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Black soldier fly as a New chitin source: Extraction, purification and molecular/structural characterization

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A R T I C L E I N F O

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A B S T R A C T

Black Soldier Fly (BSF) represents a potential chitin source that has not been fully explored in terms of characterization, extraction, and purification. In this study, different chemical and enzymatic protocols with or without pre-treatment (ultrasonication and mechanochemical milling) for chitin extraction were tested. Chitin was then accurately quantified and characterized from a molecular and structural point of view by UPLC-MS, XRD, and ESEM, and compared with chitin from shrimp shells. BSF chitin was more recalcitrant than shrimp chitin during extraction and purification, due to the strong binding of chitin to proteins. Indeed, the purity of shrimp chitin was 88.3g/100g of extract, while BSF chitin purity was 47.6–79.9g/100g. Furthermore, the chitin-bound proteins had a defined amino acid composition; their binding was also confirmed by structural characterization. Therefore, the efficiency of each step of the extraction process needs to be critically evaluated to adapt the methods used for crustaceans to insect biomass.

1. Introduction

In parallel with the use of insects as a sustainable protein source for the increasing demand for food and feed, insect chitin has recently been considered as an alternative to chitin from other sources. The prospect of increasing the production of insect biomass for protein extraction will lead to higher availability of insect chitin (Cortes Ortiz et al., 2016). Among insects, the Black Soldier Fly (BSF) is one of the most utilized thanks to its high protein and chitin content (Fuso et al., 2021). Chitin is a polysaccharide mainly composed of N-acetylglucosamine (GlcNAc) units covalently bound by β-1,4 linkages. Chitin, along with its main derivatives chitosan and chitooligosaccharides (COS), has numerous applications in agriculture, biomedicine, food, cosmetic, and pharmaceutical industries (Fuso et al., 2021). Most of these applications require a high chitin purity. Therefore, a great interest persists in the optimization of its extraction, leading to a reduction of the impurities (Percot, Viton, & Domard, 2003).

The main source of chitin is the exoskeleton of crustaceans and insects, which stabilizes the body of arthropods, acting as a physical defense against predators and desiccation (Boßelmann, Romano, Fabritius, Raabe, & Epple, 2007; Schowalter, 2016). The mechanical resistance of the exoskeleton is due to the crystalline structure of chitin, firmly bound to proteins and minerals through sclerotization (Tsurkan et al., 2021). In addition, certain proteins are bound to chitin through melanization, an immediate and localized defense mechanism that occurs mainly in insects (Khayrova, Lopatin, & Varlamov, 2021; Leni, Caligiani, & Sforza, 2019; Solano, 2014; Yang, Liu, & Payne, 2009). These aggregates, combined with the partially crystalline structure of insect chitin, make its purification intricate (Khayrova, Lopatin, & Varlamov, 2019). Therefore, a method that effectively removes proteins and minerals is needed to achieve the purity necessary for the applications of chitin and its derivatives.

The main method for chitin extraction consists of the chemical removal of these hindering substances, yielding chitin with a higher purity degree (Abdou, Nagy, & Elsabee, 2008; Caligiani et al., 2018; Triunfo et al., 2022). Chemical extraction involves two main steps: demineralization and deproteinization using strong acids (HCl or HNO_3) and strong bases (NaOH), respectively. In addition, lipids and pigments can also be removed. Otherwise, biological extraction offers an environment-friendly alternative, using specific enzymes for each fraction to be removed (Kaczmarek, Struszczyk-Swita, Li, Szczęsna-Antczak, & Daroch, 2019; Younes & Rinaudo, 2015). Furthermore, in an attempt

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to improve chitin extraction yields, pre-treatment methods were recently tested (Ravindran & Jaiswal, 2016). Pre-treatment, which can be chemical, physical, physico-chemical, or biological, causes structural and compositional changes in the substrate (Abidin, Junqueira-Gonçalves, Khutoryanskiy, & Niranjan, 2017; EL Knidri, Dahmani, Addaou, Laajeb, & Lahsini, 2019; Nakagawa et al., 2011; Vallejo-Domínguez et al., 2021; Zhang, Duan, & Li, 2021). This results in the easier removal of compounds of non-interest, increasing the porosity of the substrate and reducing crystallinity by facilitating access to the reagents or enzymes used for the extraction.

After being isolated from natural sources, chitin can be converted into chitosan and chitooligosaccharides (COS); to obtain these derivatives with a high purity, the use of pure starting chitin certainly simplifies the process. Chitosan can be obtained through the deacetylation of GlcNAc units and is more soluble in diluted acid solutions; additionally, it has useful properties such as bacteriostatic, antioxidant, and immunomodulatory activity (Wang, Xue, & Mao, 2020). Recently, there has also been increasing interest in chitin and chitosan hydrolysis products, COS, such as N,N′-diacetylchitobiose ($GlcNAc_2$) (Du et al., 2021). In the medical field, COS can be used to treat asthma, increase bone strength, and other promising bioactivities that need to be confirmed by further studies (Aam et al., 2010). COS also have food applications, being potential prebiotics (Liu et al., 2020), and agronomic applications, stimulating plant defense (Liaqat & Eltem, 2018). Thus, these properties make COS an extremely valuable product; however, they require a high degree of purity in order to be properly employed.

Chitin extraction and modification methods are now optimized for crustaceans, while they have not yet been studied extensively for insects. Considering the different nature and properties of insects and crustaceans, it is therefore necessary to understand whether the promising data obtained from crustaceans can have the same success on insects. This would increase interest in insect chitin, as purified chitin certainly has more applications and fewer limitations, e.g., due to possible allergenic responses caused by the presence of chitin-binding proteins (Pan et al., 2022).

In this paper, different systematic approaches to remove lipids, proteins, and minerals from BSF larvae chitin are explored, also in combination with physical (ultrasonication) and physico-chemical (mechanochemical milling) pre-treatments, with the aim of improving the purity of insect chitin. The data obtained for BSF were compared to the data from shrimp shells.

2. Materials and methods

2.1. Chemicals and reagents

AccQ-Fluor™ reagent kit for Amino Acid Analysis was obtained from Waters Corporation (Milford, MA, U.S.A). Amino acid standard H (2.5 mmol/L), Asparagine, Casein, Chitin Practical Grade powder, Disodium hydrogen phosphate, formic acid (>95% HCOOH), Galactosamine, Glucosamine, DL-Glutamine, hydrochloric acid (12 mol/L HCl), Monosodium hydrogen phosphate, DL-Norleucine, N-Acetylglucosamine, Protease from *Bacillus licheniformis* (0.04 μkat, SLBL2953V), Ultrapure water obtained with Milli-Q® system were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (>99% ACN) and Sodium hydroxide (>99% NaOH) were purchased from VWR (Radnor, PA, USA). Diethyl ether was purchased from Carlo Erba reagents (Carlo Erba, Milan, Italy).

2.2. Insect and shrimp shell samples

All the insect samples were provided by the Laboratory of Applied Entomology – BIOGEST-SITEIA, Department of Life Science, University of Modena and Reggio Emilia. *Hermetia illucens* L. larvae were reared and killed by blanching according to Leni and colleagues (Leni, Maistrello, Pinotti, Sforza, & Caligiani, 2022). An experimental diet composed of tomato, chickpeas, borlotti beans, green beans, and wheat bran was fed to larvae. Puparia were collected according to Luparelli and colleagues (Luparelli, Hadj Saadoun, et al., 2022). Shrimp shells from *Litopenaeus vannamei* were obtained from a seafood restaurant. Samples were ground with the IKA A10 laboratory grinder and stored at −20 °C until the beginning of the analyses.

2.3. Proximate composition analysis

The proximate composition was conducted on the BSF and shrimp shell samples and on their chitin extracts, in order to understand the degree of chitin purity and the presence of putative residues. Moisture, lipids, 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 diethyl ether as solvent. Total ash was determined after mineralization at 550 °C for 5 h. Total proteins and chitin were determined with the UPLC-MS method (Paragraph 2.6).

2.4. Chitin extraction protocols

2.4.1. One stage enzymatic extraction

The analysis was conducted following the procedure described by Caligiani and colleagues. (Caligiani et al., 2018). The defatted sample was subjected to enzymatic hydrolysis by a protease from *Bacillus licheniformis*. The hydrolysis reaction was performed at the optimal temperature and pH conditions for the enzyme, that is, 60 °C, and pH 7.5, for 16 h. 0.5 g of sample and 5 mg of enzyme (enzyme/substrate 1:100 w/w) were mixed with 4.5 ml of 10 mmol/L Na_2HPO_4/NaH_2PO_4 buffer solution (substrate/buffer 1:9 w/v), hydrolyzed for 16 h, then heated at 90 °C for 10 min to inactivate the enzyme. The protocol is summarized in Fig. 1a. The hydrolyzed substrate was centrifugated (model 5810R EPPENDORF, Hamburg, Germany) at 3900 rpm and 4 °C for 30 min. The pellet was dried in oven at 40 °C overnight. The final residue was analyzed with a UPLC-MS instrument to evaluate the amount of extracted chitin and residual protein.

2.4.2. One stage chemical extraction

The analysis was conducted following the procedure described by Caligiani and colleagues with some modifications (Caligiani et al., 2018). The protocol is summarized in Fig. 1b.

2.4.2.1. Lipid removal. The finely ground BSF larvae, puparia, and shrimp shells (5g) were subjected to lipid extraction as a first step. Lipids were removed with 60 ml of diethyl ether by Soxhlet extractor. Residual solvent was removed from the defatted samples by evaporation overnight.

2.4.2.2. Protein removal. The defatted pellet was subjected to deproteinization with 1 mol/L NaOH (1:10) in water bath at 40 °C for 1 h. The supernatant was neutralized with water and centrifuged for 15 min at 3900 rpm. The pellet was dried in oven at 40 °C overnight.

2.4.2.3. Demineralization and chitin separation. The dried pellet obtained from the previous steps was subjected to demineralization with 2 mol/L HCl (1:10) for 24 h at room temperature. Then, the sample was centrifuged for 15 min at 3900 rpm; the precipitate was washed twice with deionized water. The pellet was dried in oven at 40 °C overnight. Then, the final residue was analyzed with UPLC-MS (Paragraph 2.6) to evaluate the amount of extracted chitin and residual protein.

2.4.3. Two stage chemical extraction

The procedure is the same as the one described in paragraph 2.4.2, with a repetition of 2.4.2.2 and 2.4.2.3 twice to further improve the

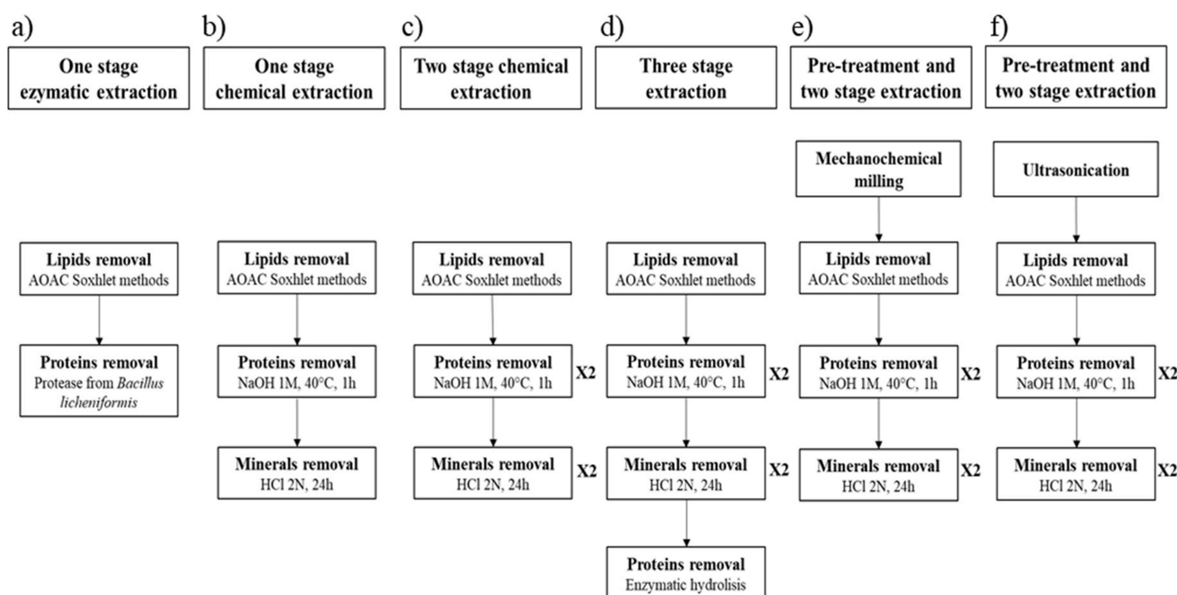

Fig. 1. Protocols for chitin extraction with different protein removal steps. a) One stage enzymatic extraction b) One stage chemical extraction c) Two stage chemical extraction d) Three stage with two stage chemical extraction and one stage enzymatic extraction; e) Mechanochemical milling pre-treatment with two stage chemical extraction; f) Ultrasonication pre-treatment with two stage chemical extraction.

removal of protein and mineral fractions from chitin. The protocol is summarized in Fig. 1c.

2.4.4. *Three stage chemical and enzymatic extraction*

After the two stage chemical extraction, a further step of enzymatic extraction was conducted as described above (paragraph 2.4.1). The protocol is summarized in Fig. 1d.

2.5. *Pre-treatment techniques*

Pre-treatment methods were tested before the two stage chemical extraction methods to increase the final chitin yield (Fig. 1e and f).

2.5.1. *Mechanochemical milling pre-treatment*

The PULVERISETTE 23 ball mill was purchased from Fritsch (Idar-Oberstein, Germany). The mechano-system consists of a milling bowl of approximately 15 mL capacity and balls of hardened, stainless steel Fe–Cr. The mechanism of mechanochemical milling is based on impact and friction. 500 mg of the whole BSF larvae were ground with one ball of 15 mm diameter at 30 Hz for 5 min, resulting in a fine powder.

2.5.2. *Ultrasonication pre-treatment*

The LABSONIC LBS2-4,5 ultrasonic bath (135 W power; 100% amplitude) was purchased from FALC INSTRUMENTS S.R.L. (Treviglio (BG), Italy). The mechanism of ultrasonication is based on the generation of many small vacuum bubbles that implode immediately (cavitation), destroying substrate aggregation. The whole BSF larvae in distilled water (10 mg/ml) were treated in the ultrasonic bath for 2h. The temperature was set at 45 °C and the frequency at 59 kHz.

2.6. *Amino acids and chitin determination with UPLC-MS*

The analysis was conducted following the procedure described by Luparelli and colleagues with some modifications (Luparelli, Leni, et al., 2022). 250 mg of whole matrix or 150 mg of extracted chitin were hydrolyzed with 6 mL of 6 mol/L HCl at 110 °C for 23 h. Then, 7.5 mL of 5 mmol/L Norleucine in 0.1 mol/L HCl, used as internal standard for total amino acid determination, were added. The hydrolysates were filtered and diluted to a final volume of 100 mL with deionized water. Then, 450 µl of hydrolysate solution were mixed with 7 µl 46 mmol/L galactosamine, used as internal standard for the quantification of N-acetylglucosamine groups of chitin (hydrolyzed to Glucosamine), and diluted to 500 µl with deionized water. The hydrolysates containing the two internal standards were derivatized with AccQ-Fluor Reagent Kit according to the method described by Leni and colleagues (Leni et al., 2019). The hydrolyzed samples were analyzed through UPLC-MS (Waters Corporation, Milford MA, USA). The following conditions have been applied for the analysis: stationary phase: Acquity BEH C18 column (1.7 µm, 2.1 × 150 mm) (Waters Corporation, Milford MA, USA); eluent A: H_2O with 0.2 % CH_3CN and 0.1% HCOOH; eluent B: CH_3CN with 0.1% HCOOH; column temperature: 35 °C; sample temperature: 18 °C; injected volume: 5 µl; flow: 0.2 ml/min; analysis time: 45 min. The elution gradient of the two solvent mixtures A and B was performed: isocratic 100% A for 7 min, from 100% A to 75.6% A by linear gradient in 21 min and washing step at 0% A (100% B) and reconditioning. Mass spectrometry data were collected in positive electrospray mode in full scan acquisition over the mass range of m/z 100−2000. Source settings were maintained using a capillary voltage, 3.2 kV; source temperature, 150 °C; desolvation temperature, 300 °C and desolvation gas flow, 650 L/h. Quantification of target analytes was performed using a calibration curve, mixing 66 µL of chitin Practical Grade hydrolysate solution (22.6 mmol/L), 10 µL of Norleucine (5 mmol/L), 7 µL of Galactosamine (46 mmol/L), 150 µL of amino acid standard H solution (2.5 mmol/L) and deionized water to a finale volume of 500 µL, to reach the final concentration of 3.0 mmol/L for chitin and 1.5 mmol/L for amino acids. The calibration curve was obtained by preparing solutions with different concentrations from 3.0 mmol/L to 0.25 mmol/L for chitin (calibration curve in Fig. S1) and 1.5 mmol/L to 0.01 mmol/L for amino acids, in triplicate.

All data were acquired with and processed by the MassLynx 4.0 software (Waters, Wilmslow, UK). Since the concentration of practical grade chitin was used for the calibration curve, the values obtained are expressed directly as chitin, even though the analytical target is Glucosamine. Data on chitin content were obtained as a triplicate analysis and are presented as mean ± SD.

2.7. *Crystallinity determination by X-ray diffraction (XRD)*

Diffraction profiles were obtained with X-ray diffractometer ARL™ X'TRA (Thermo Scientific, Waltham, MA, USA). The diffractograms of

the extracted chitin samples were collected with CuKα radiation in the range (2θ) of 5–35° with steps of 0.050°. The crystallinity degree (CD) was calculated from normalized diffractograms according to the equation below (Eq. (1)). The intensities of the peaks at [1 1 0] lattice (I_{110}, at $2\theta = 20°$ corresponding to the maximum intensity of chitin) and I_{am} at $2\theta = 16°$ (amorphous scattering) were used to calculate CD. The total scattering area of the corrected diffractogram, obtained by the subtraction of the background (Bg), was separated into crystalline (Cr) and amorphous (Am) contributions. The determination of the Cr and Am relative amounts was carried out by fitting the diffraction profile with the pseudo-Voigt function. The fitting method adopted to determine the CD of chitin is described in Fig. S2. The CD was calculated according to Ioelovich (Eq. (1)), as follows (Ioelovich, 2014):

$$CD = \int I_{cr}\, d\theta \,/\, \int I_o\, d\theta = F_{cr} \,/\, (F_{cr} + F_{am}) \qquad \text{(Eq. 1)}$$

where the intensity of I_0 is the total intensity of the corrected diffractogram after subtraction of the background, I_{cr} corresponds to the intensity of the peak related to nanocrystalline chitin; F_{cr} is the integrated area of the nanocrystalline phase diffraction and F_{am} is the area of the amorphous scattering.

2.7.1. Environmental Scanning Electron Microscopy (ESEM)

The morphology of the fine particles of the chitin samples was observed using the ESEM instrument Quanta™ 250 FEG (FEI, Hillsboro, OR) in low vacuum mode. The accelerating voltage was 7 kV, and images of the samples were acquired at 8000x.

2.8. Statistical analysis

Statistical analyses were performed using IBM SPSS v.23.0 (SPSS Italia, Bologna, Italy). The data were expressed as the mean ± SD of three measurements. The amount of chitin, protein, and the relative distribution of amino acids were subjected to one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($\alpha = 0.05$).

3. Results and discussion

3.1. Proximate composition of whole BSF, puparia, and shrimp shells

The determination of the proximate composition of BSF larvae, puparia, and shrimp shells (Table 1) is of extreme relevance for comparing the initial raw material with the sample obtained after the extraction processes, thus allowing to evaluate the extraction yield and efficiency.

BSF larvae contained 35g/100g of lipids and 31g/100g of proteins (DM basis), comparable to the data in the literature, proving to be a good source of energy and proteins (Caligiani et al., 2018). Chitin content, with a value of 14.8 g/100g, differed from the values found in the literature, especially depending on the method of analysis used for its quantification (Soetemans, Uyttebroek, & Bastiaens, 2020). It is therefore of fundamental importance to find a universal analytical method for chitin quantification. The proximate composition of the insect can be influenced by the feeding substrate (Ewald et al., 2020). The larvae analyzed in this study were not fed with a standard diet but with an experimental one, composed of tomato, chickpeas, borlotti beans, green beans, and wheat bran (Paragraph 2.2). In the study conducted by Leni and colleagues, larvae fed with these substrates showed a higher content of chitin compared to the control diet (Leni, et al., 2022), in line with our results.

Table 1
Proximate composition of BSF larvae, puparia, and shrimp shells. Values are expressed as g/100g of dry matter sample. Results from triplicate analyses.

Parameter	BSF larvae	Puparia	Shrimp shells	Method
Total protein	31.4 ± 1.0	31.7 ± 2.1	44.1 ± 1.0	From UPLC-MS of total aa[a]
Total chitin	14.8 ± 1.5	40.7 ± 3.1	27.3 ± 1.8	UPLC-MS
Crude lipid	34.6 ± 0.1	1.4 ± 0.2	1.29 ± 0.04	Soxhlet, ethyl ether
Ash	8.9 ± 0.4	24.7 ± 0.1	23.8 ± 0.1	Oven, 550 °C 5 h

[a] Total proteins were calculated from the total amino acids (aa) obtained by UPLC-MS analysis, by subtracting the molecular weight of water from the molecular weight of each amino acid.

By comparing the whole insect with its exoskeleton, or more precisely with the shell left after the emergence of the adult, called puparia, differences were detected. The puparia consisted mainly of protein, chitin, and minerals, in agreement with Luparelli and colleagues, and had a reduced lipid content compared to the whole insect (Luparelli, Hadj Saadoun, et al., 2022). This is justified as chitin, minerals, and proteins are essential for the hardening of the exoskeleton via sclerotization process, leading to higher mechanical resistance of the whole insect. The composition distribution of the puparia is closer to the distribution of the shrimp shell because the crustacean shell, similarly to the puparia, needs minerals, chitin, and protein for the hardening of the sclerotization process. One difference between the two exoskeletons is a lower chitin content and a higher protein content in the crustacean compared to the insect.

These data showed that the chitin content of the exoskeleton was higher compared to the one of the whole larvae. However, at the same time, its higher content of protein and minerals may make chitin extraction more complex than in the whole insect.

3.2. Compositional characterization of the extracted chitin

Nowadays, chitin extraction is optimized for crustaceans, while it has not been extensively studied for insects. For this reason, we have tested different extraction protocols. The extraction of BSF chitin was conducted by steps, applying different chemical or enzymatic methods, with or without pre-treatment techniques (ultrasonication and mechanochemical milling), to remove fat, protein, and minerals, and to recover chitin fractions (see paragraph 2.4). For comparison, the extraction of chitin from shrimp shells was conducted using a chemical method commonly employed to produce commercial chitin. The proximate analysis was then conducted on the extracted chitin to evaluate the presence of possible residues derived from other substances (Table 2).

With the enzymatic extraction method, proteins were not efficiently separated from BSF chitin. Indeed, the total amount of protein in the extracted sample, determined by UPLC-MS quantification, was 46.7g/100g of DM extract, meaning that 43.9% of the total protein in the BSF larvae was retained by the chitin extract. This is in agreement with the results reported in our previous work (Caligiani et al., 2018).

The chemical extraction method, with organic solvents and acid/alkaline solutions, was certainly more efficient than the enzymatic hydrolysis. However, a single step of chemical extraction did not yield a BSF chitin with sufficient purity. As a matter of fact, the chitin purity resulted in 68.8 g/100g, and the proteins were still not efficiently separated from the chitin (24.6g/100g). Therefore, a chemical extraction with two steps was required to obtain a better protein removal from the chitin, resulting in a chitin extract with 77.9g/100g pure chitin and 15.5g/100g protein. Furthermore, enzymatic extraction following the two stage chemical extraction still did not remove the remaining proteins (15.5g/100g).

The variation in the efficiency between enzymatic and chemical extraction methods may be due to the different interactions of chitin with the protein and mineral complexes of the insect. In particular, this packed chitin-protein-mineral structure could complicate the access of enzymes to proteins, which might be the reason for the lower efficiency of the enzymatic method. On the other hand, chemical methods, more aggressive, could facilitate the breakdown of these complexes, making protein removal more efficient. This hypothesis is confirmed by several

Table 2
Proximate composition of extracted chitin from BSF larvae, puparia, and shrimp shells. Values are expressed as g/100g of dry matter extract [a].

Parameter	Chitin from BSF larvae						Chitin from puparia	Chitin from shrimp shells
	One stage enzymatic extraction	One stage chemical extraction	Two stage chemical extraction	Three stage chemical and enzymatic extraction	Mechanochemical milling with two stage chemical extraction	Ultrasonication with two stage chemical extraction	Two stage chemical extraction	Two stage chemical extraction
Total protein	46.7 ± 1.6^{d}	24.6 ± 1.1^{c}	15.5 ± 0.7^{b}	15.5 ± 0.3^{b}	15.7 ± 0.7^{b}	13.0 ± 0.4^{a}	36.9 ± 0.3	2.13 ± 0.05
Total chitin	47.6 ± 1.0^{a}	68.8 ± 4.4^{b}	77.9 ± 4.4^{c}	77.9 ± 1.3^{c}	77.0 ± 3.6^{c}	79.9 ± 3.4^{c}	65.2 ± 0.9	88.3 ± 1.5
Ash	1.0 ± 0.2	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.0 ± 0.0	8.00 ± 0.05

[a] Results represent the mean ± SD of three measurements. Means in the same row with different letters are significantly different ($p < 0.05$), according to Tukey's post hoc test.

studies that compared the structure of enzymatically and chemically extracted chitin. A smooth structure with high molecular packing and a higher crystallinity index, due to greater inter- or intra-molecular hydrophobic interactions and hydrogen bonding, was previously observed for enzymatically extracted chitin in comparison with chemically extracted chitin (Marzieh, Zahra, Tahereh, & Sara, 2019). This means that the enzymatic treatment has altered the original structure less than the chemical treatment.

Despite the two stage chemical procedure appeared as the most effective extraction method, it was still not able to remove all chitin-binding proteins. Indeed, in contrast to shrimp shells, where the two-step chemical treatment removed almost all proteins, the percentage of protein remaining in the BSF extract was not negligible. For this reason, pre-treatment methods were also tested. Mechanochemical milling and ultrasonication pre-treatments were applied before the two stage chemical extraction methods, as they were supposed to facilitate the breakdown of the complexes between chitin and proteins, thus enhancing protein removal (Vallejo-Domínguez et al., 2021). However, mechanochemical milling did not lead to a purer chitin extract when compared to the traditional methods without pre-treatments, as the percentage of remaining protein was 15.7g/100g. In contrast, ultrasonication shows a slightly significant decrease ($p < 0.05$) in residual protein content (13.0g/100g) compared to the untreated and mechanically milled sample. According to Kjartansson and colleagues, who tested ultrasonication on shrimp shells to promote chitin extraction, this treatment weakens the structure of the crustacean shell, thus improving protein solubilization. In their case, the proteins contained in the chitin extract after sonication-assisted extraction were 50% less than the proteins in the non-treated extract. This differed from our data, where there was only a small increase in protein removal (17.2%). This difference in the efficiency of protein removal might be due to the different steps in which sonication was used: indeed, in our study, sonication was employed as a pre-treatment, while Kjartansson and colleagues used it for facilitating the extraction in the deproteinization step. In addition, different matrices were compared (Kjartansson, Zivanovic, Kristbergsson, & Weiss, 2006b). On the other hand, despite the percentage of chitin-bound proteins was lower in the sonicated sample, the chitin yield did not differ significantly in the sonicated and untreated samples. The reason may be that the sonication step depolymerized a minor portion of chitin due to the mechanical effects associated with cavitation, and the depolymerized chitin passed in solution (Kjartansson et al., 2006b; Kjartansson, Zivanovic, Kristbergsson, & Weiss, 2006a).

The proximate composition analyses of the BSF chitin extracts reported in Table 2 revealed that, even though several methods were tested, the amount of protein that remained bound to insect chitin was not negligible. As a matter of fact, the protein content of all BSF chitin extracts ranged between 46.7g/100g and 13.0g/100g of extract depending on the method, while in the shrimp shells, subjected to the two stage chemical extraction, the proteins were effectively removed to a final content of 2.1g/100g. These data demonstrate that the challenge of removing chitin-binding proteins is mainly related to the type of animal and not to the part of the animal from which chitin is extracted: indeed, puparia and shrimp shells, although having similar proximate composition and biological role, exhibited different behaviors during the extraction process.

To better understand the molecular characteristics of the protein fraction bound to BSF chitin, the residual amino acid composition of the BSF chitinous extract obtained by the different extraction methods tested was analyzed and compared to the amino acids present in the whole insect and in the puparia. In the residual protein fraction, some amino acids were present in a significantly higher percentage ($p < 0.05$) than the same amino acids in the insect proteins (Table 3). In particular, Tyrosine (Tyr) increased in all extracts, while Glycine (Gly) and Proline (Pro) increased in all extracts except for the enzymatically extracted sample, which is the method with the lowest total protein removal efficiency, and Valine (Val) increased only in the pre-treated extracts. This indicates that the protein fraction recalcitrant to separation from chitin has a defined amino acid composition, and it is likely to be chemically bound to chitin in the exoskeleton. Furthermore, the amino acids that remained after mechanochemical and ultrasonication pre-treatments and enzymatic treatment as an addition to chemical extraction were not significantly different ($p < 0.05$) from the conventional chemical extraction, indicating that these treatments did not change the residual amino acids composition. Interestingly, the previously mentioned amino acids were also those mostly found in puparia, confirming that the most difficult amino acids to remove are those present in the insect's exoskeleton.

Results demonstrated that insect chitin was more recalcitrant to extraction and purification than chitin from shrimp shells, mainly due to its presumed binding to proteins, which prevents its removal. In crustaceans, this binding would appear to be less present. Indeed, it is assumed that the minerals in crustacean shells replace most of the proteins in the hardening process of the exoskeleton, leading to less binding between protein and chitin. However, when comparing the exoskeleton of the BSF and the shrimp, the percentages of proteins and minerals are similar (Luparelli, Hadj Saadoun, et al., 2022). Consequently, this might lead to the assumption that the removal of chitin-binding proteins from insect chitin is not affected by sclerotization either.

Another hypothesis is that these protein fractions are bound to chitin not only by the sclerotization process but also by melanization. In most insects, the sclerotized cuticle is often much darker than the shrimp's cuticle, suggesting a higher melanin content than in crustaceans (Vavricka, Christensen, & Li, 2010). Melanization represents an essential process related to innate immunity and hemostasis in insects (Solano, 2014). In addition to providing pigmentation, melanin is used as a defense mechanism to encapsulate foreign organisms and repair wounds. During the melanization process, insect tyrosinases, copper-containing phenol oxidase enzymes, oxidize phenolic substrates, such as the tyrosine residues of peptides and proteins, generating quinones. The

Table 3
Amino acids composition (expressed as g/100 g of proteins) of the whole insect and amino acids composition of chitin residual proteins after extraction with different methods and comparison with the amino acid composition of the puparium and puparium extract[a].

Essential amino acids	Initial BSF sample	After enzymatic extraction	After chemical extraction	chemical - enzymatic extraction	Mechanochemical milling with chemical extraction	Ultrasonication with chemical extraction	Initial puparia sample	After chemical extraction
His	3.3 ± 0.1^{a}	4.2 ± 0.8^{a}	3.5 ± 0.2^{a}	3.2 ± 0.1^{a}	5.1 ± 1.2^{a}	4.2 ± 0.2^{a}	6.3 ± 0.4	6.3 ± 0.8
Thr	5.3 ± 0.1^{b}	4.25 ± 0.04^{a}	3.8 ± 0.2^{a}	3.7 ± 0.1^{a}	3.4 ± 0.2^{a}	3.5 ± 0.7^{a}	4.68 ± 0.01	3.8 ± 0.2
Val	7.01 ± 0.02^{b}	6.1 ± 0.2^{a}	7.3 ± 0.2^{b}	7.29 ± 0.01^{b}	8.2 ± 0.4^{c}	8.1 ± 0.1^{c}	10.8 ± 0.6	12.2 ± 0.1
Lys	7.3 ± 0.3^{b}	5.1 ± 1.3^{ab}	3.9 ± 0.5^{a}	4.1 ± 0.2^{a}	6.2 ± 1.4^{ab}	4.2 ± 0.4^{a}	0.9 ± 0.4	0.30 ± 0.03
Ile	5.26 ± 0.04^{c}	5.2 ± 0.1^{bc}	4.7 ± 0.2^{ab}	4.6 ± 0.1^{a}	4.3 ± 0.2^{a}	4.41 ± 0.04^{a}	4.37 ± 0.03	4.3 ± 0.1
Leu	8.8 ± 0.1^{b}	8.1 ± 0.1^{ab}	7.9 ± 0.5a^{b}	7.9 ± 0.2a^{b}	7.2 ± 0.2^{a}	7.5 ± 0.2^{a}	9.6 ± 0.1	9.6 ± 0.2
Phe	4.5 ± 0.1^{a}	7.0 ± 0.9^{b}	5.4 ± 0.4^{ab}	4.9 ± 0.1^{a}	4.5 ± 0.3^{a}	4.8 ± 0.2^{a}	1.5 ± 0.1	1.2 ± 0.1
Tyr	5.0 ± 0.1^{a}	8.0 ± 0.2^{bc}	7.8 ± 0.2^{bc}	7.0 ± 0.3^{b}	7.2 ± 0.2^{b}	8.0 ± 1.2^{c}	7.3 ± 0.4	8.2 ± 0.2
Met	1.22 ± 0.02^{a}	2.8 ± 0.1^{d}	1.58 ± 0.05^{bc}	1.5 ± 0.1^{ab}	1.4 ± 0.1^{ab}	1.9 ± 0.1^{c}	0.0 ± 0.0	0.0 ± 0.0
Non-essential amino acids								
Asp + Asn	11.6 ± 0.3^{b}	9.4 ± 0.8^{ab}	8.9 ± 0.2^{a}	9.3 ± 0.2^{ab}	7.3 ± 1.0^{a}	9.0 ± 1.5^{ab}	4.1 ± 0.1	3.1 ± 0.1
Ser	4.8 ± 0.3^{b}	3.7 ± 0.1^{a}	4.9 ± 0.2^{b}	4.93 ± 0.02^{b}	4.5 ± 0.2^{ab}	5.1 ± 0.6^{b}	8.0 ± 0.1	7.2 ± 0.3
Glu + Gln	14.1 ± 0.2^{cd}	15.2 ± 0.9^{d}	12.6 ± 0.1^{bc}	13.3 ± 0.1^{c}	11.5 ± 0.4^{ab}	9.8 ± 1.0^{a}	7.1 ± 0.2	5.8 ± 0.3
Gly	5.0 ± 0.2^{a}	6.1 ± 0.8^{ab}	7.8 ± 0.2^{bc}	7.8 ± 0.1^{bc}	8.8 ± 0.9^{c}	8.4 ± 0.8^{bc}	12.3 ± 0.3	14.6 ± 0.5
Arg	4.9 ± 0.1^{a}	6.6 ± 0.4^{a}	5.8 ± 0.9^{a}	5.8 ± 0.1^{a}	5.9 ± 0.4^{a}	6.0 ± 0.2^{a}	3.3 ± 0.1	2.4 ± 0.2
Ala	6.45 ± 0.03^{b}	4.6 ± 0.3^{a}	7.4 ± 0.3^{b}	7.5 ± 0.1^{b}	7.7 ± 0.4^{b}	6.6 ± 1.3^{b}	9.2 ± 0.2	9.9 ± 0.7
Pro	5.5 ± 0.1^{b}	4.0 ± 0.1^{a}	6.8 ± 0.3^{c}	7.09 ± 0.04^{c}	6.7 ± 0.2^{c}	8.4 ± 0.1^{d}	10.6 ± 0.1	11.20 ± 0.03

[a] Results represent the mean ± SD of three measurements. Means in the same row with different letters are significantly different ($p < 0.05$), according to Tukey's post hoc test.

de-acetylated amine groups (GlcN) of chitin and chitosan are nucleophilic and react readily with quinones leading to chitin-protein bindings (Leni et al., 2019; Sugumaran & Barek, 2016). As previously mentioned, tyrosine, the precursor of melanin, is one of the amino acids mostly present in the chitinous extract of BSF (Table 3), suggesting that a tyrosine-rich protein fraction is selectively bound to BSF chitin. This result, thus, supports the hypothesis that the melanization process is prevalent in insects and that this may be the reason for the higher protein content in chitin extracts obtained from insects compared to crustaceans.

3.3. Structural characterization of the extracted chitin and pre-treated chitin

The structure of chitin was analyzed by XRD and ESEM morphological analysis to assess possible differences between insect and shrimp shell matrices.

XRD spectra of chitin samples are shown in Fig. 2. Similarly to the commercially available chitin sample from shrimp shell, the BSF chitin showed two specific strong reflections at 2θ = 9.4° and 19.2° and minor reflections at 12.7°, 20.6°, 26.4° (Fig. 2a). This confirms that BSF chitin, as well as shrimp shell chitin, exhibits the polymorphic α-structure (Triunfo et al., 2022). In addition, the diffraction peaks indicated a more ordered crystal structure in the shrimp standard than in the extracted insect samples. Crystallinity degree (CD) from the XRD data revealed that chitin standard from shrimp shell had 83% of crystallinity degree and BSF had 27% (Table 4). According to the literature, BSF chitin from the first developmental stages has a lower crystallinity, around 35%, than shrimp shell chitin and only adult chitin reaches a crystallinity comparable to commercial shrimp chitin (Triunfo et al., 2022; H. Wang, Rehman, et al., 2020). In addition, the CD difference in the analyzed matrices could be also due to the greater fraction of proteins bound to insect chitin, responsible for the decrease of the global crystallinity. These data indicate that the complexity of chitin purification is not related to the crystallinity of the insect either, which is lower than the crystallinity of the shrimp shell, but other factors are involved in defining the observed CD. Among them it is included, as already discussed, the melanization process that binds proteins to chitin.

The surface morphologies of chitin produced from *H. illucens* and shrimp shell were observed by ESEM as shown in Fig. 2. ESEM analysis revealed, at 8000 × magnification, significant surface differences in chitin samples: α-chitin from shrimp shell showed a smooth surface without pores, whereas insect chitin showed an irregular surface. The homogeneous surface observed for shrimp shells is confirmed by the literature and is the result of the complete removal of protein and calcium carbonate from chitin (S. Wang, Fu, et al., 2020). On the other hand, the morphology of insect chitin can vary depending on several factors, including the species and the growth stage. According to Wang and colleagues (H. Wang, Rehman, et al., 2020) and Triunfo and colleagues (Triunfo et al., 2022), chitin from BSF larvae had a rough surface with broken fibers and an absence of pores. ESEM analysis performed on our samples confirmed the mentioned results. The irregularity of the surface might be due to the presence of proteins that have not been completely removed from the chitin extract.

Since the α-structure of shrimp and BSF chitin was similar while the morphology was different, the different extractability of pure chitin was likely due, in part, to the different surface morphology of the two matrices.

The structure of the insect chitin obtained from different pre-treatments, using physical and physico-chemical methods including ultrasonication and mechano-chemical milling, was then evaluated. The XRD patterns of the untreated chitin, mechano-chemically milled chitin, and the standard shrimp shell chitin are shown in Fig. 2b. The ultrasound-treated sample is not shown because it did not differ from the untreated sample. In the mechano-chemically milled sample, the peaks with higher intensities remained at the same 2θ values as the untreated

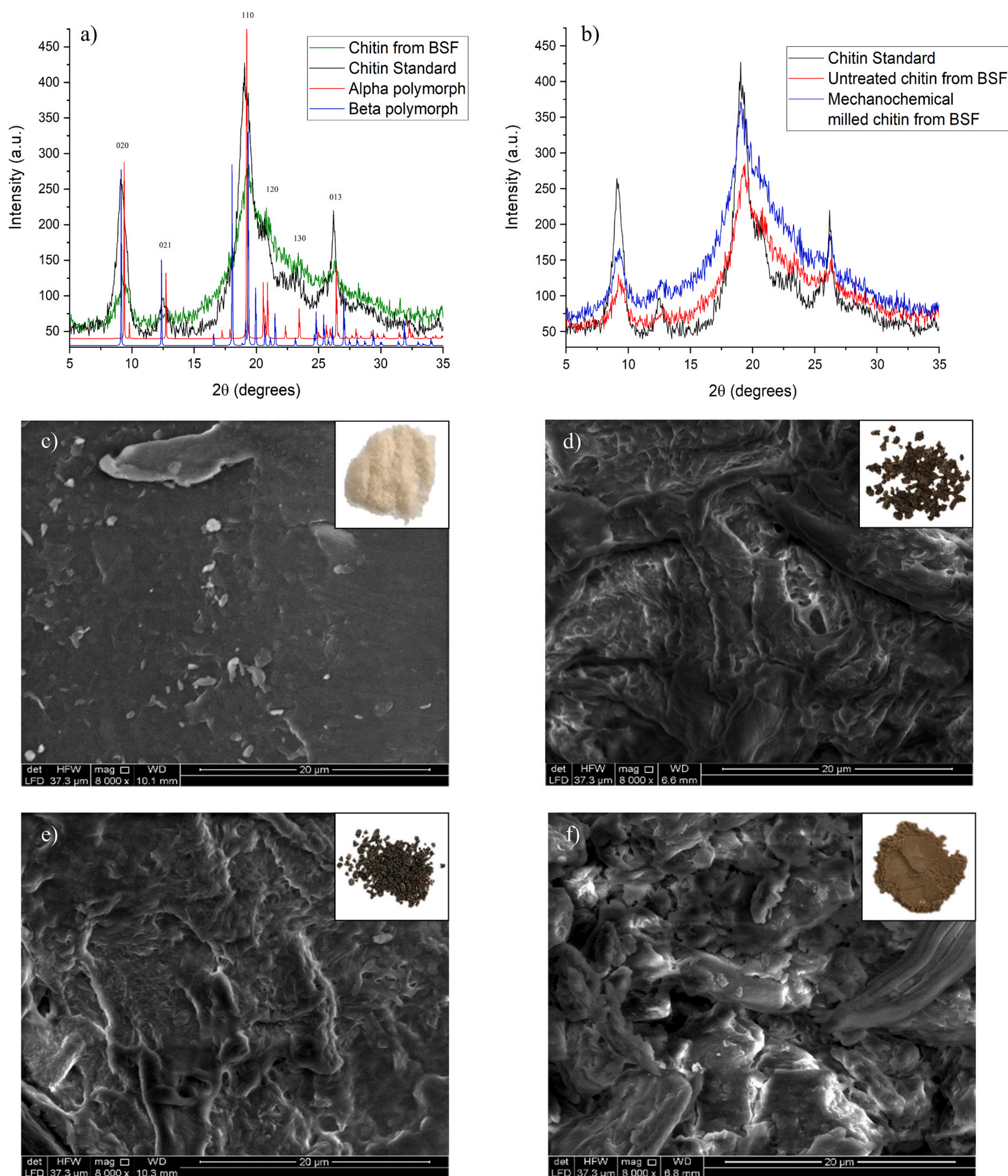

Fig. 2. XRD patterns and Environmental Scanning Electron Microscopy images (8000 ×) of different types of chitin. XRD patterns of a) chitin from BSF (green line), chitin standard from shrimp shell (black line), alpha (red) and beta (blue) chitin computationally obtained; b) Chitin standard from shrimp shell (black line), untreated chitin from BSF (red line) and mechanochemical milled chitin from BSF (blue line). ESEM of c) Chitin standard from shrimp shell; d) Untreated BSF chitin; e) Ultrasonicated BSF chitin; f) Mechanochemical milled BSF chitin.

Table 4
Crystallinity degree (CD) and amorphous percentage of the investigated samples.

Sample	Crystallinity degree (CD %)	Amorphous (%)
Chitin standard from shrimp shell	82.7	17.3
Chitin from BSF	27.0	73.0
Mechanochemical milled chitin from BSF	15.4	84.6

sample, indicating that the milled material retained its α-chitin characteristics. In agreement with the literature, the peaks from XRD of the pre-treated samples with mechano-chemical grinding were weaker, around 15%, than those corresponding to the untreated BSF sample (27%). Such difference indicates that the milling treatment alters the crystallinity because the crystalline structure of the chitin is broken. Indeed, the sample undergoes an intensive impact by the ball inside the instrument, and the generated mechanical effect changes the crystal structure (Table 4) (Y. Wang, Zhang, et al., 2020; Nakagawa et al., 2011).

Similarly, ESEM analysis of the pre-treated samples showed a change

in the case of mechano-chemical milling, where the surface appeared more fractured than the untreated and ultrasonicated samples (Fig. 2d, e, and f). This is in agreement with the fact that mechano-chemical milling makes the powder smaller and smoother, as also confirmed by Nakagawa and colleagues (Nakagawa et al., 2011).

4. Conclusion

The results of this study have highlighted some difficulties in obtaining pure chitin from BSF. With the enzymatic method, a chitin with a purity of less than 50g/100g of extract was obtained, whereas with the chemical methods, it was just under 78g/100g. This prevents a real utilization of insect chitin and its derivatives. Therefore, the efficiency of each step of the extraction process needs to be critically evaluated and optimized to adapt the methods used for crustaceans to insect biomass, paying more attention to pre-treatment methods. Among them, in our study, an improvement was obtained with ultrasonication, since it resulted in a greater removal of proteins from the extracted chitin. From a structural point of view, BSF chitin has a lower crystallinity than crustacean chitin and a different superficial morphology. From a molecular point of view, BSF chitin has a high proportion of specific tyrosine-rich chemically bound proteins. This molecular feature, combined with the dark colour, suggests the involvement of the melanization process. A better knowledge of this mechanism, especially during insect processing, could be the key to obtaining purer and thus more easily useable chitin from BSF. This is of utmost importance because, although insect chitin has similar applications to shrimp chitin, insects have greater sustainability due to their ability to bio-convert agricultural and food residues.

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CRediT authorship contribution statement

Clara Pedrazzani: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Lara Righi:** Methodology, Software, Formal analysis, Investigation, Writing – review & editing. **Ferdinando Vescovi:** Methodology, Software, Formal analysis, Investigation, Writing – review & editing. **Lara Maistrello:** Conceptualization, Resources, Writing – review & editing. **Augusta Caligiani:** Conceptualization, Validation, Resources, Writing – review & editing, Project administration, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115618.

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