Obtaining and study of physicochemical properties of chitin/chitosan-melanin complexes from *Hermetia illucens*

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Abstract. The increase of organic wastes generated worldwide has become a global issue for the past years. Saprophagous insects including black soldier fly or *Hermetia illucens* have been a topic of interest among researchers due to their ability to recycle organic wastes. Once insect species mature, their biomass can be used to extract valuable biochemicals including chitin and melanin. In this study, the extraction schemes for obtaining chitin- and chitosan-melanin complexes from pupal exuviae and dead imagoes of *Hermetia illucens* were proposed, and their physicochemical properties were investigated. It was shown that black soldier fly can serve as a unique source of covalently bound chitosan-melanin complex with melanin content greater than 14%. In addition, the antioxidant activity of chitosan-melanin complex against DPPH activity was tested and compared to chitosan and melanin. It was revealed that chitosan-melanin complex possesses an enhanced antioxidant activity. These results suggested that black soldier fly is a promising source of chitosan-melanin complex with a high potential for cosmetic and biomedical applications.

1. Introduction

With the rapid growth in global population and annual organic wastes generated worldwide, the development of new approaches for the efficient use of bioresources is of a great interest. It was reported that nowadays more than 2 billion metric tons of municipal solid wastes are generated in the world annually, and this number is expected to reach 3.4 billion by 2050 [1]. Black soldier fly (BSF, *Hermetia illucens*) is considered as an ideal insect species due to the ability of its larvae to bioconvert various decay matters in an efficient and environmentally friendly way [2]. In addition to recycling organic waste and producing protein and fat, these insects also serve as sources of other important biologically active substances, such as chitin and melanin [3].

Chitin is the second most abundant polysaccharide on Earth after cellulose. Recently, the focus on green technologies has generated interest in biopolymers because of their functionality and greater biodegradability compared to synthetic polymers. Chitosan is produced by deacetylation of chitin. This derivative has found extensive applications in biotechnology, food processing and medicine, due to its exceptional properties [4]. One of the most abundant pigments present in insect cuticles is melanin, which plays a significant role in colouration, wound healing, and innate immunity of insects [5]. It is well known that melanins have a photoprotective function - they protect the skin from the harmful effects of ultraviolet and visible radiation [6]. Research results have shown that melanin is capable of scattering 99% of absorbed ultraviolet and visible radiation, which makes it an optimal photoprotector [7]. Melanin is formed in the later stages of the BSF development and is bound to chitin by strong covalent bonds.
Based on this information, it can be assumed that, in combination with chitosan, the two biopolymers will complement and enhance each other's biological activity.

In this study, pupal exuviae and dead imagoes of *H. illucens* were examined, from which chitin- and chitosan-melanin complexes were obtained and characterised. The antioxidant activity of the obtained compounds – chitosan, melanin and chitosan-melanin complex – were also investigated.

2. Materials and methods

*H. 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 delipidation. 300 mL of 30% NaOH was added to 30 g of demineralised biomass and left in 50 °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. 9 g of chitin-melanin complex was obtained (yield=30%).

2.1.3. Deacetylation. Performed by using 80 mL of 50% 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. Delipidation. Carried out with Soxhlet method using (C₂H₅)₂O.

2.2.2. Demineralisation. 1000 mL of 1% HCl was added to 100 g of delipidated sample 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%).

2.2.3. Deproteinisation. 300 mL of 30% NaOH was added to 30 g of demineralised biomass and left in 50 °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. 2.7 g of chitin-melanin complex was obtained (yield=9%).

2.2.4. Deacetylation. Performed by using 40 mL of 50% NaOH. The alkaline solution was added to 2.7 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. 2.4 g of chitosan-melanin complex was obtained (yield=90%).

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:

\[
\%_{\text{ash}} = \left( \frac{m_2}{m_1} \right) \times 100, \quad (1)
\]

where \( m_1 \) and \( m_2 \) are weights of the initial sample and the residue, respectively [8].

3.2. Fat content determination

Fat content was estimated by Soxhlet extraction method. The fat value was measured using Equation 2:
\[
\%f_{at} = \left( \frac{m_2 - m_1}{m_1} \right) \times 100,
\]

(2)

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 Prominance 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 (\(\lambda_{abs} = 440\) nm, 570 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).

3.4. Electron spin resonance measurements
Electron spin resonance (ESR) measurements were performed with a Bruker EMX ESR spectrometer (Bruker, Germany) [9]. Spectra were recorded for dry samples weighing 50 mg, which were placed in a cylindrical quartz cuvette. ESR spectra were recorded under the following conditions: amplitude modulation, 1.25-3.0 G; scan range, 50 G; microwave frequency, 9.8 GHz; microwave power, 0.2 mW; and time constant, 100 ms. Reference sample UDA no. 5 (calibration certificate no. 905/910-2012) was used to calculate g-factors and free radical concentrations (N). Melanin contained in these samples was quantified by comparing with the amount of pure DOPA-melanin free of protein admixtures. The concentration of paramagnetic centres was determined under the same conditions and was equal to \(1.94 \times 10^{18}\) spin/g dry weight. DOPA-melanin was obtained as a result of oxidative polymerisation of dihydroxyphenylalanine in a slightly alkaline medium and purified as described in [10].

3.5. Antioxidant activity
The antioxidant activities of chitosan, melanin and chitosan-melanin complex were evaluated using a DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to [11]. One millilitre of testing samples of different concentrations (0, 0.125, 0.25, 0.5 and 1 mg/mL) were added to 4 mL of a freshly prepared 0.2 mM solution of DPPH in the absolute ethyl alcohol (99.9%). The mixture was kept at room temperature in the dark for 30 min and the reduction of DPPH radical was measured at 517 nm using a LEKI SS1207UV spectrophotometer (Leki, Finland).

Three replicates for each sample concentration were tested and the scavenging effect was measured using Equation 3 below [12]:

\[
DPPH\text{ scavenging activity (\%)} = \frac{A_C - A_S}{A_C} \times 100,
\]

(3)

where \(A_C\) and \(A_S\) are the absorbance values for the control and the test solution, respectively.

4. Results and discussion
Chitin- and chitosan-melanin complexes from pupal exuviae and dead imagoes of H. illucens were extracted according to the previous work by A. Khayrova et al. [13]. The ash, protein and fat contents are shown in Table 1. Initially milder conditions used for crustaceans and dead bees [14] were applied (5 and 10% NaOH) to extract melanin complexes, however, those were not suitable for removing protein.
and fat from the cuticle of pupae and dead imagoes. For dead imagoes a preliminary delipidation step using diethyl ether was required due to the presence of wax.

It was previously shown that treatment of BSF chitin with 50% NaOH allows obtaining chitosan with a degree of deacetylation of 90%, while treatment with a weaker alkali, 30%, does not lead to deacetylation of chitin [15]. According to Table 1 the amount of protein removed is not necessarily directly proportional to the concentration of alkali used. This can be due to the increased density and viscosity of more concentrated alkali, resulting in decreased wettability of matter. Therefore, it is more efficient to obtain chitosan-melanin complex from chitin-melanin complex, instead of demineralised material.

Electron spin resonance (ESR) analysis data (Table 2) shows the amount of melanin present in pupal exuviae and dead imagoes before and after applied treatment. It demonstrates that the content of melanin in both chitin- and chitosan-melanin complexes obtained from dead imagoes is the same exceeding 14%. This is a unique property of the black soldier fly and is not seen in other insect species. For example, chitosan-melanin complexes with a high melanin content cannot be obtained from dead bees, since melanin is removed during the deacetylation reaction [16].

The antioxidant properties of chitosan-melanin complex were compared with the properties of individual biopolymers. The extraction of melanin was carried out according to the method [17] by precipitation with concentrated hydrochloric acid. The resulting melanin was lyophilised. The antioxidant activity was evaluated by determining the free radical scavenging ability of samples via inhibition of the oxidation of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Figure 1 shows the scavenging ability of melanin, chitosan and chitosan-melanin complex of *H. illucens*, as well as crab chitosan (Sigma, USA) against DPPH radicals. Melanin and chitosan are polymeric compounds which are able to donate or accept an electron interacting with DPPH free radicals via the simple one electron transfer process thus neutralising the free radicals [18]. Initially the DPPH scavenging activity of BSF chitosan-melanin was greater in comparison to crab and BSF chitosans but lower than BSF melanin. However, after the concentration of chitosan-melanin complex was adjusted to be equal to melanin, its DPPH scavenging activity was the highest among the other testing samples. As indicated in Figure 1, melanin in combination with chitosan enhance each other’s antioxidant activity, which proves the synergistic effect of these biopolymers. Chitosan-melanin complex with improved antioxidant activity has a high potential for being used in cosmetic and biomedical areas to prevent damage or loss induced by reactive oxygen species.

**Table 1.** Extraction procedure and characteristics of chitin- and chitosan-melanin complexes from pupal exuviae and dead imagoes.

| Treatment step       | Pupal exuviae | Dead imagoes |
|----------------------|---------------|--------------|
|                      | %fat | %ash | %protein | %fat | %ash | %protein |
| Starting material    | 8.91 | 10.51 | n/a      | 27.08 | 5.16 | n/a      |
| Demineralisation (1% HCl) | 11.26 | 1.67 | n/a      | 26.63 | 2.71 | n/a      |
| 5%-NaOH              | 0.86 | n/a  | 21.78    | 21.17 | n/a  | 19.88    |
| 10%-NaOH             | 5.45 | n/a  | 11.57    | 26.63 | n/a  | 11.45    |
| 30%-NaOH             | 0.40 | n/a  | 1.69     | 7.80  | n/a  | 3.82     |
| 50%-NaOH             | 0    | n/a  | 2.83     | 5.40  | n/a  | 8.18     |
| Delipidation ((C₂H₅)₂O) | -    | n/a  | n/a      | 0     | n/a  | n/a      |
5. Conclusion
In this study, chitin- and chitosan-melanin complexes from Hermetia illucens were obtained and characterised. According to ESR analysis data, dead imagoes are unique sources of chitosan-melanin complexes with a melanin content of more than 14%. The results of the study of antioxidant activity confirms that chitosan-melanin complexes have a higher biological activity in comparison with melanin and chitosan on their own and are promising objects for future study.

6. References
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Table 2. ESR analysis data.

| Sample                        | Pupal exuviae | Chitin-melanin complex from pupal exuviae | Chitosan-melanin complex from pupal exuviae | Dead imagoes | Chitin-melanin complex from dead imagoes | Chitosan-melanin complex from dead imagoes |
|-------------------------------|---------------|------------------------------------------|---------------------------------------------|-------------|----------------------------------------|------------------------------------------|
| g-factor                      | 2.0040        | 2.0046                                   | 2.0045                                      | 2.0022      | 2.0043                                  | 2.0043                                  |
| ΔBpp                          | 6.1           | 5.9                                      | 5.8                                         | 6.0         | 5.5                                     | 5.4                                     |
| N × 10^17, spin/g             | 2.9           | 1.5                                      | 0.75                                        | 4.7         | 5.7                                     | 5.7                                     |
| %, melanin                    | 6.7           | 3.8                                      | 1.9                                         | 10.9        | 14.3                                    | 14.3                                    |

Figure 1. DPPH radical scavenging activities.
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