 % to the 60 % on dry basis, with the larval stage having more fat than the adult one (Rumpold & Schlüter, 2013a). Fats are necessary for the insect metamorphosis, since lipids serve as an energy reserve. The insect exoskeleton is mainly composed by chitin, which can range in the different species between 1 % to 14 % on dry matter basis (Rumpold & Schlüter, 2013a). Chitin is a polymer of N-acetylglucosamine and is considered as an indigestible fibre (FAO, 2013). As well as for lipid, also the protein content is extremely variable and can range between the 13 % to the 77 % on dry basis, with the adult insects which contain more (Kouřimská & Adámková, 2016). It is to be remarked that this value in many cases might be an overestimation of the real protein content, due to the presence of a significant amount of non-protein nitrogen, mainly associated to chitin (Janssen et al., 2017), which is erroneously considered as protein during standard protein analysis through Kjeldahl.

The advantage of eating insects becomes even higher when considering the edible mass (van Huis, 2013). In fact, it has been estimated that up to 80 % of a cricket is edible and digestible, compared with 55 % for poultry and swine and 40 % for cattle (FAO, 2006). This makes even less environmental impacting, beside the advantages described above, the conversion of feed into insect biomass for edible purposes.

Insect protein quality

Proteins represent one of the three macronutrient families and are complex polymers build up from different sequences of 20 amino acids, connected through peptide bonds. Amino acids are classified as non-essential and essential, based on their ability to be synthetized *de novo* by the organism or to have to be necessarily eaten with the diet. For humans, 9 amino acids are considered essential: histidine, threonine, leucine, lysine, methionine, isoleucine, phenylalanine, valine, tryptophan (Bohrer, 2017).

Nutritional properties

The nutritional value of proteins is variable and is governed by amino acid composition, essential amino acid ratio, susceptibility to hydrolysis during gastrointestinal digestion and absorption by the intestine (Friedman, 1996). Animal proteins (e.g. meat, seafood, eggs, and dairy products) are also called as “high quality” proteins, due to the large amounts of essential amino acids and their presence in the quantity required by the human body (Bohrer, 2017). On the contrary, plant proteins have poorer quality, since they do not display all the essential amino acids required for human and animal nutrition, and comprise anti-nutritional factors (Lynch et al., 2018). Among plants, legumes, and in particular soy, are considered an important source of dietary proteins but, since they are deficient in sulphurated amino acids, their fortification with another protein source which contains them is necessary (Henchion et al., 2017).

Insects are considered an interesting alternative source of animal proteins, not only for their protein content, which generally is higher (up to about 50–80 % dry weight) than most plants or many animal products, but also for the good amino acid profile (Sun-Waterhouse et al., 2016). In fact, they are an optimum source of all the essential amino acids, which also represent more than the 50 % of total ones (FAO, 2010). Rumpold & Schluter (2013) collected the data reported in literature about the compositional profile of 236 edible insects with an essential amino acids content comparable to soy and bovine casein, two important protein sources from plant and animal origin.

Table 1 Essential amino acids profile of five different insect species (*Bombyx mori, Acheta domesticus, Tenebrio molitor, Zophabas morio, Musca domestica*) compared with two important food sources of protein (egg and soybeans) (Zielińska et al., 2018). The results are expressed as mg/g of protein.

EAA (mg/g protein)	Egg	Soybeans	Silkworm (*Bombyx mori*)	House cricket (*Acheta domesticus*)	Mealworm (*Tenebrio molitor*)	Giant mealworm (*Zophobas morio*)	Common housefly (*Musca domestica*)
His	25	30	28	24	33	31	31
Ile	53	54	33	41	47	47	23
Leu	86	91	51	83	94	97	45
Lys	73	74	49	52	55	52	82
Met	30	15	13	17	16	11	37
Phe	54	58	29	31	35	35	56
Thr	44	48	30	7	38	40	36
Trp	13	16	7	34	10	9	50
Val	68	56	40	50	64	52	46
Total	469	460	288	348	400	381	410

EAA, essential amino acids; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Trp, tryptophan; Val, val

In Table 1 it is reported a comparison between the essential amino acid profile of five common edible insects (*Bombyx mori, Acheta domesticus, Tenebrio molitor, Zophabas morio, Musca domestica*) and two important food protein sources (egg and soybean). Egg and soybean contain respectively 48 % and 40 % of protein on dry weight (Eddin et al., 2019; Preece et al., 2017), significantly less than the protein content of these insect species. In fact, the five insect species reported in the table present a protein content which range between 50 %, in *Zophabas morio*, to 68 %, in *Acheta domesticus*. (Yi et al., 2013; da Silva Lucas et al., 2020). In general, the different insect species present a slightly different amino acids profile, with *Musca domestica* and *Tenebrio molitor* which are closer to soybean and egg. *Tenebrio molitor* amino acid profile fulfils all the requirement for human nutrition. The above species are rich in histidine, tryptophan and methionine, while other insects, such as termites, in lysine, tryptophan and threonine, which are deficient in certain cereal proteins (Kouřimská & Adámková, 2016). Besides amino acids composition, the protein digestibility is also an important parameter that can give information about the protein quality. This information measures of how well a human or animal can digest and absorb amino acids from dietary protein sources (Loveday, 2019). Some studies have already described the protein digestibility of insects. Longvah et al. (2011) evaluated the digestibility of silkworm and determined that it was much higher than the digestibility of different legumes food, peanut and whole wheat. Jensen et al. (2019) studied the digestibility of lesser mealworm identifying the potentiality of this insect as food source due to its high protein content and true protein digestibility (91-94 %). Marono et al. (2015) evaluated *in vitro* the protein digestibility of black soldier fly, determining a crude protein digestibility of 98 %. Nevertheless, the presence of chitin seems to significantly impair the insect protein digestibility, which could be improved by its removal during processing (Yi et al., 2016).

The protein quality could be affected not only by the species, but also by the sex, the metamorphosis stage, the composition of substrate used for feed and the processing (Oonincx et al., 2015; Spranghers et al., 2016). Protein can be extracted from insects, as well as other biomass, by many different methods, either chemicals or enzymatic (Table 2). Chemical methods, based on protein solubility at various pH and ionic strengths, can be used to obtain high yield in protein fractions (Yi et al., 2017). Alkali extraction is one of the most used, giving high final yield, even if it leads to amino acid degradation (e.g. racemisation, lysinoalanine formation, and other reactions) (Hayashi & Kameda, 1980), impairing the nutritional value of the proteins. The enzymatic hydrolysis represents a valid alternative, since milder conditions of extraction are applied, allowing to preserve the protein nutritional quality. Sometimes, a defatting step or chitin removal is applied as pre-

treatment to protein extraction, in order to maximise the final protein recovery (Belluco et al., 2013).

Table 2 Protein extraction protocols applied on insect biomass reported in literature, with information about the specific insect species, the condition applied and the protein extraction yield.

Insect species	Protein extraction	Yield	Reference
T. molitor ***Z. morio*** ***A. diaperinus*** ***A. domesticus*** ***B. dubia***	Demi water + ascorbic acid, 1 min at 20°C	23% 17% ≈20% ≈20% ≈20%	Yi et al., 2013
T. molitor	pH and NaCl (0.1 M NaCl, pH=10, overnight at 4°C + 1 h at 20°C)	100%	Yi et al., 2017
T. molitor[a]	0.25 M NaOH 40°C for 1 hour (2 times)	70%	Zhao et al, 2016
T. molitor[b] ***H. illucens***[b]	1 M NaOH, 60°C for 1 h + isoelectric precipitation with 1 M HCl pH 4 and 2	nd nd	Bußler et al., 2016
G. sigillatus	Alcalase assisted extraction (0.1% E/S, 30 min, pH 8)	88%	Hall et al., 2017
L. migratoria	Alcalase (0.5% E/S) + Flavourzyme (1% E/S) assisted extraction (2 h, 60°C, pH 8)	55%	Purschke et al., 2018

[a], protein extraction applied on defatted (EtOH for 1 h) flour; [b], protein extraction applied on defatted (Hexane for 1 h) flour; nd, not determined.

The highest extraction yield was obtained by Yi et al. (2017) on *Tenebrio molitor* flour with the combined application of salt and alkali, after a long time of reaction. Other authors reduced the time of alkali extraction by applying on the same insect a pre-defatting step with hexane or ethanol (Bußler et al., 2016; Zhao et al, 2016). As an example of a different approach, Hall et al. (2017) obtained the 88 % of yield by performing, on *Gryllodes sigillatus,* an enzymatic assisted extraction with Alcalase after only 30 minutes of reaction. It is clear that different extraction methods are being explored in order to find a good compromise between yield and economic impact, even if a direct comparison remains difficult due to the different insect species and/or technological conditions applied.

Techno-functional properties

Nowadays, the best way to obtain the benefits of insect consumption is to employ insect-based ingredients in feed and food formulations (Sogari et al., 2017). The inclusion of insect proteins as supplement requires extensive knowledge on their properties, which include

the amino acid profile, heat stability and techno-functional properties, which can directly impact on sensorial attributes of food products (e.g. texture, taste) (Lacou et al., 2016).

The techno-functional properties include protein solubility, water and oil binding, gelling, foaming and emulsifying capacity, and are influenced by the protein interaction inside the food matrix. In fact, the structural versatility of proteins and their amphiphilic nature drive their interaction with several compounds (e.g. proteins, carbohydrates) (Alves & Tavares, 2019). These interactions can occur with the formation of hydrogen bounds, hydrophobic and/or electrostatic interactions and can be affected by the applied conditions for protein extraction (Papadopoulos, 1989).

Protein solubility is important in many uses and it is a pre-requisite for formation of emulsions, foams, and gels in food technology. Generally, the determinants which affect protein solubility can be divided in intrinsic factors, such as protein structure and molecular interaction, and extrinsic factors, as pH, temperature and ionic strength. Hydrophobic and ionic interactions are the major driving forces of protein solubility property. Hydrophobic interactions, which promote the interaction between proteins, result in decreased solubility, whereas ionic interactions, which promote the interaction between protein and water, result in increased solubility (Kristinsson & Rasco, 2000). Protein structure, including amino acid sequence, three-dimensional structure and length, also affect this property. For example, reduce the protein size, such as with hydrolysis, improves solubility properties of food proteins (Kristinsson & Rasco, 2000). This phenomenon has also been observed in edible insects, when cricket and *Locusta migratoria* were subjected to enzymatic hydrolysis (Hall et al., 2017b; Purschke et al., 2018). As extrinsic factor, temperature is reported to positively influence protein solubility, up to the point where proteins start to denature (Bußler et al., 2018). After denaturation, the exposure of hydrophobic side chains influences the protein aggregation and so, protein precipitation (Sathe et al., 2018). Solubility is also affected by pH, with the isoelectric point which represents the pH in which there is the minimum protein solubility. In fact, near to this pH, the protein presents neutral net charge, affecting the water-protein interaction and improving the protein aggregation, thus resulting in protein precipitation (Sathe et al., 2018). Insect proteins demonstrated to have the same trend of common food proteins, showing maximum of solubility at extreme alkaline pH and the isoelectric point near pH 4 (Yi et al., 2013; Bußler et al., 2016; Purschke et al., 2018).

Protein gelling property is described as the proteins ability to create structured network to retain water molecules (Gravel & Doyen, 2020). Heat is a key factor for gel formation. In fact, heat induces the partial unfolding of native proteins, resulting in the exposure of non-polar residues and promoting gel formation (Munialo et al., 2017). Yi et al. (2013) evaluated

the ability of protein concentrates isolated from five different insect species to form stable gel, demonstrating their potential use as gelling agents or texturizers in food. This feasibility was demonstrated by Mariod et al. (2013) who compared the protein gelatine obtained from *Aspongubus viduatus* and *Agonoscelis pubescens,* two beetle species, with a commercial one for stabilizing ice cream and did not find significant differences.

Water and oil binding capacity define the ability of proteins to bind and retain water/oil against gravitational force. If the water holding capacity improves the final texture, the oil holding property contributes in the flavour perception, both critical points in feed and food formulations (Kristinsson & Rasco, 2000; Guichard, 2002). The different extraction methods, which can be applied for extracting proteins from insects, strictly affect the final water and oil binding capacity. For example, protein fraction obtained by alkali extraction from *Gryllodes sigillatus, Schistocerca gregaria* and *Tenebrio molitor* demonstrated to have high oil and water binding capacity. In particular, they presented value higher than pulse protein concentrate and similar to those of soy and milk protein concentrates (Zielińska et al., 2018). Purschke et al. (2017b) demonstrated the ability of enzymatic hydrolysis to improve the oil binding capacity of *Locusta migratoria* protein flour, obtaining results similar to those of common plant seed proteins. On the contrary, the hydrolysis seemed not to affect the water oil capacity, which remained lower than the refence proteins.

Emulsifying and foaming properties are defined by the ability of fat and air droplets to be dispersed in a liquid and this is connected to surface protein properties (Bußler et al., 2016). In particular, proteins are soluble surfactant, which possess the capacity to lower the interfacial tension between hydrophobic and hydrophilic compounds in foods and form a protective barrier around fat or air droplets (Zayas, 1997). Conventional proteins such as egg white, soy, casein and whey proteins from milk are commonly used as additives for their emulsifying and foaming properties (Van der Ven et al., 2001; Kinsella, 1981). Instead, insect proteins demonstrated to have lower foam and emulsion ability than conventional proteins, even if the different processing for extracting them can have a direct effect on these properties (del Valle et al., 1982; Gravel & Doyen, 2020).

Certainly, more data are needed in order to better clarify insect protein techno-functionality, and their link to the extraction/processing method. Furthermore, more comparative studies should be performed to truly compare the functionality of various insect proteins to conventional proteins within the same processing methods.

Insects as food and feed: the legal framework

In the European Union a package of legal texts, continuously being updated, establishes the limitations and permissions related to the inclusion of insects in the feed and food sector.

Since 2018 insects have joined the category of novel food, and the European Food Safety Authority (EFSA) opinion is mandatory before their marketing (EU Regulation 2015/2283, 2015). Before this date, a gap in the legal texts did not pose the limitation about insect marketing as food and as a consequence each member state decided autonomously. In the country where insects were legal (e.g. The Netherlands), the European Commission gave to food industry, which were marketing products, a window of 9 months for applying to have the opinion of EFSA.

For feed purposes the legal aspect is even more complicated. Whole insects, live or dead, and fat from them, can be used for feeding every animal species (EU Regulation 2017/1017, 2017), while insect proteins can be used to feed pet and fur animals and for aquaculture. In this last case, only seven insect species are authorized: the house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*), field cricket (*Gryllus assimilis*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), black soldier fly *(Hermetia illucens*), and the common house fly (*Musca domestica*) (EU Regulation 2017/893, 2017). Moreover, the European Union is evaluating the possibility to extend the use of insect proteins also as feed for poultry and pigs, and an amendments is expected in 2020 (Sogari et al., 2019).

Risk for insect consumption

Beside all the interesting beneficial related to the consumption of edible insects, caution needs to be exerted regarding endogenous and exogenous risk factors related to their consumption. Quite interestingly, the 50 % of scientific research with topic “safety of edible insects” have been published by European groups, even if the European Union has not an entomophagy culture, followed by African with the 30% (Murefu et al., 2019). This underlines a strong effort of western countries not only for the acceptance of edible insects, but also for the investigation on insect safety consumption.

In 2015 EFSA examined for the first time the biological and chemical risks related to the consumption of insects as feed and food (EFSA, 2015). It was outlined that the substrates

used for insect growth can be the origin of chemical (e.g. agrochemicals, mycotoxins, heavy metals) and microbiological risks. Between heavy metals, Cd and Pb were reported to be accumulated in black soldier fly and, in general, Cd to be the most impacting on insect growth performance (EFSA, 2015; Purschke et al., 2017). On the contrary agrochemicals and mycotoxins seem not to be a serious concern (Purschke et al., 2017). Few works have investigated the impact of mycotoxins on insect growth and results demonstrated the absence of any impact on growth performance (Camenzuli et al., 2018; Purschke et al., 2017; Van Broekhoven et al., 2017). Nevertheless, insects seem to metabolize mycotoxins in forms not yet known, which could be more or less toxic than the parental compound (Schrögel & Wätjen, 2019). Anyway, more research is needed in order to deeply investigate these issues.

Besides exogenous risks, insects themselves present potential hazards for the future human consumption. In particular, focusing on the protein fraction, the presence of potential allergens cannot be excluded.

Insects are well known to be responsible for non-food allergic reactions, caused by bites or stings, but in Europe no data are reported about allergic reaction after insect consumption. In China, where the entomophagy is a normal practice, edible insects, beside common allergic foods, such as nuts, vegetables, fruits, fish, meat and eggs, are deemed responsible of anaphylactic shock (Ji et al., 2009). Furthermore, the cross-reactivity between insect proteins and taxonomically related allergens could be another potential risk. In this case, clinical symptoms of cross-reactivity can occur with homologous proteins between Arthropoda species (e.g. mites, midge, crustaceans) (Downs et al., 2016). Some works have demonstrated *in vitro* the presence of cross-reactivity between Arthropoda, well known to be responsible of allergy, and different insect species (e.g. *Gryllus bimaculatus, Tenebrio molitor, Zophobas atratus, Alphitobius diaperinus* and *Hermetia illucens*) (Ribeiro et al., 2018). The potential allergenic proteins are: tropomyosin, arginine kinase, paramyosin, hexamerin, myosin, which presented more than the 80 % of similarity with known allergens (EFSA, 2015).

For the future consumption, it is important also to assess the effect of processing on insect allergens. In literature there are contrasting data on the effect of processing on allergens. The heat treatment, for example, which in normally applied to stabilize food before consumption, it was demonstrated to both increase, decrease and maintain stable the allergenic potential of insect proteins (de Gier & Verhoeckx, 2018). This different behaviour

could be related to the different target proteins, the different thermal treatments applied (temperature/time) and the different insect species.

Aim and outline of this thesis

The literature information about insect protein fractions have demonstrated the potential of using insects as a food and feed source. However, some challenges for their future employment remain:

- Investigate different method to extract protein from insect, by reducing the protein damage and preserving the protein quality;
- Evaluate the safety for future consumers of insect-based protein ingredients;
- Investigate a potential biotechnology which could reduce the allergic risk connected to insect consumption.

The objective of this PhD thesis is to investigate the protein quality and safety aspects of insects in order to gather knowledge for including insect-based protein ingredients in food and feed formulation.

In **Chapter 2,** black soldier fly (*Hermetia illucens*) larvae are evaluated as potential protein source. The amino acids profile is reported and used for properly calculating the protein content, which normally can be over-estimated by common determination based on Kjeldahl analysis. Moreover, three different protein extraction protocols were developed, differing for the final recovery and protein integrity. The different approaches are discussed and compared.

In **Chapter 3**, a metabolic study is applied in order to explore the driving force in the browning occurrence in insect proteins. The metabolic changes resulting in enzymatic browning and its effect on protein chemical and physical properties were reported.

In **Chapter 4**, the enzymatic assisted extraction is better explored and evaluated as a promising technology for the extraction of insect-based feed and food protein ingredients. Seven different enzymes were employed for extracting protein material from black soldier fly and lesser mealworm (*Alphitobius diaperinus).* Their diverse proteolytic activity is compared based on the yield of extraction, the free amino acids released and the degree of hydrolysis.

The protein hydrolysates obtained from lesser mealworm by enzymatic assisted extraction are characterized based on their techno-functionality in **Chapter 5**, in order to evaluate

their future inclusion as ingredients in food and feed matrices. The effect of degree of hydrolysis on the solubility property, oil holding ability, foaming and emulsion capacity is explored and studied.

The potential risk related to the use of insect-based protein ingredients in food and feed formulations are also investigated. In **Chapter 6** a proteomic study is deployed for the identification of prevalent allergens in both black soldier fly and lesser mealworm and confirmed by testing immunoassays. The enzymatic assisted extraction, as described in Chapter 4, is here evaluated as biotechnological tool for the production of hypoallergenic extracts. Furthermore, the exogenous risks are explored in **Chapter 7**, by evaluating the potential bioaccumulation of mycotoxins in insect grown on different substrates naturally contaminated.

Finally, the relevance of this research and implication for future application is discussed in **Chapter 8.**

CHAPTER 2

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

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

Author: Augusta Caligiani,Angela Marseglia,Giulia Leni,Stefania Baldassarre,Lara Maistrello,Arnaldo Dossena,Stefano Sforza

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Abstract

Black soldier fly (BSF, *Hermetia illucens*) represents 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 or enzymatic, to recover pure fat, protein and chitin fractions. First, exact proximate composition of the prepupae samples were determined. BSF prepupae biomass contained 32% proteins, 37% lipids, 19% minerals, 9% chitin, expressed on dry matter. The lipid fraction was easily recovered by organic solvents, while the most challenging issue was the separation of protein from chitin, due to the cross-linking between these two components. This separation was achieved first with a one-shot total protein extraction, using alkali condition. A different milder extraction method was also tested, following the stepwise method proposed by Osborne, commonly used for cereal matrix. Both methods allowed to obtain a good recovery of the protein fraction (about 90%), but only in the second one the extracted proteins preserved the integrity of their structure, as determined by calculating the degree of hydrolysis with o-phthaldialdehyde assay. As an alternative to the previous two chemical processes, proteolytic enzymes were also tested. Among the enzymes tested, the protease from Bacillus licheniformis resulted in a higher degree of nitrogen solubilisation (60 %) and lower degree of hydrolysis (6 %) in comparison to other enzymes.

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 et al., 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 et al., 2011). One of the main advantages of using BSF as waste bio converter is that adult flies do not eat, thus avoiding any disease transmission risks (Sheppard et al., 2002). In fact, after metamorphosis the adult flies lose mouth parts and digestive system, thus stopping to feed (Singh & Kumari, 2019).

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 (Cummins et al., 2017; Newton et al., 1977) and as a pet food (Bosch et al., 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 et al., 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 et al., 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 et al., 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, Mena, and Bourges (1982) performed protein extraction from *Anastrepha ludens* at pH 10 and subsequent protein precipitation at pH 5. Yi et al. (2013) performed an aqueous protein

extraction from five insect species. Recently Bußler et al. (2016) compared different protein extraction methods from *Tenebrio molitor* and *Hermetia illucens*. 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 & Hours, 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 subsequentially 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, Ltryptophan, Supelco 37 component FAME mix, chitin, glucosamine, Nacetylglucosamine, beta-phenylglucoside, Pepsin from porcine gastric mucosa (250 U/mg, 117K0811), Protease from Bacillus licheniformis (2.4 U/g, SLBL2953V), pancreatin from porcine pancreas (4xUSP, SLBM4075V), papain from papaya latex (1.5–10 U/g, SLBJ6115V), DLcystine, 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, (Section 2.3) 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 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 et al. (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, 2.1 × 150 mm). The mobile phase was composed by H2O + 0.2% CH3CN +0.1% HCOOH (eluent A) and

CH3CN + 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 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. (2001) with some modifications. Briefly, 100 mg of BSF prepupae were hydrolysed in 25 mL of 7 M 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 bistrimethylsyliltrifluorocetamide (BSTFA). The same procedure was applied to a chitin standard (Sigma Aldrich) to calculate the recovery. The solutions were split-injected (1 µL) 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, Bellefonte, 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, analysed 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%.

2.5. Extraction protocol 1: chemical method with one step protein extraction

2.5.1. 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.

2.5.2. 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% trichloroacetic acid 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 2 h.

2.5.3. 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.

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

The second protocol applied the same procedure for lipid extraction as reported in Section 2.5.1. 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 h 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 h 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 h 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 Section 2.5.3.

2.7. 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 6). 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.

2.8. 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 3). 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 3). 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 3.

2.9. Determination of the integrity of protein fraction by degree of hydrolysis determination

The protein degree of hydrolysis (DH) was calculated using ophthaldialdehyde (OPA) method described by Spellman et al. (2003) with some modifications. 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 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, Oklahoma 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.

Table 3 Proximate composition of BSF prepupae. Value are expressed on dry matter basis (DM). Moisture content: 66 ± 1%. Results from triplicate analyses.

Proximate composition	
Crude total nitrogen, % DM	6.61 ± 0.05
Total amino acids, % DM	36 ± 2
Total protein % DM	32 ± 2
Protein nitrogen, % DM	5.59 ± 0.05
Total non-protein nitrogen, % DM	1.02 ± 0.04
Total chitin, % DM	9 ± 1
Chitin nitrogen, % DM	0.62 ± 0.06
Residual nitrogen (non-protein, non-chitin compounds)	0.40 ± 0.04
Crude lipid, % DM	37.1 ± 0.1
Ash, % DM	19 ± 1

The dry matter (DM) content of BSF prepupae was found to be 34 ± 1%, in accordance with the results of previous studies (Sheppard et al., 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 et al., 2016). The protein content, as determined by Kjeldahl, 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 3), 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 (Table 4), both in absolute (g/100 g DM) as well as relative terms (mg of each amino acid/g of crude protein).

Considering the amino acid distribution as reported in Table 4, 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 et al., 2017). In order to estimate the contribution of nitrogen originating from proteins, as determined by Kjeldahl, 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 4 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.

Table 4 Total amino acid content of BSF prepupae (expressed as g/100 DM and for essential amino acids also as mg/g crude protein) with a comparison with two important food protein from animal and vegetable origin (egg white and soybean) (Young & Pellett, 1991) and with the FAO/WHO standard protein. Results are the mean of triplicate analysis and are expressed as the mean ± standard deviation.

	BSF protein (g/100 g 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	3.9 ± 0.3	110	38	91	97
Cys + Met	1.7 ± 0.1	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				
Met	0.60 ± 0.04				

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 prepupae 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 (see 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 3) coming from sources which are not protein and not chitin. This might include melanin. Indeed, according to Ushakova et al. (2017), 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. 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 extraction 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 Figure 3 and 4. Enzymatic protocol steps are reported in Figure 5. 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 Section 3.1 (Table 3). 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 Figure 3.

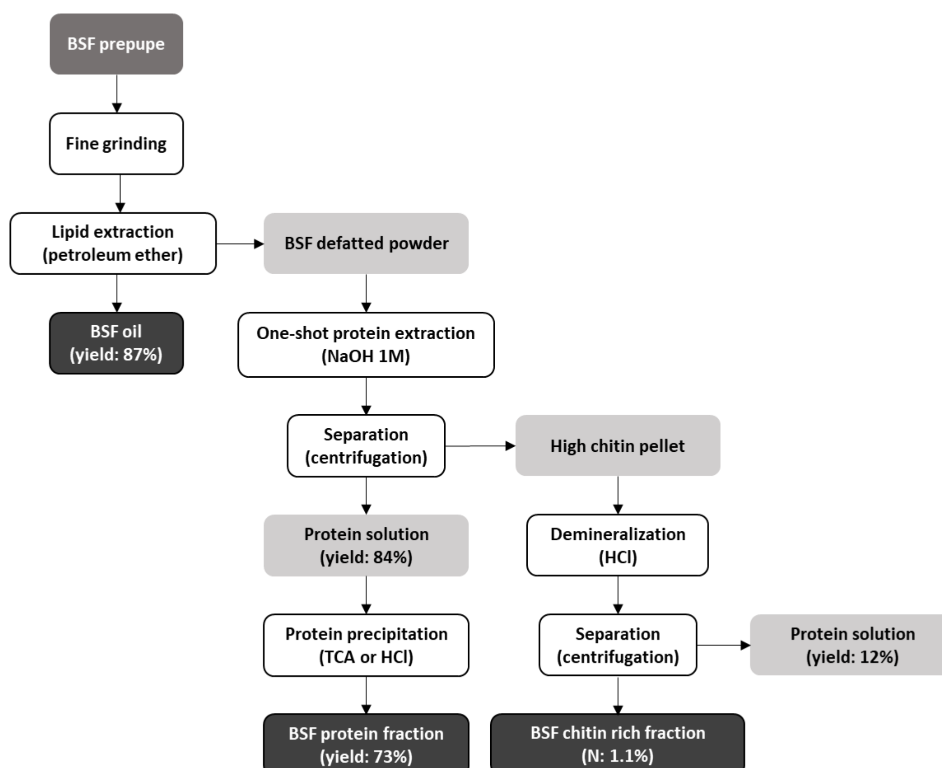

Figure 3 Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 1 (Chemical method). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

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. Then, 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 et al., 2011; Zhang et al., 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. The protein fraction extracted with NaOH was assessed for the nitrogen content: the percentage of nitrogen accounted for 4.7 ± 0.5 % on dry matter (see Figure 3), corresponding to 84% recovery of protein fraction. A residual protein fraction (N % = 0.7 ± 0.2, 12 % of protein content, Figure 3) was further solubilized during

the following acidic demineralization step. The amount of nitrogen then left in the pellet (1.1 ± 0.2 %, Figure 3) 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 5), 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 et al., 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 (Figure 4).

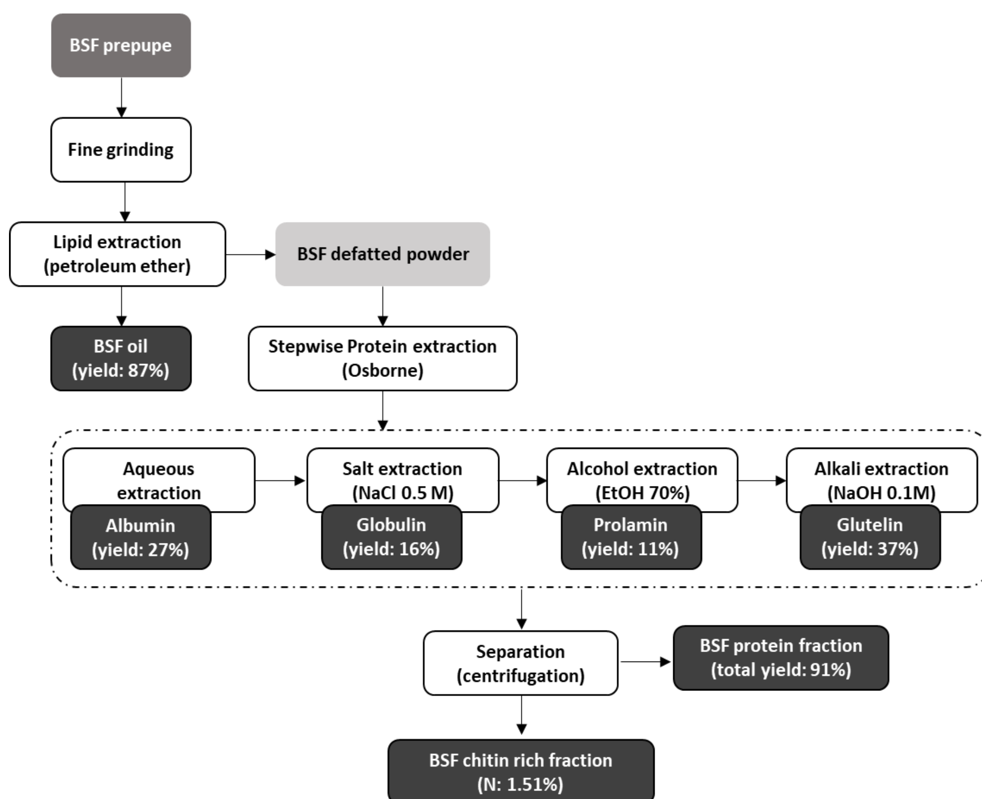

Figure 4 Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 2 (stepwise method). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

Protein extraction was based on their different solubility in different solvents. Five fractions were obtained (Table 5). 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 et al., 2009). The first fraction could have been containing also some nonprotein nitrogen soluble compounds, however their amount was low, as indicated by the free amino group determined by OPA method (Table 5).

Table 5 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/100 g 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.

The main BSF protein fractions were represented by albumins and glutelins, followed by globulins and prolamins. 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 5). 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 this method, presently developed at a laboratory scale, is likely affected by high costs when scaling it up to the industrial level due to: the high ratio water: sample employed for the extraction of each fraction; the high concentration of salts used for each extraction solution; the refrigerate temperature necessary for protein extractions.

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 (Figure 5) 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.

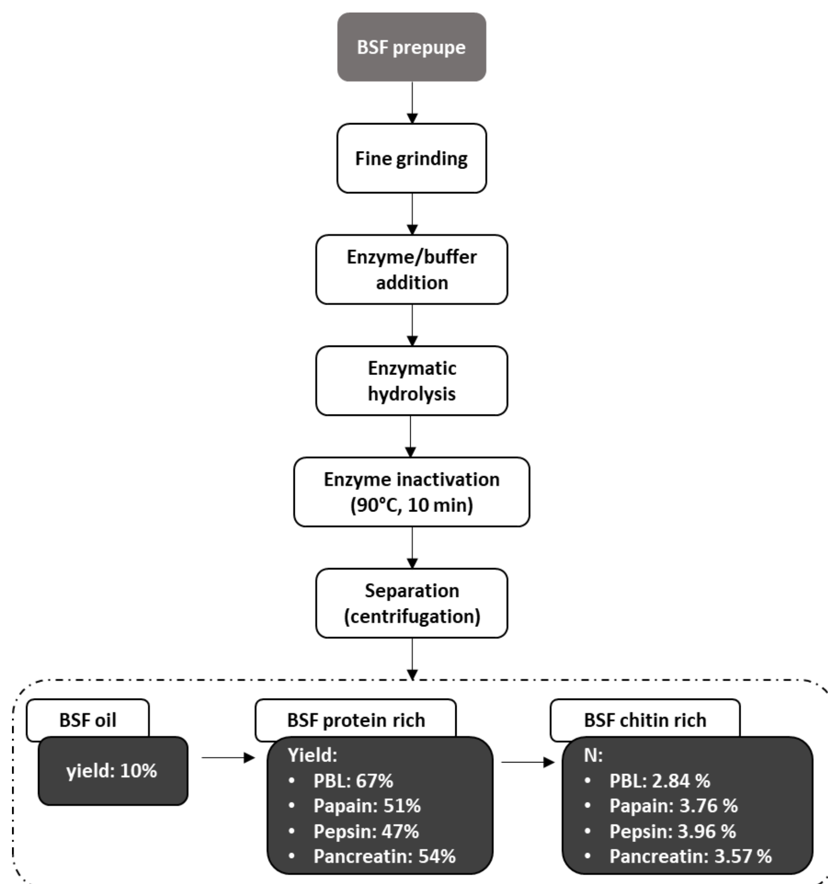

Figure 5 Schematic representation of processing and biomolecules fractionation of prepupae of BSF (*Hermetia illucens*) according to protocol 3 (enzymatic assisted extraction). Lipid yields are calculated on the total lipid content previously determined. Protein yields are calculated on the total protein content previously determined. Chitin yield is not calculated, but the amount of nitrogen contained in the fraction is reported.

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 most of the protein nitrogen. BSF prepupae were hydrolysed 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 6.

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. (2014) extracting lipids from four insect species with Soxhlet or aqueous method. 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 6). *B. licheniformis* protease was te 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.

Table 6 Solubilisation ratio (N% in solution) 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 without enzyme[a]	Solubilisation ratio 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, 2 mM 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 condition 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).

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 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.

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CHAPTER 3

Killing method affects the browning and the quality of the protein fraction of black soldier fly (*Hermetia illucens*) prepupae: a metabolomics and proteomic insight

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Author: Giulia Leni,Augusta Caligiani,Stefano Sforza

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Abstract

Insects are being explored as novel protein sources in order to overcome the future food demands connected to world growing population. Insects for food/feed uses are currently slowly killed through freezing by most insect rearing companies, and typically, enzymatic browning takes place in the insect proteins fractions. However, very little is known about the influence of these enzymatic reactions on the protein physical-chemical, nutritional and technological properties. In this work a metabolomics and proteomic study was conducted on black soldier fly (*Hermetia illucens*) prepupae, killed by two different methods: freezing (commonly used), and blanching (with the aim to inhibit the enzymatic activities). Proton nuclear magnetic resonance (^{1}H NMR) metabolomics demonstrated that slow killing methods by freezing, compared with blanching, elicit the activation of several enzymatic pathways, among them melanisation with tyrosine consumption, energetic metabolism and lipolysis. These metabolic changes have an impact also on protein nutritional quality, with a loss of cysteine and lysine, likely involved in the process of melanisation and enzymatic browning. A strong effect was also observed on protein extractability: proteins from prepupae killed by blanching were found to be more extractable in milder conditions by chemical methods, and more prone to enzymatic digestion (97 % of proteins released in solution upon proteolysis) than proteins from prepupae killed by freezing. All these data indicate that killing by blanching inhibits the browning reaction and other enzymatic changes occurring during slow killing by freezing, increasing the extractability of proteins in aqueous solutions, avoiding essential amino acid loss, and improving enzymatic digestibility.

1. Introduction

The perspectives of growing population have increased the need to find alternative protein sources for future food and feed applications (FAO, 2013). Insects represent a good possibility, since their protein percentage ranges from 35.3 % to 61.3 % (on dry matter basis) and their quality, in terms of essential amino acids, is promising in comparison to casein and soy (Rumpold & Schluter, 2013). The nutritional values of edible insects are highly variable, not only because of the wide variety of species, but also for the metamorphic stage of the insect, their habitat and diet (Spranghers et al., 2016; FAO, 2013; Rumpold & Schluter, 2013). The insects with the highest potential for industrial production in feed sector are Diptera species, such as *Hermetia illucens* and *Musca domestica*. They can grow on a broad spectrum of substrates (organic wastes included), have high reproduction rates and short life cycles (Pastor et al., 2015). Instead, *Tenebrio molitor*, coleoptera specie, is less efficient than the insects mentioned before, but considered ideal for human nutrition (Oonincx et al., 2015). All of these insects are very efficient to upgrade low value waste streams into high-value protein (Spranghers et al., 2016; Sun-Waterhouse et al., 2016; Makkar et al., 2014).

Entomophagy, the consumption of insects as food sources, is conventionally practiced in many regions of the world, such as Asia, South America, Australia and Africa, unlike Western countries (Megido et al., 2013). Insects can be consumed whole (cooked or occasionally raw), or as processed products in ground and paste forms or other less recognisable forms (e.g. extracts of protein, fat or chitin) for food and feed applications (Sun-Waterhouse et al., 2016). Studies conducted on possible future consumers have shown how westerners are disgusted towards entomophagy, when the insects are offered in a recognizable form, preferring extracts forms (Del Valle et al., 1982; Sogari et al., 2017; Varneau et al., 2016). This indicates that the extraction of proteins from insects, for food uses, is relevant for countries that do not have entomophagy's history (Yi et al., 2013).

Like most foods, extraction and processing methods applied to insects before consumption will also influence their nutritional composition (FAO, 2013; EFSA, 2015). Subsequently, insects processing should pay attention to the effects on the final protein fraction, since for food industrial purposes, next to protein content and yield, the quality of isolated fractions is also important. Among the different techniques that could be used for protein separation, chemical methods (e.g. salting out, isoelectric precipitation and solvent fractionation) are the most reported in the literature. A total protein recovery was obtained by Yi et al. (2017) using high extraction pH in combination with NaCl on *Tenebrio molitor*. In Chapter 2 it was also obtained high yields of extraction both with alkali and with Osborne

fractionation on *Hermetia illucens* proteins, albeit in the former case clear sign of degradation of the protein fractions were observed.

Usually, the insect proteins fractions, collected after water extraction, show a characteristic dark colour, possible mark of protein modification (Yi et al., 2013; Bußler et al., 2016; Zhao et al., 2016). Browning occurring during grinding of insects can influence both the visual aspects of food containing insect proteins and the proteins properties (nutritional and technological), so it could represent a hindrance for wide applications of insect proteins in foods (Sosa & Fogliano, 2017). Therefore, it is important to gain knowledge about the mechanisms involved in browning and their influence on insect protein fraction, in order to adopt the most suitable strategies to reduce or prevent this phenomenon. Some studies demonstrated the role of enzymatic activities in browning reactions and, in particular, the role of phenol oxidase as a key player in browning (Janssen et al., 2017). The dark colour observed indicates that chemical reactions took place during processing. This process arises from the oxidation by phenol oxidase (PO) of monophenols, such as L- tyrosine, which, in the presence of oxygen, are converted to o-diphenols and then to their corresponding o-quinones (Selinheimo et al., 2007) (Figure 6). Quinones, in turn, are cross linked and polymerised to complex brown, red, or black pigments called melanin (Sugumaran, 2002; Fortea et al., 2009; Sugumaran and Barek, 2016).

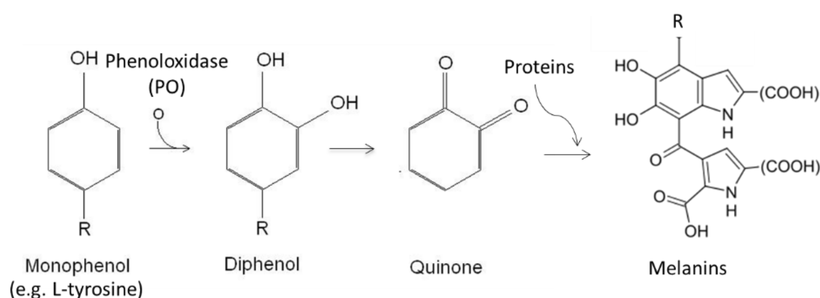

Figure 6 Melanisation process starting from the activity of phenol oxidase on monophenol.

It has been also suggested that the quinone derivatives generated by phenol oxidase might use endogenous protein components for melanin synthesis in arthropods (Lee et al., 2000). Tyrosinases are also capable of reacting with protein substrates by oxidizing phenolic residues such as tyrosine and dihydroxyphenylalanine (DOPA) (Xiaohua et al., 2009). These studies suggest that beside colour modifications, browning reaction can have direct and maybe important consequences on insect protein quality. Modifications induced by phenol oxidase (but also by other enzymes) might eventually affect protein quality, extractability and digestibility.

For many other food commodities, enzymatic browning can be inhibited by chemical inhibitors (e.g. ascorbic acid, sulphite, proteolytic enzyme) or by appropriate technological treatment (e.g. blanching, ultrafiltration, sonication) (Ozoglu & Bayındırlı, 2002). Blanching is a thermal treatment that is usually performed prior to food processes in order to preserve the quality, inactivating enzymes and destroying microorganisms that might contaminate raw products (Vandeweyer et al., 2017; Xiao et al., 2017). This heat treatment is rarely used also as killing method by entomophagy companies, being the killing by freezing the most widespread method (FAO, 2013; Makkar et al., 2014; Sun-Waterhouse et al., 2016), but clearly this might affect the extent of the subsequent enzymatic processes.

In the present work, blanching was evaluated as killing method alternative to freezing for prepupae of black soldier fly (BSF), with the aim to avoid enzymatic browning and preserve the protein quality. The protein fractions of prepupae killed by blanching and by freezing were compared to assess their composition, nutritional value, extractability, and resistance to enzymatic digestion.

2. Materials and methods

2.1. Materials

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). DL-norleucine, amino acid standard mixture, Protease from *Bacillus licheniformis* (2.4 U/g, SLBL2953V), Trypsin from porcine pancreas (1,000-2,000 BAEE units/mg, SLBF9600V), DL-Dithiothreitol, Iodoacetamide, L-Cysteic acid, N-acetyl-l-cysteine, DL-isoleucine, L-Ascorbic acid, tris-HCl, EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). D20, CDCl3 and TSP were obtained from VWR International (Milan, Italy). o-Phthaldialdehyde 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 Samples treatments

BSF prepupae were purchased alive from Smart Bugs s.s. (Ponzano Veneto, Italy). Living insects were killed in laboratory in two different ways: i) by freezing at -20°C and ii) by blanching in boiling water for 40 seconds. These two samples are visualized in Figure 7 after grinding. Furthermore, an aliquot of prepupae killed by freezing were subjected to an equivalent thermal treatment (boiling water for 40 seconds) and used as control during

protein enzymatic assisted extraction. All samples were stored at -20°C as whole prepupae and grinded before each analysis.

2.3 Enzymatic browning evaluation

The enzymatic browning was evaluated spectrophotometrically on proteins extracts from BSF samples killed by blanching and by freezing. The protein extraction was performed mixing 5 g of grinded BSF with 25 ml of deionized water under stirring at pH 6.5 and 60°C for 16 hours. Then, the supernatants were separated from pellets with centrifuge at 4000 rpm for 10 minutes at 4°C. The nitrogen content of the supernatants was determined by the Kjeldahl method (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) and appropriate dilutions were performed in order to normalize extracts to the same amount of nitrogen (0.5 mg nitrogen/mL). Then the supernatants were analysed in the range of 500-350 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, OK, U.S.A) and the absorbance at 395 nm (point of maximum) was recorded.

2.4 ^{1}H NMR metabolomics

200 mg of grinded BSF prepupae were dissolved in 1 mL of D_2O. The mixture was blended with a magnetic blender for 1 h at room temperature. To ensure a complete removal of the non-polar component, 200 µl of $CDCl_3$ were added. After centrifugation, 600 µl of the supernatant were taken for the analysis. An amount of 100 µl of TSP (20 mg/10 mL of D_2O) was used as internal standard. ^{1}H NMR spectra were recorded on a Bruker Avance III 400 MHz NMR Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic field-strength of 9.4 T. Spectra were acquired at 298 K, with 32 K complex points, using a 90° pulse length and 5 s of relaxation delay (d1). 128 scans were acquired with a spectral width of 9595.8 Hz and an acquisition time of 1.707 s. NOESY 1D sequence was utilized for water suppression.

2.5 Amino acids determination

2.5.1 Total amino acids profile

0.5 g of BSF prepupae, frozen and blanched, were hydrolysed with 6 mL of HCl 6 N at 110°C for 23h, then the internal standard (7.5 mL of Norleucine 5 mM in water) was added. For cysteine, performic acid oxidation followed by acid hydrolysis was used, in order to determine it as cysteic acid. In this case, an amount of 0.5 g of BSF prepupae 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 16h 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 HPLC/FLD after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) according to the method described by Marseglia et al. (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.

2.5.2 Free amino acids profile

0.5 g of BSF prepupae, frozen and blanched, were homogenised under stirring with 5 mL of water and internal standard (0.34 mL of Norleucine 5 mM in water), bringing to 10 mL with water as final volume. The samples were centrifuged for 15 minutes at 4000 rpm at 20°C. The supernatants were collected and filtered. The filtrates samples were analysed by HPLC/FLD after derivatization as described in point 2.5.1.

2.6 Stepwise protein extraction (Osborne fractionation)

BSF prepupae, frozen and blanched, were grinded and then defatted with Soxhlet fat extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) with diethylether (60 mL) as extraction solvent. The defatted flours were subjected to a protein extraction following the stepwise method proposed by Osborne (Osborne, 1907) with some modification, as described in Chapter 2. 250 mg of sample was mixed with 25 mL of 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 minutes 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 25 mL of 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 minutes 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 25 mL of 70% EtOH and 5 mM ascorbic acid. Then it was centrifuged for 20 minutes 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 hour and then centrifuged for 20 minutes at 4°C at 4000 rpm. The supernatant was collected as glutelin fraction and the pellet as residual. These fractions were analysed in term of nitrogen content with the Kjeldahl method (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) in order to check the mass balance and calculate the nitrogen extraction yield.

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight distribution of the insect protein fractions extracted with Osborne method. Supernatant fractions were analysed on 12% Bis/Tris Criterion™ XT Bis-Tris Gel (Bio-Rad, Hercules, CA, U.S.A) by using MES running buffer. The gel was finally stained with Coomassie Brilliant Blue, destained and finally scanned with GS-800 Calibrated Densitometer controlled by the software "Quantity one" (BIO-RAD).

2.8 Protein identification by high resolution mass spectrometry

The best nets bands were excised and tryptically digested following the In-gel digestion protocol proposed by Kinter and Sherman (2000). The dry proteins extracts were reconstituted with 50 µL of 0.2% formic acid for mass spectrometric analysis. High resolution mass spectrometry was performed on the samples for peptide identification using a µHPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific. Column: Jupiter C18 4 µ, Proteo 90 Å 150 × 0.30 mm, Phenomenex; eluent A: water + 0.1% formic acid; eluent B: acetonitrile + 0.1% formic acid; flow: 5 µL/min, gradient: 0–4 min from 100% A to 95% A, 4–60 min from 95% A to 50% A, 60–62 min from 50% A to 10% A, 62–72 min 10% A, 72–74 min from 10% A to 95% A, 74–90 min 95% A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0–75; ionization mode: ESI+; scan range (m/z): 200–1800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS + p res = 30,000 or (250.0–2000.0); (ion trap) ITMS + c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000. Protein identification was performed by using PEAKS software (Bioinformatics Solutions Inc) and INSECTA (UniProt) database. Positive hits for protein identification was arbitrarily set for all those proteins identified by the program with a score (expressed as -10lgP) >50, since such value should reduce the risk of false positives to zero.

2.9 Enzymatic hydrolysis

The enzymatic assisted extraction was performed following the method proposed in Chapter 2. The enzyme employed for samples hydrolysis was Protease from *Bacillus licheniformis* (EC 3.4.21.62). The analysis was carried out in triplicate, both on whole and

grinded insects treated as described in point 2.2. The hydrolysis reactions were performed, in terms of pH and temperature, with the optimal conditions for the enzymes (Na_2HPO_4 10 mM, pH 6.5, 60°C). 5 g of substrates were mixed with 45 ml of the appropriate solution and the enzyme was added reaching an enzyme/substrate ratio of 1:100 (w/w). The hydrolyses took place under stirring overnight, then heated at 90°C for 10 minutes to inactivate the enzyme. The hydrolysed were centrifuged at 4000 rpm at 4°C for 30 minutes. For each sample a blank control was performed: samples were subjected to hydrolysis in the same conditions but without enzymes. Three fractions were obtained, from top to bottom: the upper layer, the supernatant, and the pellet. The supernatants and the pellets were collected. In order to evaluate the nitrogen distribution in the hydrolysed and to verify the mass balance, Kjeldahl method was used to calculate the total nitrogen in the supernatants and pellets.

2.10 Determination of the degree of Hydrolysis

The degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved with respect to the total number of peptide bonds, was calculated using o-phthaldialdehyde (OPA) method described by Spellman et al. (2003) with some modification. 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 borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed to stir 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. The absorbance of this solution 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 buffer used for the 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^{-1}). The degree of hydrolysis (DH) was calculated as the ration between the free nitrogen groups after hydrolysis and the total nitrogen groups: DH%= $(N_{free} / N_{total}) \times 100$. The first value was calculated by the OPA reactivity by subtracting the reactivity in the blanks (same samples without enzymes). The total moles of nitrogen atoms in solution before hydrolysis were calculated by the ratio of total grams of proteins and the average of residual amino acids molecular mass (Mw 110). The total grams of proteins were calculated from total amino acids analysis (paragraph 2.5.1).

2.11 Statistical analysis

All experiments applied to the two batches of larvae (blanched and frozen) 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., USA). The data were subjected to Independent T-Test or to one-way analysis of variance (ANOVA) to determine the differences between samples. Significant differences were compared at a level of $p < 0.05$.

3. Results and Discussion

3.1 Effect of blanching or freezing as killing methods on browning

During the grinding step of BSF prepupae, samples killed by freezing or by blanching developed different colours, and in particular, insects subjected to the heat treatment were definitely characterized by a lighter colour (Figure 7).

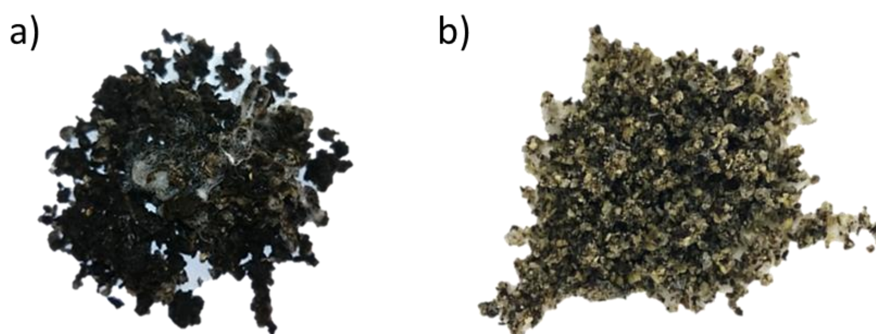

Figure 7 Pictures of ground BSF prepupae killed in different ways: a) by freezing at -20°C and b) by blanching in boiling water for 40 seconds.

In order to understand if the different colours of grinded larvae were correlated to the colour of the protein fraction, a protein aqueous extraction protocol was applied. The protein extracts were first visually compared (Figure 8a) and then the brown colour was measured by spectrophotometric analysis (Figure 8b).

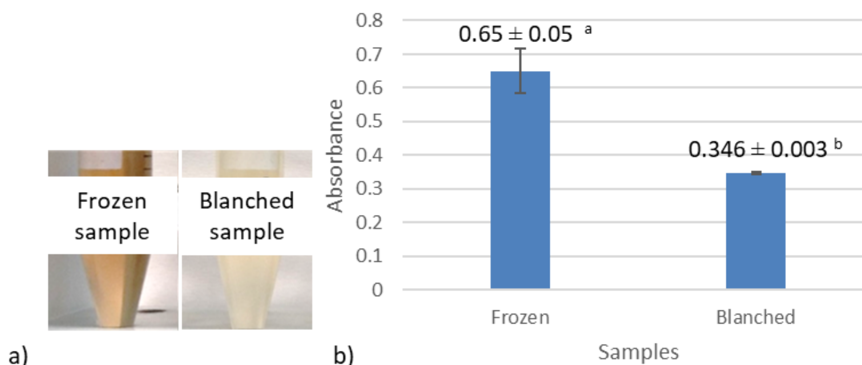

Figure 8 Colour comparison between water extracts (16 hours pH 6.5 and 60°C) of grinded BSF prepupae: a) visual comparison of extract and b) absorbance values determined at 395 nm on 0,5 mg/mL of proteinaceous nitrogen. Results are the means of triplicate analysis with a p-value lower than 10^{-5}. Significant differences were compared at a level of $p < 0.05$.

From a simple visual inspection (Figure 8a) it appears that extracts from prepupae killed by freezing presented a darker colour than blanched ones. This was also confirmed by the absorbance values measured at 395 nm (point of maximum absorbance) reported in Figure 3b. These data suggest that blanching results in significantly less colour development during subsequent processing. Thus, blanching seems to be an efficient way to keep enzymatic browning under control.

3.2 Effect of blanching or freezing as killing methods on insect metabolome

In order to get deeper insights in the metabolic changes induced by killing by blanching or freezing, and to identify the main metabolites and possibly the metabolic pathways involved or correlated to the browning reaction, the aqueous extracts of BSF samples killed in different ways were analysed by ^{1}H NMR, obtaining a profile of the principal polar metabolites (Figure 9).

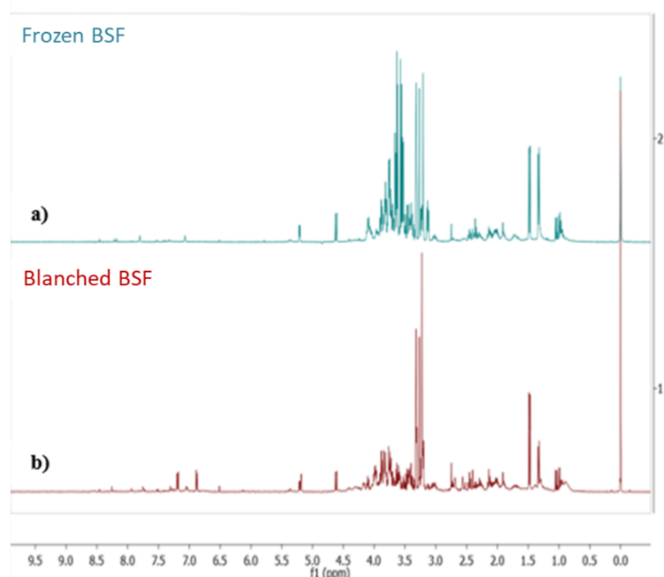

Figure 9 ^{1}H NMR spectra of D_2O extracts of ground BSF prepupae killed by a) freezing and b) blanching.

The identified metabolites, their chemical shifts and their relative amounts are reported in Table 7.

Table 7 Metabolites identified in ^{1}H NMR spectra of water extracts of BSF prepupae killed by freezing or blanching. Quantitative data are reported as relative area of the specific spectral signal, referred to the internal standard (TSP) area. nd: not detectable.

Chemical shifts range (ppm)	Metabolite	Relative area, frozen	Relative area, blanched
1.35 - 1.31	Lactic acid	119.64	45.51
5.22 - 5.20	Glucose	14.11	6.98
5.19 - 5.16	Alpha-glucose oligomer/ N-Acetylglucosamine	nd	12.72
4.64 - 4.58	Glucose	21.22	14.01
3.27 - 3.26	Choline	71.38	52.64
3.21 - 3.19	Phosphocholine derivative 1	81.25	nd
3.23 - 3.21	Phosphocholine derivative 2	nd	113.93
2.74 - 2.50	Citric acid	27.4	74.60
2.41 - 2.39	Succinic acid	10.8	11.57
1.51 - 1.44	Ala	87.8	71.71
1.06 - 0.92	Leu. Ile. Val	96.7	73.24
6.91 - 6.84	Tyr	0.27	17.81
3.67 - 3.51	Glycerol	481.07	n.d.

Important differences were indeed detected in the intensity of some metabolites. ^{1}H NMR metabolite fingerprints indicate that during slow killing by freezing different metabolisms are activated by BSF prepupae, involving the hydrolysis of glucose from storage polymers/oligomers, production of lactic acid (occurring during anaerobic step of energy production), consumption of citric acid, and release of glycerol from triglycerides (lipolysis). All the reactions indicate many enzymatic activities related to energetic metabolism that are not present in sample of BSF prepupae killed by blanching, likely due to the quick killing and to the thermal inactivation of enzymes. In addition, one of the most evident metabolite change concerns tyrosine, which is clearly visible in blanched sample spectrum, whereas disappears completely in the frozen sample spectrum. Tyrosine indeed represents a substrate for phenol oxidase activity. This enzyme, known also as tyrosinase, hydroxylates monophenols, such as L-tyrosine, to *o*-diphenols, subsequently oxidized into *o*-quinones. These compounds can further react producing melanins, pigments responsible of browning. The complete consumption of tyrosine is perfectly consistent with the hypothesis that the darker colour characterizing the aqueous extracts obtained from frozen larvae is indeed due to melanisation process. On the other hand, the blanched prepupae, which present lighter protein fraction, still show in ^{1}H NMR spectrum the abundant presence of tyrosine, indicating that no enzymatic browning reaction leading to its consumption took place, and demonstrating that blanching is actually an effective method to inhibit reactions involving tyrosine.

These data confirmed a strong involvement of phenol oxidase during killing by freezing and grinding. Since this enzyme is reported to be also able of reacting with protein substrates (Xiaohua et al., 2009), it has been then studied the effect of the browning reaction on the protein nutritional value and on the protein behaviour during different extraction processes.

3.3 Effect of blanching or freezing as killing methods on free and total amino acids profile

The impact of the melanisation process on the free amino acid content, already evidenced by ^{1}H NMR, was also checked by Liquid Chromatography (LC) analysis of free amino acids. The data are reported on Table 8.

Comparing the results in Table 8 it is possible to observe immediately that the data about tyrosine found by ^{1}H NMR were confirmed: in blanched prepupae the free tyrosine was much higher than in frozen prepupae, going from 0.23 g/100g DM to not detectable, the highest difference among all amino acids. Also other free amino acids showed variation among the two set of samples, albeit no one as much important as the one of tyrosine. In

general, free amino acids were less abundant in frozen larvae than in blanched ones (1.22 g against 1.34 g on 100 g DM).

Table 8 Free amino acids profile of black soldier fly (BSF) prepupae killed by freezing and by blanching, expressed as g/100 g DM.

	Frozen BSF	Blanched BSF	P-values
Asp/Asn	0.050 ± 0.001	0.01 ± 0.01	0.01
Ser	0.046 ± 0.002	0.09 ± 0.01	0.01
Glu/Gln	0.08 ± 0.01	0.021 ± 0.003	0.02
Gly	0.034 ± 0.004	0.01 ± 0.01	0.04
His	0.10 ± 0.02	0.21 ± 0.01	0.02
Arg	0.13 ± 0.03	0.23 ± 0.03	0.05
Thr	0.08 ± 0.01	0.05 ± 0.01	0.09
Ala	0.22 ± 0.05	0.23 ± 0.03	0.83
Pro	0.23 ± 0.04	0.14 ± 0.01	0.09
Tyr	nd	0.23 ± 0.02	-
Val	0.08 ± 0.01	0.07 ± 0.01	0.36
Met	0.013 ± 0.003	nd	-
Lys	0.03 ± 0.01	nd	-
Ile	0.036 ± 0.001	0.020 ± 0.003	0.02
Leu	0.06 ± 0.01	0.01 ± 0.01	0.02
Phe	0.045 ± 0.003	0.038 ± 0.001	0.07
Cys	nd	nd	-
Sum	1.22 ± 0.16	1.34 ± 0.07	0.45

As a further step, the nutritional quality of protein fraction was also assessed, by evaluating the total amino acid profile on frozen and blanched BSF prepupae (Table 9). The sum of the total amino acids, expressed on 100 g of dry matter, was not significantly different between the two sets of samples: 40.7 ± 4.9 g for frozen prepupae, and 37.9 ± 2.9 g for blanched prepupae. These values are in line with those of Chapter 2 and Janssen et al. (2017). The essential amino acids profile of BSF prepupae, when compared with reference pattern from egg white, soybean and the human requirement proposed by FAO/WHO/UNU (FAO/WHO/UNU, 1985), indicates that they contain all the essential amino acids in more than adequate amounts for humans.

Table 9 Total amino acid profile of black soldier fly (BSF) prepupae killed by freezing and by blanching (expressed as g/100 DM and for essential amino acids as mg/g crude protein) compared with other food protein (Young and Pellett, 1991) and with the FAO/WHO standard protein.

	Frozen BSF	Blanched BSF	P-values	Frozen BSF	Blanched BSF	Reference protein*	Egg white	Soybean
	(g/100g DM)				(mg/g protein)			
Essential Amino acids								
His	1.6 ± 0.2	1.4 ± 0.1	0.30	41	39	15	22	25
Thr	1.7 ± 0.3	1.6 ± 0.1	0.49	42	41	23	47	38
Val	2.8 ± 0.5	2.5 ± 0.3	0.53	68	65	39	68	43
Lys	2.1 ± 0.1	2.5 ± 0.1	0.03	51	66	45	70	63
Ile	1.8 ± 0.1	1.7 ± 0.2	0.51	45	45	30	53	47
Leu	3.1 ± 0.4	2.8 ± 0.2	0.39	77	73	59	88	85
Phe+Tyr	5.2 ± 0.6	4.80 ± 0.2	0.46	133	132	38	91	97
Cys + Met	2.9 ± 0.2	3.3 ± 0.1	0.10	69	90	22	66	68
Non-essential amino acids								
Asp/Asn	3.7 ± 0.4	3.1 ± 0.3	0.19					
Ser	1.9 ± 0.3	1.7 ± 0.2	0.49					
Glu/Gln	4.0 ± 0.4	3.5 ± 0.5	0.38					
Gly	2.7 ± 0.4	2.5 ± 0.4	0.68					
Arg	2.2 ± 0.2	2.2 ± 0.1	0.97					
Ala	2.5 ± 0.4	2.2 ± 0.3	0.48					
Pro	2.6 ± 0.5	2.2 ± 0.1	0.49					
Tyr	2.8 ± 0.3	2.8 ± 0.1	0.79					
Cys	1.9 ± 0.1	2.5 ± 0.1	0.04					
Sum	40.7 ± 4.9	37.9 ± 2.9	0.55					

However, when comparing blanched with frozen prepupae, it is clear that the killing by freezing lead to some alteration of the total amino acid fraction, with the notable decrease of Cys, Lys and Met. These amino acids are likely involved in the process of melanization, reacting with quinones with their side chains, also when they are protein-bound, thus their available amount decreases. This is of no secondary importance, given their primary role as essential and semi essential amino acids, and in many cases even the limiting ones. Quite notably, and a bit unexpectedly, total tyrosine seemed not to decrease from blanched to frozen prepupae, indicating that the melanization process mostly concerns free tyrosine.

3.4 Effect of the killing method on protein extractability

One of the main criteria to allow food applications of insect proteins is the possibility to isolate them from the other insect components, mainly lipids and chitin. Since the influence of the killing methods on protein extractability was completely unexplored, we performed protein extraction on BSF prepupae killed by freezing or killed by blanching, applying both chemical and enzymatic assisted extractions.

3.4.1 Stepwise chemical protein extraction and protein characterization

According to a method described in Chapter 2, the stepwise protein extraction proposed by Osborne (1907) was applied to defatted insect biomass, in order to obtain four intact protein fractions: albumin, globulin, prolamin and glutelin. The same procedure was applied both to prepupae killed by freezing and killed by blanching. A schematic representation of the method is reported in Figure 10.

Each fraction was subjected to Kjeldahl analysis in order to check the nitrogen content and the extraction yields of each fraction, expressed as percentage of nitrogen. Moreover, the results previously obtained from amino acids analysis also allowed to know the specific contribution of protein and chitin on total nitrogen. The specific nitrogen conversion factor, calculated from the total amino acid amount, was found to be 5.89 ± 0.01. This allowed to calculate, on the total nitrogen, the contribution of proteins, obtaining a calculated amount of proteinaceous nitrogen of 5.9 ± 0.8 % for frozen prepupae and of 5.5 ± 0.5 % for blanched ones on DM, which corresponds respectively to 78 % and 81 % of total nitrogen. This means that the remaining amount of nitrogen (about 20 %) was included in chitin. With this information, we were able also to calculate the specific yield of protein extraction respect to total protein fraction.

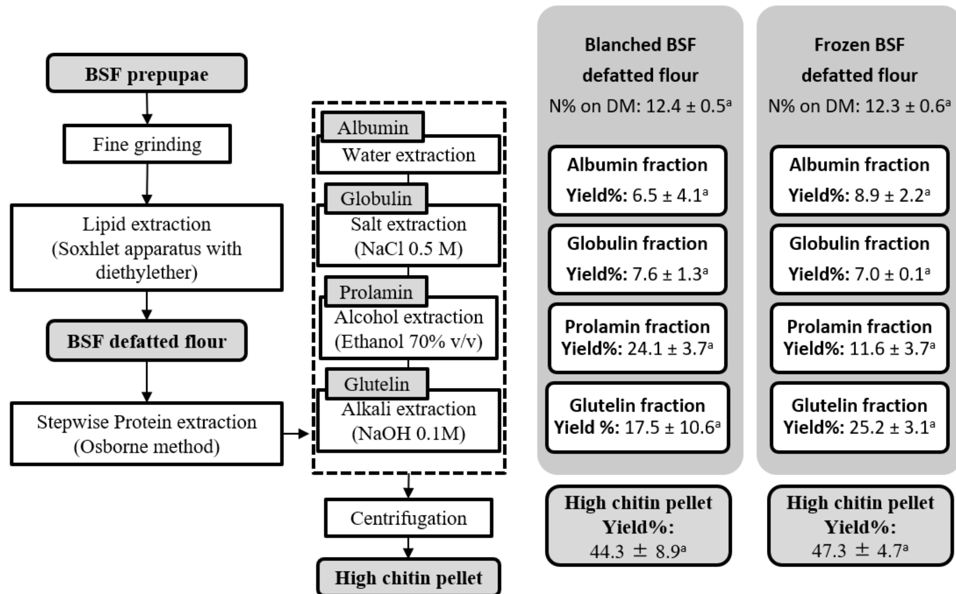

Figure 10 Schematic representation of Osborne fractionation of prepupae of BSF with nitrogen yield calculated on total nitrogen content. Results are the means of three independent extractions. Values followed by the same letter within one row are not significantly different (p > 0.05).

Applying the Osborne fractionation, we were able to extract 71 ± 11 % of total proteinaceous nitrogen from blanched prepupae, and the 67 ± 6 % from the frozen ones. Even if the overall yield was not significantly different, it is possible to observe in Figure 10 that, in frozen prepupae, the fraction containing most of the nitrogen was represented by the glutelin, the fraction only soluble in alkali solutions (32 ± 4 % of total proteinaceous nitrogen), whereas in the blanched ones the prolamins (proteins extractable in hydroalcoholic solution) were the most abundant (31 ± 5 % of total proteinaceous nitrogen). Thus, apparently, the melanisation has the effect to change the solubilisation properties of proteins, decreasing their solubility and extractability in milder conditions, a possible sign of protein aggregation.

In both cases, the method yielded quite different results than those obtained in Chapter 2. First, yields were lower, both for blanched and frozen prepupae. Furthermore, also distribution among the different fractions was different than those reported in Chapter 2. The main difference is obviously the starting biomass: in Chapter 2 the samples were constituted by prepupae already killed when bought in the market, in frozen state, without any indication nor on the way of killing, nor on the time of storage after killing. In the present work, the prepupae were bought alive, killed in our laboratory, and almost immediately analysed. This strongly outlines that the extractability of the proteins and their

behaviour change considerably according to the killing methods and to the storage conditions and time.

Due to the different distribution of protein fraction, an SDS-PAGE analysis of all the fractions extracted was also performed, in order to identify the most abundant proteins and deeply understand the different behaviour of BSF samples killed in different ways (Figure 11). By comparing the same fraction in frozen and blanched samples, it is apparent that both overall intensity and the band pattern tend to be quite different. Going from blanched to frozen prepupae, the first effect is an apparent depletion of protein bands in all fractions (Figure 11).

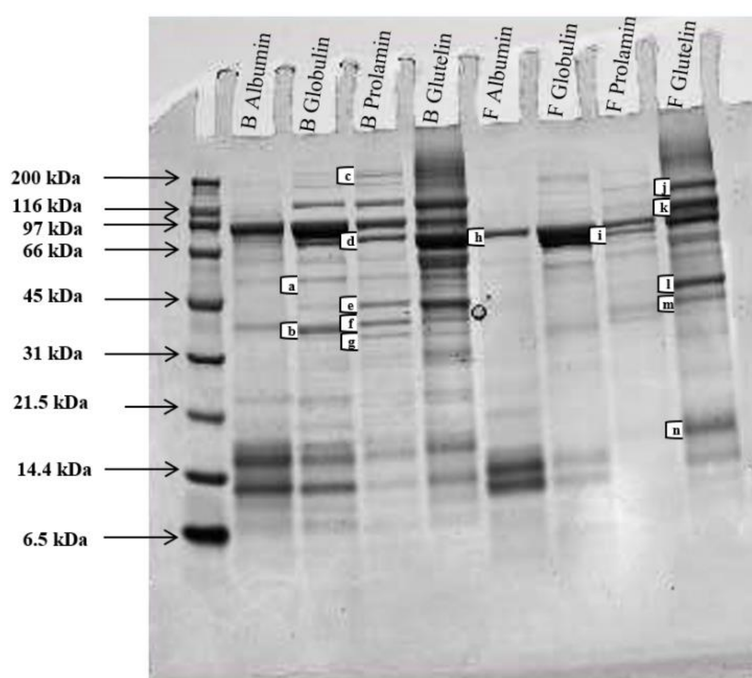

Figure 11 SDS-PAGE analysis of BSF prepupae protein fractions. Samples from right to left: albumin, globulin, prolamin, glutelin from blanched prepupae; albumin, globulin, prolamin, glutelin from frozen prepupae. B: blanched; F: frozen. The letters from "a" to "n" indicate the bands identified by HPLC-LTQ-Orbitrap.

In order to identify the most abundant proteins present in the extracts, the most intense bands were excised from the gel, tryptically digested and identified by high resolution mass spectrometry using an LTQ-Orbitrap instrument. The software PEAKS for protein identification gave a list of potential candidates which were characterized by a specific score (-10lgP) that defines the reliability of the result. Conventionally, we reported only the positive ones with the best score (higher than 50) and also being significantly higher than

other possible hits. The main proteins identified for blanched and frozen prepupae were shown in Table 10.

Overall, the most abundant proteins identified were muscle proteins, including putative Tropomyosin-2 (177 kDa), Myosin heavy chain (235 kDa), Actin-87E (42 kDa), for blanched BSF prepupae, and Actin (32 kDa), Actin-87E (42 kDa), Troponin C (18 kDa), for frozen BSF prepupae. Furthermore, Hexamerin F1 (81 kDa), a hemolymphatic protein, was identified in both samples. The main difference is the fraction where these muscle proteins were extracted: in frozen prepupae they were extracted with alkali condition, while in blanched ones mainly with salt solutions and alcohol. This strongly indicates that the reactions happening during and after killing by freezing strongly influence protein chemical-physical properties. The different distribution of proteins in the fractions, together with the loss of few essential amino acids, strongly hints to a protein aggregation linked to the melanisation process.

3.4.2 Effect of blanching or freezing as killing methods on the susceptibility of insect proteins to enzymatic proteolysis

Enzymatic hydrolysis of proteins has multifold implications in protein technologies, ranging from extractability to bioavailability after digestion. Enzymatic proteolysis has been shown to modify technological properties and liberate biologically active peptides from certain proteins (Adler-Nissen, 1979; Kristinsson and Rasco, 2000; Spellman et al., 2003; Klompong et al., 2007; Pacheco-Aguilar et al., 2008; Rossi et al. 2009). In Chapter 2, the enzymatic hydrolysis was used as a way to extract insect proteins. The best extraction yield was obtained using protease from *Bacillus licheniformis*, reaching anyway a level of protein solubilisation not higher than 70%. In the present work, the same enzyme was used to carry out enzymatic proteolysis on prepupae of BSF, killed both with blanching and freezing method, in order to assess the effect of the killing method on protein susceptibility to proteolysis. For each enzymatic hydrolysis a control sample was obtained extracting the samples in the same conditions (buffer, time and temperature) but without enzyme.

Table 10 Most abundant protein identified in frozen and blanched BSF prepupae. separated with SDS-PAGE. identified by tryptic digestion and LTQ-Orbitrap analysis. Only protein identified with a score -10lgP > 50 are reported.

Bands	Protein fraction	Most abundant protein	UniProt accession	Score (-10lgP)	Mol. weight (kDa)	Family and domain databases
			Blanched BSF prepupae			
a	Globulin	*Uncharacterized protein*	A0A1I8MYN4_MUSDO	188.17	45	Troponin
b	Globulin	Putative Tropomyosin-2	A0A1J1HVT4_9DIPT	201.46	177	
c	Prolamin	Myosin heavy chain	A0A0M4ECL7_DROBS	246.66	235	
d	Prolamin	Hexamerin F1	Q86G30_MUSDO	59.36	81	
e	Prolamin	Actin-87E	ACT5_DROME	330.17	42	
f	Prolamin	*Uncharacterized protein*	A0A1I8MRC6_MUSDO	264.85	33	Tropomyosin
g	Prolamin	*Uncharacterized protein*	D2A2S3_TRICA	59.60	37	Myofilin
			Frozen BSF prepupae			
h	Albumin	Hexamerin F1	Q86G30_MUSDO	90.91	81	
i	Prolamin	Hexamerin F1	Q86G30_MUSDO	92.61	81	
j	Glutelin	Actin (Fragment)	F1C3P4_TIMCA	72.78	32	
k	Glutelin	*Uncharacterized protein*	K7J804_NASVI	134.99	38	Actin/actin-like_CS
l	Glutelin	Actin-87E	ACT5_DROME	321.31	42	
m	Glutelin	Actin muscle	A0A151WIN4_9HYME	240.15	42	
n	Glutelin	Troponin C	D3TPS7_GLOMM	53.50	18	

Moreover, experiments were carried out both on whole and grinded insects, in order to verify the possibility to avoid the grinding step. The efficiency of the hydrolytic cleavage was determined by measuring, with Kjeldahl method, the amount of nitrogen released in solution and left in the pellet, on the assumption that a more efficient proteolysis releases more nitrogen in solution in form of amino acids or small peptides. The results are reported in Table 11.

Table 11 Solubilisation ratio and degree of hydrolysis of black soldier fly (BSF) prepupae killed by freezing and blanching and treated with Protease from *Bacillus licheniformis* in the optimum conditions of pH and temperature as indicated by the enzyme supplier (pH 6.5, T=60°C).

Sample	Solubilisation ratio without enzyme*	Solubilisation ratio with enzyme*	DH % with enzyme**
Blanched BSF (whole)	7.4 ± 0.5 ^{a}	19 ± 6 ^{a}	10 ± 3 ^{ab}
Frozen BSF (whole)	7 ± 2 ^{a}	10 ± 1 ^{a}	2 ± 1 ^{b}
Blanched BSF (ground)	28.4 ± 0.4 ^{b}	97 ± 1 ^{c}	32 ± 4 ^{c}
Frozen BSF (ground)	35 ± 1 ^{b}	68 ± 1 ^{b}	16.5 ± 0.1 ^{a}
Frozen and blanched BSF (ground)	31 ± 3 ^{b}	77 ± 6 ^{b}	18 ± 2 ^{a}

Results are the mean of three separate hydrolysis experiments. Values followed by different letters within one column are significantly different ($p < 0.05$).
*** Same condition reported applied with and without enzymes for 16 h. Results from three replicates, average standard deviation of 2%.**
**** 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).**

The nitrogen content in the samples treated with the enzyme shows the positive effect of proteolytic enzyme on the solubilisation of insect proteins, linked to its ability to break the peptide bonds. Two factors mostly emerged as affecting enzyme performance in a significantly way. First, the physical state of insects: whole insects, compared with grinded ones, showed lower levels of nitrogen released in the supernatants, as expected given the lower contact surface. A second considerable and most important difference emerged by considering the effect of the killing method. Blanched prepupae released in solution more nitrogen than frozen ones (about 79 % vs. 53 %). To assure that this effect on protein extractability was due to the killing method and not only to a simple thermal denaturation of protein, an aliquot of prepupae, killed by freezing where then blanched in the same conditions of the blanched BSF sample. This sample showed an intermediate behaviour between the previous two (62 % of nitrogen released during enzymatic extraction), which resulted to be significantly different only with the blanched prepupae. This indicates that

protein denaturation plays a role on enzyme accessibility to peptide bonds, but the significant effect is related to the thermal treatment applied as a killing method.

Considering that nitrogen distribution between protein and chitin, as discussed previously, is about 80:20, it was possible to calculate the yield of protein nitrogen solubilized by enzymatic hydrolysis respect to total protein nitrogen. It was possible to estimate that on blanched prepupae 97 % of the total protein content was released in solution, whereas this value for frozen prepupae went down to 68 % of total protein content. This last value is in agreement with that obtained in Chapter 2. This result is of outmost importance in the view of a total biorefinery of BSF, because it demonstrates that it is possible to fully separate protein and chitin by enzymatic extraction acting on the pre-treatment (blanching) of the insect biomass.

The degree of hydrolysis (DH) was also calculated in order to evaluate the level of protein degradation for the proteins released in solution. DH is defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis (Hernandez et al., 1990). In this work, DH was evaluated by OPA assay on the proteins released in solution (details in the material and methods), and the results are shown in Table 11. The samples having the highest level of protein solubilisation also had the highest DH %, indicating that the breaking of the amide bonds is the key event to allow protein solubilisation. Thus, blanched grinded prepupae showed the higher value of DH (32 %), which is significantly different from the other samples subjected to hydrolysis. These values were significantly lower when dealing with whole insects, mirroring the low solubilisation obtained.

All the data on enzymatic hydrolysis are consistent with the hypothesis that in insects killed by freezing the proteins underwent more aggregation, making them less susceptible to enzymatic attack. This aggregation is likely linked to the melanisation process, but also possibly to the other metabolic changes induced by the insect stress response to the low temperature. On the other side, blanching not only blocks melanisation and other protein aggregation phenomena, but likely it also has the effect to denature proteins, making them more susceptible to the enzymatic digestion. This has strong implications not only in the extractability of proteins in solution using enzymes, but also on the eventual digestibility of the protein fractions when used as ingredients for food or feed. Indeed, this data suggests that a protein fraction extracted from insects killed by freezing could possibly have a worse digestibility profile, which would however require further investigations.

4. Conclusion

In this study, for the first time, the effect of two different killing methods, blanching and freezing, on BSF prepupae protein fraction, was compared. It was demonstrated, by applying a metabolomics approach, that slow killing methods by freezing elicit the activation of several enzymatic pathways, among them melanisation. Identified metabolic changes include tyrosine consumption, hydrolysis of glucose from storage polymers, production of lactic acid, consumption of citric acid, and lipolysis. Consistently, insects killed by freezing (and protein fractions obtained by them) are more black. The effect on the protein fraction was the partial consumption of Lys, Cys and Met, essential amino acids, most likely involved in the melanisation process, and the aggregation of proteins, changing their extractability profile. In this context, blanching is not only a killing method, but also represents a way to inactivate phenol oxidase, fully inhibiting browning reaction and other metabolic pathways activated by the stress induced by the slow killing by freezing. Proteins from prepupae killed by blanching were found to be more extractable in milder conditions, less nutritionally impaired and more susceptible to enzymatic hydrolysis than proteins from those killed by freezing. These results are of high relevance for the incorporation of protein from insects into food and feed products, since they demonstrate that the killing method affects their extractability, their processability and their final nutritional profile.

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CHAPTER 4

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

The content of this chapter has also been submitted as:

Giulia Leni, Lise Soetemans, Johan Jacobs, Stefaan Depraetere, Natasja Gianotten, Leen Bastiaens, Augusta Caligiani, Stefano Sforza. Protein hydrolysates from *Alphitobius diaperinus* and *Hermetia illucens* larvae treated with commercial proteases.

Abstract

Recently, 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 DM) 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. 1.5 kg of AD were hydrolysed with the Protease from Bacillus licheniformis and 600 g of protein hydrolysate were obtained. The final product resulted to be rich in protein (60 % on DM) with a DH % of 10 %. This work support enzymatic hydrolysis as an effective and easy 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 (due to the degradation of full protein to shorter peptides) and making them more accessible for their future use as feed/food supplements.

1. Introduction

A generalized 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 (Sánchez-Muros et al., 2016; Xiaoming et al., 2010). From the legal point of view, Regulation (EC) No 999/2001 authorizes 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 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 (European Commission, 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 & 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 Chapter 2, more than 90 % of the total proteins in black soldier fly (BSF) was extracted from delipidated prepupae by applying the Osborne fractionation, normally used

for cereals. 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. (2018 a) 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 *Tenebrio 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 BSF.

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., 2015). 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. (2018 b) compared the ability of different enzymes, used at a different concentration and for a different hydrolysis time, to solubilize proteins from a commercial locust protein flour. With the addition of enzyme, they were able to significantly increase the amount of solubilized 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 Chapter 3, a thermal treatment as killing method was combined with an enzymatic hydrolysis in order to extract 97 % of total proteins from *Hermetia*

illucens in form of peptides. 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 *Alphitobius diaperinus* (AD) and *Hermetia illucens* (HI) 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

2.1. Material

Kjeldahl defoamers and catalyst were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit and AccQ-TagTM were obtained from Waters (Milford, U.S.A.). 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, U.S.A.). All the other solvents, salts, acids and bases (analytical grade) were purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

2.2. Insect samples

Black soldier fly (*Hermetia Illucens*, HI) larvae were provided by Circular Organics (Turnhout, Belgium), whereas Lesser Mealworm (*Alphitobius 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 minutes with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Staufen, Germany). Three different trials were performed for each insect species.

2.3. 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.

2.3.1. Total amino acids analysis of insects

25 mg of dilapidated ground insects and 3 mL hydrolysis reagent (phenol (1g/L)-HCl (6 M)) were mixed and heated at 110 °C for 23 hours. For cysteine measurements, an additional sample was oxidized prior to hydrolysis with performic acid/phenol. After heat treatment, the samples were centrifuged for 5 min at 3000g to remove solid particles. Amino acids were analyzed 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 (2x250 nm) and Dionex AminoPac PA-10 Guard (2x50 mm) at 30°C; mobile phases (A) milliQ water, (B) 250 mM NaOH, (C) 1M 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 10µL.

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 4000 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. The mobile phase was composed by H2O + 0.2% CH3CN + 0.1% HCOOH (eluent A) and CH3CN + 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 ionization mode, capillary voltage 3.2 kV, cone voltage 30V, source temperature 150°C, desolvation temperature 300°C, cone gas flow (N2): 100 L/h, desolvation gas flow (N2): 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 1s

2.3.2. Determination of chitin

Quantification of the chitin content was performed as described by D’Hondt et al. (D’Hondt et al., submitted). 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.

2.4. 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 the Table 12.

Table 12 Optimum condition of pH and temperature for the enzymes tested.

Enzyme	pH	Temperature	Solution buffer
PBL	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

PBL: protease from *Bacillus licheniformis.*

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 (see Table above) and mixed with a magnetic stirrer. The hydrolysis reaction was carried out for 18 hours in order to reach the plateau phase. The enzymes were inactivated by heating at 90°C for 5 minutes. The hydrolysates were then centrifuged (Eppendorf, 5810/ 5810 R) at 2683g at 4°C for 30 minutes. 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.

2.4.1. 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.

2.4.2. 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) SDS, stirred for 20 minutes 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 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 minutes at 280g. The absorbance of the obtained solution was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, U.S.A.) 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).

2.4.3. 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 hours. The volume was then brought to 10 mL with the addition of deionized water and then centrifuged for 30 minutes at 4°C at 2683 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 deionized 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 Co., Milford, U.S.A.) were mixed and then heated at 55 °C for 10 min. The derivatised samples were diluted with 100 µL of deionized water before injecting in the UPLC/ESI-MS system. The conditions of analysis were the same described for Trp analysis.

2.5. Process scale up

Process scale up was performed on AD larvae by using a protease from *Bacillus 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 hours) 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 minutes, while checking the pH (ProfiLine pH 3310, WTW, Xylem Analytics LLC, 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 minutes for the endogenous and exogenous enzymes inactivation and subsequently centrifuged at 4°C for 30 minutes at 3220g. 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 characterized in terms of proximate composition and DH% as previously described.

2.6. 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, 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 *Alphitobius diaperinus* (AD) and *Hermetia illucens* (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.

3.1. Insects composition

The proximate composition of HI and AD larvae, in terms of dry matter, ash, lipid, protein and chitin was first determined (Table 13).

Table 13 Proximate composition of *Hermetia illucens* and *Alphitobius diaperinus*.

Composition (%) [a]	*Hermetia illucens*	*Alphitobius 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 AA (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

[a] 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 characterized 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 14). 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 in Chapter 2 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 DM (Janssen et al., 2017).

Table 14 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).

Essential AA (g/100 g protein) [a]	*Hermetia illucens*	*Alphitobius 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	

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

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 in Chapter 2, Chapter 3 and by Janssen al. (2017) (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 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 (Chapter 2). 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.

3.2. 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 *Bacillus licheniformis* (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 12). 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 solubilized nitrogen as compared to the total protein nitrogen (see above).

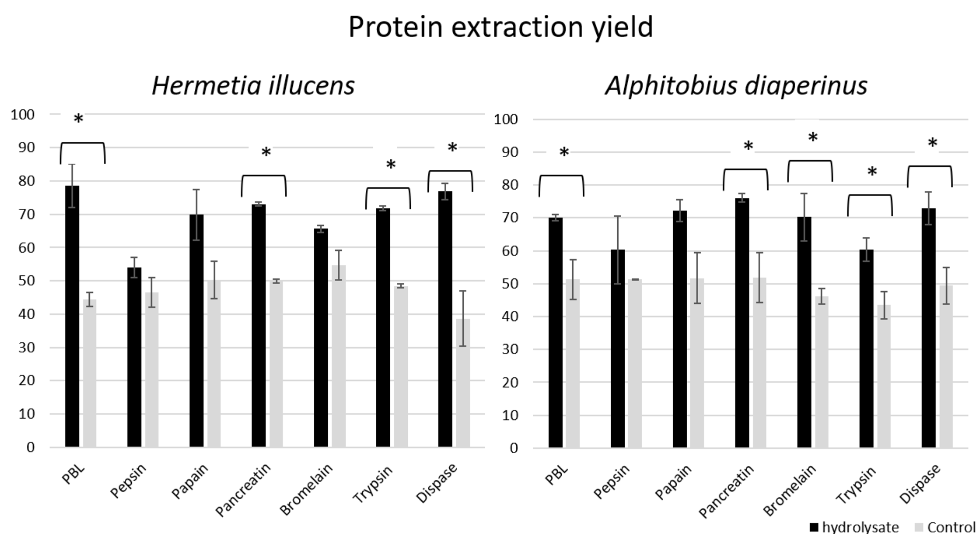

Figure 12 Protein extraction yields obtained by performing enzymatic hydrolysis with seven different enzymes (black columns) to ground *Hermetia illucens* (HI, on left) and *Alphitobius diaperinus* (AD, on right). The protein extraction yields for the blanks (buffers with no enzymes) are also reported (grey columns). The results are the mean of three separate hydrolysis experiments.

The extraction yield is reported in Figure 12, as a measure of the ability of proteolytic enzymes to extract and solubilize proteins. The yields obtained with the same solutions, but without enzymes, are also reported. Even in absence of enzymes, insect proteins were

partially solubilized 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 what reported in Chapter 2, where an analogue yield (43 %) was reached when extracting albumins and globulins from HI. With the addition of enzymes, the amount of solubilized proteins showed an average 20 % increase, supporting the enzymatic hydrolysis as an efficient way to extract and solubilize proteins from AD and HI.

3.2.1. 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 solubilized 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 15. The DH% of the solutions obtained without enzymes were not measured, since in Chapter 2 they have already been demonstrated to be very low. This also indicates a lack of a consistent endogenous protease activity.

Table 15 Degree of hydrolysis (%) and average peptide chain length (100/DH%, uncertainty included in the range) of protein hydrolysates obtained from *Hermetia illucens* and *Alphitobius diaperinus* larvae subjected to different enzymatic extraction.

	Hermetia illucens		***Alphitobius diaperinus***	
Enzyme	***DH%***	***Average peptide chain length***	***DH%***	***Average peptide chain length***
PBL	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

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 amount reachable), the cross inhibition exerted by the generated peptides, the presence of specific inhibitors. Purschke et al. (2018 b) reported, with the same enzyme/substrate ration, a comparable DH% for the hydrolysis of migratory locust with PBL (19 % to 31 % from 8h to 24h of hydrolysis), but a higher DH% for the one with Papain (13 % for only 8h 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 1500 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 (Commission Directive 2006/141/EC).

3.2.2. Free amino acids profile of protein hydrolysates

The hydrolysates and the untreated larvae were also analysed in order to evaluate the free amino acids (FAAs) 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 (see Supplementary Material) was Ala, for both AD and HI, ad

also Leu only for AD. The amount of free amino acids in the supernatants, compared to the total protein (deduced from Kjeldahl) also allowed to estimate the amount of amino acids in bound form (Figure 13). The HI extracts obtained with dispase I gave the highest relative proportion of free amino acids (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 free amino acids (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.

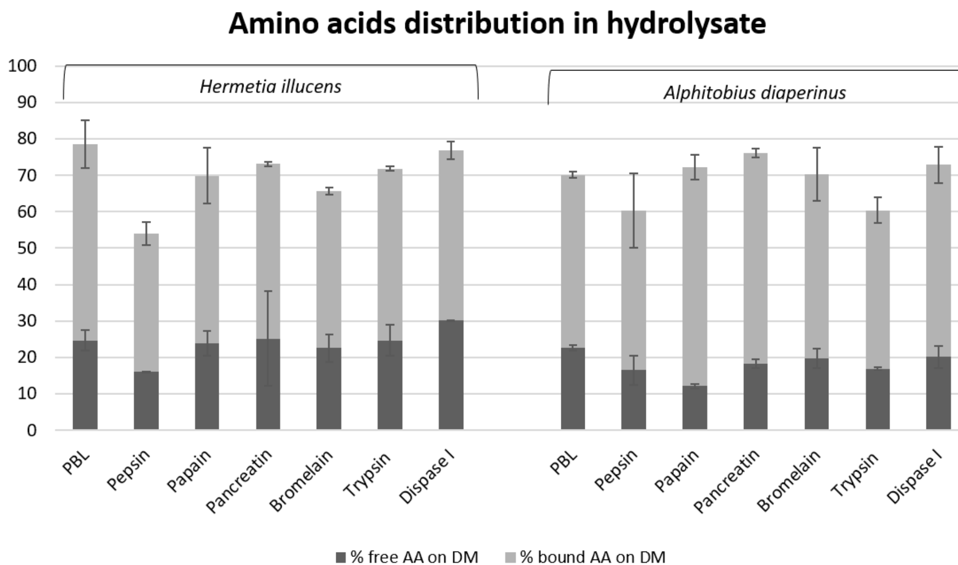

Figure 13 Distribution of bound and free amino acids in the supernatant after enzymatic hydrolysis. The full bar indicates the protein extraction yield (as reported in Figure 12) in the supernatant. The darker part of the bar indicates the amino acids present in free form and the light part of the bar the amino acids involved in peptide bond

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 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 14 (with the only exception of cysteine released with trypsin). This indicates that free amino acids 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. FAAs released 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). Indeed, the high level of FAAs released could be limited by reducing the time of hydrolysis and so, enriching the protein hydrolysates in oligopeptides, known to have higher nutritional value than FAAs.

The high levels of oligopeptides and free essential amino acids (more digestible than intact proteins) enhance the value and potential of insect hydrolysates for feed and/or food formulations. Moreover, the different cleavage specificity of proteases could be exploited for obtaining protein hydrolysates with diverse nutritional value and final taste.

3.3. 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 hours, at optimal temperature and pH conditions. The obtained hydrolysate was freeze-dried, yielding 600 g of protein hydrolysate. This protein hydrolysate was then characterized in order to define the proximate composition (Table 16).

Table 16 Bulk composition expressed on dry matter (DM 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). 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 optimization 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. Conclusion

The enzymatic-assisted protein extraction here presented clearly represents an effective method to extract and isolate protein from two different edible insects, *Hermetia illucens* and *Alphitobius diaperinus*. 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 free amino acids 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 free amino acids, 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 *Alphitobius diaperinus* larvae, 600 g of dry hydrolysate containing more than 60 % proteins, in form of peptides and free amino acids, 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 optimized protocol will be needed to verify this feature.

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CHAPTER 5

Degree of hydrolysis affects the techno-functional properties of lesser mealworm protein hydrolysates

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Giulia Leni, Lise Soetemans, Augusta Caligiani, Stefano Sforza, Leen Bastiaens. Degree of hydrolysis affects the techno-functional properties of lesser mealworm protein hydrolysates.

Abstract

Protein hydrolysates from Lesser mealworm (*Alphitobius diapeerinus*, LM) were obtained by enzymatic hydrolysis with protease from *Bacillus licheniformis*. Preliminary test performed for five hours of hydrolysis generated an insect protein hydrolysate with 15 % of degree of hydrolysis (DH), optimum solubility property and oil holding capacity, but emulsifying and foaming ability were completely impaired. In order to investigate the potential implication of DH on techno functional properties, a set of protein hydrolysates with a different DH was obtained by sub-sampling at different time points during three hours of enzymatic hydrolysis process. An increase in DH% had positive effects on solubility property and oil holding ability, while a reduced emulsifying ability was observed up to five hours of hydrolysis. These results demonstrated that the enzymatic hydrolysis, if performed under controlled condition and not for a long period, represents a valid method to extract high quality protein from insects with increased techno functionality, in order to produce tailored ingredients for feed and food purpose.

1. Introduction

According to the last FAO reports, a huge effort must be deployed to meet the future food demand connected to the increasing world population. The world in 2050 will host about 9 billion people, 30 % higher than nowadays, and a strong lack of farmland, water, nutrients and non-renewable energy is expected (FAO, 2009). Along the food chain, the meat production represents the most impacting field and for these reason novel food protein sources have been envisioned to meet the future demand (Boland et al., 2013).

Insects represent a good source of proteins for feed and food application. In fact, the amount of proteins in insects can range between 30 % and 60 % on dry matter basis, with a high-quality profile rich in essential amino acids (Yi et al., 2013). Furthermore, insects, in comparison to the common livestock, are characterized by many environmental advantages, such as less land use, feed and water requirement, fewer greenhouse gas emission and high food conversion rate (Rumpold & Schlüter, 2013). In the European Union the legalisation of insect proteins as food and feed has been granted by several regulations which include them, respectively, in the Novel Food category and permit their use for pet, fur and aquaculture (Regulation EU 2015/2283, 2015; EU Commission, 2017; Regulation EC No 999/2001).

As future protein source, in the recent years, insects have been studied and different extraction protocols for proteins were explored. For examples, Yi et al. performed a chemical extraction on *Tenebrio molitor* by combining concentrated salt and alkali pH for an overnight extraction (Yi et al., 2017). Bußler at al. and Zhao et al. applied an alkali extraction only after defatting the insects with hexane or ethanol (Bußler et al., 2016a; Zhao et al., 2016). Soetemans at al. reported the use of organic acids to obtain protein and lipid enriched fractions from black soldier fly larvae, after a mechanical removal of chitin (Soetemans et al., 2019). In Chapter 2, it was compared the ability of three different protocols to fractionate and separate the main components of insect: chitin, lipid and protein. The protein fraction of black soldier fly was recovered by both chemical and enzymatic process. Indeed, the enzymatic method constitutes an essential part of the processes used by modern companies to produce, from complex matrices, a large and diversified range of products for human and animal consumption (Nielsen et al., 2002). The use of exogenous enzymes, instead of chemicals, allows not only to control the process, but, due to the milder reaction condition, also to prevent the protein degradation. Proteases are efficient to separate proteins from lipids and insoluble compounds (e.g. fibres) by hydrolysing peptide bonds and releasing peptides and free amino acids in solution with high efficiency, as demonstrated in Chapter 4. Peptides, compared to the parental proteins, are characterised by an enhanced in gastro-intestinal digestibility and

bio-accessibility (Tavano, 2013). In literature many studies have investigated the bioactivity of protein hydrolysates obtained from insects, such as antioxidant, ACE-inhibitory, antidiabetic and antihypertensive activity (Nongonierma & FitzGerald, 2017). On the other side, only limited number of works are available regarding the techno functional properties of insect protein hydrolysates. Purschke et al. (2017) demonstrated the ability of targeted enzymatic hydrolysis to produce protein hydrolysate from *Locusta migratoria* protein flour with tailored techno functionality. They observed an increase in protein solubility, emulsifying activity, foam ability and oil binding capacity in a broad spectrum of pH. Hall et al. (Hall, Jones, O'Haire, & Liceaga, 2017) proved that Alcalase hydrolysis could represent an efficient biotechnology tool to improve the techno functionality of cricket proteins. They determined the solubility property, the emulsion activity and foam ability of different hydrolysates obtained by modifying the time of hydrolysis and the enzyme concentration, without underlining any correlation between proteolysis and techno-functionality.

The enzymatic assisted extraction has been demonstrated to be a valid method to extract proteins from insects in form of peptides. In Chapter 4, seven different enzymes from microbial, vegetable and animal origins, have been tested on LM by performing at a laboratory scale an end-point hydrolysis. The hydrolysates were characterized for the DH %, the yield of extraction and the presence of free amino acids but, for their future involvement as insect-based protein ingredients in food or feed formulations, it is necessary to assess also their techno-functional properties. For the first time, the present work investigates the techno-functional properties of protein hydrolysates isolated from lesser mealworm larvae (*Alphitobius diaperinus*, LM), focusing on the effect of the degree of hydrolysis.

2. Material and methods

2.1. Insect samples

Lesser mealworm larvae (*Alphitobius diaperinus*, LM), provided by Protifarm (Ermelo, The Netherlands), were reared as described in Chapter 4. Larvae were killed by liquid nitrogen, packed under vacuum sealed and freezing at -20°C. After one week, samples were freeze-dried (Christ, gamma 1-16 LSC, 36 hours) and stored at -20°C for the future analysis. Samples were grinded for 2 minutes with a laboratory grinder at maximum speed before each analysis.

2.2. Enzymatic hydrolysis for protein extraction

2.2.1. Preliminary enzymatic assisted extraction

The enzymatic assisted extraction of proteins was carried out by commercial protease from *Bacillus licheniformis* (≥ 2.4 U/g; EC Number 3.4.21.62) at optimal conditions for hydrolysis as suggested by the supplier. More specifically, 200 g of dried ground larvae were mixed with 1 L of a buffer solution (Na_2HPO_4 10 mM) and 0.25 % of enzyme in a 2 L reactor, combined with the pH-STAT system (Metrohm, Varese, Italy) to control the pH during the reactions by the addition of NaOH 1 M. By knowing the amount of NaOH added, it was possible to back-calculate the degree of hydrolysis as described by Butrè et al. (2014). The hydrolysis reaction was performed for five hours and after this time the solution was heated at 90°C for 5 minutes for enzyme inactivation. The hydrolysates were then centrifuged (Eppendorf, 5810/ 5810 R) at 2683 g at 4°C for 30 minutes. The supernatant was separated from the pellet and lyophilized with LIO-5PDGT freeze-dryer (5pascal, Milano, Italy). The freeze-dried protein hydrolysate was then defatted with diethyl ether, then quantified for protein content by Kjeldahl analysis as described in Chapter 4.

2.2.2. Set of protein hydrolysates collected at different time-points

The enzymatic hydrolysis as previously described, was performed again in order to obtain a set of hydrolysates collected at different time-points. More specifically, 1.5 kg of dried ground larvae were mixed with 7.5 L of a buffer solution (Na_2HPO_4 10 mM) in a 10 L flask at pH 7.5 and at 60°C. Before addition of enzyme, the mixture was homogenised in an incubator at 130 rpm for 30 minutes, while checking the pH (ProfiLine pH 3310, WTW, Xylem Analytics LLC, Germany). The pH fluctuations were adjusted by adding concentrated NaOH. After this period, 1.5 L of solution was subsampled and collected as control (time 0). Next, 0.25 % of enzyme was added and the hydrolysis was performed for 180 min, with sub-sampling of 1.5 L aliquots of hydrolysate at different time points, being after 30 min, 60 min, 120 min, 180 min. Each sub-sample, control included, was heated at 90 °C for 5 minutes to inactive the endogenous and exogenous enzymes and subsequently centrifuged at 4°C for 30 minutes at 3220 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. Each hydrolysis reaction was performed in duplicate. The supernatants were used for characterisation.

2.2.2.1. Bulk composition

The dried supernatants and the intact larvae were characterised in terms of humidity, lipids, total N and ash. The dry matter content was determined after drying of the samples

in oven at 105 °C for 24 h. Total ash was determined after mineralization at 550 °C for two times 5 h. For crude lipid quantification an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Italy) was used with diethyl ether. The total N content was measured by Vario EL Cube (Elementar, Germany) instrument by the supplier. Briefly, the sample was burned in an oxygen rich environment at 1150 °C in the combustion tube. All the burning gasses flowed through the reduction tube (helium as support gas) and were reduced to N_2, CO_2, H_2O and SO_2. These different components were adsorbed at Selective Trap Columns and separated liberated (purge and trap technique). The detection of the components was performed with a thermoconductivity detector (TCD) cell. The proteinaceous N contribution was separated from the chitinous one, assuming that 87 % of total N in LM was from protein origin, and then multiplied for the N to protein conversion factor 5.67, as described in Chapter 4.

2.2.2.2. Degree of hydrolysis

The degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds in a protein hydrolysate, was calculated using o-phtaldialdehyde (OPA) as described in Chapter 4 with some modification. In particular, the hydrolysates were diluted in a 2 % (w/v) sodium dodecyl sulphate, stirred for 20 minutes 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 50mM OPA (in methanol) and 10 mL of NAC 50 mM, 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 to protect it from light and allowed to stir for at least 1 h before use. The OPA assay was carried out (in triplicates) by the addition of 5 µL of sample (or standard) to 215 µL of OPA/NAC reagent in microplates. The absorbance of this solution was measured, after 10 minutes of shacking, at 340 nm with Tecan Infinite® 200 PRO spectrophotometer (Tecan, Switzerland) against a control cell containing the reagent and 5 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% = (N free /N total) × 100. The first value was calculated by the OPA reactivity. The total moles of nitrogen atoms involved in peptide bonds before hydrolysis were calculated by considering that the 87 % of total N in insects is from protein origin and is involved in peptide bonds (Chapter 4).

2.3. Techno functional properties

2.3.1. Solubility

The protein solubility was determined at pH 3, pH 5 and pH 7. The supernatants collected at different time points, were diluted with demi water till a final concentration of 1 % (w/w). The pH was adjusted with NaOH and HCl and the solution was mixed with an overhead shaker (Trayster digital, IKA, Germany) for 30 minutes. The pH was checked and adjusted if needed and the samples were placed on an overhead shaker for another 30 min. After that, the tubes were weighted and centrifuged at 5910 g for 20 min at 4°C. The supernatant was separated and collected for the analysis in order to quantify the soluble N and the ionic strength. The total N was determined by a chemiluminescence detector (Multi N/C 3100 Analytik Jena). Briefly, the sample was oxidized by catalytic combustion in an oxygen atmosphere at 800°C, to N oxides. The formed measuring gas was dried and entered in the reaction chamber of the chemiluminescence detector. There, the N monoxide present in the measuring gas, was oxidized with ozone into activated N dioxide. By emitting light photons (luminescence) the molecules of the N dioxide returned to their original state. The luminescence, proportional with the N monoxide concentration, was detected using a photomultiplier. The ionic strength was measured with a conductivity meter (ProfiLine Cond 3310, WTW, Germany).

The protein solubility was calculated as described in equation (1):

$$(1)\ \text{Protein solubility } (\%) = \frac{\text{g N in the supernatant}}{\text{g proteinaceous N in the sample}}\ \text{x } 100$$

The N-content in the supernatant measured by chemiluminescence analysis was assumed to be only from protein origin, whereas the amount of proteinaceous N in the sample was determined as described in 2.2.2.2. The analysis was performed in triplicate.

2.3.2. Emulsification properties

The emulsification property was determined following the method proposed by Purschke et al. (2017) with some modification. Briefly, the supernatants were diluted with demi water at a concentration of 0.1 % (w/w) and mixed with an overhead shaker for 30 min. The ionic strength was measured with a conductivity meter (ProfiLine Cond 3310, WTW, Germany). The solution was centrifuged at 3220 g for 15 min and the supernatant mixed with commercial seed oil (1:1 v/v) and emulsified using a homogenizer (ULTRA-TURRAX® T18, IKA, Germany). An aliquot of the emulsion was immediately transferred into scaled tubes and centrifuged at 3220 g for 15 min at 20 °C. The height of the resulting emulsified

layer (HeL) and the total height of solution (Hs) were used to calculate the emulsification ability as described below (2). The analyses were performed in triplicate.

$$(2)\ \text{Emulsifying activity } (\%) = \frac{\text{Hel}}{\text{Hs}} \text{ x } 100$$

2.3.3. Oil holding capacity

For the oil holding capacity (OHC), 1 g of supernatant was transferred to a falcon tube and 10 g of demi water was added. The solution was mixed with an overhead shaker for 5 min at 55 rpm. After 30 min, the tube was centrifuged at 3000 g for 30 min at 20°C. The sample was re-weighed after 10 min of decantation upside-down (45° angle) and the holding capacity calculated as described in the equation below (3):

$$(3)\ \text{OHC}_{(\text{g oil/ g sample})} = \frac{\text{W2}-\text{W1}}{\text{W0}}$$

Where W0 was the weight of the sample, W1 was the weight of the tube and the sample, W2 was the weight of the tube after decantation. The analyses were performed in triplicate.

2.3.4. Foaming properties

The foaming properties were measured with a homemade apparatus, composed by a graduated glass cylinder, in which was placed the solution, and a pump which fluxed air inside the mixture. The supernatant was suspended in demi water at a final concentration of 1 % and mixed on an overhead shaker for 30 min. The ionic strength was measured with a conductivity meter (ProfiLine Cond 3310, WTW, Germany) and then the solution was transferred in the foam tube and the starting volume (in cm) was noted. The air flow was bubbled through the sample at a flow rate of 2 L/h for 1 minutes and the final volume reached by the foam was red and reported. The analysis was performed in triplicate. The foam capacity was measured with the following equation (4):

$$(4)\ \text{Foam capacity } (\%) = \frac{\text{Volume foam}}{\text{Volume start}} \text{ x } 100$$

2.3.5. Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL., USA). The data were subjected to one-way analysis of variance (ANOVA) to determine the differences between samples. Significant differences were compared at a level of $p < 0.05$.

3. Results and Discussion

3.1. Techno functional assay of protein hydrolysate

Insect protein hydrolysate was obtained from LM with the protease from *Bacillus licheniformis.* The whole insect starting material presented 52 ± 0.2 % of proteins on dry matter basis. After five hours of hydrolysis, a hydrolysate with a DH of 14.9 ± 0.2 % was obtained. The final protein hydrolysate, after defatting step, contained 58.2 ± 1.3 % of proteins on dry matter basis, corresponding to the 46.8 ± 0.9 % of total protein content in the starting material (Table 17). The protein hydrolysate presented a high solubility property, with 95 ± 4 % of total proteins soluble at pH 3, 5 and 7, explicable by the presence of peptides and free amino acids. Further, the protein hydrolysate presented also good capacity to hold oil, with 6.7 ± 0.6 g oil per g of sample. This value was 5 times more than the ability evaluated for casein and egg white. On the contrary, the protein hydrolysate did not display foaming ability and only a slight capacity to form emulsions. In order to better explore if the high DH % reached could have affected foaming and emulsifying property, a new enzymatic hydrolysis was performed, and different hydrolysates collected at different time-points.

3.2. Generation of protein hydrolysates with different degrees of hydrolysis

Via subsampling during enzymatic assisted hydrolyses of LM starting material, water soluble protein hydrolysates with a DH ranging between 2.9 % and 9.8 % were obtained. The water-soluble fraction of the control sample, even if obtained without the enzyme addition, presented itself a small DH % which could be related to a mild denaturation and hydrolysis occurred during the heat inactivation. This is in accordance with the findings of Purschke et al. (2018) and Hall et al. (2017), who both demonstrated an initial DH of about 5 %. No significant differences were determined on DH% from 30 min to 60 min of hydrolysis, while a significant increase was monitored from 60 min till 180 min.

The proximate composition of the soluble fraction of the control and the hydrolysates was determined in terms of humidity, protein, lipid and ash content and is reported in Table 17. All samples presented a similar compositional profile, rich in protein (66 ± 4 % on DM on average) and with a slight amount of lipid (on average 15 ± 4 % on DM basis).

The addition of exogenous enzyme significantly increased the protein extraction yield from a 6.8 ± 0.9 % for the control sample to the 42.1 ± 4.3 % after 180 min of hydrolysis. This extraction yield could be further improved by increasing the time of hydrolysis, as determined before for the 300 min of hydrolysis, or the enzyme concentration.

Table 17 Bulk composition of freeze-dried soluble protein hydrolysate samples collected at different time points during the enzymatic hydrolysis with information about DH% and protein extraction yield.

Hydrolysis time	Protein % on DM	Lipid % on DM	Ash % on DM	DH%°	Protein extraction yield %
			Preliminary test		
300 min*	58.2 ± 1.3[a]	nd	nd	14.9 ± 0.2[e]	46.8 ± 0.9[e]
			Detailed test		
Control (0 min)	68.8 ± 4.9[b]	16.3 ± 0.9[ab]	10.7 ± 0.1[a]	2.9 ± 0.5[a]	6.8 ± 0.9[a]
30 min	69.2 ± 4.5[bc]	10.9 ± 6.3[a]	9.3 ± 1.3[a]	4.8 ± 0.5[b]	14.5 ± 1.8[ab]
60 min	65.4 ± 1.1[bc]	12.7 ± 2.3[ab]	9.6 ± 0.8[a]	5.7 ± 0.5[b]	23.3 ± 2.4[bc]
120 min	63.5 ± 1.7[cd]	16.04 ± 2.36[ab]	8.66 ± 1.98[a]	8.3 ± 0.5[c]	32.7 ± 3.2[cd]
180 min	62.1 ± 0.3[d]	17.3 ± 2.6[b]	9.1 ± 0.6[a]	9.8 ± 0.7[d]	42.1 ± 4.3[d]

Results are expressed as the mean ± standard deviation (n=6). Values followed by different letters within one column are significantly different ($p < 0.05$). Abbreviation: degree of hydrolysis, DH; dry matter, DM; lesser mealworm, LM; nd, not determined;

° For samples collected from 0 to 180 min DH% was calculated by OPA assay, for sample obtained after 300 min of hydrolysis DH% was calculated from pH-STAT method;

****hydrolysate produced in a separated enzymatic hydrolysis as described in 2.2.1.***

3.3. Functional properties

3.3.1. Solubility

The solubility property in function of pH is an important parameter of the protein hydrolysates in view of their potential industrial application. Its driving force is the ionic interactions between proteins and water. In the present work, all hydrolysates were more soluble than native proteins at both pH 3 and pH 7 with values higher than 82 %, even if only after 60 min of hydrolysis the solubility started to significantly increase. In general, all samples, control included, presented better solubility at these pH than at pH 5 (Table 18). The lower solubility at pH 5 is due to the proximity to the isoelectric point, in accordance with the findings of Purschke et al. (2018), who determined the isoelectric point *of Locusta migratoria* hydrolysate at pH 4. The proximity to the isoelectric point could also justify the high standard deviation (15 % on average) of trials performed at pH 5. In fact, at isoelectric pH, the zero net charge reduces the repulsive electrostatic forces, whereas the attraction forces predominate and cause the protein aggregation and precipitation. In the hydrolysate produced after 300 min of hydrolysis no significant differences were identified at pH 3, 5 and 7. This is in agreement with wat reported by Hall et al. (2017) where cricket hydrolysate produced after 90 min with 1.5 % of Alcalase enzyme did not present big differences at pH 3, 7, 8 and 10.

The % of solubilized protein in the current study was higher compared to Purschke et al., (2018) where the solubility of *Locusta migratoria* protein hydrolysate did not exceed the 60 %. The differences could be related to the condition of hydrolysis, which allowed us to reach higher protein extraction yield. Instead, some similarities where identified with the results reported by Hall et al. (2017), where the solubility of cricket protein hydrolysates exceeded the 87 % at neutral and alkali condition.

Table 18 Techno functional properties of freeze-dried samples collected at different time: protein solubility (pH 3, 5, 7), emulsification ability, oil holding and foaming capacity. Information about casein and egg white functionality are also reported.

Sample	Protein Solubility %			Emulsification activity %	Oil holding capacity g oil/g sample	Foaming capacity %
	pH 3	pH 5	pH 7			
			Preliminary test			
300 min*	94.8 ± 4.8^{ab}	96.1 ± 4.2^{b}	94.3 ± 0.6^{d}	1.2 ± 0.3^{a}	6.7 ± 0.6^{d}	0
			Detailed test			
Control	86.7 ± 0.3^{a}	60.3 ± 28.6^{a}	81.04 ± 0.95^{a}	20.99 ± 4.06^{e}	1.4 ± 0.1^{a}	0
30 min	91.1 ± 2.1^{ab}	67.8 ± 22.4^{a}	82.1 ± 1.6^{ab}	11.8 ± 3.1^{d}	1.4 ± 0.1^{a}	0
60 min	92.7 ± 1.8^{b}	58.1 ± 12.9^{a}	85.8 ± 1.7^{bc}	6.7 ± 2.7^{c}	1.7 ± 0.3^{ab}	5.3 ± 4.2^{a}
120 min	93.6 ± 1.6^{b}	67.1 ± 17.4^{a}	90.4 ± 2.7^{cd}	3.1 ± 1.1^{bc}	1.99 ± 0.25^{bc}	12.59 ± 7.02^{a}
180 min	94.9 ± 2.6^{b}	69.9 ± 2.6^{a}	90.9 ± 2.5^{d}	2.03 ± 0.36^{b}	2.2 ± 0.2^{c}	73.6 ± 16.1^{b}
			Standard protein			
Casein	nd	nd	nd	34.3 ± 2.3	1.52 ± 0.03	43 ± 5
Egg white	nd	nd	nd	52.5 ± 3.5^{g}	1.1 ± 0.2	123 ± 24

Results are expressed as the mean the mean ± standard deviation (n=6). Values followed by different letters within one column are significantly different ($p < 0.05$). Nd: not determined.

[*]hydrolysate produced in a separated enzymatic hydrolysis as described in 1.2.1.

In Figure 14 the % of solubilized protein at different pH was plotted against the DH % and a positive correlation was observed for solubility at pH 3 and 7 (respectively, r = 0.842 and r = 0.876), whereas close to the isoelectric point, protein solubility stayed mostly unchanged.

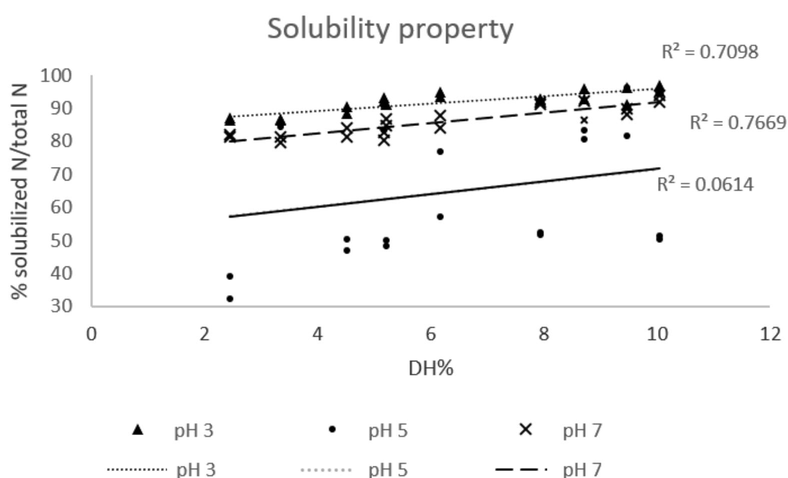

Figure 14 Solubility properties of 1 % of protein hydrolysates (average ionic strength 2.8 ± 0.2 mS/cm) reported as % of solubilized N in function of DH%

An increase in solubility with increasing DH-values may be associated with an increase of small peptides, that exposes more ionisable amino and carboxylic groups. These groups promote the formation of hydrogen bonds with water molecules and, as such, solubility improvement. This positive correlation was also determined by Purschke et al. when Alcalase, Neutrase and Papain were used to produce protein hydrolysates from locust, while Flavourzyme did not show any correlation at pH 3, 5, 7 and 9. The latter maybe due to the different proteolytic activity of this enzyme (Purschke et al., 2018). The same correlation was determined also for other animal and vegetable matrices, such as salmon and egg hydrolysed with Alcalase (Gbogouri et al., 2004; Bao et al., 2017), rice endosperm and chickpea hydrolysed respectively with endoprotease and with Alcalase (Nisov et al., 2020; Ghribi et al., 2015). At pH 5 we did not determine any correlation, due to the instable peptide solubility near the isoelectric point. The same phenomenon was underlined for sardinella hydrolysed with Alcalase, where no correlation between DH and solubility was determined near the isoelectric point determined at pH 3 and 4 (Souissi et al., 2007).

3.3.2. Emulsifying activity

Emulsions consist of two immiscible phases, and the oil in water emulsions are the most common in food products. In the present work, the control sample presented the highest emulsifying ability (EA), which significantly decreased immediately after the enzyme addition. In particular, as illustrated in Figure 15, a negative correlation (r = 0.887) was recorded between emulsifying activity and DH% underlining the loss of this property during the hydrolysis, till manifesting the complete coalescence of oil droplets and phases separation.

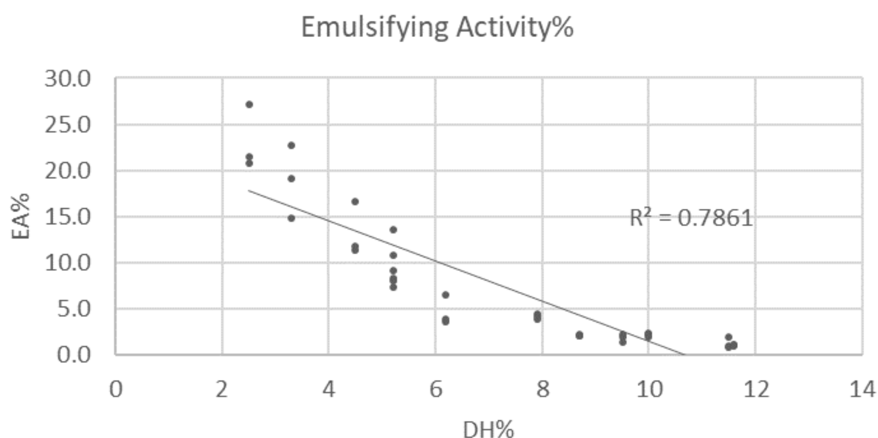

Figure 15 Emulsifying activity of 0.1 % of protein samples (average pH 7.5 ± 0.2 and ionic strength of 1.3 ± 0.1 mS/cm) reported in function of DH%. The analysis was done in triplicate.

The protein emulsification mechanism is attributed to their migration to the surface of freshly formed oil droplets during homogenization. Proteins are able to form a protective film promoting oil-in-water emulsion due to their duality for the presence of hydrophilic and hydrophobic groups. The shorter peptides, released from proteins after hydrolysis, may migrate to the interface oil/water more rapidly than proteins, but they are less efficient to reduce the interfacial tension between the two phases and to create a strong interfacial film round fat droplets (Van der Ven et al., 2001). Similar results were found by Quaglia & Orban (1990), Kristinsson & Rasco (2000) and Purschke et al. (2018) working with sardine, salmon and cricket, respectively. In this last case, a significant loss in emulsifying activity in comparison to the control sample was observed when cricket protein flour was hydrolysed with Neutrase and Flavourzyme mainly at acidic pH, whereas at alkali pH this property was maintained. At neutral pH the two enzymes displayed a different behaviour, with Neutrase negatively affecting the emulsify ability, while Flauvourzyme improved it, maybe due to the different specificity of proteolytic enzymes. Hall et al. (2017) identified

the same correlation when 0.5 % of enzyme/substrate concentrations were used, while at higher enzyme concentrations (3 %) no correlation were identified.

3.3.3. Oil holding capacity

The oil holding capacity (OHC) of control and hydrolysates was correlated to the different DH% and reported in Figure 16. The lowest value, 1.4 ± 0.1 g oil/g sample, was determined for the control sample. This amount increased with an increasing DH % till the 2.2 ± 0.2 g oil/g sample only after 180 min of reaction and has expected to continuously increase. In fact, as determined in the preliminary test, after 300 min of reaction the capacity to hold oil increased till 6.7 ± 0.6 g oil/g sample.

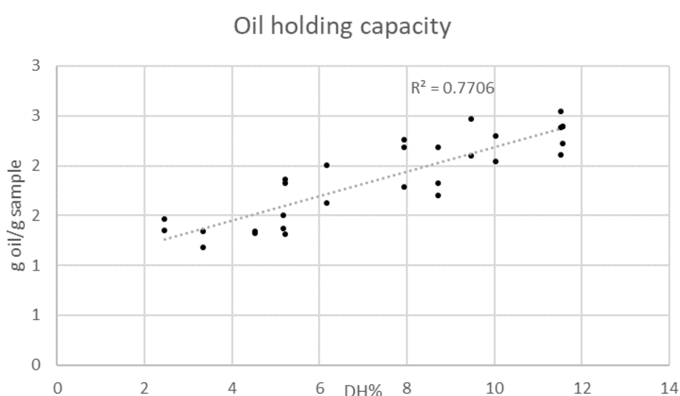

Figure 16 Oil holding capacity of the different hydrolysates produced expressed as g oil/g sample in function of DH%. The analysis was done in triplicate

This raise could be explained by the modification of protein structure and the exposure of more hydrophobic side chain of amino acids, which, before the hydrolysis, were trapped in the protein folding, promoting the physical entrapment of oil (Mune, 2015). Purschke et al. demonstrated the improvement in OHC of protein hydrolysates when compared to the unhydrolyzed samples even if no correlations were calculated (Purschke et al., 2018). Souissi et al. obtained results in agreement with Purschke after the hydrolysis of sardinella (Souissi et al., 2007). However, several authors observed in plant protein hydrolysates an initial increase in OHC upon hydrolysis (Wouters et al., 2016). In general, a critical point exists at which the liberation of polar ionizable groups has a larger impact on OHC than the increased availability of hydrophobic regions.

3.3.4. Foaming

Foam is a colloidal system comprising a continuous aqueous phase with dispersed gas. Proteins, due to their amphiphilic nature, represent a good surfactant with the hydrophobic portion oriented to the air bubbles and the hydrophilic part to the watery phase (Kristinsson & Rasco, 2000). In Figure 17, the foaming capacity of the different hydrolysates, with a comparable average pH and ionic strength, was plotted to their DH%, along with the control and an exponential correlation determined. The control sample did not present foaming capacity, which on the contrary seemed to appear in the hydrolysates collected after 60 min of hydrolysis, reaching their maximum after 180 min of hydrolysis. Nevertheless after 300 min of hydrolysis no foam capacity was determined, defining this exponential trend only up to limit value of DH, which was between 10 % to 15 %.

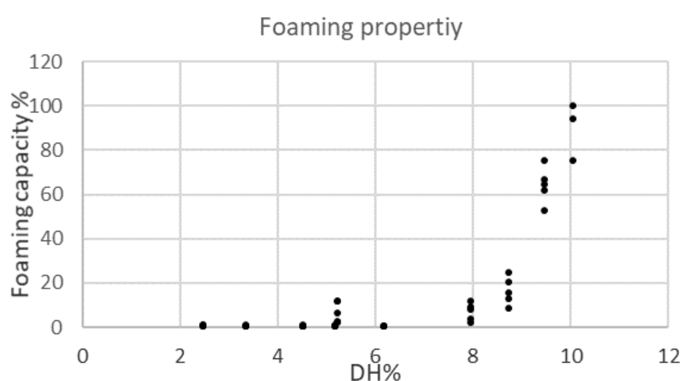

Figure 17 Foaming capacity of 1 % of protein samples (average pH 7.9 ± 0.3 and ionic strength of 2.8 ± 0.2 mS/cm) reported in function of DH% with the trendline. The analysis was done in triplicate

An increase in DH% probably led to a more pronounced amphiphilicity, which may enhance the interfacial interaction with air bubbles until a DH of 10%. Even if the foaming capacity appeared after 60 min of hydrolysis, the formed foams did not display any stability property and after 1 min they started to collapse. These results were in line with what reported for other protein hydrolysates obtained from edible insects (Purschke et al., 2018; Hall et al., 2017).

4. Potential applications

In the present work, lesser mealworm (LM), a common edible insect, was used to produce protein hydrolysates with the commercial protease from *Bacillus licheniformis*. The techno functional properties of protein hydrolysates with a DH ranging between 3 % and 15 % are summarised in Table 18. The potential correlation between DH % and techno functionality was explored for the second set of hydrolysis in which the same processing condition have

been applied. Nevertheless, the results collected from the hydrolysate obtained after a long hydrolysis reaction (300 min) are also here considered for the potential application.

The enzymatic hydrolysis led to an improvement of solubility and oil holding ability, while it reduced the emulsifying property. The foaming capacity was found to improve somehow with increasing DH reaching the maximum after 180 min of hydrolysis, but the foam stability remained very low. By considering the 300 min long hydrolysis, this property was completely impaired. Furthermore, it was determined that functional properties could be tailored according to their DH value. In particular, the solubility of protein hydrolysates at pH 3 could be implemented with only 30 min of enzymatic hydrolysis, since no significant differences were evaluated after that time, while at pH 7 only after 120 min of hydrolysis no significative improvement was observed in solubility property. On the contrary, at pH 5 the near to isoelectric point impaired the solubility properties of both control and hydrolysates. With the increase in DH% we determined also a slight, albeit significant, improvement of LM proteins to hold oil till after 180 min of hydrolysis, becoming even more pronounced after 300 min of hydrolysis. Compared to casein and egg white, the OHC was high, implying a high added value to insect protein hydrolysates in this perspective. This is not of a secondary importance since the oil holding capacity is a property appreciated especially for the meat industry. In fact, the higher the OHC, the higher the ability of a food or feed formula to retain flavours and improve the palatability. The high solubility property at pH 3 and 7, and the increase in oil holding capacity demonstrated the potential for using LM hydrolysates in acidic food systems, such as sports beverages and acidified sauces, and in feed system as replacement of milk for weanling animals. The enzymatic assisted extraction affected the ability of LM proteins to act as surfactants for marinating oil and air droplets dispersed in an aqueous solution. In fact, immediately after 30 min from the enzyme addition the emulsifying ability of LM protein hydrolysates started to significantly decrease. Furthermore, the emulsify properties evaluated for all the insect samples were far to the one calculated for egg white, which is known to be a good emulsifier. For this reason, for feed and food emulsions, which will be prepared with insect hydrolysates, it could be necessary to add emulsifiers to stabilize the formulation. All the technological properties described till now showed linear correlation with the different DH %. On the contrary, the foaming capacity presented an exponential correlation with the DH % till 180 min of hydrolysis. In particular, the ability of LM proteins to foam began after 60 min of enzymatic hydrolysis and significantly increased after 120 min of hydrolysis. After 300 min of hydrolysis no foaming ability was determined. The increasing in hydrolysis time, and so DH %, could have impaired the ability of peptides to arrange round air bubbles, due to the shorter length. The maximum foam ability was determined after 180 min of hydrolysis, overcoming the foam ability of casein. Nevertheless, the formed foams did not

show stability and, immediately after 1 min from their constitution, they started to collapse. For this reason, insect protein hydrolysate could not be used as foaming agents but, due to the absence of foam stability, could be included in food or feed beverages.

5. Conclusions

In conclusion, this work provides for the first time information about the influence of DH % on techno functional properties of protein hydrolysates produced from LM under process condition that are scalable for the industrial production. These results demonstrated that the functional properties of LM could be tailored by enzymatic assisted extraction. Nevertheless, deep investigations are needed in order to evaluate how these properties could be affected when insect protein-based ingredients will be included in food/feed complex matrices.

Acknowledgements

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CHAPTER 6

Shotgun proteomics, *in-silico* evaluation and immunoblotting assays for allergenicity assessment of lesser mealworm and black soldier fly and their protein hydrolysates

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Giulia Leni, Tullia Tedeschi, Andrea Faccini, Federico Pratesi, Claudia Folli, Ilaria Puxeddu, Paola Migliorini, Natasja Gianotten, Johan Jacobs, Stefaan Depraetere, Augusta Caligiani, Stefano Sforza. Shotgun proteomics, *in-silico* evaluation and immunoblotting assays for allergenicity assessment of lesser mealworm and black soldier fly and their protein hydrolysates.

Abstract

Since 2018, insects have belonged the category of Novel Foods and the presence of allergens represents one of the main hazards connected to their consumption, also due to the potential cross-reactivity with Arthropoda pan-allergens. In the present work, the allergenicity assessment of black soldier fly and lesser mealworm was performed with a shotgun bottom-up proteomic approach combined with *in-silico* assessment, followed by IgG- and IgE-immunoblotting experiments. The peptides identified, filtered for their abundance and robustness, belonged mainly to muscle proteins, which represented the most abundant protein group. The relevant potential allergens were *in-silico* identified by sequence similarity to known allergens, and among them tropomyosin resulted the most abundant insect allergen. IgG-immunoblotting analysis with anti-Tropomyosin I antibodies and IgE-immunoblotting assay with serum from patient allergic to crustacean tropomyosin were performed in order to assess the immunoreactivity in both insects. The immunoassays were carried out also on protein hydrolysates extracted by treating insects with protease from *Bacillus licheniformis* (1%, 60°C, pH 7.5). While IgG-immunoblotting demonstrated the loss of immunoreactivity for both hydrolysates, IgE-immunoblotting showed a partial immunoreactivity preservation, also after hydrolysis, in the case of black soldier fly hydrolysate, and a total loss of immunoreactivity for lesser mealworm hydrolysate.

1. Introduction

Novel food protein sources are being studied in order to meet the future requirement for food, in connection to the perspectives of growing population (Boland et al., 2013). Insects represent a good source of proteins, not only because of the high protein content in some species, but also for their nutritional quality in terms of essential amino acids profile. Not of a secondary importance, insect breeding, in comparison to common livestock, is characterized by many environmental advantages, such as less land use, feed and water requirement, fewer greenhouse gas emission and high feed conversion ratio (Rumpold et al., 2013).

From January 1st of 2018, insects have been included in the category of novel foods and the European Food Safety Agency (EFSA) opinion is mandatory before their marketing (EU Regulation No 2015/2283). In 2015, EFSA assessed for the first time the safety related to insect consumption. The potential hazards connected to the use of insects in food or feed were deemed to be related to exogenous and endogenous factors, which could be influenced also by harvesting and processing methods (EFSA, 2015). The presence of allergens represents one of the main endogenous risk related to the consumption of insects. Insect-based food ingredients could cause an allergenic response either due to a primary sensitization, or to a cross-reaction. Primary sensitization is related to the ability of insect proteins to elicit an allergic reaction not related to other food allergies. This risk is widespread mainly in regions where edible insects are commonly used (e.g. China) (Ji et al., 2009). A cross reaction, more likely where insects are not commonly consumed, like western countries, is related to an IgE cross-reactivity between insect proteins and known allergens belonging to species taxonomically related (other Arthropoda such as mites and crustaceans) (Fels-Klerx et al., 2018). In the last five years the potential allergic risk of whole insects' consumption has been studied and evaluated. Many authors demonstrated a cross-reactivity between insects and other Arthropoda (crustaceans, mite) identifying as pan-allergens different proteins involved in the muscle contraction (actin, tropomyosin, troponin C), in enzymatic pathway (arginine kinase, fructose diphosphate aldolase) or part of the hemolymphatic system (hexamerin 1B) (Gier et al., 2018; Downs et al., 2016).

The texture and appearance of insects are perceived as strong barrier for future consumers, moving the industrial interest to processed insect (e.g. flour, extracted proteins) (Sogari et al., 2017). It is well known that technological processes may alter the allergenic properties of food proteins (Verhoeckx et al., 2015), but in literature there are only few data about the impact of technological process on insect allergenicity.

Broekman et al. (2015) and van Broekhoven et al. (2016) evaluated the effect of different thermal treatment (e.g. blanching, baking, frying, microwave heating) on mealworm immunoreactivity and concluded that heat processing did not lower the allergenicity of mealworm proteins but affected their solubility properties. In contrast, Pali-Schöll et al. (2019) demonstrated the ability of thermal treatment to reduce the potential allergic risk of *Locusta migratoria*. This difference could be due to the diverse combination time/temperature applied in the process and the different insect species tested. Pali-Schöll demonstrated also the ability of enzymatic hydrolysis to reduce insect immunoreactivity.

Enzymatic hydrolysis is indeed widely used in the food/feed sector in order to extract proteins from vegetables and meat by-products and is exploited also to obtain ingredients with bio and techno functional properties (Del Mar Contreras et al., 2019). In Chapter 4 this biotechnological tool was explored on insects, deeply investigating the composition of protein hydrolysates obtained. The protease assisted extraction represents also an effective approach to reduce the allergenicity of different food matrices (Clemente, 2000; Tavano 2013; Meinlschmidt et al., 2017) and was demonstrated useful also for crickets, mealworms and locust species (van Broekhoven et al., 2016; Pali-Schöll et al., 2019; Hall et al., 2018).

In the present study, for the first time the proteome of lesser mealworm (*Alphitobius diaperinus*, LM) and black soldier fly (*Hermetia Illucens*, BSF) larvae was characterized by a shotgun proteomic approach, evaluated in order to find potential allergens, and to estimate their abundance, via *in-silico* assessment and *in-vitro* immunoassays. Furthermore, the enzymatic hydrolysis was explored for these two insects as a possible way to reduce the allergenic risk related to the consumption of insect proteins.

2. Material and methods

2.1. Insect sample

BSF larvae were provided by Circular Organics (Turnhout, Belgium), whereas LM larvae by Protifarm (Ermelo, The Netherlands). BSF and LM larvae were reared in temperature and humidity-controlled rooms, with standard temperature ranging between 28 and 32°C and humidity above 60%. Larvae were fed daily with standard rearing feed and at the end of the rearing cycle (15 days for BSF and 28 days for LM) were separated from the substrate. Larvae were killed by packing them vacuum sealed and freezing at -30°C. After one week, samples were freeze-dried and stored at -20°C for the future analysis. Samples were grinded for 2 minutes with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Germany) before each analysis.

2.2. Protein extraction

Grinded insects were defatted with diethyl ether following the method proposed in Chapter 2 using a Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy). Defatted flours were subjected to the protein extraction protocol proposed by Abdel Rahman et al. (2013) with some modifications. Briefly, 0.5 g of sample were homogenized in an ice-bath with 10 mL of a Tris buffer (25 mM Tris-HCl pH 8.0, 1M KCl, 50 mM DTT and 0.5 mM EDTA) using Ultra Turrax homogenizer (11000 rpm) for 1 minute. The mixture was left in an ice bath under agitation for 7 hours. The slurry was than centrifuged at 2683 g for 30 minutes at 4 °C and the supernatants collected were subjected to desalting using 3kDa Amicon® Ultra Centrifugal Filters (Merck Millipore). The final protein concentration was determined using the Qubit Protein Assay Kit and the Qubit 2.0 fluorometer (Invitrogen, California, USA). The protein extraction was performed in duplicate and the extracts were used for further protein molecular characterization and allergenicity assessment.

2.3. In-solution tryptic digestion

The protein identification was carried out with a Shotgun proteomic approach following the method proposed by Kinter et al. (2000). In particular, the defatted sample was suspended in 6 M Urea, 100 mM Tris-HCl pH 7.8 at a final protein concentration of 10 mg/mL. The mixture was homogenized at 4 °C and centrifuged at 2683 g for 30 minutes at 4 °C. 100 µL of the supernatant was mixed by gentle vortex with 5 µL of a reducing solution (200 mM DTT, 100 mM Tris-HCl pH 7.8) in order to reduce the disulfide bonds. After 1 hour, 20 µL of an alkylating reagent (200 mM iodoacetamide, 100 mM Tris-HCl pH 7.8) was added and mixed by gentle vortex. After 1 hour, 20 µL of the reducing solution was added to consume any unreacted iodoacetamide and mixed by gentle vortex. After 1 hour, the urea concentration was reduced by diluting the mixture with 775 µL of MilliQ® water and combined with 100 µL of a trypsin solution (200 ng/µL trypsin and 100 mM Tris-HCl pH 7.8). The samples were mixed with gentle vortex and the trypsin digestion carried out overnight at 37 °C. The reaction was stopped decreasing the pH below 6 by adding acetic acid. The digests were dried under nitrogen before the mass spectrometry analysis.

2.3.1. Protein identification by High Resolution Mass Spectrometry on LTQ-Orbitrap Instrument

The dry protein extracts were reconstituted with 50 µL of 0.2% formic acid solution for mass spectrometric analysis. High resolution mass spectrometry was performed on the samples for peptide identification using a µHPLC DIONEX Ultimate3000 interfaced with an LTQ-Orbitrap XL Thermo Fisher Scientific. Column: Jupiter C18 4 µ, Proteo 90 Å

150 × 0.30 mm, Phenomenex; eluent A: water +0.1% formic acid; eluent B: acetonitrile +0.1% formic acid; flow: 5 µL/min, gradient: 0–4 min from 100% A to 95% A, 4–60 min from 95% A to 50% A, 60–62 min from 50% A to 10% A, 62–72 min 10% A, 72–74 min from 10% A to 95% A, 74–90 min 95% A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0–75; ionization mode: ESI+; scan range (m/z): 200–1800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS + p res = 30,000 or (250.0–2000.0); (ion trap) ITMS + c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000. Protein identification was performed by using PEAKS software (Bioinformatics Solutions Inc) and INSECTA (UniProt) database. Positive hits for protein identification was arbitrarily set for all those proteins identified by the program with a score (expressed as -10lgP) >50, all those peptides with a score (-10lgP) >20 and ppm in the range ± 6, since such value should reduce the risk of false positives to zero.

2.3.2. *In-silico* analysis for the prediction of allergenicity

The UniProt database (www.uniprot.org) was employed to retrieve information about the sequences identified in BSF and LM protein extracts by HRMS. For the prediction of allergenicity it was used Allermatch™ tool (www.allermatch.org) by which the amino acid sequence of a protein of interest can be compared with sequences of allergenic proteins. Peptides identified, after data filtering, were entered in the webtool and analyzed with the wordmatch method. Positive hits as potential allergens were arbitrarily taken for all the peptides having more than 50% of exact wordmatches with known allergens.

2.4. Enzymatic assisted extraction

Protease from *Bacillus licheniformis* (≥ 2.4 U/g; EC Number 3.4.21.62) was used in order to produce a peptide rich fraction from grinded LM and BSF larvae. The hydrolysis reactions were carried out as described in Chapter 4 in the optimal condition for enzyme (pH 7.5 and temperature of 60°C). For the hydrolysis, 5 g of ground insects were mixed with 45 mL of a phosphate buffer (Na_2HPO_4 10 mM) of the specific solution and 0.05 mL of enzymes in a 50 mL plastic falcon. The falcon was placed in a heating bath at the correct temperature for the enzymatic activity and mixed with a magnetic stirrer. The hydrolysis reaction was carried out for 18 hours in order to reach the maximum enzyme activity and then at 90°C

for 5 minutes for inactivating the proteases. The hydrolysates were then centrifuged (Eppendorf, 5810/5810 R) at 2683 g at 4°C for 30 minutes. The supernatants were separated from the pellets and collected at -20°C for subsequent analysis.

2.4.1. Degree of hydrolysis

The degree of hydrolysis (DH), which is defined as the percentage of cleaved peptide bonds in a protein hydrolysate, was calculated using o-phtaldialdehyde (OPA) method described in Chapter 4. The hydrolysed sample was diluted in a 2% (w/v) SDS, stirred for 20 minutes 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 50mM OPA (in methanol) and 10 mL of NAC 50 mM, 5 mL of 20% (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was protected from light by using aluminium foil 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 minutes at 1000 rpm. The absorbance of the obtained solution was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, U.S.A.) against a control cell containing the reagent and 20 µL of the buffer used for the sample. The intrinsic absorbance of the sample 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 first value was calculated by the OPA reactivity. The total proteinaceous N of proteins was calculated by considering the total N and by separating the protein N contribution from the chitin one, as described for these two insect species in Chapter 4.

2.5. SDS–PAGE and IgG-immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight distribution of the insect protein extracts (paragraph 2.2) and protein hydrolysate (paragraph 2.4). Protein fractions were analysed on 12% Bis/Tris Criterion™ XT Bis-Tris Gel (Bio-Rad, Hercules, CA, U.S.A) by using MES running buffer. The gel was finally stained with Coomassie Brilliant Blue, destained and finally scanned with GS-800 Calibrated Densitometer controlled by the software “Quantity one” (BIO-RAD). IgG-immunoblotting was performed on the same samples used for SDS-PAGE, which were separated on gels and electro-transferred onto nitrocellulose membranes. The assays were performed using anti-TPM1 Polyclonal Antibody (Thermo Fisher PA5-29846) produced against the highly conserved 92-273 AA sequence of human tropomyosin. The membrane was incubated under agitation overnight with 5% skimmed milk powder in incubation

buffer TT (0.3% Tween 20 in TBS pH 9.6). The blot was washed three times using the TT buffer and then incubated with primary antibody anti-TPM1 polyclonal Antibody (1:250 dilution in TT buffer) at room temperature for 1 hour under agitation. The blot was washed three times with TT buffer and then incubated with Rabbit IgG Secondary Antibody (polyclonal) conjugated with DyLight 680 (1:5000 dilution in TT buffer) for 1 hour at room temperature under agitation. The membrane was washed extensively with TT buffer and then preserved in 50 mM Tris pH 7.5 until the detection with Li-Cor Odyssey Infrared Imaging System.

2.6. IgE-Immunoblotting

Twenty µL of insect protein extracts (paragraph 2.2) and hydrolysate (paragraph 2.4) were analyzed using 15 % acrylamide/Tris–HCl gels (Criterion, Biorad, Germany). After electrophoretic run, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% (w/v) of BSA in incubation TT buffer (0.05 % Tween in TBS) for 60 min after which the membrane was incubated overnight at 4°C with diluted sera (1:10 in TT, BSA 5%) from non-allergic person and patient who showed a food allergy sensitisation to crustacean, as determined by skin test, and, in particular, with IgE positivity to tropomyosin (Pen a 1). After incubation the blot was washed three times with TT buffer and then incubated with Anti-human IgE diluted 1:10000 in TT buffer for 90 min. Bound IgE was detected using a chemiluminescent peroxidase substrate kit, blots were scanned using a BioRad Versadoc 1000 image scanner (Bio-Rad) and images analysed using Quantity One BioRad software.

3. Results

3.1. Shotgun characterization of insect proteome

A shotgun proteomic approach was applied in order to evaluate the major determinants of the proteome of LM and BSF by HRMS on LTQ-Orbitrap instrument. Peptide identification was achieved by comparing the tandem mass spectra, derived from peptide fragmentation, with theoretical tandem mass spectra generated from *in-silico* digestion of *Insecta* protein database. The use of this targeted database, which only comprises insect proteins, increased the sensitivity of protein identification. A total of 261 and 107 peptides were identified, respectively in LM and BSF protein extracts. In order to reduce the presence of false positive, a data filtering was performed and the cut off arbitrarily set at 20 (-10lgP parameters in the PEAKS software® measuring the statistical significance of peptide-spectrum match) for the score and at ± 6 ppm for mass accuracy. After data filtering, 127 and 67 peptides for LM and BSF, which were respectively mapped to 20 and 17 proteins,

were retained and reported in detail in the Table S2 and S4 in the Supplementary Material. Indeed, the application of such restricted parameters reduced the amount of identifiable peptides, but also allowed to focus our characterization on the more confident hits and most abundant proteins. In Figure 18 we reported, with a schematic representation, the peptide distribution according to their functionality.

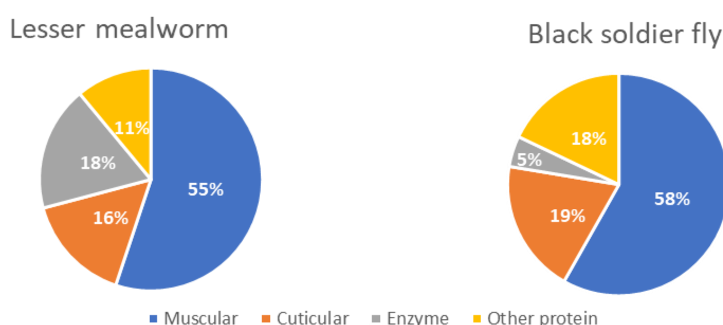

Figure 18 Distribution of peptides identified in LM and BSF protein extracts based on their functionality: muscular, cuticular, enzyme and other protein.

The main proteins identified by HRMS, both for BSF and LM, were muscle proteins (in particular actin, tropomyosin, myosin, troponin), which represented more than the 50% of identified proteins, followed by cuticular and metabolic proteins (enzymes and other proteins). It is important to underline that the *Insecta* database, used for the identification, is not complete, which implies a lesser amount of identified proteins, also in consideration to the strict cut off applied. In the Table 19 is reported a list of all the proteins identified, the number of peptides which covered the sequence and the peptide average Area. This last parameter was used to order the protein list according to their abundance, from the most abundant to the least abundant.

Table 19 The main proteins identified in both insects, lesser mealworm and black soldier fly, with information about the number of peptides, the average abundance and the protein functionality. We reported only the proteins represented by more than 2 peptides and isoforms of the same protein were grouped together under the parental protein.

	Lesser mealworm				**Black soldier fly**		
Protein	**n° peptides**	**average Area**	**Function**	**Protein**	**n° peptides**	**average Area**	**Function**
Actin	8	1.07E+06	Muscular	Actin	10	7.13E+04	Muscular
Myosin	39	5.67E+05	Muscular	Cuticle protein	12	6.14E+04	Cuticular
ADFb like protein	2	5.56E+05	Other	Hexamerin	2	3.86E+04	Other
Arginine kinase	5	5.28E+05	Enzymatic	Tropomyosin	20	3.68E+04	Muscular
Cuticle protein	18	3.99E+05	Cuticular	Ca binding	2	2.69E+04	Other
Tropomyosin	11	3.41E+05	Muscular	Troponin	2	2.55E+04	Muscular
Apolipophorin protein	2	3.40E+05	Other				
Larval serum protein	6	3.15E+05	Other				
ATP synthase	11	2.71E+05	Enzymatic				
Paramyosin	5	2.71E+05	Muscular				
Troponin	5	2.45E+05	Muscular				
Chitin binding	2	2.12E+05	Cuticular				
Catalase	2	7.51E+04	Enzymatic				

For both LM and BSF, peptides from actin presented the highest average Area, clearly representing the most abundant protein. Actin is a multifunctional protein which express its function after the creation of a microfilament with other proteins. Actin is involved, with myosin, tropomyosin and troponin, in the formation of muscular myofibrils, engaged in the muscle contraction (de Gier et al., 2018). Myosin represented the protein with the highest amount of identified peptides in LM (39 peptides), whereas tropomyosin was the one having most peptides identified in BSF (20 peptides). Both proteins belong to the category of muscle proteins. All the above results demonstrate how the group of muscle proteins were the most abundant proteins, with the highest number of peptides identified (Figure 1). Cuticle proteins, in both insects, were also detected with a high number of peptides, 18 peptides in LM and 12 in BSF. These proteins characterize the insect external coating and creates a complex with chitin, which is the main constituent of the exoskeleton (Andersen et al., 1995).

In literature, especially in the last years, many authors have explored the proteome of edible insects, but only few of them have applied a shotgun approach. Rabani et al. (2019) evaluated the protein profile of BSF and blow fly (*Lucilia serenica*) for their future non-food applications. They performed a protein extraction by the trichloroacetic acid and acetone method, followed by tryptic digestion before the mass spectrometry analysis (by NanoLC-Ultra). Compared to the present work, they were able to identify a higher number of proteins, possibly due to the different extraction protocol and to the less strict criteria used for data filtering. Nevertheless, our method allowed to highlight that muscle proteins, which in Rabani et al. (2019) represented only a small proportion of the total, are indeed the most abundant. The protein patterns here determined were more similar to the ones described by Yi et al. (2016) and Barre et al. (2019) for Yellow mealworm (*Tenebrio molitor*). Li et al. (2016) applied for protein extraction a Filter Aided Sample Preparation (FASP) (involving the use of SDS, Urea and DTT). Barre and colleagues extracted the *Yellow mealworm* proteome in a tris-buffered saline solution and performed the mass spectrometry analysis with an LTQ-Orbitrap instrument. Furthermore, they also studied the distribution of detected proteins, according to their functionality, by identifying mainly enzymatic and functional proteins (65 % of total protein identified). The difference in functional protein distribution with the present work could be related to the different species, and to the strict data filtering here applied, which focused on the most robust results and hence on the most abundant proteins.

By our knowledge, the protein profile of LM was described for the first time and also compared with the BSF pattern. This detailed protein identification constitutes the basis for the *in-silico* assessment of cross reactivity with known allergens.

3.2. *In silico* allergenicity assessment by Allermatch™ tool

Bioinformatic tools are used to compare the amino acids sequence of a protein with the sequence of known allergens in order to determine sequence similarity. Based on the results of this alignment it is possible to discover the presence of potential allergens. In fact, FAO/WHO 2001 and Codex Alimentarius 2003 reported that 35% sequence identity to known allergen over a window of at least 80 amino acids is considered a minimal requirement to regard a protein allergenic in nature (Codex Alimentarius, 2003). In the present work, we decided to focus our attention on the peptide sequences actually identified and not to the potential parental protein from which they occur, in order to avoid a less robust allergenicity assessment, due to the incomplete *Insecta* database. The identified peptides were matched with allergen sequences using Allermatch™ tool and we obtained a positive hit for 32 peptides from LM and for 25 peptides from BSF. In order to avoid false positive results, we performed a data filtering considering only peptides with more than the 50% of wordmatches (Table 20).

After data filtering, we identified positive hits for 16 peptides from LM and 18 from BSF, corresponding respectively to the 13% and 27% of total peptides identified. The relevant allergens detected after alignment belong mainly to two distinct classes, associated to two distinct animal groups: allergens belonging to crustaceans, so very relevant for allergic response after ingestion as food, or allergens belonging to insects already known as being responsible for inhalatory allergies or after stinging (other insects and mites). Furthermore, the identified peptides from LM and BSF showed high sequence identity with tropomyosin and arginine kinase, well known allergens for both classes (Faber et al., 2017). The 73% of potential allergenic peptides presented a complete wordmatch (100%) with the amino acids sequence of known allergens. This indicates not only a close similarity of this protein regions between species taxonomically related, but also the risk of cross-reactivity for persons allergic to crustaceans and house dust mites.

Table 20 Identified peptides which presented a positive hit with known allergens. The *in-silico* assessment was carried out with Allermatch™ on both lesser mealworm and black soldier fly peptide identified. After data filtering, we reported only the results which presented more than the 50% of wordmatches.

Peptide	Protein	% wordmatches	Species	Allergen	Class
Lesser mealworm					
LIDDHFLF	Arginine kinase	100	*B. germanica*	Bla g 9	Insect
IVELEEELR	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
MYDGIAELIK	Arginine kinase	100	*B. mori*	Bomb m 1	Insect
YKEIGDDLD	Tropomyosin	100	*C. kiiensis*	Chi k 10	Insect
VIQSGLENHDSGIGIYAPDAD	Arginine kinase	56	*B. germanica*	Bla g 9	Insect
FLAEEADKKYDEVAR	Tropomyosin	100	*C. kiiensis*	Chi k 10	Insect
LQLIEEDLER	Tropomyosin	80	*L. destructor*	Lep d 10	Mite
IQLLEEDLER	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
IMELEEELK	Tropomyosin	100	*L. saccharina*	Lep s 1	Insect
MDALENQLK	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
VSSTLSGLEGELK	Arginine kinase	100	*B. germanica*	Bla g 9	Insect
LEEVASKF	Arginine kinase	67	*B. mori*	Bomb m 1	Insect
QLQEQEGMSQQNVTR	House dust mite allergen	50	*D. pteronyssinus*	Der p 11	Mite
LAEASQAADESER	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
STAGDTHLGGEDFDNR	Heat shock protein 70	100	*D. farinae*	Der f 28	Mite
NALEQANKDLEEKEK	Myosin	80	*A. aegypti*	Aed a 10	Insecta
Black soldier fly					
DRLEDELGLNK	Tropomyosin	50	*L. saccharina*	Lep s 1	Insect
DRLEDELGINK	Tropomyosin	100	*L. saccharina*	Lep s 2	Insect
IVELEEELR	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
IQLLEEDLER	Tropomyosin	100	*M.rosenbergii*	Mac r 1	Prawn
IMELEEELK	Tropomyosin	100	*L. saccharina*	Lep s 2	Insect
MDALENQLK	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn

MDQLTNQLK	Tropomyosin	100	*L. saccharina*	Lep s 2	Insect
MVEADLER	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
LAFVEDELEVAEDR	Tropomyosin	100	*A. aegypti*	Aed a 10	Insect
LLAEDADGK	Tropomyosin	75	*L. saccharina*	Lep s 1	Insect
LSEASQAADESER	Tropomyosin	75	*M. rosenbergii*	Mac r 1	Prawn
FRAAVPSGASTGVHEALELR	Beta-enolase	100	*S. salar*	Sal s 2.0101	Fish
LAMVEADLER	Tropomyosin	100	*M. rosenbergii*	Mac r 1	Prawn
QLIEEDLER	Tropomyosin	100	*L. destructor*	Lep d 10	Mite
QLLEEDLER	Tropomyosin	100	*M. rosenbergii*	Lep d 10	Prawn
LEVSEEK	Tropomyosin	100	*M. rosenbergii*	No name	Prawn
ALGFPFDR	Haemolymph	66.67	*B. germanica*	Per a 3	Insect
SLEVSEEK	Tropomyosin	100	*M. rosenbergii*	No name	Prawn

Between the two potential allergens identified, tropomyosin resulted the most impacting, due to the high similarity with other known tropomyosin allergens. In fact, tropomyosin from shellfish, house dust mite and insects (American cockroach) share a high degree of similarity, round 75–80 % (Downs et al., 2016). This similarity raises the possibility of reactivity to shellfish tropomyosin due to sensitization from inhalatory insect sources. The allergenicity risk for both insects could be related to ingestion and inhalation. Given the inhalator aspect, this risk is to be considered not only for potential consumers, but also for workers having to deal with insect rearing and fractionations.

Thus, all the above data hints at tropomyosin as the potential prevalent allergen in eventual allergic reactions due to insect consumption.

3.3. IgG- and IgE-immunoblotting experiments

In order to deeply explore the allergenic potential of tropomyosin from LM and BSF we performed IgG- and IgE-immunoblotting analysis. For IgG-immunoblotting, the assay was performed using anti-Tropomyosin I (TPM1) antibody, produced from the 92-273 AA sequence of human Tropomyosin. This antibody has been chosen since it has been obtained against an amino acid sequence that includes immunogenic regions highly conserved in all tropomyosin forms. Thus, in this way we maximized the chances to recognize, with this antibody, also insect tropomyosin, whatever form was present. As a matter of fact, scarce information is present in literature about how many forms of tropomyosin come from alternative splicing. The presence of alternative isoforms might also justify the presence of several bands of various dimensions in the blotting analyses.

The protein profile of LM and BSF presented some similarity (Figure 19.1). In particular, major bands were visible in both lines: one band at 45 kDa and two at 66 kDa, which could be related respectively to myosin, hexamerin and cuticle protein. Under 45 kDa other bands described the protein pattern of both insects, especially for LM. The band round 31 kDa could be related to tropomyosin, even if the presence of a lot of isoforms made the correct association difficult (Reese et al., 1999). The IgG-immunoblotting scan are reported in Figure 19.2. Anti-TMP1 antibody was able to bind a protein in the extract from raw material (LM and BSF lines). In LM extract the TMP1 antibody was able to bind a protein with a MW between 45 and 31 kDa, whereas in BSF the antibody was able to bind two proteins in the range between 66 and 45 kDa.

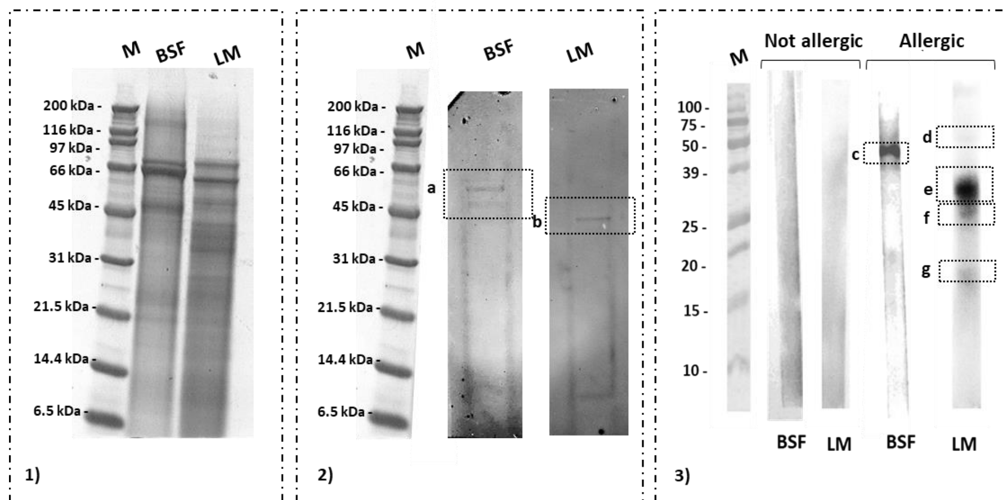

Figure 19 1) SDS-Page of: black soldier fly (BSF) and lesser mealworm (LM) larvae; 2) IgG-immunoblotting of the samples separated by SDS-Page, followed by incubation with anti-Tropomyosin I IgG; 3) IgE-Immunoblotting results after incubation with sera from non-allergic patient and person allergic to crustacean tropomyosin. Response from IgG binding can be visible in a and b, while from IgE binding in c, d, e, f and g. M: marker.

In order to really demonstrate that TPM may actually be relevant in allergy, we performed an immunoassay with serum from allergic person to crustacean tropomyosin (Pen a 1) (Figure 19.3). In both insects we identified the presence of a reactivity with IgE from serum of allergic patient, while in the control (person without allergy) no reactivity was determined. In the BSF profile a strong reactivity was determined between 50 and 39 kDa, while in the LM profile between 39 and 25 kDa, which matched with the ones detected in the IgG-immunoblotting assays. For LM, we identified other two minor reactivity at 50 kDa and between 20 and 15 kDa.

The above data definitely confirmed the potential allergenicity of insect tropomyosin, also in cross reactivity with crustacean allergens.

3.4. Influence of enzymatic hydrolysis on allergenic properties

The allergenic risk of insects represents a critical point for their future consumption and for this purpose we explored the enzymatic hydrolysis as a biotechnology tool for decreasing the allergenic potential of both LM and BSF. The enzyme employed was the Protease from *Bacillus licheniformis*, which was used as a technological adjuvant not only to extract the protein fraction, but at the same time also to reduce the protein molecular size. Van Broekhoven et al. (2016) demonstrated the ability of gastro-intestinal enzymes to reduce the immunoreactivity of LM, while Hall et al. (2018) and Pali Schöll et al. (2019) obtained

the same results but by processing different insects with Alcalase. In the present work, for the first time, BSF was subjected to enzymatic hydrolysis and the potential reduction in immunoreactivity evaluated.

The enzymatic hydrolysis was performed as described in Chapter 4. The hydrolysates were rich in protein compounds, since 79 ± 7 % and 70 ± 1 % of total proteins were extracted respectively from BSF and LM. The protein fractions obtained were mainly composed of peptides and the degree of hydrolysis, calculated by OPA assay, was 10.4 ± 2.3 % for BSF and 21.8 ± 0.5 % for LM. From the reverse of these values it was possible to theoretically calculate the average peptide length, which resulted in 8 to 12 amino acid residues for BSF and an average peptide length of 4 to 5 for LM. As described by Nagodawithana et al. (2008) the peptide length directly influences the allergenicity properties of a protein hydrolysates, demonstrating that an average molecular weight lower than 1500 Da can reduce the allergenicity property of a food product. Assuming an average molecular mass for residual amino acid of 110 Da, this means that the hydrolysates here could potentially be defined as hypoallergenic, even if the following results from IgE-immunoblottig (Figure 20.3) revealed the presence of immunoreactivity in BSF hydrolysate, outlining the presence of residual intact proteins. Indeed, as reported by a European Union directive, the hypoallergenicity has to be assessed by showing the absence of orally sensitisation after animal administration (Commission Directive 2006/141, 2006), but certainly the possibility to degrade the allergenic proteins to small peptides might help to achieve this result.

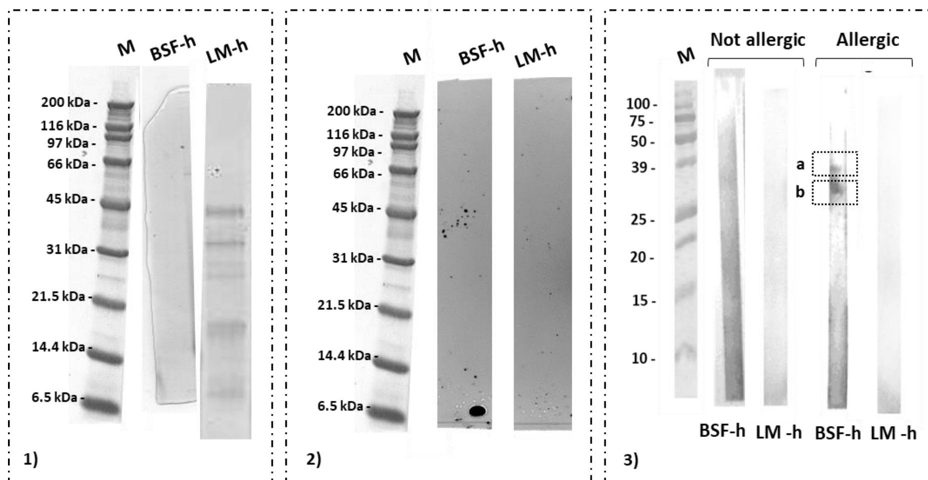

Figure 20 1) SDS-Page of hydrolysates obtained from proteolysis activity of the protease from *Bacillus licheniformis* on black soldier fly (BSF-h) and lesser mealworm (LM-h) protein hydrolysates; 2) IgG-immunoblotting of the samples separated by SDS-Page, followed by incubation with anti-Tropomyosin I IgG; 3) IgE-Immunoblotting results after incubation with sera from non-allergic patient and person allergic to crustacean tropomyosin. Response from IgE binding in a and b. M: marker.

In Figure 20.1 it is reported the protein profile of LM hydrolysate, where three major bands are visible round 45 kDa, 21.5 kDa and 14.4 kDa. No proteins were displayed at molecular weight higher than 45 kDa, which were present in the whole LM pattern instead. In BSF hydrolysate no net bands were identified in the gel.

IgG-immunoblotting assay was performed also on these protein hydrolysates, and the results are reported in Figure 20.2. In both LM and BSF hydrolysates, no reactivity with the anti-TPM1 antibody was identified. The enzymatic hydrolysis apparently eliminated the antibody ability to bind the target sequence in tropomyosin. This result was in line with what reported by Pali- Schöll et al. (2019), Hall et al. (2018) and van Broekhoven et al. (2016), respectively for locust, crickets and mealworms, where after enzymatic hydrolysis there was a visible reduction of reactivity between insect proteins and the antibodies in crustaceans and house dust mite allergic patients. The absence of reactivity was also underlined in the IgE-immunoblotting assay of the protein hydrolysate obtained from LM, where the reactivity determined in the protein extract here was not evident (Figure 20.3). IgE-immunoblotting displays a more allergen-relevant environment than IgG-immunoblotting, since it is performed with a pool of different antibodies available in the allergic patient serum. On the contrary, the BSF protein hydrolysate kept an evident reactivity with the IgE from allergic patient between 39 and 25 kDa, which could be due to fragments released during the enzymatic hydrolysis from the intact protein, but still retaining the ability to be recognized by IgE antibodies (Figure 20.3). The enzymatic assisted extraction, as here performed on BSF, was then not sufficient to completely eliminate the cross-reactivity between insect tropomyosin and the human IgE.

In general, the enzymatic assisted extraction here presented could be a good strategy to obtain hypoallergenic extracts not only from LM, as already reported in literature (Broekman et al., 2015), but also from BSF, even if its real efficacy in all cases will have to be carefully further studied and optimized in order to achieve a sufficient DH able to suppress IgE reactivity. Furthermore, the less efficient reactivity of the enzyme and the variability of the results can be ascribed to the fact that the hydrolysates have been here obtained by ground insects, not by soluble homogeneous protein extracts. Inhomogeneous solid material is hydrolysed much less efficiently than soluble proteins, and other factors, beside protein susceptibility, play an important role (such as chitin or lipid presence).

4. Conclusion

In the present work the allergenicity assessment of two common edible insects, LM and BSF, was evaluated by both proteomics and *in-vitro* assays, and the enzymatic hydrolysis

performed as a biotechnological tool to produce hypoallergenic fractions from these two insect species.

Major proteins in the proteome of both insects were determined with a shotgun proteomic approach by HRMS on LTQ-Orbitrap instrument, followed by a severe data filtering of the identified peptides in order to focus on the most abundant and the most certainly identified insect proteins. The peptides identified mainly belonged to the category of muscle proteins, with actin, tropomyosin and myosin representing the most abundant ones. The proteomic characterization posed the basis for the subsequent *in silico* allergenicity assessment with the Allermatch™ tool, which evaluated the presence of similarity between the identified peptides and the sequence of known allergens. Tropomyosin was identified as the major pan-allergen, underling a strong similarity with other tropomyosin from crustaceans and dust mites, known to be responsible of food and inhalatory allergy. An IgG-immunoblotting analysis conducted by using antibodies directed against tropomyosin I, recognized specific bands in extracts from both insects. An IgE-immunoblotting experiment, performed with serum of patient allergic to crustacean tropomyosin, then confirmed in a more allergen-relevant environment, the potential of tropomyosin to be the prevalent insect allergen.

In view of the future expected use of insects as food sources, we explored the enzymatic hydrolysis as a way to reduce the allergenic properties of LM and BSF. For BSF, this approach was explored for the first time in the present work. The protein hydrolysates demonstrated complete disappearance of immunogenic reactivity in IgG-immunolotting experiments, while complete disappearance in LM and partial reduction in BSF of immunogenic reactivity in IgE-immunoassay analysis. In particular, in BSF protein hydrolysate we identified two protein fragments maintaining the immunoreactivity.

In conclusion, the data here presented confirms the allergenic potential of insect proteins. For the first time a proteomic approach clearly indicated tropomyosin as the prevalent potential allergen. The cross-reactivity for the subjects already allergic to crustacean tropomyosin was clearly proven by immunoblotting experiments. Enzymatic hydrolysis was confirmed as effective strategy to reduce LM allergenic risk and demonstrated potentially valid also for BSF, even if deeper investigations are needed in order to adjust the condition of hydrolysis, achieving a sufficient DH to obtain effective hypoallergenic properties. The results obtained are relevant since they indicate different immunoreactivity can still remain in different species, even when subjected to the same enzymatic hydrolysis. It was also demonstrated clearly that the simple measure of DH is not enough to assess hypoallergenicity and that IgE reactivity and possibly *in vivo* challenges are needed.

Founding sources

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CHAPTER 7

Impact of Naturally Contaminated Substrates on *Alphitobius diaperinus* and *Hermetia illucens*: Uptake and Excretion of Mycotoxins

The content of this chapter has also been published in:

Leni Giulia, Cirlini Martina, Jacobs Johan, Depraetere Stefaan, Gianotten Natasja, Sforza Stefano, Dall'Asta Chiara (2019). Impact of Naturally Contaminated Substrates on *Alphitobius diaperinus* and *Hermetia illucens*: Uptake and Excretion of Mycotoxins. *Toxins,* 11(8):476, DOI: 10.3390/toxins11080476. Permission of use of the content in the present Thesis has been granted by the Editor.

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Abstract

Insects are considered a suitable alternative feed for livestock production and their use is nowadays regulated in the European Union by the European Commission Regulation No. 893/2017. Insects have the ability to grow on a different spectrum of substrates, which could be naturally contaminated by mycotoxins. In the present work, the mycotoxin uptake and/or excretion in two different insect species, *Alphitobius diaperinus* (lesser mealworm, LM) and *Hermetia illucens* (black soldier fly, BSF), grown on naturally contaminated substrates, was evaluated. Among all the substrates of growth tested, the *Fusarium* toxins deoxynivalenol (DON), fumonisin 1 and 2 (FB1 and FB2) and zearalenone (ZEN) were found in those based on wheat and/or corn. No mycotoxins were detected in BSF larvae, while quantifiable amount of DON and FB1 were found in LM larvae, although in lower concentration than those detected in the growing substrates and in the residual fractions. Mass balance calculations indicated that BSF and LM metabolized mycotoxins in forms not yet known, accumulating them in their body or excreting in the faeces. Further studies are required in this direction due to the future employment of insects as feedstuff.

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1. Introduction

Given the large growth of the World population expected in the coming years, it has been estimated that the demand for food will raise of about the 60 % in 2050 (FAO, 2019). Along the food chain, the meat production represents the field with the most impact, with serious consequences on the demand of feed supply. Edible insects have been explored as an alternative to common livestock, and their use is encouraged for their sustainability and the minimal environmental impact applied in their breeding (Veldkamp et al., 2012; van Huis et al., 2017). Furthermore, edible insects have been proposed as a promising alternative nutrient source due to the high content and quality of their macronutrients (Charlton et al., 2015). In general, they have a well-balanced nutrient profile, high in polyunsaturated fatty acids and essential amino acids which meet the requirement for humans and livestock, rich in micronutrients and vitamins (Rumpold et al., 2013).

In the European Union, the use of insects in the feed and food sector is nowadays regulated by a package of legislative texts. Insects are included in the category of Novel Foods and the European Food Safety Authority (EFSA) authorization is mandatory before their marketing in the European Union (EU) [Regulation EU 2015/2283, 2015). As alternative source for feedstock, they may substitute the sources of protein and fat normally added in animal feed, as soy, maize, grain and fishmeal (Raamsdonk et al., 2017). Insects can be used as whole or processed. Whole insects, alive or dried, and fat fraction can be employed for livestock feed, except for aquaculture (Commission Regulation EU 2017/893). Whereas, the protein fraction isolated from insects can be used to feed pet and fur animals, but not for ruminants and monogastrics (Regulation EC 2001/999, 2001). Recently, the European Commission (EC) has expanded also to the aquaculture sector, the addition of processed proteins from seven insect species: black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) (Commission Regulation EU 2017/893).

One of the limitations in the use of insects as feed and food is certainly linked to safety aspects. The potential hazards are related to exogenous and endogenous factors, which could be influenced also by harvesting and processing methods, and which could be achieved during all the insect life-cycle (Charlton et al., 2015; EFSA, 2015). Exogenous factors may occur from the behaviour and from the substrate of growth which, as feed, must meet the requirements of the current regulation that fixed the maximum limits of undesirable substances in feedstock (European commission 2002/32, 2002; Commission recommendation 2006/576, 2006). In particular, the potential hazards could be heavy metal residues, pesticides and mycotoxins.

Mycotoxins are a wide range of different substances, produced by the secondary metabolism of various species of fungi, that can infect cereal or vegetable crops at pre- or post-harvest (Hussein & Brasel, 2001). Mycotoxins are characterized by a large chemical diversity and may exert a broad spectrum of adverse effects in animals and humans (Steinkellner et al., 2019; Binder et al., 2007). In addition, they can undergo biotransformation in plants, microbes and animals (Berthiller et al., 2011) leading to the uptake of modified forms which may account for a similar toxicity compared to parent compounds (Steinkellner et al., 2019).

Although mycotoxin occurrence in food and feed is extensively covered by regulation/guidelines at EU level, their presence at concentration levels not exceeding the EU limits cannot be ruled out in rearing substrates obtained from vegetable waste. Therefore, insects are potentially exposed to mycotoxins when reared on a contaminated substrate and, at least from a theoretical point of view, they could accumulate mycotoxins (or modified forms) at levels exceeding the legal limits.

However, despite a growing interest, only few papers have addressed the possible uptake and biotransformation of mycotoxins in insects so far (De Zutter et al., 2016; van Broekhoven et al., 2017; Niermans et al., 2019; Camenzuli et al., 2018; Purschke et al., 2017; Sanabria et al., 2019). Although information on the possible biotransformation and/or excretion mechanisms are still scattered, all the studies performed to date consistently demonstrated that parent mycotoxins are not bioaccumulated by insects grown in contaminated substrates. In addition, many of these studies were done on substrates artificially contaminated with mycotoxins, possibly not exactly describing the situation arising in the case of a natural contamination.

The aim of this research, in the framework of the EU project InDIRECT, was to investigate the possible uptake and excretion of mycotoxins in two species of insect larvae, lesser mealworm (LM) and black soldier fly (BSF), produced on different naturally contaminated substrates, obtained from vegetable and cereals waste.

2. Materials and methods

2.1 Materials

Mycotoxin standard solutions of aflatoxins B1, B2, G1 and G2, fumonisin B1 and B2, A and B trichothecenes (nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, fusarenone X, diacetoxyscirpenol, T-2 toxin, HT-2 toxin), zearalenone, ochratoxin A, and patulin were obtained from Romer Labs (Tulln, Austria). All the solvents applied for both the extraction

and analysis steps, methanol, acetonitrile formic acid and acetic acid, were HPLC-grade and were purchased from Sigma-Aldrich (Milan, Italy), while Bi-distilled water was produced in-house by using a Milli-Q System (Millipore, Bedford, MA, USA). Salts used for extraction as for the preparation of the eluents as sodium chloride and ammonium acetate were obtained from Sigma-Aldrich (Milan, Italy).

2.2 Insect treatments and sampling

For the experiments described herein, two insect species were selected: *Alphitobius diaperinus* (lesser mealworm, LM) and *Hermetia illucens* (black soldier fly, BSF). The larvae were grown on different substrates prepared using different feed materials, prevalently coming from the processing of cereals (wheat, corn, rice and rapeseed) and vegetable (apple and olive), as indicated in Table 22. The choice of these substrates was driven by the seasonality, the availability and the cost of different by-products of agriculture sector. The substrates were analysed previously for mycotoxins presence, then also larvae were extracted and analysed using the same protocol applied for feed samples. Moreover, rests of insects grown on substrates resulted positive to the presence of mycotoxins were also collected and analysed. Insects were grown on naturally contaminated feed, and in particular 2 substrates were used for BSF cultivation, while 13 different feeds were prepared for LM production (Table 23). The insects rearing was conducted as indicated in Chapter 4. Briefly, BSF eggs were initially placed in a specific incubator at 28°C for 2 days, then the eggs were transferred in the rearing bins and the new-born larvae treated for 2 days with a started feed composed of chicken feed, the feed materials selected for the experiment and water, with a total dry matter of about 30%. During this time the temperature was set at 28-32°C and humidity at 60% minimum. After that, the growth substrate was removed and substituted with that selected for the experiment, provided ad libitum. The larvae were reared under these conditions for 15 days. After this period, larvae were removed and quantified, and samples of remaining fractions were also collected as listed in Table 24. Similarly, LM larvae were reared utilizing the selected feed materials chosen for the experiments, under the same controlled conditions of temperature and humidity used for BSF growth, providing feed daily ad libitum. In this case, larvae were harvested for 28 days and then collected. As for BSF, also samples of remaining fractions were recovered and weighted. Larvae of BSF and LM were killed at -18°C and stored at the same temperature before each analysis. At the same time, samples of growth substrates and remaining fraction were stored at -18°C until analyses.

2.3 Mycotoxins extraction and purification

Mycotoxins class is represented by several organic compounds with different chemical and physical properties. For this reason, we decided to apply different extraction protocols, selected for a specific class of toxins. In addition, all the samples of insect larvae and samples of remaining fractions were subjected to a lyophilization process (Freeze dryer Lio-5P, 5Pascal, Milano, Italy) for 48h and milled using a laboratory miller. The dried powders obtained from these steps were stored at -20°C until extraction and analysis. For the extraction of aflatoxins, 1g of sample added with 0.2 g of NaCl was extracted using 4 mL of a mixture of methanol/bi-distilled water, 80/20 v/v on a shaker at room temperature, at 200 strokes/min for 90 min. After that, the extract was centrifuged at 10621 g, at 25°C for 10 min. 1 mL of the supernatant was transferred in a tube, diluted with 4 mL of bi-distilled water and submitted to a purification step using immuno-affinity columns (VICAM, Afla Test®, mycotoxin testing system). The cartridges were conditioned with 10 mL of bi-distilled water and subsequently with 10 mL of pure methanol. The diluted extract was then eluted through the column and, after the elution, a washing step with 10 mL od bi-distilled water was performed. The analytes were recovered with 1 mL of pure methanol. The purified sample was dried under a gently nitrogen flow, suspended in 1 mL of bi-distilled water/methanol, 80/20 v/v, and analysed by HPLC-FLD technique. The contemporary extraction of fumonisins, ochratoxin A, zearalenone, patulin and A and B trichothecenes was performed on the basis of different protocols with slight modifications (Sulyok et al., 2006; Dall'Asta et al., 2008). Briefly, 1 g of sample was extracted adding 4 mL of a solution composed of bi-distilled water/acetonitrile/methanol 50/25/25 v/v. The sample was positioned on a shaker at room temperature, at 200 strokes/min for 90 min. After that, the extracts were centrifuged at 10621 g, for 10 min at 25°C. 1 mL of the supernatant was collected and dried under a gently nitrogen flow. The residue was then dissolved in 1 mL of bi-distilled water/methanol 80/20 v/v. The samples were then subjected to UHPLC-MS/MS analyses.

2.4 Mycotoxins analysis

Aflatoxins B1, B2, G1 and G2 were analysed on a HPLC Waters Alliance 2695 separation module, coupled with a FLD detector (Waters, Multi λ Fluorescence detector 2475) and an UV detector (Waters, Dual λ Absorbance Detector 2489). The analytes separation was achieved on a C18-RP XTerra (Waters; 250x2.1mm, i.d. 5 mm) column using as eluents bi-distilled water (A) and methanol (B), in isocratic conditions (65% A and 35% B). The flow was set at 0.25 mL/min and the column oven temperature was kept at 30°C. A volume of 10 µL was injected. For the detection of aflatoxins, the UV detector was set at λ=365nm, while for the FLD λ =365nm and λ =425nm were chosen as the typical wavelength of

absorbance and of emission, respectively. For the quantitative determination of aflatoxins, a calibration curve was prepared starting from the commercial standard which contained AFB1 and AFG1 at the concentration of 2 mg/Kg and AFB2 and AFG2 at the concentration of 0.5 mg/Kg. Starting from this solution, 5 different dilutions in pure methanol were performed obtaining AFB1 and AFG1 at 0.5, 0.75, 1, 1.5 and 2 µg/Kg, while for AFB2 and AFG2 concentrations of 0.125, 0.187, 0.25, 0.375 and 0.5 µg/Kg, obtaining a good linearity (R2 > 0.99) for the both calibration ranges. Fumonisins B1 and B2, ochratoxin A, zearalenone, patulin and A and B trichothecenes (nivalenol, deoxynivalenol, 3-acetyl-deoxinivalenol, fusarenone X, T2 toxin, HT2 toxin and deacetoxyscirpenol) were determined on an UHPLC–MS/MS apparatus consisted of an UHPLC Ultimate 3000 separation module (Dionex, Sunnyvale, CA, USA), coupled with a TSQ Vantage triple quadrupole (Thermo Fisher, Waltham, MA, USA) equipped with an ESI interface. The separation of the analytes was achieved on a RP-C18 EVO Kinetex column (2.6µ, 100A; 100 x 2.10 mm) from Phenomenex (Torrance, CA, USA). Ammonium acetate 5 mM in bi-distilled water and methanol were used as eluent A and B respectively, both acidified with the 0.2% of acetic acid. A gradient was applied as follows: the elution started with 2% of B and these conditions were maintained for 1 min, then at 2 min the percentage of B was increased at 20 % and kept for 6 min, at 17 min the column was flashed with the 90 % of B for 3 min, then in 1 min the initial conditions were ri-established and the column was ri-equilibrated for 9 min, with a total run time of 30 min. During the analyses the column temperature was maintained at 40°C while samples were maintained at 20°C. The flow was 0.35 mL/min and for each sample 4 µL were injected into the system. PAT, NIV, DON, 3ADON, FUSX, and ZEN were monitored in negative ion mode with a spray voltage of 3500 V, a capillary temperature of 270°C, a vaporizer temperature of 200°C, a sheat gas flow of 50 units and an auxiliary gas flow of 5 units. T2, HT2 toxins, DAS, FB1, FB2, and OTA were monitored applying a positive ionization mode, with the following parameters: spray voltage of 3000 V, a capillary temperature of 270°C, a vaporizer temperature of 200°C, a sheat gas flow of 50 units and an auxiliary gas flow of 5 units. All the other parameters as S-Lens RF amplitude values were obtained and set by tuning methanolic solutions of each considered molecule (1 mg/kg).

Table 21 Characteristic transitions monitored for the target mycotoxins: fumonisins B1 and B2 (FB1, FB2), ochratoxin A (OTA), zearalenone (ZEN), patulin (PAT) and A and B trichothecenes (nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON), fusarnone X (FUSX), T2 toxin, HT2 toxin and deacetoxyscirpenol (DAS).

Compound	Ionization mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (V)	LOD (µg/Kg)
PAT	Negative	152.9 $[M–H]^-$	109/81	-12/-12	100
NIV	Negative	371.1 $[M+CH3COO]^-$	311.1/281.1/59.1	-10/-32/-48	10
DON	Negative	355.1 $[M+CH3COO]^-$	295.1/265.1	-13/-16	10
3ADON	Negative	397.1 $[M+CH3COO]^-$	307.1/59	-18/-20	20
FUSX	Negative	413.3 $[M+CH3COO]^-$	353.6/262.9/59.1	-14/-22/-10	20
OTA	Positive	404.5 $[M+H]^+$	238.7/220.7/101.7	21/31/68	20
FB1	Positive	722.3 $[M+H]^+$	704.7/352.1/334.1	26/35/38	25
FB2	Positive	706.5 $[M+H]^+$	688.4/336.3	51/51	25
DAS	Positive	384.2 $[M+NH_4]^+$	307.2/105.1	17/61	10
T2	Positive	484.3 $[M+NH_4]^+$	215.0/185.0	19/22	10
HT2	Positive	442.0 $[M+NH_4]^+$	263.1	11	10
ZEN	Negative	317.0 $[M–H]^-$	175.0/131.0	-26/-32	10

Detection of all the considered analytes was performed in SRM modality (Single Reaction Monitoring) monitoring the characteristic transitions for each considered mycotoxin (Table 21). In order to quantify these mycotoxins, a calibration curve containing all the considered analytes was prepared starting from the commercial standard. For this purpose, 6 different dilutions were prepared considering the following concentrations: 50, 100, 200, 500, 750 and 1000 µg/Kg, obtaining a good linearity ($R^2 > 0.99$) for the calibration range.

2.5 Mass balance calculation

The mass balance was calculated as described by Camenzuli et al. (2018) on the basis of the amount of substrates used for insects growth, the amount of harvested larvae and of the residual fractions (frass). Furthermore, the accumulated, extracted and potential metabolized mycotoxins were calculated as follow:

$$\%\ \textbf{accumulated} = \frac{\textbf{amount of harvested insects x concentration mycotoxin detected in insects}}{\textbf{amount of substrates x concentration mycotoxin detected in substrates}} x\textbf{100}$$

$$\% \text{ excreted } = \frac{\text{amount of frass x concentration mycotoxin detected in frass}}{\text{amount of substrates x concentration mycotoxin detected in substrates}} x\ 100$$

$$\% \text{ metabolyzed } = 100 - \% \text{ excreted mycotoxin} - \% \text{ accumulated mycotoxin}$$

Metabolized mycotoxins were referred to the undetected compounds which could be metabolized in different structures not yet identified. All the measurements of mycotoxin below the LOD were considered as equal to LOD values.

3. Results

Different feed materials were selected for the experiments, in order to ensure optimal growth of the larvae on the basis of preliminary trials. These wastes derived from the processing of cereals, as wheat, corn, rice, while others were chosen among vegetables, as olive and apple pomace, rapeseed and chopped carrots (Table 22).

Samples were analysed for all the regulated mycotoxins, according to the possible occurrence. In particular, samples were analysed for Fusarium toxins (deoxynivalenol (DON), fumonisins 1 and 2 (FB1 and FB2), zearalenone (ZEN)) as well as aflatoxins, while the possible presence of patulin was checked in vegetable-based samples.

Table 22 Substrates used for feed formulations with results about their mycotoxin contamination deoxynivalenol (DON), fumonisins 1 and 2 (FB1 and FB2), zearalenone (ZEN)). Results showed the detected mycotoxins and are reported as mean of two different replicates ± standard deviation.

		Mycotoxin amount (µg/Kg)			
Sample code	**Description**	**DON**	**FB1**	**FB2**	**ZEN**
WM	Wheat middlings	938 ± 100	< LOD	< LOD	< LOD
CDR	Corn distillation residues	779 ± 5	573 ± 3	441 ± 3	< LOD
CG	Corn gluten feed	1207 ± 43	727 ± 6	294 ± 5	173 ± 4
RB	Rice Bran	< LOD	< LOD	< LOD	< LOD
RW	Rapeseed wastes	< LOD	< LOD	< LOD	< LOD
OP	Olive pomace	< LOD	< LOD	< LOD	< LOD
AP	Apple pomace	< LOD	< LOD	< LOD	< LOD
CC	Chopped carrots	< LOD	< LOD	< LOD	< LOD

All samples underwent ochratoxin (OTA) analysis, in consideration of its possible synthetisation postharvest in all the considered feed materials. As shown in Table 22, only Fusarium toxins were found in three out of eight matrices, at concentration levels in agreement with the EU limits for cereal-based feed. While only DON (938 ± 100 g/kg) was found in wheat middlings, the contemporary presence of DON, FB1, FB2 and ZEN was

detected in corn wastes. In order to optimize and promote the insect growth, 15 feed formulations were obtained by mixing these substrates (originated from the same batch) in different percentages. In particular, corn distillation residues were mixed with chopped carrots, olive and apple pomace, while wheat middlings with corn gluten feed, rice bran and rapeseed, as indicated in Table 23. According to the contamination pattern observed in raw waste materials, BSF and LM larvae were analysed for the target mycotoxins and results are reported in Table 23. No mycotoxin uptake was observed in BSF larvae, while DON was detected in 6 out of 13 LM larvae samples, being one contaminated by FB1 as well.

In particular, DON was detected in samples of LM larvae grown on substrates prepared with high percentages of wheat and/or corn residues, and the amount of contamination ranged between 416 ± 28 μg/Kg of larvae produced on 100% of wheat wastes and 755 ± 134 μg/Kg of insects cultivated on wheat (90 %) added with rice (10 %). Low concentrations of FB1 (127 ± 6 μg/Kg) were detected in LM larvae grown on a substrate composed of 100% of corn gluten feed.

Table 23 Larvae of black soldier fly (*Hermetia illucens*, BSF) and lesser mealworm (*Alphitobius diaperinus*, LM) reared on the feed formulations with results about the target mycotoxins occurrence. Results are the mean of two different replicates and are reported as mean ± standard deviation. Abbreviations: deoxynivalenol, DON; fumonisins 1 and 2, FB1 and FB2; zearalenone, ZEN; corn distillation residues, CDR; olive pomace, OP; apple pomace, AP; wheat middlings, WM; corn gluten feed, CG; rice bran, RB; rapeseed wastes, RW.

		Mycotoxin amount (μg/Kg)			
Sample code	**Description**	**DON**	**FB1**	**FB2**	**ZEN**
BSF-100%CDR	BSF larvae grown on: 100% CDR	<LOD	<LOD	<LOD	< LOD
BSF-79%CDR-10.5%OP/AP	BSF larvae grown on: 79% CDR, 10.5% OP, 10.5% AP	<LOD	<LOD	<LOD	< LOD
LM-100%WM	LM larvae grown on: 100% WM, 0% CG	416±28	<LOD	<LOD	< LOD
LM-75%WM-25% CG	LM larvae grown on: 75% WM, 25% CG	608±59	<LOD	<LOD	< LOD
LM-50%WM-50% CG	LM larvae grown on: 50% WM, 50% CG	<LOD	<LOD	<LOD	< LOD
LM-100% CG	LM larvae grown on: 100% CG	726±164	127±6	<LOD	< LOD
LM-100%CDR*	LM larvae grown on: 100% CDR*	468±181	<LOD	<LOD	< LOD
LM-95%WM-5%RB	LM larvae grown on: 95% WM, 5% RB	<LOD	<LOD	<LOD	< LOD
LM-90%WM-10%RB	LM larvae grown on: 90% WM, 10% RB	755±134	<LOD	<LOD	< LOD
LM-85%WM-15%RB	LM larvae grown on: 85% WM, 15% RB	<LOD	<LOD	<LOD	< LOD
LM-80%WM-20%RB	LM larvae grown on: 80% WM, 20% RB	<LOD	<LOD	<LOD	< LOD
LM-95%WM-5%RW	LM larvae grown on: 95% WM, 5% RW	<LOD	<LOD	<LOD	< LOD
LM-90%WM-10%RW	LM larvae grown on: 90% WM, 10% RW	<LOD	<LOD	<LOD	< LOD
LM-85%WM-15%RW	LM larvae grown on: 85% WM, 15% RW	557±237	<LOD	<LOD	< LOD
LM-80%WM-20%RW	LM larvae grown on: 80% WM, 20% RW	<LOD	<LOD	<LOD	< LOD

∗ small amount of chopped carrots was arbitrarily added in order to get the desired water content for optimal insect growth.

In order to evaluate the uptake and possible excretion of mycotoxins, the residual fractions were also analysed. All the results are listed in Table 24. Interestingly, the contemporary presence of DON, FB1, FB2 and ZEN was observed in the residual fraction obtained from BSF larvae grown on 100 % corn residues, while DON and FB1 were detected in residual fractions from LM corn-based growing substrates. No residual contamination was observed in wheat-based residual fractions, in spite of the DON occurrence detected in wheat waste.

Table 24 Residual fractions harvested from insects resulted positive to the presence of target mycotoxins and results about their concentration level expresses as µg/Kg. Results are the mean of two different replicates and are reported as mean ± standard deviation. Abbreviations: black soldier fly, BSF; lesser mealworm, LM; deoxynivalenol, DON; fumonisins 1 and 2, FB1 and FB2; zearalenone, ZEN; corn distillation residues, CDR; olive pomace, OP; apple pomace, AP; wheat middlings, WM; corn gluten feed, CG; chopped carrots, CC; rice bran, RB; rapeseed wastes, RW.

		Mycotoxin amount (µg/Kg)			
Sample code	**Description**	**DON**	**FB1**	**FB2**	**ZEN**
REST-BSF-100%CDR	Rests of BSF larvae grown on: 100% CDR	1473±197	951±152	344±64	334±44
REST-BSF-79%CDR-10.5%OP/AP	Rests of BSF larvae grown on: 79% CDR, 10.5% OP, 10.5% AP	<LOD	<LOD	<LOD	<LOD
REST-LM-100%WM	Rests of LM larvae grown on: 100% WM, 0% CG	<LOD	<LOD	<LOD	<LOD
REST-LM-75%WM-25% CG	LM larvae grown on: 75% WM, 25% CG	<LOD	<LOD	<LOD	<LOD
REST-LM-100% CG	Rests of LM larvae grown on: 100% CG	827±61	728±7	<LOD	<LOD
REST-LM-100%CDR*	Rests of LM larvae grown on: 100% CDR*	587±73	224±8	<LOD	<LOD
REST-LM-90%WM-10%RB	Rests of LM larvae grown on: 90% WM, 10% RB	<LOD	<LOD	<LOD	<LOD
REST-LM-85%WM-15%RW	Rests of LM larvae grown on: 85% WM, 15% RW	<LOD	<LOD	<LOD	<LOD

* small amount of chopped carrots were arbitrarily added in order to get the desired water content for optimal insect growth.

In order to better investigate the amount of mycotoxins and its distribution in the larvae and in the residual fraction, the mass balance was calculated for larvae which had been resulted positive to the presence of mycotoxins, or grown on contaminated substrates, as in the case of BSF. This calculation was performed on the basis of the concentration of contaminants found in feed, in larvae and in the respective residual fractions. The samples for which the amount of mycotoxins resulted below the LOD were considered as equal to LOD values. Results are represented in Figure 21.

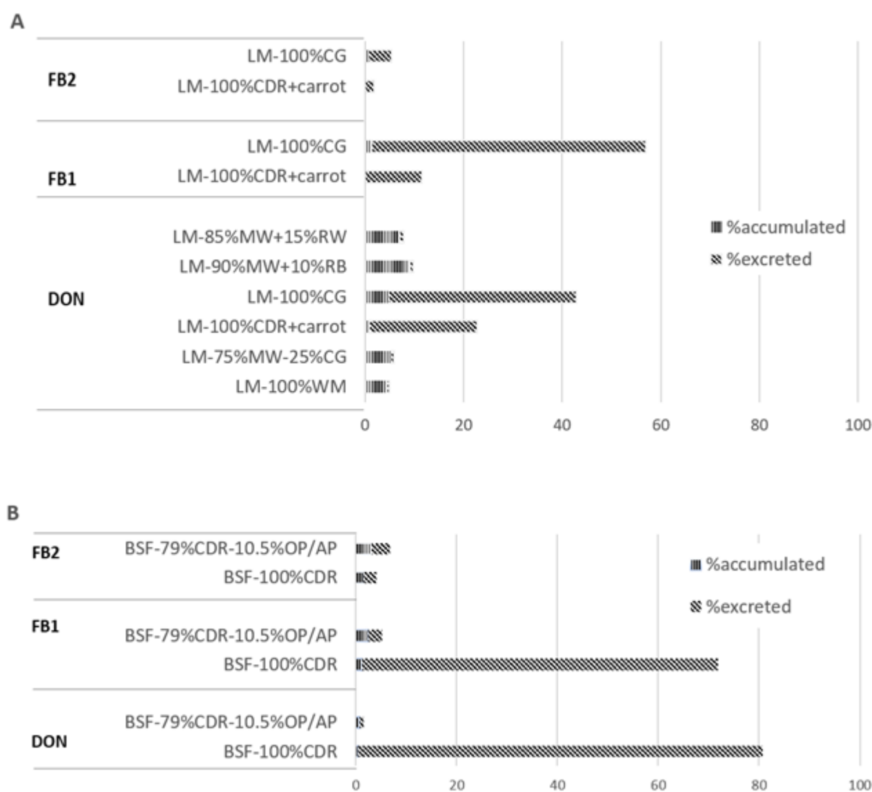

Figure 21 Mass balance of deoxynivalenol (DON), fumonisins 1 and 2 (FB1 and FB2) in lesser mealworm (*Alphitobius diaperinus*, LM) (panel A) and black soldier fly (*Hermetia illucens*, BSF) (panel B) treatments. Abbreviations: corn distillation residues, CDR; olive pomace, OP; apple pomace, AP; wheat middlings, WM; corn gluten feed, CG; rice bran, RB; rapeseed wastes, RW.

As far as DON concerned, the mass balance ranged from 2 to 81 % in BSF, while it was found to be between 1 and 43 % in LM. The average mass balance of fumonisins in BSF ranged between 4% and 72 %, and it was ranged from 1 to 57 % in LM for FB2 and FB1 respectively. Almost the total amount of ZEN resulted excreted in BSF (data not shown in figure).

4. Discussion

This study is focused on the uptake of mycotoxins in BSF and LM larvae, grown on naturally contaminated substrates, obtained from residues of cereal and vegetable processing. In particular, BSF were reared on two different substrates based on corn distillation residues, which had been found to be contaminated by DON, FB1 and FB2. In agreement with other studies form the literature (Purschke et al., 2017), no uptake of mycotoxins in BSF larvae was observed for both trials, while DON, FB1 and FB2 were found only in the residual fractions collected from the BSF reared on 100% of pure substrate. In this case, the concentration of DON, FB1 and FB2 found in the rests were higher as compared to the

concentrations detected in feed. Interestingly, ZEN, lower than LOD in the initial feed, was detected in the residual fraction at 334 ± 44 µg/kg. Since the insects were exclusively grown on the selected substrate, the occurrence of ZEN after harvesting suggested its possible cleavage from the matrix, due to a hydrolytic activity carried out by the insect. An overall increase of ZEN in BSF larvae growing substrates was already observed by Camenzuli et al. (2018), although the authors measured the parent compound together with its major phase I metabolites, α- and β-ZEL.

It is well-known that *Fusarium* mycotoxins can be biotransformed by plants into phase I and phase II metabolites, being the glycosylation the most common pathway (Berthiller et al., 2011). Conjugates mycotoxins can be however cleaved by microbial enzymes, as reported by several authors (Berthiller et al., 2011; Dall'Erta et al., 2013; Gratz et al., 2017). Although a hydrolytic activity in insects towards modified mycotoxins has never been described so far, it cannot be ruled out and can be a possible explanation for the observed data. In addition, it is known that *Fusarium* mycotoxins are often associated to the matrix, and this binding strongly affect the extractability (Rychlik et al., 2014; Damiani e t al., 2019). Therefore, taking into consideration the increase of mycotoxins from starting materials to post-growing residues, it can be argued that BSF larvae may induce the substrate degradation upon growing, thus increasing the overall extractability of mycotoxins.

In contrast with BSF larvae, DON was found in LM larvae grown on contaminated substrates, mainly in those containing wheat middlings in combination with corn gluten. When 100% corn gluten was used as growing substrate, DON, FB1 but not ZEN were transferred to LM larvae. The lack of ZEN uptake in LM is in agreement with the literature (Niermans et al., 2019), while the possible transferal of DON and FB1 in LM larvae grown on naturally incurred substrates, was observed in this study for the first time. In case of LM residual fractions, the overall mass balance never exceeded 60 %, clearly indicating that mycotoxins are partially metabolized by the larvae to unknown compounds, in agreement with the literature (Niermans et al., 2019; Purschke et al., 2017). It should be noticed that the occurrence of DON in residual fractions was lower than the one reported in previous studies performed on Yellow mealworm larvae under similar conditions (Van Broekhoven et al., 2017). However, uptake, biotransformation and excretion of mycotoxins in insects could be affected by a range of factors, among them the substrate, the species, and the dose, as well as the use of naturally incurred or spiked growing material (Camenzuli et al., 2018).

Differently from other studies in which larvae were grown on a substrate artificially contaminated by mycotoxins, at higher concentrations than those found in the substrate samples considered in this study (Van Broekhoven et al., 2017; Camenzuli et al., 2018), the

present work clearly demonstrated that DON and FB1 can be found in LM (but not in BSF) larvae. The amount of mycotoxins anyway never exceeds the starting levels, indicating that there is no active uptake in insects, and there is rather a degradation or an excretion.

In addition, the mass balance calculation clearly indicated that biotransformation is rather the operating mechanism instead of simple excretion. Further experiments will be needed in order to investigate the mycotoxin biotransformation pattern in insects.

5. Conclusions

The present study reported on the possible uptake and/or excretion of mycotoxins in two insect species, LM and BSF, reared on naturally contaminated substrates. As feed, organic side streams recovered from cereal and vegetable processing were considered under a circular economy perspective. Collected data clearly indicated that transfer from the waste to the insect of mycotoxins is possible, but without uptake into insects, and rather with an overall decrease of their amount. LM larvae were found able to transfer DON and FB in low amount from naturally incurred growing substrates. Data were consistent with the possible biotransformation of mycotoxins in unknown metabolites in insects. ZEN was detected in BSF residual fractions but not in starting materials, suggesting a possible hydrolytic activity carried out by larvae upon growing. Taking all together, our results proved the urgency of better deciphering the ability of insects to uptake, transform, and excrete mycotoxins, in view of a safer use of insects as alternative protein source.

Founding sources

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CHAPTER 8

General Discussion and Perspectives

Introduction

By 2050 the world population will reach 9.7 billion people, more than the 30 percent higher than today (UN DESA, 2019). As a consequence, it has been estimated that the future food production must increase by 70 % (FAO, 2009), with a huge impact on the meat sector. The meat consumption in developed countries is expected to increase by 9 % in 2030 and this number is estimated to triplicate for developing countries (Chadd et al., 2002). This high numbers are related to the shifts in the composition of global food demand, due to the changing lifestyles, rising incomes, food preference and rapid urbanization (van Huis, 2013).

Indeed, the raise in meat consumption has a cascade effect on the livestock production and feed supply, exerting increased pressures on natural resources. Between the different livestock species, the main sources of animal proteins are poultry, cattle and pig. To produce 1 kilogram of these animals, it is estimated that are necessary 2.5 kilogram of feed for poultry, 5 kg for pork, while 10 kg of feed for beef (FAO, 2009). Nowadays the main feed sources are represented by grass, crops and by-products, such as meals from the oil crop sectors (e.g. soybean, rapeseed and sunflower) (FAO, 2015). Cereals represent the main energy supply for feed production, while soybeans the main protein supply (Westhoek et al., 2011). But the increase in feed supply production in turn needs more water and agricultural lands, promoting deforestation and biodiversity reduction (Henchion et al., 2017). Furthermore, even if new technologies will be developed to increase the efficiency of crop production, a negative impact on greenhouse gas emission is expected (FAO, 2009). In parallel, the increase in livestock production results in an increase in land occupied, water consumption and greenhouse gas emissions (Herrero et al., 2011). In this scenario develops the need to find new more sustainable protein sources to alternate/substitute the common ones used for both feed and livestock production.

One possible solution could be the replacement of animal proteins with plant proteins, shifting the population habits to a plant-based diet (Lynch et al., 2018). From a nutritional point of view, plant proteins have poorer quality in comparison to animal proteins and must be well balanced in the diet in order to reach the correct nutritional profile. In fact, plant protein does not display all the essential amino acids required for human and animal nutrition, which cannot be synthesized by the organism and so, must be assumed with diet (Boland et al., 2013). In addition, the presence of anti-nutritional factors could reduce their absorption (Friedman, 1996). Another solution could be the introduction of novel protein sources, such as microalgae, which present high protein content and have amino acid pattern suitable for human nutrition (Becker, 2007). Nevertheless, due to high production

costs and technological efforts to incorporate algae-based ingredients in food products, the propagation of algal proteins is still at the beginning (Becker, 2007). Besides micro-algae, in the last years, also insects have been explored as promising source of proteins to be introduce in the feed and food sector.

Insects can be produced more sustainably and with a much smaller environmental impact than common livestock. The amount of feed required to produce 1 kg of insects is about 2 kg, significantly much lower than the quantity previously described for other vertebrates (Gahukar, 2016). Insects can be fed with different types of organic side streams, urban wastes or agricultural by-products included indeed less environmentally impacting than the common feed, cereal- and soy-based. Insect could be explored also for reducing the burden on the environment, due to waste and by-products disposing. For this reason, insects can be perfectly introduced in a circular economy perspective, where they could provide high added value to low quality side-streams (Pastor et al., 2015).

Main findings of this PhD thesis

In the recent year, insects have been explored as source of novel proteins. In this PhD thesis, insect-based protein ingredients for food and feed application have been investigated.

In Chapter 2, the first systemic approach to extract insect protein fraction for its potential future use is reported. Before starting with the different fractionations, black soldier fly (*Hermetia illucens*, BSF) was characterized and the main components correctly quantified, filling a gap existing in the literature. BSF prepupae biomass contained 32 % proteins, 37 % lipids, 19 % minerals, 9 % chitin, expressed on dry matter basis. The lipid fraction was easily recovered by organic solvents, with petroleum ether which allowed to extract 87 % of total fat. For the protein separation, two different chemical methods were applied on defatted insects. The first one, which might be called "one shot", is based on an alkali extraction allowing to recover 96 % of total proteins. The second one, a "stepwise method", is based on the Osborne fractionation, normally applied for cereals. Four protein fractions, with different solubility properties, were collected and a total extraction yield of 91 % reached. Even if the alkali extraction ensured to extract a higher amount of proteins, the extreme pH condition of extraction damaged protein quality. A third fractionation based on assisted enzymatic extraction was also described. This alternative method, cheaper and environmentally friendly, was performed directly on insects, without any defatting pre-treatment. Protease from *Bacillus licheniformis*, allowed to recover 60 % of total proteins in form of peptides. The protein fractions were characterized based on the protein content. In the future steps, deep investigations will be necessary to evaluate also their essential

amino acid profile and nutritional quality. Furthermore, since the fractionations have been performed at laboratory scale, it will be necessary to optimize the condition of extraction, making the protein extraction scalable for industrial production and more cost-efficient.

In Chapter 3, the browning of insect proteins, also observed in Chapter 2 due to the phenol oxidase activity, was here investigated. In particular, the potential feasibility of blanching as killing method to reduce/inhibit the enzymatic browning was evaluated. A spectrophotometric analysis demonstrated that blanching reduced the formation of melanin pigments, allowing to obtain a “white” protein fraction. On the contrary, slow killing methods by freezing elicit the activation of several enzymatic pathways which affect metabolic changes. These have an impact also on protein nutritional quality, with a loss of cysteine and lysine, likely involved in the process of melanisation and enzymatic browning. A strong effect was also observed on protein extractability, since proteins from prepupae killed by blanching were found to be more extractable by chemical methods and more prone to enzymatic digestion than proteins from prepupae killed by freezing. Blanching is not only a killing method, but also a way to inhibit the browning reaction and other enzymatic changes occurring during slow killing by freezing. Nowadays, other killing methods, besides blanching and freezing, are employed by insect rearing farmers, such as freeze-drying, liquid N, vacuum packing and mechanic squeeze. All these treatments should be better investigated in order to evaluate the potential implication on the final nutritional quality of insect meals.

The enzymatic approach, even if less efficient than the chemical extractions, was considered the most promising method, deserving some deepening and optimization. Firstly, 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 (Clemente, 2001). Finally, the peptides released during the hydrolysis might present bio-functional activities (Nongonierma & FitzGerald, 2017). In Chapter 4, the proteolytic activity of seven different enzymes from different origins (Papain, Pancreatin, Dispase I, Pepsin, Protease from *Bacillus licheniformis*, Bromelain and Trypsin) was compared, and their ability to extract proteins from both BSF and lesser mealworm (*Alphitobius diapherinus*, LM) evaluated. The enzymatic assisted extractions, performed at a laboratory scale, allowed to extract on average the 71 ± 8 % of total proteins from BSF, and 67 ± 6 % from LM. The proteins extracted were in form of peptides and free amino acids, the latter accounting for more than 30% of the extracted protein fraction and released according to their abundance in the initial protein pool. In order to demonstrate the scalability of these process, the conditions of hydrolysis were modified and a final amount of 600 g of protein hydrolysate from 1.5 kg of LM were obtained by using protease

from *Bacillus licheniform*is. Even if a lower protein extraction yield (42.1 ± 4.3 %) was obtained than the preliminary laboratory tests, the potential scalability of the process was demonstrated obtaining protein hydrolysates containing 62.1 ± 0.3 % of protein material. The data demonstrated that the hydrolysate composition can be tailored by adjustment in process conditions (time, concentration of enzyme, substrate/buffer ration). Furthermore, a full factorial design should be performed for determining the most promising process conditions and to scale up the full process.

The protein hydrolysates obtained during the enzymatic assisted extraction scale up were characterized in Chapter 5 as far as techno functional properties are concerned. Preliminary tests demonstrated that, after five hours of hydrolysis (pH 7.5, 60 °C, enzyme concentration 0.25 %), insect protein hydrolysates presented optimum solubility property, but oil holding capacity, emulsifying and foaming ability were completely impaired. The objective of this chapter was to tailor a degree of hydrolysis (DH) allowing good techno functional properties. For this purpose, sub-samples at different time points (30, 60, 120, 180 min) of the hydrolysis were collected. With the increase in hydrolysis time, an increase in both protein extraction yield and DH was demonstrated. Solubility assay demonstrated that 95 ± 3 % of total proteins were solubilized at alkali pH, after 3 hours of hydrolysis. On the contrary, at pH 5, due to the near to the isoelectric point, no correlation was determined. Also oil holding capacity demonstrated to be positively affected by the DH. The driving force in this case was attributable to the exposure, after hydrolysis, of hydrophobic side chains, which promote the oil entrapment. On the contrary, emulsifying abilities resulted to be impaired by the increased DH. It has to be considered that protein functionality was here assessed in simple model systems, which did not mimic the real food/feed products that are generally much more complex. For these reason, deep investigation will be necessary in order to evaluate how these techno functionalities could be affected by the presence of other food/feed ingredients.

Finally, in Chapter 6 and 7 the safety related to the future consumption of insect-based ingredients was evaluated for two specific aspects. In particular, in Chapter 6, the presence of potential allergens was deeply investigated by performing a proteomic characterization, followed by an *in silico* evaluation of allergen presence. Tropomyosin was identified as the prevalent insect allergen, and immunoblotting assays underlined the immunoreactivity of insect proteins also in connection with crustacean allergy. Furthermore, the enzymatic hydrolysis was explored as a biotechnological tool for the reduction of immunoreactivity. The results clearly indicated that insect consumption can represent a serious risk for people with crustacean allergy, albeit enzymatic assisted extraction could reduce the immunoreactivity. More investigations are necessary to investigate how other technological process, and gastro-intestinal digestion itself, could affect the insect immunoreactivity, in order to fill the gap which existing now in literature and to assess the

real allergenic risk posed by insect based food, and also the technologies necessary to lower it. In Chapter 7 the potential bioaccumulation of mycotoxins in LM and BSF was investigated, when insects are reared on substrates naturally contaminated by DON and FBs (common feed mycotoxins). The presence of mycotoxins did not impair the growth performance of both insects, which, moreover, did not bioaccumulate mycotoxins into their body. In fat, the mycotoxins contamination detected in insects resulted lower than the one quantified in substrates. On the contrary the frass fractions (remaining material) were determined to be contaminated by mycotoxins in an increased amount than in insect body, defining the potential excretion from insects. Mass balance calculation delineated the potential mycotoxins metabolization of insect, making necessary further investigations on the eventual metabolic derivatives.

Future perspective and research directions

In this PhD thesis, the evaluation of insects as future source of protein-based ingredients has been investigated. In order to meet the future demand of animal proteins, LM and BSF have been determined as promising source of novel proteins, not only for the high-quality protein profile, but also for their ability to grow on a different spectrum of substrates. For these reasons, inside a circular economy perspective, insects can be involved as a "biorefinery" tool for valorising low value side streams (e.g. urban wastes or agricultural by-products) into final products with high-added value (Figure 22).

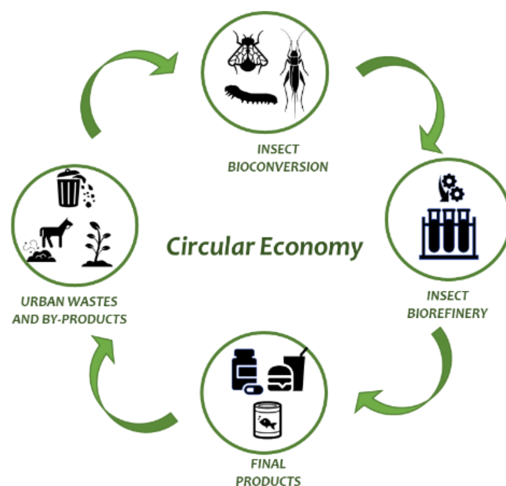

Figure 22 Insect biorefinery in a circular economy perspective

This is not of a secondary importance since, due to the increased number in world population, it is expected also an increase in urban wastes and agri-food industrial by-products. Nowadays, most insect rearing companies employs broiler standard feed for

insect breeding, but the widening possibilities of using new substrates can play a key role in enhancing the circularity of insect production. This will make the insect farming more cost efficient and sustainable. Indeed, deep investigations are needed between stakeholders involved for identifying the potential by-product substrates which could be used for insect breeding, by considering also the seasonal availability.

Processing plays an important role in the future perspective inclusion of insect protein-based ingredients in the feed and food chain. In this PhD thesis, it is described how technologies could affect the final protein quality, and the killing step itself has been described as first key factor. Besides killing by freezing and by blanching, the insect rearing companies can apply many different technologies which can affect the quality of insect compounds. The quality of insect proteins could be affected by the presence of endogenous enzymes which, if not properly inactivated, could damage the protein structure. Extensive studies should first evaluate the potential causal relationship between other killing method and insect nutritional quality and then identifying the metabolites responsible. After killing, insects are subjected to other technological process which allow the extraction of interesting biomolecules. In this PhD thesis, different protocols for protein extraction have been proposed and developed in a laboratory scale, but for their scaling up more investigations are needed for optimizing the condition in a more economic and sustainable view. The enzymatic assisted extraction has been demonstrated a promising biotechnology for extracting protein from insect biomass, nevertheless the low protein recovery obtained in this PhD thesis suggested the need of extensive studies. There are many factors which could affect in a different manner the hydrolysates protein content (e.g. amount of substrate, enzyme, buffer, pH, temperature, time of hydrolysis) and which can be tailored in order to increase the final protein recovery. Furthermore, different implication on bio and techno functionality are expected, which can give added value to the final product for its inclusion in feed and food formulations. Indeed, it is necessary to develop prototype formulations in order to analyse the combining effect of different compounds to the final product quality. In addition, processing can induce reactions which alter flavour or nutritional properties, such as the release of bitter peptides (Adler-Nissen, 1984), which could compromise the final use and so, has to be assessed.

Nowadays one of the limitations in the use of insects as food is certain linked to safety aspects. In this PhD thesis, the presence of allergens has been discovered as the main risk related to the human insect consumption, in particular for people who already present crustacean allergy. The enzymatic assisted extraction has been determined a valid tool to reduce the immunoreactivity of LM but, on the contrary, the conditions applied were not sufficient to reduce also the immunoreactivity of BSF and so, has to be adjusted to reach the same goal. This clearly demonstrates the different susceptibility of insect species when

processed under the same conditions. Indeed, more investigations are necessary to study the effect of other insect processing (cooking, mechanic separation, alkali extraction, solid state fermentation) on immunoreactivity in order to fill an existing gap in literature. Furthermore, the future marketable products should be correctly labelled for informing the future consumers about the potential allergic risk related to insect consumption. In the next future, if allergic reactions will occur, it could be necessary to include them in the food allergic list, besides for examples cereals, crustaceans, peanuts, milk and their by-products.

In this PhD thesis, it has been demonstrated the ability of insects to grow on substrates contaminated by mycotoxins and to excrete them in the frass fraction. For this finding, some authors proposed the potential use of insects to convert contaminated substrates exceeding the allowed level of mycotoxins and pesticides into insect biomass for feed or non-food application (Purschke et al., 2017). Nevertheless, the results obtained in this PhD thesis suggested that this assumption must be critically evaluated, since the results here presented suggested the insect mycotoxin metabolization in forms not yet known. For these reasons it will be necessary to deep investigate not only the new metabolic forms, but also evaluate their potential toxicity.

The potentiality of insect protein-based ingredients has been assessed, but for the final inclusion in the food chain it is necessary to make huge efforts for sensitizing the future new consumers, who disgust the entomophagy practise. For this reason, it is important to correctly inform the future consumer about the economic and environmental sustainability related to the substitution of common animal protein with insect protein. Moreover, the inclusion of insects in food formulation in form not recognisable, such as protein ingredient, will help the consumers to accept this novel food.

Conclusion

In conclusion, insects can represent an interesting nutritional source of proteins with high nutritional quality, which can substitute the protein sources that are nowadays employed for both feed and food sector. The inclusion of insect protein-based ingredients in the food chain has been demonstrated to represent a clear benefit, with associated potential risk for consumers with crustacean allergy. In fact, the high homology between Arthropoda proteins defines the possibility of cross-reactivity between insect proteins and known food allergens. Anyway, the enzymatic assisted extraction has been demonstrated a valid biotechnology tool for reducing the insect immunoreactivity. This technology has been proven to be also a promising method to extract protein from insects in a cheap and environmentally friendly way. Furthermore, the tailoring of enzymatic hydrolysis

conditions allows to modulate the techno functional property and the nutritional value of the final hydrolysates, based on the product end-use.

The results contained in this thesis constitute then an important piece in the building up and shaping a new, more sustainable, food and feed production, having high nutritional value but low risk, based on insects. These new food and feed will help the world to become a better place for the billions of people that will require a quality diet for longer and healthier life.

Supplementary Material

The free amino acids profiles of the untreated larvae and the hydrolysates were reported in Table S1 for the fractions originating from *Alphitobius diaperinus* and in Table S2 for the ones from *Hermetia illucens*.

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).

mg/g	AD larvae	Pepsin	Pancreatin	PBL	Trypsin	Papain	Bromelain	Dispase I
His	0.32 ± 0.12 ^{c}	4.7 ± 0.1 ^{ab}	5.6 ± 0.4 ^{b}	4.98 ± 0.42 ^{b}	4.01 ± 0.08 ^{ab}	2.7 ± 0.1 ^{a}	4.8 ± 1.4 ^{ab}	4.02 ± 0.04 ^{ab}
Thr	0.051 ± 0.002 ^{b}	3.8 ± 1.9 ^{a}	3.12 ± 0.05 ^{a}	1.9 ± 2.2 ^{a}	0.9 ± 0.5 ^{a}	2.1 ± 0.2 ^{a}	1.8 ± 0.9 ^{a}	2.90 ± 0.97 ^{a}
Val	0.091 ± 0.007^{e}	11.0 ± 0.5 ^{bcd}	11.5 ± 1.4 ^{cd}	12.4 ± 0.3 ^{cd}	8.07 ± 0.05 ^{b}	4.1 ± 0.3 ^{a}	13.3 ± 0.8 ^{d}	9.69 ± 0.99 ^{bc}
Lys	0.08 ± 0.03 ^{d}	1.37 ± 0.02 ^{ab}	2.05 ± 0.13 ^{b}	3.56 ± 0.21 ^{c}	0.85 ± 0.01 ^{a}	0.7 ± 0.21 ^{a}	1.35 ± 0.52 ^{ab}	1.22 ± 0.45 ^{ab}
Ile	0.058 ± 0.002 ^{e}	6.6 ± 0.4 ^{bc}	8.4 ± 0.9 ^{bcd}	8.7 ± 0.3 ^{cd}	6.33 ± 0.03 ^{b}	2.2 ± 0.3 ^{a}	8.91 ± 0.01 ^{d}	6.8 ± 1.1 ^{bcd}
Leu	0.11 ± 0.01 ^{d}	13.8 ± 1.4 ^{bc}	13.7 ± 0.9 ^{bc}	14.1 ± 0.4 ^{bc}	10.6 ± 1.1 ^{b}	6.4 ± 0.7 ^{a}	14.9 ± 0.179 ^{c}	13.4 ± 1.2 ^{bc}
Trp	0.31 ± 0.09 ^{d}	3.2 ± 0.1 ^{bc}	3.6 ± 0.3 ^{c}	3.1 ± 0.4 ^{abc}	2.5 ± 0.3 ^{ab}	2.1 ± 0.1 ^{a}	3.7 ± 0.2 ^{c}	3.2 ± 0.1 ^{bc}
Arg	0.29 ± 0.09 ^{c}	1.1 ± 01 ^{a}	1.1 ± 0.3 ^{a}	0.91 ± 0.02 ^{a}	0.86 ± 0.01 ^{a}	4.96 ± 0.14 ^{b}	1.08 ± 0.03 ^{a}	0.97 ± 0.02 ^{a}
Ala	0.21 ± 0.05 ^{e}	16.05 ± 0.37 ^{cd}	15.37 ± 1.03 ^{c}	17.1 ± 0.6 ^{cd}	11.98 ± 0.18 ^{b}	7.21 ± 0.51 ^{a}	18.87 ± 0.74 ^{d}	14.62 ± 1.57 ^{bc}
Gly	0.09 ± 0.01 ^{d}	3.9 ± 0.4 ^{b}	3.9 ±0.3 ^{b}	5.7 ± 0.3 ^{c}	3.7 ± 0.5 ^{b}	1.9 ± 0.2 ^{a}	5.8 ± 0.3 ^{c}	3.7 ± 0.5 ^{b}
Ser	0.08 ± 0.01 ^{b}	2.86 ± 1.15 ^{a}	1.21 ± 0.45 ^{a}	0.2 ± 0.3 ^{a}	0.16 ± 0.07 ^{a}	2.36 ± 0.31 ^{a}	0.19 ± 0.2 ^{a}	2.04 ± 1.29 ^{a}
Pro	0.31 ± 0.07 ^{d}	12.7 ± 1.1 ^{bc}	10.5 ±0.9 ^{abc}	11.3 ± 1.3 ^{abc}	9.8 ± 0.7 ^{ab}	8.4 ± 0.6 ^{a}	13.6 ± 0.9 ^{c}	12.05 ± 0.03 ^{bc}
Met	0.17 ± 0.06 ^{e}	2.4 ± 0.2 ^{b}	3.25 ± 0.03 ^{d}	3.04 ± 0.19 ^{cd}	1.75 ± 0.01 ^{a}	1.6 ± 0.2 ^{a}	2.8 ± 1.7 ^{bcd}	2.5 ± 0.2 ^{bc}
Phe	0.32 ± 0.09 ^{c}	7.7 ± 0.2 ^{b}	9.04 ± 0.31 ^{b}	8.9 ± 0.8 ^{b}	5.99 ± 0.59 ^{ab}	2.43 ± 2.01 ^{a}	8.6 ± 0.9 ^{b}	7.7 ± 0.6 ^{b}
Glu	0.66 ± 0.09 ^{b}	12.1 ± 0.2 ^{a}	15.4 ± 0.3 ^{a}	13.99 ± 0.58 ^{a}	12.9 ± 2.6 ^{a}	7.3 ± 0.4 ^{a}	7.6 ± 1.7 ^{a}	11.5 ± 4.9 ^{a}
Gln	0.66 ± 0.22 ^{d}	5.1 ± 0.2 ^{ab}	4.1 ± 0.6 ^{ab}	11.4 ± 3.2 ^{c}	2.15 ± 0.01 ^{a}	7.7 ± 0.5 ^{bc}	3.83 ± 0.98 ^{ab}	11.1 ± 0.6 ^{c}
Asp	0.54 ± 0.19 ^{d}	6.7 ± 1.4 ^{b}	2.8 ± 0.5 ^{a}	2.66 ± 0.39 ^{a}	6.71 ± 0.44 ^{b}	3.86 ± 0.12 ^{a}	10.29 ± 0.13 ^{c}	8.51 ± 0.01 ^{bc}
Asn	0.011 ± 0.004 ^{d}	0.53 ± 0.04 ^{bc}	0.7 ± 0.2 ^{c}	0.05 ± 0.01 ^{a}	0.2 ± 0.1 ^{ab}	0.07 ± 0.03 ^{a}	0.27 ± 0.05 ^{ab}	0.3 ± 0.1 ^{ab}

Cys	0.32 ± 0.09 [b]	1.31 ± 0.01 [a]	1.6 ± 0.4 [a]	1.4 ± 0.2 [a]	1.1 ± 0.2 [a]	2.9 ± 0.3 [a]	1.32 ± 0.04 [a]	1.3 ± 0.1 [a]
Tyr	0.38 ± 0.09 [e]	3.6 ± 0.4 [bc]	3.9 ± 0.8 [cd]	1.11 ± 0.03 [a]	1.7 ± 0.2 [ab]	5.6 ± 0.5 [d]	1.7 ± 0.1 [ab]	1.6 ± 0.7 [a]
Sum	5.07 ± 1.6 [d]	120.2 ± 5.2 [c]	120.9 ± 1.04 [c]	126.6 ± 6.3 [c]	92.3 ± 0.4 [b]	76.7 ± 3.3 [a]	123.95 ± 3.81 [c]	119.1 ± 0.7 [c]

Table S2 Free amino acids composition of hydrolysates from *Hermetia illucens* (HI) 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 ($p<0.05$).

mg/g	HI larvae	Pepsin	Pancreatin	PBL	Trypsin	Papain	Bromelain	Dispase I
His	0.34 ± 0.02 [e]	3.6 ± 0.4 [ab]	2.6 ± 0.1 [a]	3.8 ± 0.7 [bc]	5.25 ± 0.02 [d]	3.7 ± 0.1 [ab]	4.9 ± 0.1 [cd]	5.3 ± 0.1 [d]
Thr	0.044 ± 0.002 [c]	3.6 ± 0.1 [a]	2.8 ± 1.4 [a]	4.99 ± 0.25 [ab]	7.3 ± 0.1 [b]	4.5 ± 0.1 [a]	7.4 ± 0.9 [b]	7.5 ± 0.4 [b]
Val	0.09 ± 0.01 [e]	7.4 ± 0.2 [ab]	5.96 ± 3.36 [a]	8.9 ± 0.3 [abc]	14.26 ± 0.95 [cd]	10.2 ± 1.6 [abcd]	13.3 ± 1.4 [bcd]	15.6 ± 1.4 [d]
Lys	0.094 ± 0.008 [f]	0.58 ± 0.01 [a]	0.79 ± 0.02 [ab]	2.08 ± 0.09 [e]	1.63 ± 0.04 [d]	1.02 ± 0.04 [bc]	0.82 ± 0.21 [ab]	1.38 ± 0.09 [cd]
Ile	0.06 ± 0.01 [d]	4.8 ± 0.1 [ab]	4.09 ± 1.98 [a]	6.6 ± 0.1 [abc]	10.4 ± 0.1 [c]	7.5 ± 1.7 [abc]	8.99 ± 0.21 [bc]	10.3 ± 0.5 [c]
Leu	0.102 ± 0.016 [e]	7.25 ± 0.01 [ab]	6.5 ± 1.7 [a]	9.9 ± 0.1 [bc]	15.1 ± 0.3 [d]	12.1 ± 0.1 [cd]	12.7 ± 1.1 [cd]	14.5 ± 0.6 [d]
Trp	0.35 ± 0.03 [c]	2.5 ± 1.4 [ab]	1.7 ± 1.4 [a]	3.6 ± 0.5 [ab]	5.61 ± 0.02 [b]	4.1 ± 0.2 [ab]	5.06 ± 0.03 [b]	5.1 ± 0.7 [b]
Arg	0.27 ± 0.03 [c]	1.37 ± 0.01 [a]	1.01 ± 0.36 [a]	2.7 ± 0.7 [b]	1.5 ± 0.1 [a]	1.59 ± 0.02 [ab]	1.79 ± 0.04 [ab]	1.52 ± 0.02 [a]
Ala	0.299 ± 0.052 [d]	12.46 ± 0.45 [ab]	9.26 ± 2.62 [a]	12.09 ± 0.19 [ab]	19.48 ± 0.58 [c]	17.06 ± 2.46 [bc]	19.84 ± 1.19 [c]	19.71 ± 0.01 [c]
Gly	0.13 ± 0.02 [d]	4.7 ± 0.1 [ab]	3.8 ± 1.8 [a]	4.2 ± 0.6 [a]	7.9 ± 0.4 [bc]	5.62 ± 0.98 [abc]	7.8 ± 0.5 [bc]	8.2 ± 0.97 [c]
Ser	0.05 ± 0.01 [b]	1.2 ± 1.4 [a]	1.15 ± 1.52 [a]	5.79 ± 0.12 [a]	0.16 ± 0.05 [a]	2.73 ± 3.53 [a]	0.2 ± 0.1 [a]	0.8 ± 0.9 [a]
Pro	0.13 ± 0.03 [e]	6.5 ± 0.4 [abc]	4.5 ± 3.1 [ab]	3.61 ± 0.04 [a]	11.3 ± 0.9 [cd]	6.99 ± 2.31 [abcd]	10.8 ± 0.7 [bcd]	12.9 ± 1.3 [d]
Met	0.19 ± 0.02 [b]	2.21 ± 0.03 [a]	1.5 ± 0.1 [a]	1.8 ± 1.2 [a]	3.1 ± 0.4 [a]	2.6 ± 0.3 [a]	2.9 ± 0.2 [a]	3.1 ± 0.2 [a]
Phe	0.32 ± 0.06 [e]	1.47 ± 0.01 [a]	3.99 ± 0.48 [b]	6.5 ± 0.4 [c]	8.8 ± 0.5 [d]	6.4 ± 0.2 [c]	7.72 ± 0.05 [cd]	7.7 ± 0.9 [cd]
Glu	0.61 ± 0.05 [c]	5.9 ± 0.6 [a]	6.25 ± 2.05 [a]	8.5 ± 0.2 [ab]	14.03 ± 0.51 [b]	10.7 ± 2.6 [ab]	13.1 ± 0.7 [b]	9.8 ± 2.9 [ab]
Gln	0.73 ± 0.07 [c]	6.9 ± 0.9 [a]	5.8 ± 0.4 [a]	10.2 ± 0.7 [ab]	9.15 ± 3.03 [ab]	10.47 ± 2.01 [ab]	4.99 ± 0.1 [a]	12.8 ± 0.2 [b]
Asp	0.56 ± 0.05 [e]	4.65 ± 0.34 [ab]	3.63 ± 1.12 [a]	6.01 ± 0.44 [bc]	3.01 ± 0.18 [a]	7.92 ± 0.45 [cd]	2.87 ± 0.02 [a]	8.7 ± 0.3 [d]
Asn	0.012 ± 0.001 [d]	0.1 ± 0.1 [a]	0.07 ± 0.01 [a]	2.8 ± 0.2 [b]	0.2 ± 0.1 [a]	0.12 ± 0.04 [a]	0.13 ± 0.02 [a]	3.8 ± 0.1 [c]

Cys	0.37 ± 0.03 [b]	1.9 ± 0.1 [a]	2.35 ± 1.99 [a]	1.84 ± 0.05 [a]	2.1 ± 0.1 [a]	3.2 ± 1.8 [a]	2.1 ± 0.2 [a]	1.9 ± 0.1 [a]
Tyr	0.33 ± 0.02 [b]	1.7 ± 0.2[a]	2.7 ± 2.8 [a]	3.2 ± 2.3 [a]	1.82 ± 0.03 [a]	2.1 ± 0.2 [a]	2.2 ± 0.1 [a]	1.7 ± 0.1 [a]
Sum	4.8 ± 0.5 [f]	80.8 ± 3.3 [a]	70.6 ± 8.8 [a]	109.2 ± 0.4 [b]	141.98 ± 0.38[de]	120.5 ± 0.8 [bc]	129.6 ± 7.4 [cd]	152.3 ± 2.1[e]

CHAPTER 6

Peptide sequences identified by High Resolution Mass Spectrometry on LTQ-Orbitrap instrument in *Hermetia illucens* (Table S3) and *Aplhitobius Diaperinus* (Table S4).

Table S*3* Peptide sequences identified in *Hermetia illucens* protein extract by High Resolution Mass Spectrometry on LTQ-Orbitrap instrument. The characterisation reported information about the precision of the MS identification (10lgP and ppm), the peptide abundance (Area), the Uniprot accession number and also the name of the parental protein.

Peptide	-10lgP	ppm	Area	Accession	Protein
NLGTDAEAIVVR	22.97	3.6	2.44E+05	B0XA27_CULQU	Larval cuticle protein 8.7
VADENGFQPEGAHIPK	30.21	2.5	1.98E+05	B0XA27_CULQU	Larval cuticle protein 8.8
VAPEEHPVLL	26.7	3.3	1.73E+05	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding)
VDGFDLK	21.29	4.1	1.71E+05	A0A0M4EJM5_DROBS	Fringe glycosyltransferase
SFKDDFLEK	23.61	4.2	1.43E+05	A0A182SYB4_9DIPT	Uncharacterized protein (GNEFA)*
EITALAPSTIK	38.65	3.2	1.02E+05	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding)
EITALAPSSIK	24.72	3.2	1.02E+05	F1C3P6_TIMCA	Actin
WGFETSDGK	22.85	3.1	9.65E+04	C0H6J6_BOMMO	Putative cuticle protein
DRLEDELGLNK	59.11	3.9	9.60E+04	E2A6N1_CAMFO	Tropomyosin-1
DRLEDELGINK	59.11	3.9	9.60E+04	A0A158NXC8_ATTCE	Uncharacterized protein (tropomyosin)
ALLPDGK	26.53	4.5	9.17E+04	E2C0X6_HARSA	NHL* repeat
IVELEEELR	44.82	2.9	6.68E+04	B4QYK2_DROSI	GD19006 (Tropomyosin)
AGFAGDDAPR	46.01	3.5	6.60E+04	A0A1A9ZNP6_GLOPL	Uncharacterized protein (Actin ATP binding)
VDLVEGK	24.06	3.8	6.60E+04	B0WYN8_CULQU	Dipeptidyl - peptidase

FPLDMSEAH	21.75	3.4	6.18E+04	U5EWU6_9DIPT	Putative hexamerin 2 beta
LADENGFQPEGAHLPR	22.46	4.1	6.13E+04	A0A1A9Z940_GLOPL	Uncharacterized protein (cuticle protein)
LEDELVIEK	37.64	3.7	5.78E+04	TPM_LOCMI	Tropomyosin
LEDELVLEK	37.64	3.7	5.78E+04	B0X3L6_CULQU	Tropomyosin invertebrate
DSYVGDEAQSK	53.04	3.8	5.59E+04	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding)
GIITNWDDMEK	48.22	3.2	5.58E+04	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding)
IQLLEEDLER	42.01	3.4	5.07E+04	B0X3L6_CULQU	Tropomyosin invertebrate
SGGTTMYPGIADR	26.13	4.5	5.01E+04	A0A1A9ZNP6_GLOPL	Uncharacterized protein (Actin ATP binding)
QEYDESGPGIVHR	45.35	3.2	4.95E+04	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding)
SLEVSEEK	23.97	3.7	3.99E+04	A0A0Q9WML9_DROVI	Uncharacterized protein (tropomyosin isoform J)
LDLGLNK	22.68	4.5	3.95E+04	K7JVQ9_NASVI	Uncharacterized protein (Zinc ion binding)
GEGDPEFIK	28.33	3.7	3.94E+04	TNNT_DROME	Troponin T, skeletal muscle
IMELEEELK	31.51	3.1	3.64E+04	A0A1J1HZZ6_9DIPT	CLUMA_CG005729, isoform B (putative Tropomyosin 2)
LEYPELK	26.03	2.9	3.55E+04	A0A182IRM9_9DIPT	Uncharacterized protein (Ca binding)
MDALENQLK	42.36	4	3.21E+04	B0X3L6_CULQU	Tropomyosin invertebrate
VVDNGSGMCK	35.6	3.4	3.04E+04	A0A1B0CWE9_LUTLO	Uncharacterized protein (Actin ATP binding
SYELPDGQVITI	33.45	3.9	2.86E+04	A0A1A9ZNP6_GLOPL	Uncharacterized protein (Actin ATP binding
QLIEEDLER	30.89	5.3	2.79E+04	A0A158NXC8_ATTCE	Uncharacterized protein (Tropomyosin)
QLLEEDLER	30.67	5.3	2.79E+04	A0A182XV85_ANOST	Uncharacterized protein (Tropomyosin)
AYDVQDALTGDSK	46.87	3.3	2.77E+04	R4G8D1_RHOPR	Putative cuticle protein
VQQIEEDLEK	44.08	3.4	2.74E+04	A0A1L8EHE5_HAEIR	Putative tropomyosin-2 isoform x1
AYDVQDAITGDSK	42.58	4.7	2.61E+04	A0A026VY81_CERBI	Cuticle protein
GFETSDGK	22.89	4.3	2.19E+04	C0H6J6_BOMMO	Putative cuticle protein
MDQLTNQLK	28.79	3.5	2.04E+04	A0A1J1HX79_9DIPT	CLUMA_CG005729, isoform F (tropomyosin 2)
MVEADLER	32.92	3.4	1.84E+04	B0X3L6_CULQU	Tropomyosin invertebrate
TPDYLLR	28.53	3.6	1.83E+04	A0A1B6CX71_9HEMI	Uncharacterized protein (calcium ion binding)
TPDYILR	28.53	3.6	1.83E+04	B4LFD9_DROVI	Uncharacterized protein (Cuticle protein)

LAFVEDELEVAEDR	48.2	3.5	1.81E+04	A0A1J1HX79_9DIPT	CLUMA_CG005729, isoform F (tropomyosin 2)
NVVHSDK	31.82	2.6	1.73E+04	E9JC17_SOLIN	Aminopeptidase
LEVSEEK	23.21	4.6	1.73E+04	A0A1J1HX79_9DIPT	CLUMA_CG005729, isoform F (tropomyosin 2)
DLLIACTICGK	24.17	5.8	1.62E+04	B0W4Y6_CULQU	Serendipity locus protein delta
ALGFPFDR	30.41	3.9	1.53E+04	U5EWU6_9DIPT	Putative hexamerin 2 beta
LLAEDADGK	41.32	3.8	1.30E+04	A0A1J1HZZ6_9DIPT	CLUMA_CG005729, isoform B (putative Tropomyosin 2)
LSEASQAADESER	51.57	3.4	1.25E+04	B4QYK2_DROSI	GD19006 (Tropomyosin)
DEEVDEMIR	35.23	4.4	1.18E+04	H9IVN8_BOMMO	Uncharacterized protein (calmodulin)
LVQVEADLVSSK	36.07	3.2	1.16E+04	T1GWE6_MEGSC	Uncharacterized protein (Tropomyosin)
ILEELIEEVDEDK	46.75	3.1	1.15E+04	A0A0L0BTD6_LUCCU	Troponin C, isoform 3
LEDEQSVVGK	29.63	3	1.08E+04	A0A139WE70_TRICA	Myosin heavy chain, muscle-like Protein
VADEYDPHPQY	23.02	3.4	1.08E+04	R4G8D1_RHOPR	Putative cuticle protein
DVQDSLTGDSK	29.68	3.4	1.06E+04	N6UFH9_DENPD	Uncharacterized protein (chitin binding protein)
DVQDSITGDSK	29.68	3.4	1.06E+04	K7IX09_NASVI	Uncharacterized protein (cuticle protein)
AYDVQDSLTGDSK	42.31	4.3	1.06E+04	T1GYP1_MEGSC	Uncharacterized protein (cuticle protein)
AYDVQDSITGDSK	42.31	4.3	1.06E+04	K7IX09_NASVI	Uncharacterized protein (cuticle protein)
HEIASTR	25.31	1.9	1.00E+04	A0A026WSH0_CERBI	Protein scabrous
FRAAVPSGASTGVHEALELR	31.67	4.3	9.60E+03	A0A411G6M9_9HYME	Putative enolase isoform X1
LTQEAVADLER	38.94	3.8	9.59E+03	A0A139WE70_TRICA	Myosin heavy chain, muscle-like Protein
TGYGPLGK	28.6	3.2	9.46E+03	Q0IF51_AEDAE	Protein HIRA
LAMVEADLER	39.22	3.9	8.73E+03	B0X3L6_CULQU	Tropomyosin invertebrate
IEELEEEVEAER	42.35	3.3	7.40E+03	A0A139WE70_TRICA	Myosin heavy chain, muscle-like Protein
AQQELEEAEER	41.11	3.6	6.84E+03	A0A139WE70_TRICA	Myosin heavy chain, muscle-like Protein
HNDAVAEMAEQVDQLNK	28.1	3.5	6.48E+03	W4VRL5_9DIPT	Putative myosin class i heavy chain
ADMAEQAISK	27.42	3.8	6.02E+03	A0A0R1DVF3_DROYA	Myosin heavy chain, isoform D
TGEELQAAEDK	33.08	2.6	4.89E+03	A0A0R1DVF3_DROYA	Myosin heavy chain, isoform D

* GNEFA: guanyl-nucleotide exchange factor activity; NHL: tripartite motif-containing protein 71.

Table S4 Peptide sequences identified in *Alphitobius diaperinus* protein extract by High Resolution Mass Spectrometry on LTQ-Orbitrap instrument. The characterisation reported information about the precision of the MS identification (10lgP and ppm), the peptide abundance (Area), the Uniprot accession number and also the name of the parental protein.

Peptide	-10lgP	ppm	Area	Accession	Protein
SGGTTMYPGIADR	58.22	1	3.79E+06	D6WF19_TRICA	Actin-87E-like Protein
IEELEEEVEAER	60.31	-0.9	3.55E+06	N6T0X7_DENPD	Uncharacterized protein (Myosin)
GIITNWDDMEK	52.01	-0.9	2.97E+06	D6WF19_TRICA	Actin-87E-like Protein
LTQEAVADLER	52.06	-1.1	1.54E+06	N6T0X7_DENPD	Uncharacterized protein (Myosin)
LAEAEETIESLNQK	59.93	0.4	1.53E+06	N6T0X7_DENPD	Uncharacterized protein (Myosin)
AAVPAGSGLEGQWIPDINEK	70.44	-1.1	1.39E+06	Q7M478_TENMO	Cuticle structural protein
TVVADEYDPHPQYS	67.23	-0.5	1.31E+06	R4G8D1_RHOPR	Putative cuticle protein
TTGIVLDSGDGVTHTVPI	42.53	-2.2	1.12E+06	D6WF19_TRICA	Actin-87E-like Protein
GAYEEGQEQLEAVR	62.96	-0.7	1.00E+06	N6T0X7_DENPD	Uncharacterized protein (Myosin)
IEDEIAKLEEK	52.04	-1.3	1.00E+06	N6T0X7_DENPD	Uncharacterized protein (Myosin)
LIDDHFLF	31.04	-1.6	9.32E+05	A0A139WNX9_TRICA	Arginine kinase 1
IVELEEELR	45.47	-0.8	9.32E+05	U4U063_DENPD	Uncharacterized protein (Trpomyosin)
LSIENSDLLR	43.92	-0.5	9.26E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
DTQTALEEEQR	49.34	0.7	9.11E+05	A0A139WE70_TRICA	Myosin heavy chain, muscle-like Protein
ELQAALEEAEAALEQEENKVLR	30.12	-0.7	9.01E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
GMESCGIHETVY	23.21	-1	8.78E+05	D6WF19_TRICA	Actin-87E-like Protein
MYDGIAELIK	32.32	-0.9	8.66E+05	I4DIQ0_PAPXU	Arginine kinase
SDLESQLSETQDR	62.76	-1.3	8.64E+05	A0A139WE70_TRICA	Myosin
LEDEQSVVGK	51.5	-0.2	8.36E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
IADENGFQPH	51.31	-0.5	7.73E+05	U4UAT3_DENPD	Alpha-mannosidase
LFEGGYEEIIK	32.29	2	7.65E+05	V5GZG5_ANOGL	Troponin T
FAYDVQDGLTGDSK	34.45	1	7.34E+05	A0A194QZ16_PAPMA	Cuticle protein

QLEEAESQVNQLSK	61.67	0.9	6.92E+05	V9ICZ0_APICE	Myosin (+ isoform 7)
DLEESNIQHEGTLANLR	68.3	-0.5	6.87E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
TVEYTADPVNGF	61.71	0.8	6.49E+05	R4G8D1_RHOPR	Putative cuticle protein
LYDDGSYKPELTPIPL	67.84	0.1	6.44E+05	D6WQN1_TRICA	ADFb like protein
SIIFEDPHPV	38.56	-0.1	6.34E+05	A0A139WGR3_TRICA	Apolipophorins-like protein (lipid transporter activity)
LDEAEANALK	43.86	0.1	6.28E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
LLNEDLEIER	44.14	-1.8	6.06E+05	A0A0U2P8E2_9CUCU	Paramyosin
WWNELEAK	28.85	-1.2	5.93E+05	A0A0C5D652_TENMO	Chemosensory protein CSP6 mRNA
KLEADINEL	22.7	-1.1	5.91E+05	N6T0X7_DENPD	Uncharacterized protein (myosin)
AGFAGDDAPR	47.89	-1.2	5.62E+05	D6WF19_TRICA	Actin-87E-like Protein
IINVIGEPIDER	54.51	-0.8	5.54E+05	A0A034VA91_BACDO	ATP synthase subunit beta
VTPEEFVQDSFK	48.97	-1.3	5.48E+05	D6WUQ7_TRICA	Larval serum protein 2-like Protein
YSYETSNGLSSDEQGEVKNEGR	25.26	-0.1	5.08E+05	D6WMB1_TRICA	Larval cuticle protein 8-like Protein
DIEDLELNIQK	53.96	-1.2	5.04E+05	A0A0M4ECL7_DROBS	Myosin
IQEKEEEFENTR	62.14	0.5	4.96E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
YKEIGDDLD	37.35	0.1	4.94E+05	U4U063_DENPD	Tropomyosin (+ ISOFORMS C, A, B, G)
HFETSMRDPA	20.02	4.1	4.76E+05	D6WUQ7_TRICA	Larval serum protein 2-like Protein
LELSVPAGSGLEGQWIPDVNEK	66.95	-1	4.68E+05	D6WQM8_TRICA	ADFb like protein
TLNDFDAAK	38.14	-0.9	4.46E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
LEEAEGGAESQFEINK	76.89	-1.1	4.36E+05	A0A0U2P8E2_9CUCU	Paramyosin
QLMDHDKDGIITK	32.48	1.4	4.30E+05	D6WZU7_TRICA	Myosin regulatory light chain 2-like Protein
VIQSGLENHDSGIGIYAPDAD	41.28	0.5	4.24E+05	A0A139WNX9_TRICA	Arginine kinase 1
ELEELGER	21.6	0.2	4.10E+05	N6T0X7_DENPD	Uncharacterized protein (myosin)
YFGELNDLR	45.63	-0.3	4.00E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
GNLIEGNPDSPYYKYYGAYQVFARHLLG	25.58	-1.4	3.99E+05	D6WUQ7_TRICA	Larval serum protein 2-like Protein

FLAEEADKKYDEVAR	34.2	-0.1	3.99E+05	U4U063_DENPD	Tropomyosin (+ ISOFORMS C, A, B, G)
TIAMDGTEGLVR	62.55	-0.7	3.55E+05	A0A034VA91_BACDO	ATP synthase subunit beta
KFENENIGVDGY	47.57	0.1	3.51E+05	D6WMB2_TRICA	Larval cuticle protein 8-like Protein
TADPIHGF	20.28	0.2	3.48E+05	Q16UU4_AEDAE	AAEL009796-PA (cuticle protein)
ADLAEQAISK	46.09	-1.2	3.46E+05	A0A0M4ECL7_DROBS	Myosin
LQLIEEDLER	38.49	-0.6	3.39E+05	N6UBK5_DENPD	Tropomyosin
IQLLEEDLER	38.49	-0.6	3.39E+05	U4U063_DENPD	Tropomyosin (+ ISOFORMS C, A, B, G)
TVDYTADPHNGF	53.34	-0.3	3.36E+05	A0A194QZ16_PAPMA	Cuticle protein
ANALQNELEESR	50.65	0.3	3.34E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
YGPNELPAEEGK	43.61	-1.6	3.30E+05	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)
DLQIEVDR	25.89	-1.3	3.28E+05	A0A139WE70_TRICA	Myosin
DLQLEVDR	25.89	-1.3	3.28E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
EAALEQEENKVLR	30.55	0	3.27E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
IEDLELNIQK	31.95	-0.2	3.22E+05	A0A0M4ECL7_DROBS	Myosin
MVGPIEEVVQK	51.09	-1.6	3.15E+05	A0A034VA91_BACDO	ATP synthase subunit beta
IMELEEELK	30.74	0	3.02E+05	A0A0A1E5I3_MONAT	Tropomyosin (isoform 1)
KQEGEDPDPTPY	37.14	0.7	3.01E+05	A0A1L8E579_9DIPT	Myosin
TEMSETEEIKTPL	46.71	-0.4	2.95E+05	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)
GEYIGDGDYHGEGLAEA	62.36	-0.3	2.89E+05	D6WT50_TRICA	structural constituent of cuticle
LSILEEESMFPK	58.64	-1.2	2.77E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
MDALENQLK	33.38	0.8	2.69E+05	U4U063_DENPD	Tropomyosin (+ ISOFORMS C, A, B, G)
TVEYTADPINGF	40.66	-0.3	2.68E+05	CUA1A_TENMO	Larval cuticle protein A1A
TVVADEYDPNPQYSFGYDVQD	63.74	-0.7	2.62E+05	CUA1A_TENMO	Larval cuticle protein A1A
IMDPNIIGQEHY	41.78	0.2	2.55E+05	A0A034VA91_BACDO	ATP synthase subunit beta

KITDVGDVVVD	25.22	-0.9	2.53E+05	A0A1B6E9S2_9HEMI	Calcium-transporting ATPase
FDRPVY	30.59	0.4	2.47E+05	D6WUQ7_TRICA	Larval serum protein 2-like Protein
ETGNGIAAQEQGQLK	53.42	-0.9	2.42E+05	A0A139WHF4_TRICA	Chitin binding protein
IGVFGEDEDTTGK	55.84	-3.1	2.39E+05	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)
LLAEDADNKSDEVSR	61.95	-0.7	2.32E+05	A0A0A1E5I3_MONAT	Tropomyosin (isoform 1)
VRELENELD	20.92	0.3	2.29E+05	N6T0X7_DENPD	Uncharacterized protein (myosin)
SLVDPDGTR	29.75	-0.7	2.19E+05	CUA1A_TENMO	Larval cuticle protein A1A
VSSTLSGLEGELK	39.65	1.8	2.16E+05	A0A139WNX9_TRICA	Arginine kinase 1
LEEVASKF	28.62	0	2.01E+05	D5LG83_LYSTE	Arginine kinase
VEEGAGDPEFIKR	46.99	0.2	2.00E+05	D6W953_TRICA	Troponin T
IDQSILTGESVSVIK	61.36	0.1	1.99E+05	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)
QEQEVNFDGSYH	39.81	0.2	1.81E+05	A0A139WHF4_TRICA	Chitin binding protein
WMSGEEFNLK	27.57	-0.2	1.72E+05	D6WUQ7_TRICA	Larval serum protein 2-like Protein
VAVADEYDPHPQYS	43.47	-1.3	1.61E+05	D6W8Q5_TRICA	Pupal cuticle protein
NLNDEIAHQDELINKL	23.97	-3.3	1.59E+05	N6T0X7_DENPD	Uncharacterized protein (myosin)
YPDVHELAK	30.88	0.7	1.42E+05	tr|A0A1A9YR78|A0A1A9YR78_GLOFF	Phosphofructokinase
SDLDEQLR	41.59	0.5	1.42E+05	V5GZG5_ANOGL	Troponin T
LKVDDLAAELD	37.21	-0.8	1.36E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
SYELPDGQVITIGNER	53.6	-2.1	1.32E+05	D6WF19_TRICA	Actin-87E-like Protein
AQQELEEAEER	47.71	-0.7	1.30E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
FTEEQLR	35.57	-0.1	1.22E+05	C9X4E7_9CUCU	Muscular protein 20
GAPEGVLER	26.1	-0.6	1.17E+05	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)

LEEAGGATSAQIELNK	62.83	-1.6	1.14E+05	N6T0X7_DENPD	Uncharacterized protein (Myosin)
IQEKDEEIEAIRK	22.84	-1.7	1.14E+05	A0A0U2P8E2_9CUCU	Paramyosin
LQEKDEEIEAIRK	22.84	-1.7	1.14E+05	E0W1N5_PEDHC	Paramyosin, long form, putative
FGYDVQDGLTGDSK	65.1	1.6	1.04E+05	CUA1A_TENMO	Larval cuticle protein A1A
NALEQANKDLEEKEK	22.14	0.3	1.04E+05	A0A1J1HVX0_9DIPT	Uncharacterized protein (myosin)
FETSDPISR	40.49	0.1	1.02E+05	D6WMB3_TRICA	Larval cuticle protein
GFTQEEKDNIYK	35.93	1	9.97E+04	Q178Y3_AEDAE	Actin filament binding
AGVLGQMEELR	40.44	-0.2	9.31E+04	N6T0X7_DENPD	Uncharacterized protein (Myosin)
HVGDLGNVEAGGDGVAK	66.17	-0.6	9.13E+04	A0A076G467_TENMO	Superoxide dismutase [Cu-Zn]
DGPQAINNQGGAPNYHPN	52.77	0.1	8.82E+04	A0A139WLA9_TRICA	Catalase
IGELENQKY	28	-0.5	8.76E+04	A0A139WJG4_TRICA	Troponin
SSLEGEKGSLSEVQER	44.97	-1.1	8.68E+04	A0A139WE70_TRICA	Myosin
QLQEQEGMSQQNVTR	57.56	2	8.57E+04	E0W1N5_PEDHC	Paramyosin
SNDIHDYY	32.31	-0.7	8.49E+04	A0A1L8E579_9DIPT	Myosin
PEEHPVL	20.89	-0.4	8.17E+04	D6WF19_TRICA	Actin-87E-like Protein
KHNDAVSEMGEQLD	51.04	-0.4	7.52E+04	H9JXG1_BOMMO	Myosin
KHNDAVSEMGEQID	51.04	-0.4	7.52E+04	N6T0X7_DENPD	Uncharacterized protein (Myosin)
VIVITGDNK	38.26	-1.2	7.40E+04	ATC1_ANOGA	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (+ ISOFORMS A,B)
GMDFQPR	24.76	1.6	6.94E+04	D2A424_TRICA	Phenoloxidase subunit A3-like Protein
DGDVVHGSY	47.46	0	6.90E+04	R4G8D1_RHOPR	Putative cuticle protein
IWIDGTGEYVR	42.53	-1.9	6.74E+04	A0A067R5C5_ZOONE	Glutamine synthetase
LAEASQAADESER	67.79	-0.2	6.66E+04	U4U063_DENPD	Tropomyosin (+ ISOFORMS C, A, B, G)
KSFDENGTIDSER	28.77	0.8	6.60E+04	D6WZU7_TRICA	Myosin regulatory light chain 2-like Protein
AADFIQER	32.91	-1.3	6.20E+04	A0A139WLA9_TRICA	Catalase
STAGDTHLGGEDFDNR	60.04	-0.1	5.37E+04	I6SMI7_BICAN	Heat shock cognate 70

LGPKYDEYGR	23.52	-2.9	4.96E+04	D6WUQ7_TRICA	Larval serum protein 2-like Protein
AQIPAGVDAR	26.3	-1.1	4.94E+04	D6WT50_TRICA	structural constituent of cuticle
SQNSFLPR	29.87	-0.4	4.55E+04	A0A0L7LKM3_9NEOP	Apolipophorin protein
FENVKESKNI	21.07	-1.3	3.94E+04	Q6NL43_DROME	GM10157p (Zinc finger_CCHC)
DNAQDKADAMEGQAK	26.38	-0.9	3.39E+04	A0A139WAP9_TRICA	Tropomyosin (Isoform 2)
GEYSYVGPDGK	25.42	-2.3	2.94E+04	D6WMB2_TRICA	Larval cuticle protein 8-like Protein
LIAEDVQGR	35.12	-1.9	2.93E+04	A0A0L7KSL2_9NEOP	40S ribosomal protein S3a
LNSQVNDLR	24.46	-2.7	2.90E+04	A0A139WJG4_TRICA	Uncharacterized protein (troponin)
KQEYDESGPGIVHR	31.3	0.5	2.69E+04	D6WF19_TRICA	Actin-87E-like Protein

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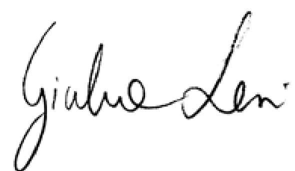

About the Author

Curriculum Vitae

Giulia Leni was born on September 1st, 1991 in Parma (Italy) and she has performed her academic studies at the University of Parma obtaining both Bachelor and Master's Degree in Food Science and Technology at the Department of Food Science. Three years ago, in 2016, she started the PhD in Food Science at the Department of Food and Drug of the University of Parma, under the supervision of Prof. Stefano Sforza and Dr. Leen Bastiaens. Her doctoral research aimed at the evaluation of edible insects as alternative protein sources for feed and food application. Part of her PhD has been developed in the framework of a European BBI project, InDIRECT, which aims to investigate direct and Indirect biorefinery technologies for conversion of organic side-streams into multiple marketable products. From January 10th, 2019 she worked for four months as visiting researcher at Flemish Institute for Technological Research (VITO), Belgium, where she acquired competence in the food technology sector, analysing the techno functionality of protein fractions obtained from edible insects.

She is author of scientific articles published in ranked journals and official deliverable reports for the European project.

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- **Leni G.**, Cirlini M., Jacobs J., Depraetere S., Gianotten N., Sforza S., Dall'Asta C. (2019). Impact of Naturally Contaminated Substrates on *Alphitobius diaperinus* and *Hermetia illucens*: Uptake and Excretion of Mycotoxins. *Toxins,* 11(8):476, DOI: 10.3390/toxins11080476.
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