Bulgarian Chemical Communications, Volume 51, Special Issue A (pp. 108 - 112) 2019

Comparative evaluation of the radical scavenging activity of cocoon extracts from different silkworm breeds

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Received March 15, 2019; Revised April 16, 2019

The aim of this study is to investigate the biological activities of water-soluble extracts, obtained from different silkworm cocoon breeds (Bombyx mori), by various extraction methods. We determine the scavenging capacity, using ABTS and DPPH tests, of four silkworm breeds, having different cocoon colours (white, yellow, greenish and orange). The used methods of aqueous extraction were – (1) 16 h water bath at 22°C, (2) 60 min water bath at 65°C, (3) 30 min ultrasonic extraction at 22°C and (4) 30 min ultrasonic extraction at 22°C and 1 h subsequent incubation at 65°C. The UV/Vis spectra of the solutions were obtained. The extracts with the highest antioxidant potential were obtained by the last method (4), and smallest by the first one (1). The greenish cocoons have the strongest scavenger activity, while the white one have the weakest. The extract from greenish cocoons, obtained by method (1) demonstrated elevated scavenger effect compared to extract from white cocoons by method (4). Comparison between the spectra of the pure sericin and the obtained extracts shows that the results depend not only on the amount of the extracted protein, but also on other components in the extract, probably flavonoids, which may contribute. The UV/VIS spectra confirmed that the differences in the antioxidant activity are related with the other cocoons' components.

Keywords: Bombyx mori, antioxidant activity, ABTS, DPPH, UV/Vis spectroscopy

INTRODUCTION

Studies on the biological activities of extracts of different silkworms have shown that extracts obtained by various methods contain components with antioxidant activity. Initially, the focus of the researches was directed to the protein fibroin, and subsequently to sericin contained in the aqueous extract. The sericin is a waste product in the silk production, with various biological activities and can be used in cosmetics, food industry, polymer and biomaterial production and other [1-4]. Gradually the focus of studies is redirected to other components in the extract, which also have biological activities [5, 6].

The animals' food during its larva phase influences the composition of the resulting shell. Feeding with mulberry leaves suggests a rich flavonoid diet associated with a pronounced antioxidant effect. The components with antioxidant potential in the diet, via metabolism of the insect, pass into the extract. The amount of biologically active components presented in the extract depends also on the extraction conditions: solvent type, extraction temperature, time, etc. Another factor determining the content of the extract and its antioxidant potential is the silkworm breed. Different breeds can produce cocoons of different colour, which implies a different composition. It is known that the yellowish tinge of cocoons is associated with the presence of carotenoids in it, and the greenish ones are due to flavonoids (mainly kaempferol and quercetin) [7, 8]. The both types of substances have antioxidant properties, which would suggest that the resulting extracts would also have an antioxidant effect [9].

The purpose of this study is to determine the scavenging capacity of water-soluble extracts of cocoons from 4 silkworm breeds having different colour: white, yellow, green and orange as a new source of natural antioxidants. They can have different medical uses and the presence of additional components, other than the protein compounds, with anti-oxidants properties can be studied. Additionally different aqueous extraction conditions were tested. The link between potential radical-capturing properties and components other than sericin was estimated using UV/Vis spectra of the extracts.

MATERIALS AND METHODS

Cocoon samples and aqueous extractions

The analysis was carried out using cocoons from different breeds – white, yellow, greenish and

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orange in colour. White cocoons are monovolitine race, yellow cocoons are bivoltine race, both introduced from China. Greenish cocoons are polivoltine race, introduced from Japan. Orange cocoons are introduced from Egypt, uni-bivoltine race. Feeding and rearing of silkworms was done according to the requirement for highly productive breeds in experimental centre at the section of Sericulture at the Faculty of Agriculture of Trakia University. Its only food was mulberry leaves.

The cocoon shell is cut into small pieces about 2-3 mm^2. The pieces are soaked in bidistilled water and every milliliter of water contains 30 mg of dry cocoon. Thus, the prepared samples are subjected to extraction.

Soft extraction conditions were selected to allow extracts with maximum preserved anti-oxidant capacity (AOC). In our previous study [10], we found that the application of temperature extraction for a long time leads to loss of AOC if the temperature exceeds 70 °C and the time is over 60 min. The extraction conditions applied in the present study allow optimization of the extraction process, seeking for the extract with the greatest AOC without necessarily leading to the extract with the highest protein content (sericin content). The primary objective is to determine the anti-radical effect according to the colour of the cocoons. This study was conducted under different conditions to obtain additional data about the possible variants of extraction procedure.

Samples for measurement of radical scavenging activity were prepared in 4 different types of extraction: (1) incubation at 22 °C for 16 h; (2) incubation at 65 °C for 60 minutes; (3) ultrasonic extraction (US) at a power of 80 W for 30 minutes; (4) ultrasonic extraction at a power of 80 W for 30 min and subsequent incubation at 65 °C for 60 min. The first 3 extraction methods are carried out independently of each other. In the 4th method, we combined sequentially two extraction methods. The aim is to verify the classic method of hot extraction can result in an extract with better antioxidant properties with combination with ultrasonic extraction. The extraction conditions were kept identical for the four breeds of cocoons.

The radical scavenging activity was tested on the extracts thus obtained by ABTS and DPPH assays. UV/Vis spectra of each of the extracts were taken.

Spectrophotometric model systems

To determine the antioxidant properties of the cocoons extracts we used the commonly used for study of complex samples ABTS and DPPH tests [11, 12]. This combination gives information about the possible mechanisms of the neutralisation: Single Electron Transfer (SET) for ABTS and Hydrogen Atom Transfer (HAT) for DPPH. Both methods are based on the ability of the tested samples to scavenge the radicals in the ABTS or DPPH solution. The effect was determined by measuring extent of decolonization (inhibition of the absorbance) of the radical solution at specific wavelength. Stronger antioxidant properties correspond to higher extent of decrease of the absorbance. Details about both methods are given in our previous studies [10, 13]. All analyses were performed by tree independent measurements for each sample. The obtained results expressed as percent of the control containing only radical were presented as means ± S.D.

RESULTS AND DISCUSSION

A pure sericin, produced from Seiren Co., Ltd., was dissolved in distilled water in different concentrations. The sericin spectra show that elevating concentration of sericin leads to increase the peak at 205 nm and at the same time it shifts to larger wavelengths, while the peak at 276 nm is flatter and changes only in the amplitude (Fig. 1.). This behaviour will help to analyse the spectra obtained for the cocoon extracts. Absorbance after 310 nm in the extract's spectrum is not connected to sericin, since the spectrum does not show uptake after this wavelength.

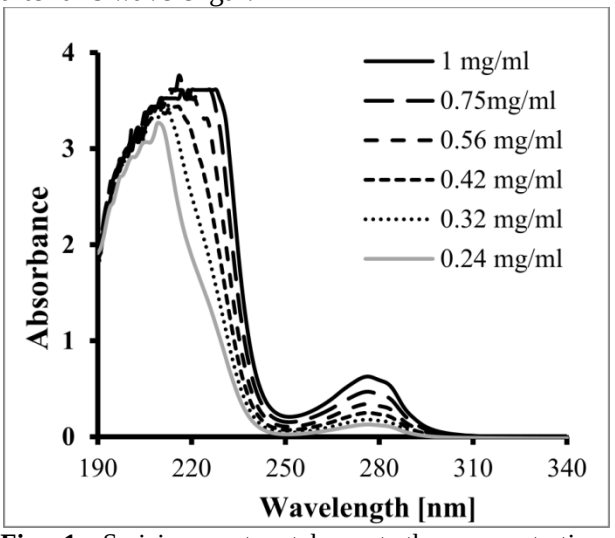

Fig. 1. Sericin spectra taken at the concentrations indicated in the figure.

It was found that there is a linear dependence between the absorbance at 276 nm and the concentration of sericin: $c = 0.6169*A_{267}$, $R^2 = 0.997$. Through this calibration the concentration of extracted sericin in the samples can be determined.

Fig. 2. shows the spectra of the extracts obtained from cocoons with different colours. Comparing the spectra obtained from the different coloured cocoons under the same conditions, it is seen that

the peak at about 205 nm does not substantially change its position for the different cocoons, but only slight changes in the amplitude. Comparing the result to the spectra on Fig.1, where there is a significant change in its position, we can assume that the considerable differences in the cocoons spectra do not depend on the difference in the amount of extracted sericin. It is seen that the spectrum of the extracts couldn't be only explained by the presence of sericin in the samples. This conclusion applies definitely to the spectra above 300 nm, where sericin absorbance is zero. According to the literature the absorption spectra of other substances with antioxidant capacity, which are expected to be contained in the extracts, are in this area [7].

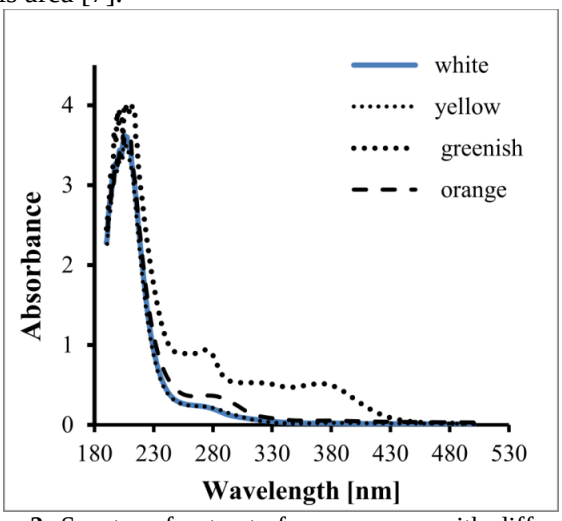

Fig. 2. Spectra of extracts from cocoons with different colour.

There is evidence that the colour of the yellow-orange cocoons is due to carotenoids (absorption is expected in the range of 400-550 nm), whereas the greenish colours are associated with the presence of flavonoids quercetin and kaempferol whose absorption spectra have a maximum of 365 nm and a second significant peak of about 255 nm. The green cocoons spectrum is most intensive, showing the presence of additional components, probably of plant origin, that would increase the anti-oxidant activity (AOA) of green cocoons extracts compared to the one from yellow, orange and white cocoons.

The literature study [14] shows that the extraction conditions largely determine the antioxidant capacity of the obtained extracts. Using water as an extraction medium gives extracts with better qualities (amount of protein and antioxidant capacity) than extracts obtained in methanol and ethanol. The water is closer to the setting of the human body that's why our studies have been conducted in an aquatic environment. The experimental conditions, which are varied in our experiments, are the temperature and the incorporation of Ultrasound waves as an extraction method. Previous studies [10] have shown that extraction at temperatures above 70 °C leads to reduction in the scavenging capacity of the extracts. Prolonged extraction at high temperatures also leads to reduction in the AOA, although the yield of sericin increases. On the basis of the previous experience, four extraction conditions were selected, which can generally be described as soft.

The measurements were provaded with 100 µl and 200 µl of the extracts added to 2 ml radical. Using 100 µl the dilution is 22 times, with 200 µl dilution is 11 times (100 µl extract from 30 mg dry cocoon/ml to 2.2 ml radical is equal to 1.36 mg dry cocoon/ml).

Fig. 3 shows the results for AOC obtained by ABTS test for the four cocoon colours and four extracting conditions – after incubation for 16 hours at 22 °C, incubation for 1 hour at 65 °C, US for 30 minutes and US for 30 minutes plus subsequent incubation at 65 °C. It is evident that incubation at 22 °C for a long time results in an extract with a smaller scavenging capacity compared to the extracts obtained by incubating at 65 °C for 1 hour. This result is observed in all extracts. The difference is less than 10% for white, orange and yellow cocoons and significant for green cocoons – from 45% relative to control at 22 °C, the scavenging capacity reaches about 20% of that of the controls at 65 °C for 1 hour incubation.

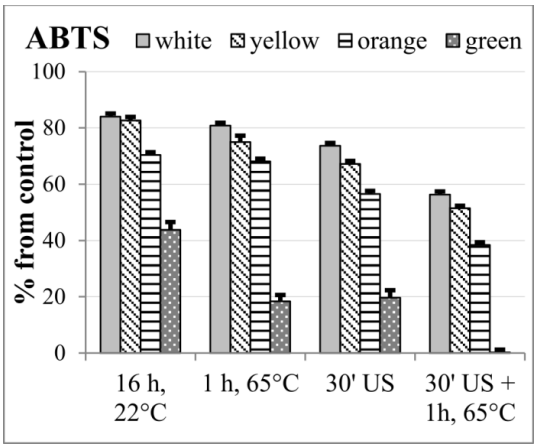

Fig. 3. The results for the scavenger effect obtained by the ABTS method under different extraction conditions. The control value corresponds to 100%.

Treatment with US for 30 minutes yields an extract with better AOC compared to incubation at 65 °C for 1 hour for white, orange and yellow cocoons of about 8 to 15% and practically does not change the AOC of the green cocoon extract that retains its high AOA over 80%. The production of extracts with better AOA by US extraction is probably due to the shorter extraction time, which limits the loss of AO capacity as well as increased extraction of components with AOC due to the

energy of the ultrasound waves that favour extraction from a greater depth and speeding up the diffusion process of the components from the cocoon to the solution.

Combination of two extraction methods – US for 30 minutes and incubating at 65 °C for 1 hour, results in extracts with significant AOC. Extracts of white, yellow and orange cocoons eliminate more than 40% of the radicals in the system, while green cocoon extract eliminates virtually all ABTS radicals at the same concentration and the samples showed complete decolourization.

From the separately used extraction methods, the sounding gives the best results for AOA. The dependence between the AOA of different coloured cocoons is retained: the least activity is from the white then the yellow, the orange and the green cocoons.

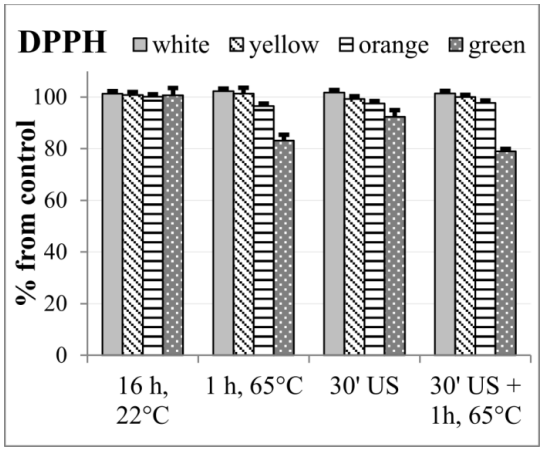

Fig. 4. The results for the scavenger effect obtained by the DPPH method under different extraction conditions. The control value corresponds to 100%.

Experiments with the DPPH method showed that the extracted components predominantly demonstrate better effectiveness in the system supposing SET activity comparing to the HAT one. The data shown in Fig. 4 shows an absence of statistically significant differences between the control value and samples containing white and yellow cocoons extracts in every method. For orange one, there was a slight decrease in the amount of DPPH radicals using method (3) and (4). Significant radical's reduction of 20% is compared to control is observed only for green cocoon extracts obtained by incubation at 65 °C and combination with US extraction. The same extracts using the ABTS test eliminate more than 80% of the radicals. This result shows the both extraction methods produce more components as quantity and type (components with SET and HAT type molecular mechanism of radical elimination). In the experiments with sericin by DPPH method was not found scavenging effect, but there was indirect evidence that AOA for the extracts of green cocoons is not associated with obtaining a higher amount of this protein in the extraction process. The arrangement of the potency of the AOA of the cocoons is white, yellow, orange, green and it is maintained under the different conditions.

CONCLUSIONS

Extracts obtained using different experimental conditions during the extraction process showed different antioxidant activity. The extracts with the highest antioxidant potential were obtained by the ultrasonic extraction at a power of 80 W for 30 min and subsequent incubation at 65 °C for 60 min, and smallest by the incubation at 22 °C for 16 h. In all cases the extract from greenish cocoons demonstrated elevated scavenger effect compared to extract from another tested cocoons. The orange cocoons showed a higher AOA than the yellow. The smallest was scavenger effect of extract from white cocoons.

Comparison between the UV/VIS spectra of the pure sericin and the obtained extracts shows that the results depend not only on the amount of the extracted protein, but also on other components in the extract which may contribute. We assume that the green cocoons' AOA is due to substances (whether of protein or other origin) which are water soluble and are extracted after soaking in water. The heating or sounding is likely to increase the amount of extracted components with AO properties and/or new components with such properties.

Acknowlegement. *This work is partially supported by Bulgarian National Research Programme "Young scientists and postdoctoral students".*

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