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Physico-chemical and colloidal properties of protein extracted from black soldier fly (*Hermetia illucens*) larvae

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ABSTRACT

The black soldier fly larvae (BSFL), *Hermetia illucens* (Linnaeus), has been largely utilized for animal feed. Due to its interesting composition, BSFL has great potential to be further implemented in the human diet. Herein we compared the flour and protein extract composition based on their moisture, ash, amino acids, mineral, and protein content. To have wide knowledge on protein profile and behavior, SDS-page electrophoresis, Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were used to give information about protein structure and thermal stability, respectively. The flour and protein extract contained respectively 37.3% and 61.1% of protein. DSC graph reported a glass transition temperature around 30 °C, recognizable by a shift in the curve, and an endothermic peak for solid melting at around 200 °C. FTIR analysis showed the main amide bands (A, B, I, II, III) for the flour and protein extract. The foam properties of BSFL protein extract were explored under different temperatures treatment, and the best foam stability was reached at 85 °C with 15 min of treatment. The data highlight the promising techno-functional properties of BSFL protein extract, and that the nutritional composition might be suitable for further use of BSFL as food fortification system.

1. Introduction

The world's population is expected to reach 9 - 10 billion in 2050 [1]. This perspective directly impacts food consumption by means of agricultural and livestock growth which might increase deforestation, water consumption, and greenhouse gas emission [2]. To mitigate the risks of climate change caused by traditional agriculture and livestock production, there has been an increase in the search for new sustainable protein sources. In recent years, insects have gained increased attention by the FAO as an underexploited sustainable protein source [3] due to their high protein content and the presence of all required amino acids for human consumption [4]. Insects have a high feed conversion efficiency and a low environmental footprint when compared to livestock production and most conventional plant agriculture practices [5]. Among all edible insects, *Hermetia illucens*, popularly known as black soldier fly (BSF), has been considered as one of the most promising insects for the commercial production of proteins, with an effective sustainable approach for human consumption [6]. Due to the presence of a selection of enzymes in their gut, BSFL can consume a variety of substrates and then reduce agricultural wastes by feeding on them and synthesize high-quality proteins [7]. BSFL have revealed the ability to feed on abattoir waste, animal waste, spent grains and general organic waste from crop agriculture [4,8]. This ability can lower the cost of farming and unveil BSFL as a sustainable biotechnological tool for waste valorization [9]

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Besides livestock and poultry, the larvae of black soldier fly have been applied in feed formulation in the aquaculture sector [10]. the insect meal was used to feed salmon in their early stage and no difference in fish zootechnical performance was observed compared to traditional diets [11] BSFL meal also offered a good alternative to fish meal and soya when tested for rainbow trout, catfish, tilapia and Pacific white shrimp [12].

Protein content of this insect is reported to be between 37 and 63% of dry matter, and fat content can vary from 7 to 39% [13].The variation in nutrient content in BSFL can come from a variety of factors, including diet quality and quantity and larvae density [13]. *H. illucens* are rich in saturated fatty acids, in particular, lauric acid (C12:0) [14]. A large amount of structural and muscular proteins have been identified in BSFL, corroborating with the possibility of using insect proteins as an alternative to replace meat and soybean proteins [15,16]. Moreover, antimicrobial peptides and some enzymes such as trypsin and chymotrypsin were identified, which could be, in the near future, of industrial interest [17]. Besides protein and fat, BSF larvae contain of around 2 to 9% of chitin, a modified polysaccharide that contains nitrogen [4].

Despite the reduced negative impact on the environment as well as the interesting nutritional properties in insects, the visual aspect and the association of insects with vermin and as disease vectors [18] have been considered the main limiting factors, particularly in the western world, for consumer acceptance of insects as a food [19]. An interesting, proposed alternative to increase user's acceptance is the extraction of insect proteins to be further used as food fortification ingredients. To extract a high protein content, different methods can be applied, and each process can cause protein modifications resulting in changes of the final techno-functional properties, protein thermal stability and its subsequent application [6,20–22]. Some authors have sought to optimize the protein extraction of BSF, exploring a common alkaline extraction method after microwave oven drying process. The optimization was considered for alkaline solution to sample ratio, temperature of extraction and time. The authors also reported that defatting also enhanced the protein content of BSF meal [6]. Buβler et al. [21] investigated the influence of protein extraction and defatting process in the functional properties of BSFL and *Tenebrio molitor*. The authors produced three different samples, high protein fraction, low protein fraction (insoluble) and defatted meal. The study suggested that the techno-functionalities can be effectively manipulated depending on the method of protein extraction and consequently the protein composition.

Therefore, knowing the protein profile, the protein functionality and consequently its quality can be an important strategy to increase consumer's acceptance of insect ingredients used in food formulation development. Some papers in the literature have focused on the nutritional composition of insects, including the use of BSF flour and protein extract for feed ingredients [23,24]. The techno-functional properties of some insects have also been highlighted over the literature, based on the water/oil holding capacity, gelling, foaming, and emulsifying properties [22,25–29]. However, considering BSFL protein extract, there is still a lack of information on its protein characterization, thermostability and functional properties. Herein, we aimed to evaluate black soldier fly larvae (BSFL) flour and its protein extract composition, considering the nitrogen-protein conversion factor of 5.62 for protein extract and 4.67 for larvae flour, instead of the general value of 6.25, according to Janssen et al. [30]. Furthermore, in order to investigate the nutritional value of BSFL flour and BSFL protein extract, amino acid analysis and mineral composition were evaluated. Beyond nutritional aspects, the thermal stability by differential scanning calorimetry (DSC) and the protein structure by Fourier-transform infrared spectroscopy (FTIR) was studied for BSFL flour and protein extract. Finally, the foaming properties after heat treatment was evaluated, to evidence the advantage of protein extraction for food technological application.

2. Material and methods

2.1. Preparation of defatted insect powder

The BSFL flour was kindly provided by nextProtein, Tunisia. Briefly, nextProtein raises BSFL in a plant located in Grombalia, Tunisia. BSFL were fed on formulas of feed mixtures obtained from biowaste, in compliance with EU regulations. Once larvae reached their harvesting stage insects were separated from the substrate by sieving. They were then blanched in hot water (70 °C) for 5 min and softly dried for 3-4 h until a moisture content <7% was obtained. Dried BSFL were then pressed to extract the oil and obtain a protein meal, partially defatted. The protein meal was then further ground to obtain a powder, named "protein flour" in this paper. The chemicals were purchased from Sigma-Aldrich. To remove the remaining lipids, the BSFL flour was stirred in heptane extractor solvent (ratio 1:5, w/v) for 1 h at 40 °C. The mixture was then decantated and the fat extract (liquid fraction) was recovered with a paper filtration (N° 00H, 7 cm, Sweden). After solvent evaporation by a rotary evaporator (Laborota 4000-efficient, Heidolph instruments GMBH & CO KG), the recycled solvent was used for a second extraction in order to remove any residual fat remaining in the insect flour.

2.2. Protein extraction

After fat extraction, the insect defatted flour was left in a fume hood overnight to evaporate the residual solvent. It was then dissolved in 0.25 M NaOH (ratio 1:15, w/v) according to Zhao et al. [31] and stirred (Polymix Buch & Holm, DK) at 300 rpm, 40 °C for 1 h. After centrifugation (Sigma Laborzentrifugen GmbH 4-16KS, Germany) at 2493 × *g* 4 °C for 20 min, a 2nd alkali extraction followed by centrifugation was made on the solid fraction. This remaining solid fraction was stored and referred to as the chitin extract. The supernatants from both extractions were combined. The pH of this obtained liquid was adjusted to the isoelectric point 4.3– 4.5 with 2 M HCl to precipitate the proteins. After decantation, the solution was centrifuged at 1272 ×*g* 4 °C for 15 min to recover the precipitate. Finally, the precipitate was washed twice with distilled water and freeze-dried overnight.

2.3. Moisture, ash, and protein content

Moisture and ash of the flour and the protein extract were determined after drying in the oven for 24 h, respectively at 105 °C and 600 °C, according to the AOAC standard method [32]. Dry matters Eq. (1) and ashes Eq. (2) were calculated as follows:

$$\text{Dry matter } (\%) = \frac{\text{dry matter weight}}{\text{initial weight}} \times 100 \quad (1)$$

$$\text{Ashes } (\%\text{DM}) = \frac{\text{ash weight}}{\text{dry matter weight}} \times 100 \quad (2)$$

The protein content in BSFL flour and protein extract was measured with Dumas method, a quantitative analysis of the protein content based on the amount of nitrogen. A nitrogen-to-protein conversion factor (Kp) of 4.67 and 5.62 were used for protein flour and protein extract respectively, according to Janssen et al. [30]. Kp value is given by the sum of all anhydrous amino acids and the percentage of nitrogen (Nt), provided by Dumas method. Nt for BSFL flour and BSFL protein extract were 7.70% and 12.06%. The protein content of the flour and protein extract were measured in duplicate, using 150 mg in crucibles. Aspartic acid and wheat flour were used to calibrate the instrument.

2.4. Amino acid composition

Acid hydrolysis (6 M HCl) in an oven for 18 h at 110 °C using 10 mg sample per mL HCl was applied for amino acid profile measurement.

Samples were then cooled at room temperature (25 °C) and used both without dilution and after a 3-fold (1 + 2) dilution with 6 M HCl in the remaining procedure to quantify both high and low abundant amino acids. During acid hydrolysis, cysteine and tryptophan are destroyed and therefore they have not been reported in the results. Then 100 μL was diluted with 1.5 mL 1 M $NaCO_3$ and filtered in a 0.2 μm syringe filter (Q-max PTFE, Ø13 mm, Frisenette ApS, Knebel, Denmark) before derivatization using the EZ: Faast™ Amino Acid Analysis kit from Phenomenex® (Torrance, CA, USA). 50 μL of samples were then analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technology) [33].

2.5. Mineral composition

The mineral composition was measured by inductively coupled plasma mass spectrometry (Thermo iCAPq ICPMS, Thermo Electron, Bremen, Germany) following microwave-assisted digestion (Multiwave 3000, Anton Paar, Graz, Austria) using concentrated nitric acid (SPS Science, Paris, France). The sample digests were diluted with ultrapure water (milli-Q) before analysis. Quantification was performed using yttrium as the internal standard. All standards were prepared from certified stock solutions (SPS Science). Quality assurance of the analytical results was done by analysis of the certified reference material (DORM-4 fish protein, NRCC, Canada).

2.6. SDS-gel electrophoresis

Polyacrylamide gel electrophoresis was performed in order to determine the insect protein profile. The method was applied in a Mighty Small (Hoefer) slab cell using 12% acrylamide (C = 2.6% (w/w)) slab gels (1.5 mm thick). 2 mL 1% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT) and 60 mM Tris HCl (pH 8.3) were used for extraction of the dry sample (50 mg). The sample was previously prepared under shaking at room temperature for 1 h. Then it was homogenized (Polytron PT 1200, Kinematica) for 30 s, boiled for 2 min, and set at room temperature (25 °C) for 30 min. For supernatant collection, the sample had undergone a homogenization process, boiled again (2 min), and was centrifuged for 15 min at 20 °C at 20,000 × g. Gel wells were loaded with 10 μL (40 μg protein), and the extract aliquot was diluted with sample buffer (125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA) and bromophenol blue. 100 *V* for 15 min constant voltage was applied followed by 150 V for 1 h (max. 40 mA per gel). Finally, the gel was stained using colloidal Coomassie Brilliant Blue as described by [34] Rabilloud and Charmont (2000). Molecular weight Mark12™ from Novex was applied for further analysis.

2.7. Differential scanning calorimetry

Differential scanning calorimetry DSC 250 (TA Instruments, New Castle, Delaware, USA), equipped with Refrigerated Cooling System 90, was used to understand insect products' thermal stability. Calibration required distilled water (melting point (m.p.) = 0 °C; DHm = 334 J/g) and indium (m.p. = 156.5 °C; DHm = 28.5 J/g). 30 L empty aluminum pans, hermetically sealed, were used as reference. Nitrogen at a flow rate of 50 mL/min was used as a carrier gas. The DSC was measured on the flour and protein extract using approximately 4 mg of sample in the pans, in duplicate. The samples were cooled to -90 °C and then scanned from 10 °C to 250 °C at a heating rate of 5 °C/min, to obtain the heat flow in the function of the temperature. The thermograms present characteristic peaks, which can be associated with glass transition, thermal unfolding, and solid-melting according to Al-Saidi et al. [35]. The interpretation of the curves was made using Trios software, determining glass transition from the mid-point of the shift in the curve, and unfolding and melting with the enthalpy and maximum of the peak.

2.8. Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of insect flour and protein extract were recorded in using a Perkin-Elmer Spectrum 100 spectrometer (Waltham, Massachusetts, USA), based on a Universal Attenuated Total Reflectance sensor 125 (UATR-FTIR). ATR FTIR was used to record the FTIR spectra in the transmission mode over a range of 4000–650 cm^{-1}. Spectra were plotted as absorbance (AU) as a function of wavenumber (cm^{-1}). Automatic signals were recorded in 4 scans. The measurements were performed in triplicate.

2.9. Turbidity measurements

As an indicator of turbidity, the absorbance at 400 nm of protein dispersion was evaluated according to Shen et al. [36]. Measurements were performed in a quartz sample cell with a UV–Vis spectrophotometer (Hitachi, U-1500, Japan) Milli-Q water was used as a blank. All measurements of each sample were performed in duplicate.

2.10. Particle size by dynamic light scattering (DLS)

Particle size distribution was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (MPT-2, Malvern Instruments, Worcestershire, UK), according to Chili et al. [37], with modifications. Briefly, protein dispersions were diluted at 0.1% w/v with 10 mM phosphate buffer (pH 7), followed by filtration using 0.45 μm syringe filters (Pall, New York, USA). Measurements were carried out at 25 °C, at a scattering angle of 173°. Each sample was analyzed in triplicate, and the particle size was expressed as hydrodynamic diameter (d_h). Eq. (3).

$$d_h = \frac{KT}{3\pi\eta D} \tag{3}$$

where D is the translational diffusion coefficient; K is the Boltzmann's constant; T is the thermodynamic temperature; η is the dynamic viscosity.

Polydispersity Index (PdI) was also reported for each sample, data was measured in triplicate, Eq. (4).

$$PdI = \left(\frac{\sigma}{2\alpha}\right)^2 \tag{4}$$

where it is represented by the standard deviation (σ) of the particle diameter distribution divided by the mean particle diameter.

2.11. ζ-potential and isoelectric point

Protein extract was dissolved in Milli-Q water, and using a titration instrument (MPT-2, Malvern Instruments, Worcestershire, UK) the pH of the sample (10 mL) was adjusted in a range from 2 to 9 by adding 1 M NaOH or 1 M HCl. The analysis was performed on the 8 solutions using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) with capillary cells. The isoelectric point (pI) was determined graphically, as being the pH corresponding to a ζ-potential of zero. ζ-Potential was calculated from the electrophoretic mobility (μ) using the Henry equation, Eq. (5):

$$\zeta = \frac{3\eta\mu}{2\epsilon f(kRh)} \tag{5}$$

where η is the viscosity of the buffer (1.033×10^{-3} Pa s^{-1}), ϵ is the medium dielectric constant (dimensionless), Rh is the complex radius (nm) and f(kRh) is Henry's function. A value of 1.5 was adopted for f (kRh), referred to as the Smoluchowski approximation, as the analysis was performed in aqueous media.

2.12. Foaming properties

Foaming properties of the samples were measured according to Jarpa- Parra et al. [38], with some modifications. Insect flour and protein extract were rehydrated in distilled water (C = 10 g/l) and left for 12 h at 25 °C for swelling. Ultraturrax mixer (Colonial Scientific, DI 25 basic yellow line, Richmond, USA) was used to prepare foam through homogenization of 40 mL of solution at 9500 rpm for 1 min. The homogenized solution was poured into a 100 mL graduated cylinder sealed with parafilm. The foaming capacity (FC) was calculated according to Eq. (6). V_t and V_0 represent the formed foams after homogenization, and the volume of the protein solution, respectively. The foaming stability (FS) was evaluated based on Eq. (7), V_{foam} is the foam volume at specified times (t_n) of 5, 10, 15, 30, 60 min at 20 °C and V_f is the foam volume immediately after mixing at time zero (t_0).

$$FC = \frac{V_t - V_0}{V_0}\ x\ 100\% \tag{6}$$

$$FS = \frac{V_{foam}\ (t_n)}{V_f\ (t_0)}\ x\ 100\% \tag{7}$$

The whole experiment was performed by the same person and conducted in triplicate at room temperature (25 °C), 75 °C, and 85 °C.

2.13. Statistical analysis

All statistical tests were done using ANOVA, the level of significance was $p < 0.05$, followed by Tukey's test using the Statistical Package for the Social Sciences software (SPSS 22.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Nutritional composition

The nutritional composition of BSFL flour and protein extract were analyzed including dry matter, ash, and protein content, and are reported in Table 1.

The dry matter of the flour and protein extract were 94.8% (w/w) and 97.4% (w/w) respectively. The ash content of flour was 9.2% (w/w) and 14.4% (w/w) for the protein extract. The results for flour and protein extract ashes were similar to those reported by Mintah et al. [6], at 8.5% and 12.4% (w/w), respectively. After extraction, 50 g of BSFL flour yield 22.7% of protein extract, 18.9% of chitin extract and 9% of lipid extract. The lipid and chitin extracts were stored and the protein extract was considered in this study for protein quantification and characterization. Using a conversion factor of 4.67 and 5.62 for insect flour and protein extract, based on Dumas method, the protein content of raw BSFL flour was 37.3% and after alkaline extraction, protein extract had 61.1% of proteins. The data was in accordance with the results found by Janssen et al. [30], at 36.7% and 67.6% for BSFL flour and BSFL protein extract, respectively. The mineral content was lower in the protein fraction (23.0 g/kg) compared to the flour (33.2 g/kg) (Table 1), as the extraction process separates the proteins from the chitin and minerals. For most individual minerals, the content decreased after the protein extraction process (e.g. Mg: 3.04 g/kg in the flour, 0.17 g/kg in the protein extract). Further studies need to be performed in order to understand how different methods of extraction can alter the mineral content. Regarding the flour, the comparison with other studies suggests that the mineral content could vary depending on the feeding substrate of the larvae, yet this variation might be minor according to Spranghers et al. [39,40]. It is known that BSFL accumulate calcium and manganese during life, but not sodium or sulphur which is in accordance with the results herein reported [40]. In general, different parameters might alter the nutritional composition of BSF in different stage of life, for example, a study using a mixture of waste and specific amount of protein/fat showed that using more protein in their diet produce a more proteinaceous insect, however this correlation was not very clear for fat [41]. The processes used to prepare the flour also can influence the nutritional composition such as the defatting step can be performed by some methods and using a variety of solvents such as n-hexane, ethanol, ethyl acetate and etc. Ravi et al. [42] showed that each method will affect the lipid composition and the quality of proteins at the end of extraction. Therefore, the obtained results for nutritional composition here presented, is directly related to all the parameters of processing applied in this study and can be altered if modifications occur.

Table 1
Dry matter, ash, protein content, and mineral composition for BSFL flour and protein extract.

Composition	Elements	Flour	Protein extract
Dry matter (%)	–	94.8	97.4
Ash (%)	–	9.2	14.4
Protein (%)	–	37.3[a]	61.1[b]
Macrominerals (g/kg)	Na	0.90	20.2
	Mg	3.04	0.17
	K	12.4	1.34
	Ca	15.6	0.49
Microminerals (mg/kg)	Cr	0.84	1.05
	Mn	124	6.65
	Fe	916	776
	Co	0.10	0.06
	Ni	0.57	0.55
	Cu	12.1	32.9
	Zn	92.4	29.7
	As	0.13	0.02
	Se	0.41	0.71
	Sr	150	8.41
Heavy metals (mg/kg)	Cd	0.14	0.09
	Pb	0.49	0.27
	U	0.02	0.03
	Hg	0.01	0.02
Total minerals (g/kg)		33.2	23.0

[a] Nitrogen-to-protein conversion factor of 4.67.
[b] Nitrogen-to-protein conversion factor of 5.62.

Table 2
Amino acid (AA) composition (mg/g of crude protein) and total AA content (w/w %dry weight) of the Black Soldier Fly larvae flour and protein extract, compared with the adult daily requirements.

Amino acid	Flour	Protein extract	Adult daily requirements 1985 FAO/WHO/UNU
Histidine	14.5	23.6	15
Leucine	49.9	78.9	59
Isoleucine	45.1	64.8	30
Lysine	39.7	47.1	45
Methionine	11.8	18.1	22 (Met + Cys)
Phenylalanine + Tyrosine	51.6 (24.4+ 27.2)	91.1(44.8+ 46.3)	38 (Phe + Tyr)
Threonine	21.5	21.7	23
Valine	61.7	78.9	39
Tryptophane	nd	nd	6
Sum essential AA	**295.8**	**424.2**	**277**
Alanine	59.2	67.4	
Arginine	20.9	20.9	
Aspartic acid	52.4	75.6	
Glutamic acid	80.5	77.4	
Glycine	39.9	44.8	
Hydroxyproline	1.3	0.7	
Proline	62.2	65.2	
Serine	26.7	26.8	
Sum non-essential AA	**343.1**	**378.8**	
Sum AA	**638.9**	**803**	

(Met: methionine, Cys: Cysteine, Tyr: Tyrosine, Phe: Phenylalanine, nd: not detected)

3.2. Amino acid composition

Table 2 reports the amino acid composition, expressed in mg of amino acid per gram of crude protein where the essential amino acids: threonine, methionine, valine, histidine, lysine, leucine, phenylalanine, and isoleucine, can also be found. The flour and the protein extract contained all essential amino acids (EAA) measured (tryptophan was not measured). Quantitatively, the sum of all the EAA, 295.8 mg/g, and 424.2 mg/g, for flour and protein extract, respectively, meets the requirement set out by the World Health Organization (minimum of 277 mg/g) [43]. BSFL flour showed low values for some EAA such as threonine, methionine, lysine, and leucine, according to the FAO [4,22]. However, except for threonine, BSFL protein extract showed higher levels for all EAA which makes the method of extraction interesting when nutritional value is considered. Spranghers et al. [39] have shown that concentration of each amino acid might have small differences according to the BSFL diet. Herein, we have reported that the amino acid content might also vary after protein extraction and previous processing such as the killing method [4]. Mintah et al. [6] has also reported changes in the amino acid (AA) content based on different protein extraction methods for BSFL. When compared to other protein sources such as meat and chicken, BSFL protein extract can be considered as a sustainable alternative source, based on the greater values for some EAA. In developing countries, lysine is of particular interest as it shows low content in people's diet [4], and the recommended concentration (45 mg/g) can be easily reached by using BSFL protein extraction as a food fortification ingredient.

3.3. Protein composition SDS-page

SDS-PAGE technique was performed under reducing conditions, with dithiothreitol (DTT) reagent (Fig. 1). In Fig. 1 the image of the marker, from the same gel, was added on the side of protein extract and flour line, in order to make it easier to identify the bands. Two main regions could be noticed for protein extract distribution, one band right below 66 kDa (according to the marker reference) and another region below 31 kDa. Ravi et al. [44] have already reported a similar protein distribution under different product processing (conventional drying, frozen, microwave drying, scalding, and blanching). The authors matched the lower protein bands to the cleavage of disulfide bonds, under reducing conditions, which guides to protein denaturation and further breakdown into peptides. Rabani et al. [17] have also stated the large presence of structural and muscular proteins showing stronger bands in the region between 75 and 50 kDa. This range of molecular weight, for insect samples, has been linked to enzymes, muscle proteins and exoskeleton proteins in other previous works for *H. illucens* and *Tenebrio molitor* [22,42]. The low molecular weight proteins, under 31 kDa might also refer to glutelin proteins from actin family, with Troponin C the most abundant according to Leni et al. [45]. Hexamerin F, a common protein in larvae of insects that functions as storage protein [46], was identified by Leni et al. [45] as the main protein between 116 kDa and 66 kDa bands. The protein extraction, defatting, drying, and killing methods applied to BSFL samples are known to exhibit a different protein profile and must be considered for further analyzes [42,44]. A darker area is seen on the top of the gel in the protein extract area, which might correspond to proteins that did not fully run through the gel, due to protein aggregation that caused an increase in size. Gels performed with bigger pores might facilitate the separation of such proteins and identification, in the future. According to BSFL flour and protein extract, it is proposed that insects could be a promising source for fortification and technological investigation purposes [15–17].

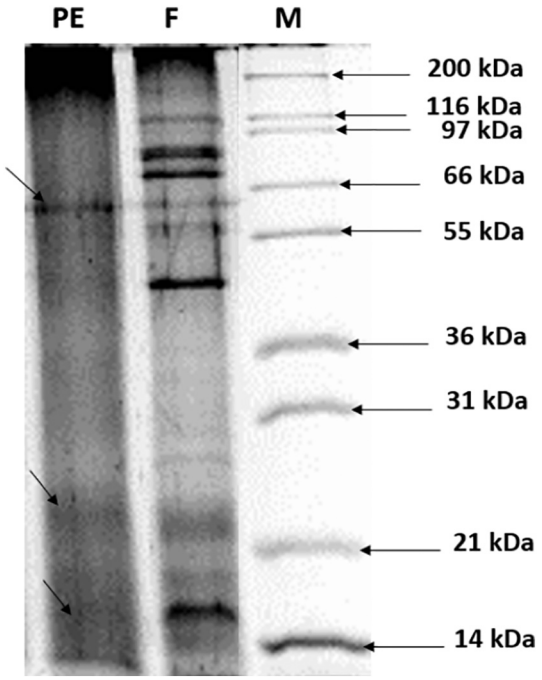

Fig. 1. SDS-PAGE analysis of BSFL protein extract and flour, where PE = Protein extract; F = Flour and M = Marker.

3.4. Turbidity

Absorbance at 400 nm was applied to calculate the turbidity (optical density, OD) of the dispersion. Three different treatments of temperature, untreated (25 °C), 75 °C, and 85 °C were used to observe any change in turbidity. An increase in turbidity might be due to protein aggregation that remained suspended in the liquid matrix [47,48]. Fig. 2 shows that OD clearly increases after each treatment and the difference was statistically significant according to Tukey's test for $p < 0.005$. Even though the concentration used was low (1% w/v) it was enough to reveal some protein aggregation and increase in turbidity. Untreated group had OD of 0.297±0.001, 75 °C group had OD of 0.368± 0.004 and 85 °C 0.414±0.001. All measures were performed in duplicate In accordance, we reported an increase in particle size after temperature treatment which can be directly correlated to the increase in turbidity, where the highest turbidity was verified in the sample treated at 85 °C that showed the highest particle size. In addition, LaClair and Etzel [49] have reported a complete study on protein aggregation and increase in turbidity upon heating treatment of whey protein in beverages over storage.

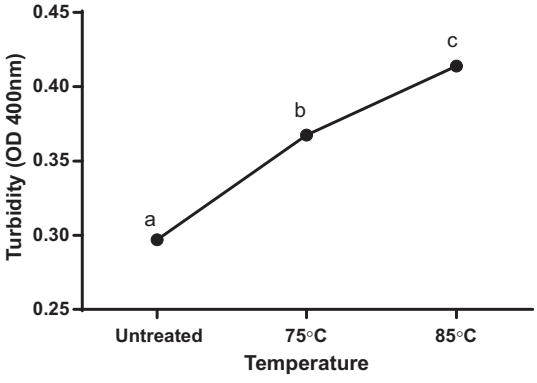

Fig. 2. Turbidity OD 400 nm of BSFL protein extract dispersion (1% w/v), untreated, heat-treated (15 min) at 75 °C and 85 °C. Different letters mean a significant difference for $p < 0.05$ among samples.

3.5. Thermal stability

DSC analysis gives detailed information about the qualitative and quantitative thermal properties of a solid material such as the melting and the degradation temperature, and specifically heat-induced denaturation of proteins [49]. The thermograms present the heat flow (W/g) in function of temperature (°C). The DSC graphs for the BSFL flour (A) and BSFL protein extract (B) are shown in Fig. 3. All thermograms are marked with an X, Y, and Z letters for glass transition (Tg), unfolding, and solid-melting, respectively. Tg was observed as a shift in the curve between 20 and 30 °C for the BSFL flour and 30 to 50 °C for BSFL protein extract, and the results are similar to the previous data reported for Tg of *H. illucens* protein extract by Barbi et al. [50]. The range in Tg temperature might be due to different protocols of protein extraction. An exothermic peak, right before the unfolding peak (Y), temperature range between 110 °C to 140 °C, appears in the flour graph (Fig. 3A) but not in the protein graph (Fig. 3B). This led to the hypothesis that this peak was characteristic for remaining components such as chitins and lipids rather than the proteins from BSFL flour [51,52]. BSFL protein extract thermogram, Fig. 3B, showed an endothermic peak (Y), which is defined at 150 °C, resulting from the thermal unfolding of the proteins [33,53]. Both the BSFL flour and protein extract also showed an evident endothermic peak (Z) at around 200 °C, which has been previously reported as the melting point [33], and restrained weight loss due to moisture content [50]. The complete degradation of BSFL proteins can be reached at 500 °C according to Barbi et al. [50] and Wang et al. [54].

3.6. FTIR spectra

In order to obtain a biochemical fingerprint of BSFL proteins and to verify if the extraction process might have altered the molecular bonds from BSFL proteins, FTIR analysis was applied in the flour and in the protein extract (Fig. 4). The technique might provide solid information about molecular composition, functional groups, and the secondary structure of proteins. In general, five distinct FTIR spectra regions can be identified corresponding to the Amide group due to the presence of peptide bonds between amino acids (Table 3). For FTIR protein extract analyses, a clear N—H free stretching vibration could be noticed in the range of 3500–3200 cm^{-1}. However, referring to insect powders some authors have linked the peaks in the same range to vibrations of O—H group stretching [55,56]. Amide *B* group, 3100–2900 cm^{-1} seems to be represented by a split of two peaks. This wavelength result represents the asymmetric and symmetric stretching of C—H. The next Amide *I* and II bands are the most prominent and characteristic vibrational bands from the protein back-bone in the FTIR spectra [57]. Amide *I* band is identified in the range of 1700–1600 cm^{-1} which is caused by the stretch vibrations of carbonyl group C═O from peptide linkage. The frequency of Amide *I* is also correlated to the secondary structure of proteins [57].

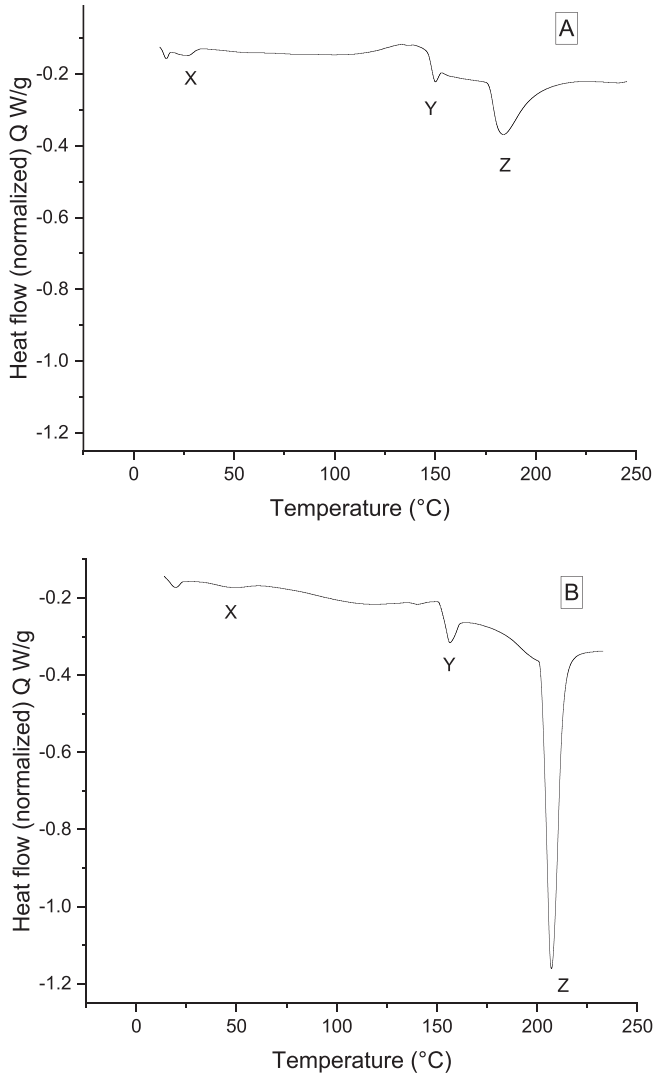

Fig. 3. DSC thermograms of (A) BSFL flour and (B) BSFL protein extract.

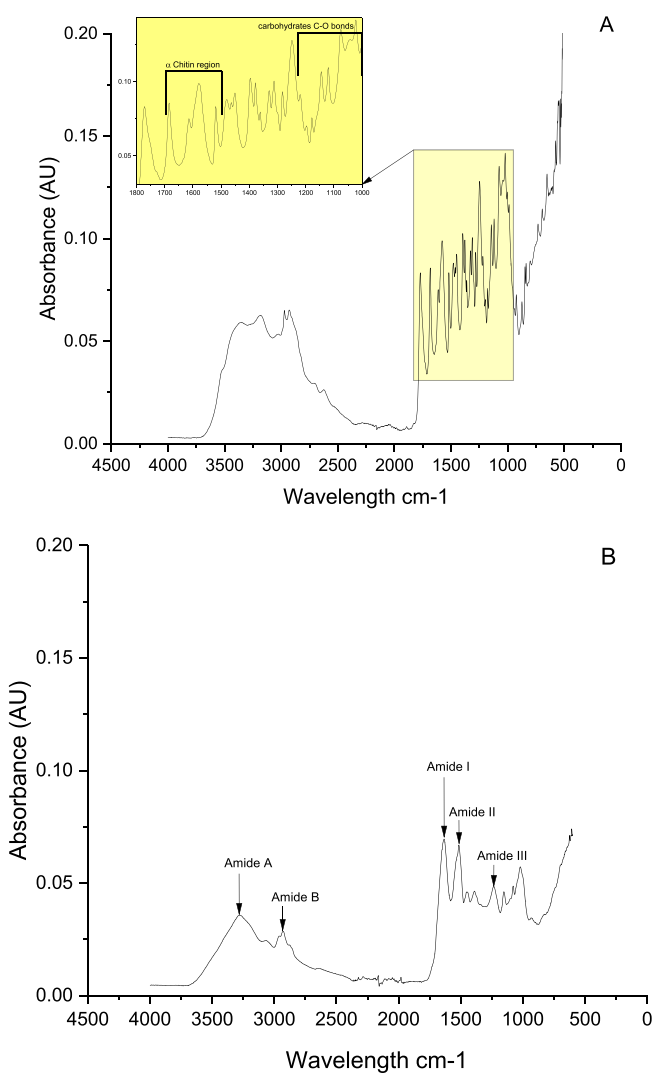

Fig. 4. FTIR spectra of (A) BSFL flour and (B) BSFL protein extract.

Table 3
Characteristic infrared band frequencies and their corresponding functional groups in *H. illucens* protein extract.

Designation	Found frequency (cm^{-1})	Description
Amide A	3274	N-H stretching
Amide *B*	2929	C-H stretching
Amide *I*	1634	C=O stretching
Amide II	1514	C-N stretching, N—H bending
Amide III	1233	C-N stretching, N—H bending

The result herein reported (Fig. 4 B) shows high absorbance at 1634 cm^{-1}, and according to Kong & Yu [57], the region 1640–1620 cm^{-1} has been assigned to β-sheet structure. In contrast, Amide II band in the range of 1550–1500 cm^{-1} derives from in-plane N—H bending and CN stretching vibration and although it might be correlated to protein secondary structure, it shows less protein conformational sensitivity compared to Amide I [57]. C—O stretching vibrations between 900 and 1200 cm^{-1} are normally caused by carbohydrates [55]. The band near 1040 cm^{-1} was observed to correspond to some polysaccharides such as galacturonic acid and glucuronic acid [55]. Amide III band was identified at 1233 cm^{-1} according to Nagarajan et al. [55]. This region represents the combination between peaks of C—N stretching vibrations and N—H deformation from amide linkages, and also absorptions from CH_2 wagging *v*ibrations, arising from two amino acids, glycine and proline side-chains. The FTIR spectra of the protein extract were well defined according to the reported bands. However, due to the more complex composition, the flour FTIR spectra had more bands in the same range (Fig. 4A). In the insect flour FTIR spectra the absorbance intensity in the range of 900-1200 cm^{-1}, which is associated with carbohydrates C—O bonds, was higher for flour than for the protein extract. In addition, the sharp bands between 1500 and 1700 can be related to the presence of α-chitin, as previously reported for other insects [51,58]. BSFL flour has a very complex composition, with high content of chitin, lipids and other elements that made it difficult to point out the specific region for proteins in the FTIR spectra. However, all amide bands where well identified in the protein extract showing the efficiency of the extraction process.

3.7. Foam capacity and foam stability

Fig. 5 shows the protein extract foamability and foam stability at 1% w/v of protein content, under different temperature treatments. The lowest foamability (Fig. 5B) was found for untreated dispersion (65%) and the highest value for samples treated at 85 °C (72%). However, no statistical difference between all groups was observed ($p < 0.05$). Foam stability is reported in Fig. 5A. The result represents how foam stability might increase according to the heating treatment on the protein matrix, due to the capacity of unfolded proteins with exposed hydrophobic groups, and form a more cohesive surface film at the air-water interface [59]. After 5 min, the lowest foam stability was found for untreated samples (66%) followed by the sample heated at 75 °C/ 15 min (83%) and finally the sample at 85 °C/15 min (96%). A statistically significant difference could be noticed between the untreated and 85 °C/15 min samples, during the first 20 min of the experiment. According to Lawal et al. [60], high foam stability could be a result of the formation of stable molecular layers at the air-water interface which will consequently cause benefits in texture, stability, and elasticity. This result was in accordance with the size and ζ-potential data, which suggests charge decrease and protein aggregation under heat treatment causing the particle size to increase. The graphic shows results for foam stability up to 60 min. However, the experiment was carried out for more than 12 h, and no reduction in foam volume was observed for all groups after this point (data are not shown). Santiago et al. [48] have also reported the foamability and foam stability for black cricket protein isolate. The authors showed an increase in foamability after heat treatments of 75 °C/15 min and 95 °C/15 min, with the highest value for samples treated at 95 °C. The foam properties of *H. illucens* from two different protein extracts under different pH, were previously reported by Mintah et al. [6]. The study revealed the best foamability at pH 6 and foam stability at pH 4, and in the Isoelectric point region, no temperature treatment was mentioned.

3.8. Particle size distribution and ζ-potential

The particle size was expressed as the hydrodynamic diameter (d_h) of proteins in an aqueous dispersion, and it was determined by the dynamic light scattering method, stated in Table 4. Samples were considered as a polydispersed system which suggests that there were a few populations within the sample. Polydispersity index (PdI) was also reported in

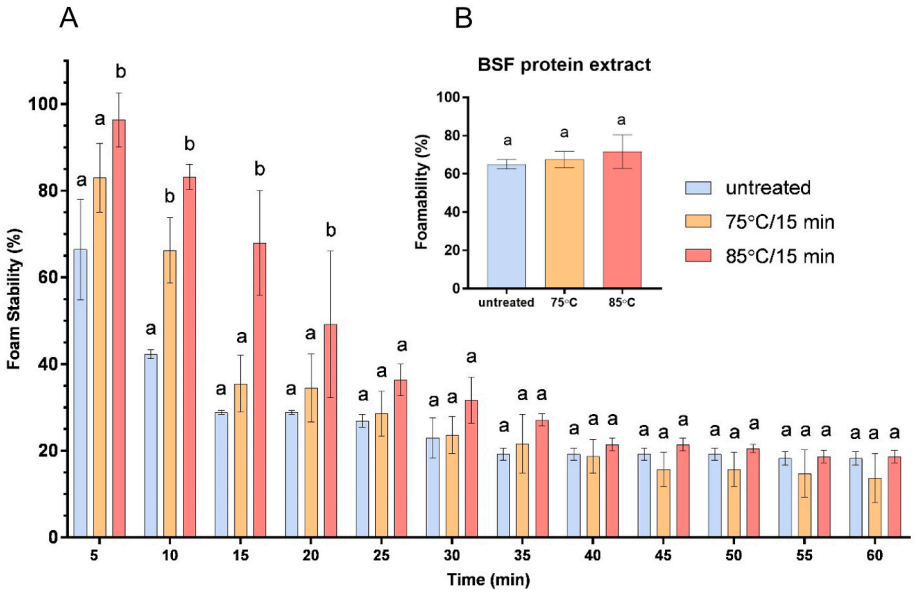

Fig. 5. Foam stability (A) and foamability (B) of BSFL protein extract (1% w/v) concentration under three different conditions: untreated, 75 °C/15 min and 85 °C/15 min.

Table 4
Hydrodynamic diameter (d_h, nm), ζ-potential (mV) and Polydispersity index (PdI) of BSFL protein extract before and after heat treatment. Different letters in the same column show a significant difference ($p < 0.05$) among samples, according to Tukey's test.

Sample	d_h (nm)	ζ- potential (mV)	PdI
Untreated	174.76±1.61[a]	−15.63±0.4[a]	0.262±0.003
75 °C/15 min	202.80±7.30[b]	−5.96±0.4[b]	0.467±0.024
85 °C/15 min	251.56±5.50[c]	−6.51±0.7[b]	0.245±0.012

Table 4 in order to estimate average uniformity of samples. Samples are considered as polydisperse when PdI is higher than 0.1, which matches with the data herein stated for all samples [61]. It might be confirmed by the d_h of 174.76±1.61 nm for the untreated sample, which was larger than the upper size for typical proteins. However, after the heating treatments, it was clear to see how aggregation was induced and particle size extensively increased. Samples heated for 15 min at 75 °C showed an increase up to 202.80±7.30 nm and the highest d_h was found for samples heated at 85 °C, 251.56±5.50 nm. Upon heating, treated proteins might unfold and cause exposure of hydrophobic side chain groups. This may lead to a reduced solubility and formation of intermolecular hydrophobic bonds that cause further protein aggregation and particle size to increase [6,62,63]. Mishyna et al. [27] have shown the heat-induced aggregation of proteins for *Apis millifera* protein extract at temperatures of 55, 70, 85, and 100 °C, with the highest protein coagulation reached at 85 °C. ζ-potential is the difference between the mobile dispersion medium and the stationary layer of fluid, the slipping plane, attached to the dispersed particle [64]. A numerically high ζ-potential, close to ±30.00 mV, is considered as an indication of stability and lower values, close to zero, indicates protein instability as it might favor hydrophobic-hydrophobic interactions and cause protein aggregation [33,53]. The ζ-potential data showed that all samples were negatively charged, and the highest values were found for the untreated sample −15.63±0.4 mV. After the heat treatment, a significant difference was observed between untreated and treated samples, where samples 75 °C/15 min and 85 °C/15 min showed a very low value of −5.96±0.40 mV and −6.51±0.7 mV, respectively. This reduction in charge might help to explain the previously reported particle size increase, and lower ζ-potential indicates less repulsion between proteins and more probability of protein aggregation [65]. Fig. 6 revealed the ζ-potential variation according to the pH range from pH 2 to 9. The data showed that all samples were positively charged in pH range 2–4 and negatively charged from pH 5-9. The isoelectric point (p*I*) value was established by the intersection of the curve on X-axis at 0 mV. The Isoelectric point was defined at 4.5 which was similar to other previous studies of insect protein values [29].

4. Conclusions

BSFL protein extract revealed a higher protein content (61.1%) compared to the flour (37.3%). BSFL protein extract and flour were physiochemically characterized. FTIR was applied to identify the main amide bands of insect protein extract and to correlate them to the secondary structure of the proteins. All five amide bands were identified in the BSFL protein extract and the region corresponding to β-sheet structure was also addressed as dominant in amide I band. DSC was applied to verify the glass transition (Tg), unfolding, and solid-melting temperature of the samples. These results give information about the sample's thermal stability, mainly for the protein extract, that might be suitable for food formulation. Also, the techno-functional property, foam stability, showed improved stability after 15 min of heating at 85 °C. This result was explained by DLS and ζ-potential data, where particle size seems to be increased under heating and ζ-potential decreased under the same treatment. The treatment might provide more protein aggregation and thus a more stable viscoelastic film on the air-water interface of bubbles. Moreover, higher optical density was observed at temperatures 75 and 85 °C when compared to the untreated sample. The data give more information on protein stability and physicochemical characterization which could make the techno-functionalities of BSFL protein extract more interesting to explore beyond their nutritional value. This study indicates that BSFL can be utilized to prepare protein-rich formulations to be further used in the production of food and feed. The protein composition of BSFL revealed an interesting foam capacity and stability which under temperature treatment could perform greater results. Besides temperature, other parameters such as, pH, salt concentration, method of protein extraction and fat removal can be further investigated to better understand how they can improve the foam properties of BSFL proteins. The data showed that BSFL proteins can be a good candidate, to be used as foaming agents, in order to gradually reduce the use of proteins from non-sustainable source. The techno-functionality, sustainability and nutritional value herein reported for BSFL preparation can add value to the product and help improving consumer acceptance, which is an important step towards the development of sustainable products. Further studies must be considered in the colloidal and molecular field, to explore the air/oil-water interfacial properties of these proteins, and how they might migrate to and stabilize the foam and emulsion structure.

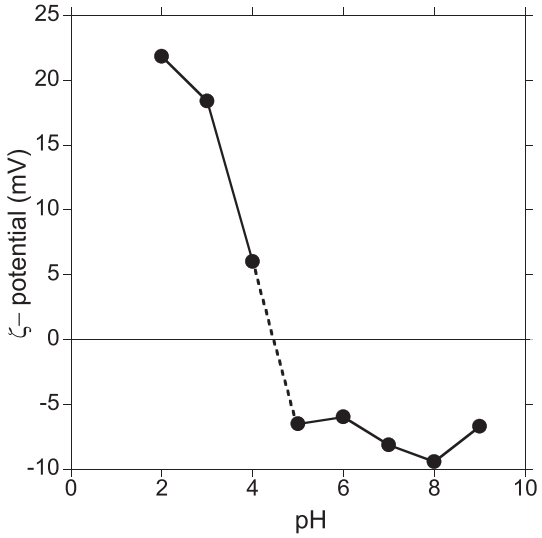

Fig. 6. ζ-potential as a function of the pH.

CRediT authorship contribution statement

Lucas Sales Queiroz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing. **Jens J. Sloth, Heidi Olander Petersen, Fatemeh Ajalloueian, Marine Regnard:** Formal analysis**. Flemming Jessen, Chloé Marie Charlotte Brouzes, Wael Fraihi, Heather Fallquist:** Conceptualization, Methodology, Validation. **Mohammad Amin Mohammadifar, Antonio Fernandes de Carvalho and Federico Casanova:** Supervision and Project administration.

Declaration of competing interest

NextProtein produces and commercializes insect-based protein for animal feed. Heather Fallquist and Chloé M.C. Brouzes are full time employees of nextProtein France. Wael Fraihi is a full-time employee of nextProtein Tunisia. The Technical University of Denmark received free samples of insect-based protein flour from nextProtein to perform the analyses of this study.

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