Obtaining Chitin/Chitosan-Melanin Complexes from Black Soldier Fly Hermetia Illucens

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Abstract. Chitosan, obtained by deacetylation reaction of chitin, is a regular biopolymer with unique properties, including high biological and sorption activities. Melanin is considered as a natural photo and radio protector. Depending on the source and the isolation method, melanin possesses various physicochemical properties that can complement the radioprotective activity of chitosan and be used to solve important scientific and practical issues. Insects are relatively understudied natural sources of chitin-melanin complexes, whereas these components are linked by strong covalent bonds. This study developed a technology for the production of this natural conjugate, based on which its deacetylated derivative, the chitosan-melanin complex, was obtained, taking the black soldier fly as an example.

1. Introduction

The discovery of new and sustainable approaches for efficiently utilising biomass resources is a global priority, with the world population predicted to reach 9 billion in 2050. Due to its ability to feed on a wide range of organic waste, the black soldier fly Hermetia illucens has emerged as the most important insect for bioconversion [1]. Therefore, this insect species is becoming a valuable tool for protein production and waste management, and several companies around the world are interested in exploring its potential [2]. Insects, in addition to being natural recyclers of organic waste, are efficient producers of protein, fat and other important bioactive substances such as chitin and melanin. Other advantages of insects include their positive environmental, economic and ecological impacts. Farming insects has many benefits compared with conventional livestock farming approaches such as increased feed-conversion efficiency, lower greenhouse gas emission (by up to 100 times) and water pollution, and reduced land use with low environmental contamination [3]. Chitin is the second most common polysaccharide in nature after cellulose. The recent emphasis on environmentally friendly technology has led to increased interest in biopolymers and bio-based polymers. They are more beneficial compared to synthetic polymers due to increased functionality and biodegradability. Chitosan can be obtained from chitin by deacetylation reaction, offering a wide range of applications in many fields including biotechnology, food industry, and medicine due to its exceptional properties such as biocompatibility, biodegradability, non-toxicity, and chelation of metal ions [4].
The exoskeleton of insects provides structural support and is made up of cuticle, an extracellular matrix comprising chitin fibers, cuticular proteins, lipids, and pigments [5]. One of the most common pigments found in insect cuticle is melanin, which is crucial for colouration, wound healing and innate immunity [6]. Within the animal kingdom, melamins are classified based on the presence of a group, into eumelanin (which imparts brown to black colouration) and phleomelanin (red to yellow colouration) [7]. It is known that melamins are photo-protective, providing the protection for skin against the harmful effects of ultraviolet (UV) and visible radiation [8]. As a result, its optical properties have been widely investigated for the past years. Being able to dissipate 99% of the absorbed UV and visible radiation through non-radiative means, melanin is considered as an optimal photo-protector [9]. Based on this information, it can be assumed that when complexed with chitosan, melanin would exhibit increased biological activity. In this study, pupal exuviae and dead imagoes of the black soldier fly *Hermetia illucens* were investigated as melanin has already been developed at these stages and covalently bound to chitosan.

2. Materials and methods

*Hermetia illucens* pupal exuviae and dead imagoes were provided by Entoprotech LLC, Russia. Samples consisting of pupal exuviae and dead imagoes were powdered into 35-70 mesh size.

2.1. Obtaining chitin/chitosan-melanin complexes from pupal exuviae

2.1.1. Demineralisation. 1000 mL of 1% HCl was added to 100 g of pupal exuviae and left at 20 °C for 2 h. The solid residue was separated through glass filter. It was washed with distilled water and lyophilised afterwards. 74 g of product was obtained (yield=74%).

2.1.2. Deproteinisation and defatting. 300 mL of 5% (w/v) KOH was added to 30 g of demineralised biomass and left in 50 °C water bath for 2 h with occasional stirring. The suspension was warmed in 100 °C water bath for 2 h with occasional stirring. The solid residue was separated through glass filter, washed with distilled water until neutral pH and lyophilised. 23 g of product was obtained (yield=77%). Treatment with stronger alkali, 10% (w/v) KOH and 50% (w/v) NaOH, was carried out in the same way. 10 g (yield=34%) and 9 g (yield=30%) of product was obtained, respectively.

2.1.3. Deproteinisation, defatting and deacetylation. Performed by using 80 mL of 50% (w/w) NaOH. The alkaline solution was added to 4 g of chitin-melanin complex and left at room temperature for 30 min. The suspension was warmed in 100 °C water bath for 2 h with occasional stirring. The suspension was cooled, washed until neutral pH and lyophilised. 1.2 g of chitosan-melanin complex was obtained (yield=29%).

2.2. Obtaining chitin/chitosan-melanin complexes from dead imagoes

2.2.1. Demineralisation. 1000 mL of 1% HCl was added to 100 g of dead imagoes and left at 20 °C for 2 h. The solid residue was separated through glass filter. It was washed with distilled water and lyophilised afterwards. 87 g of product was obtained (yield=87%).

2.2.2. Deproteinisation and defatting. 300 mL of 5% (w/v) KOH was added to 30 g of demineralised biomass and left in 50 °C water bath for 2 h with occasional stirring. The suspension was warmed in 100 °C water bath for 2 h with occasional stirring. The solid residue was separated through glass filter, washed with distilled water until neutral pH and lyophilised. 7.2 g of product was obtained (yield=24%). Treatment with stronger alkali, 10% (w/v) KOH and 50% (w/v) NaOH, was carried out in the same way. 4.5 g (yield=15%) and 2.7 g (yield=9%) of product was obtained, respectively.

2.2.3. Deproteinisation, defatting and deacetylation. Performed by using 40 mL of 50% (w/w) NaOH. The alkaline solution was added to 4 g of chitin-melanin complex and left at room temperature for 30 min. The suspension was warmed in 100 °C water bath for 2 h with occasional stirring. The suspension was cooled, washed until neutral pH and lyophilised. 0.72 g of chitosan-melanin complex was obtained (yield=18%).
3. Characterisation

3.1. Ash content determination
The ash content was determined gravimetrically by incinerating the sample (0.6-6 g) in a muffle furnace at 650 °C for 4 h. The value of ash content was found as a proportion of weight of the residue to the weight of the sample according to Equation 1 below:

\[
%\text{ash} = \left(\frac{m_2}{m_1}\right) \times 100,
\]

where \(m_1\) and \(m_2\) are weights of the initial sample and the residue, respectively [10].

3.2. Fat content determination
Fat content was estimated by Soxhlet extraction method. The fat value was measured using Equation 2:

\[
%\text{fat} = \left(\frac{m_2-m_1}{m_1}\right) \times 100,
\]

where \(m_1\) is the initial mass of the sample, while \(m_2\) – mass of the sample after being dissolved in diethyl ether.

3.3. Amino acid concentration determination
Ion-exchange chromatography coupled with post-column derivatisation of samples with ninhydrin was used to determine the total content of amino acids (free and bound). Acid hydrolysis (with the exception of tryptophan) in 6 M HCl solution was used for sample preparation, with norleucine added as an internal standard. The hydrolysis was carried out at 110 °C for 24 h. The samples were oxidised with 50% n-formic acid (HCOOOH) to determine cystine and methionine. Alkaline hydrolysis in Ba(OH)\(_2\) solution was used for tryptophan determination, with norleucine added as an internal standard at 110 °C for 20 h.

The analysis was performed on Shimadzu LC-20 Prominence high performance liquid chromatography (HPLC) system (Japan), with a reaction module for post-column derivatisation with ARM-1000 ninhydrin (Sevko & Co, Russia) equipped with an absorption detector \((\lambda_{abs} = 440\ \text{nm}, 570\ \text{nm})\) and column with ion exchange resin 4.6 x 150 mm (Sevko & Co, Russia). Ready-to-use buffer solutions (Sevko & Co, Russia) were provided. The concentration was calculated using a standard amino acid sample (Sykam, Germany).

4. Results and discussion
Pupal exuviae and dead imagoes of *H. illucens* were subjected to demineralisation, deproteinisation, defatting and deacetylation steps. The results of physicochemical analyses are presented in Table 1. It is important to note that the impurities in the chitin/chitosan-melanin complexes have not been previously evaluated in the literature [11].

It was assumed that the conditions (1-10% NaOH), previously used for crustaceans and bees, were suitable for removal of protein and fat from pupal exuviae and dead imagoes [12]. Two concentrations of potassium hydroxide were initially used – 5 and 10% (w/v) – after a demineralisation step in 1% HCl. However, this treatment did not allow to remove the majority of protein and fat residues from the chitin-melanin complexes, therefore, the alkali concentration was increased to 50% (w/v) and (w/w). The protein content is shown in Table 2 and was determined as a total amount of amino acids present in a sample. Dead imagoes contained a particularly high amount of fat (21-27%) after the alkali treatment. This can be explained by the fact that the fat in dead imagoes differs in composition and may contain wax. Hence, an additional defatting step using an organic solvent was introduced after demineralisation. As a result of treatment with diethyl ether, the fat from the chitosan-melanin complex was removed. Previously, we have shown that the treatment of chitin from *H. illucens* with 50% (w/w) alkali allows to obtain chitosan with degree of deacetylation of 90%, whereas treatment with weaker alkali (50% (w/v)) does not lead to deacetylation of chitin [13]. It was observed that as the concentration of NaOH
increased, the protein content of the dead imagoes also increased. It could be due to the fact that the more concentrated alkali is denser and more viscous, which does not allow it to effectively wet the material and penetrate into it. Based on these findings, it is preferred to obtain chitosan-melanin complex out of chitin-melanin complex, instead of the demineralised material.

In addition, it was observed that the pupal exuvia has a two-layer structure. The inner white layer is chitin, and the outer dark layer is the chitin-melanin complex. (Figure 1) The inner chitin layer can be removed by direct extraction using phosphoric acid [14]. A mixture of chitosan and chitosan-melanin complex can be obtained by deacetylation reaction. Chitosan can be separated from the chitosan-melanin complex by dissolving it in acetic acid.

**Table 1.** Physicochemical analyses of pupal exuviae and dead imagoes at various treatment stages.

| Treatment                      | Pupal exuviae | Dead imagoes |
|-------------------------------|---------------|--------------|
| Starting material             |               |              |
| Demineralisation HCl (1%)     | 8.91%         | 27.08%       |
| 5% (w/v) KOH                  | 11.26%        | 26.63%       |
| 10% (w/v) KOH                 | 5.45%         | 21.17%       |
| 50% (w/v) NaOH                | 0.86%         | 21.78%       |
| 50% (w/v) NaOH                | 1.67%         | 7.80%        |
| Defatting ([(C,H,O)]         | -             | 0            |

**Table 2.** Amino acid profiles of pupal exuviae and dead imagoes after the alkali treatment.

| Amino acid          | Pupal exuviae | Dead imagoes |
|---------------------|---------------|--------------|
| Aspartic acid (Asp) | 0.40%         | 0.93%        |
| Threonine (Thr)     | 0.05%         | 0.14%        |
| Serine (Ser)        | 0.06%         | 0.08%        |
| Glutamic acid (Glu) | 0.15%         | 0.69%        |
| Glycine (Gly)       | 0.10%         | 0.13%        |
| Alanine (Ala)       | -             | 0.07%        |
| Valine (Val)        | 0.17%         | 0.41%        |
| Isoleucine (Ile)    | 0.05%         | 0.14%        |
| Leucine (Leu)       | 0.12%         | 0.41%        |
| Tyrosine (Tyr)      | -             | -            |
| Phenylalanine (Phe) | -             | 0.26%        |
| Histidine (His)     | 0.49%         | 0.40%        |
| Lysine (Lys)        | -             | 0.42%        |
| Arginine (Arg)      | -             | 0.34%        |
| Proline (Pro)       | 1.10%         | 3.68%        |
| Cysteine (Cys)      | 0.14%         | 0.08%        |
| Methionine (Met)    | -             | -            |
| Total               | 2.83%         | 8.18%        |

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Figure 1. The picture of pupal exuviae (on the left), chitin (bottom right) and chitin-melanin complex (upper right).

5. Conclusion
In this work chitin- and chitosan-melanin complexes from Hermetia illucens were obtained and characterised for the first time. Optimal conditions for the removal of impurities were adjusted. It is expected that chitin/chitosan-melanin complexes may have higher biological and sorption activities compared to pure chitin and chitosan and are of great interest for future studies.

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7. References
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| Your Name        | Title*                  | Research Field                                                                 | Personal website                                               |
|------------------|-------------------------|--------------------------------------------------------------------------------|-----------------------------------------------------------------|
| Adelya Khayrova  | PhD student             | Obtaining chitosan and chitosan-melanin complexes from insects and investigation of their physicochemical and biological properties |                                                                  |
| Sergei Lopatin  | PhD in Chemistry        | Metal-chelate affinity chromatography, HPLC, obtaining and analysis of chitin and chitosan |                                                                  |
| Valery Varlamov  | PhD in Chemistry, Full professor | Biopolymers, chitin, chitosan, enzymes, proteins, chromatography                 | https://www.fbras.ru/en/about/nauchnye-podrazdeleniya/laboratoriya-inzhenerii-biopolimerov |