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Antioxidant properties of extracts from Daizo silkworm cocoons and relationship with near infrared spectra of intact cocoons

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The aim of this study is to investigate antioxidant properties of water-soluble extracts from silkworm (*Bombyx mori*) cocoons, breed Daizo and their relation to near-infrared (NIR) spectra of intact cocoons. The process of extraction with water was carried out in two stages: an ultrasonic extraction for 30 min and subsequent incubation at 60°C for 1 h. The antioxidant activity (AOA) was determined after each stage by ABTS and DPPH test. It was found that the extract exhibits radical-scavenging properties in both model systems. The incubation at 60°C increases twice the yield of substances with antioxidant properties. The measurement of the standard sericin in the same conditions shows that the AOA of the obtained extracts is not only due to the sericin, but to the other extracted components, which contributes to the observed biological effect. Comparison between the AOA of the extracts with NIR spectra of intact cocoons shows a good correlation between these properties and spectral characteristics – obtained errors of estimation were low and correlation coefficients higher than 0.96.

Key words: *Bombyx mori*, Daizo, Antioxidant activity, ABTS, DPPH, NIR spectroscopy

INTRODUCTION

Silkworm (*Bombyx mori*) is an insect grown by people for production of silk, extracted from its cocoon, which is part of its lifecycle. The cocoon shell is composed from 70% silk fibroin fibers, 25% sericin and 5% non-sericin components. The sericin and non-sericin components are concentrated in a layer surrounding the silk fibroin and acting like a glue to stick the fibers together. Non-sericin components consist of carbohydrates, salt, wax, flavonoids and flavonoid derivatives and vary according to the silkworm strain [1]. The sericin, which is considered as waste product in the production of raw silk, is highly hydrophilic with a molecular weight that ranges from 20 to 400 kDa. It consists of 18 amino acids, including essential ones, and structurally it has a globular structure. In hot water above 50-60°C the protein adopts its soluble form.

Recent studies related to the extracts of the cocoons have proved the different beneficial effects associated with the protein sericin and have shown its potential use in the field of polymers, biomaterials, cosmetics, food industry [2], cryopreservation, wound healing and as a vehicle designed for drug delivery [3]. Sericin has many bioactivities, including antioxidant [4] and antitumor [5, 6] properties, skin care [7], UV protection [8] and other. The dietary sericin can protect mice against diabetic complication [9] and it is reported to have beneficial effects on lipid and carbohydrate metabolism of rats on a high-fat diet [10].

Especially for the medical applications of the sericin it is important to have information about antioxidant properties of its water extracts obtained from the cocoons, their dependence from the conditions of extraction and the presence of other water-soluble components. For silk production, the cocoons are boiled at 100°C and our preliminary tests showed very low radical scavenging activity (RSA) of such extract. We have studied two different mild methods – ultrasonic extraction and incubation at 60 °C, having better antioxidant properties [11].

In this paper we also investigated the correlation between the antioxidant properties and the near-infrared (NIR) spectra of the intact cocoons. Such correlations are used for example for fast identification of silkworm gender inside the cocoon [12]. As the main contribution to antioxidant properties comes from sericin, a non-destructive and fast method like NIR spectroscopy (NIRS) can be used in the breeding process in order to produce cocoons with higher content of this protein, like the one reported by Mase *et al.* [13].

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EXPERIMENTAL

Cocoon samples

The analysis was carried out using cocoons from breed Daizo. It is a polyvoltine race, introduced from Japan in 1998. The cocoons are green yellow in color and spindle-shaped. Feeding and rearing of silkworms was done according to the requirement for highly productive breeds in the Experimental centre at the section of Sericulture at the Faculty of Agriculture of Trakia University. Its only food is mulberry leaves. Each of the cocoons was tested separately.

Each of the cocoons was cut into small pieces and distilled water was added to it to obtain a concentration of 30 mg dry material per ml. The process of extraction with water was carried out in two stages: ultrasonic (US) extraction at a power of 80 W for 30 min and subsequent incubation at 60°C for 1 h. After each stage, a sample was taken for measurement of radical scavenging activity.

Using the same conditions, a solution of pure sericin, produced from Seiren Co., Ltd., in distilled water in a concentration of 10 mg/ml was prepared.

Total antioxidant activity evaluation

To determine the total antioxidant capacity we tested the extracts in two model systems with stable ABTS radical cation [14] and DPPH radical [15]. Both methods are widely used together and are complementary to each other because of the different mechanisms of interaction of the radicals with complex samples – SET and HAT type antioxidant activity (AOA), respectively.

They are based on the measurement, at a specific wavelength, of decolorization of the initial solution of the radical, due to the interaction between the radical and the substances with AOA in the investigated sample. We have observed linear dependence between the absorbance and the amount of radical in the solution. The observed reduction of the absorbance is proportional to the radical scavenging activity of the sample added to the radical and can be used to evaluate it. The $ABTS^{\bullet+}$ solution was prepared with initial absorbance of 0.700 ± 0.002 at 734 nm. The $DPPH^{\bullet}$ solution had initial absorbance of 0.900 ± 0.003 at 517 nm.

The absorption spectra of the extracts were measured with UV-Vis spectrophotometer in the range of 190-1100 nm to check that there are no absorption lines around the above wavelengths.

In both methods, different amounts of extracts were added to 2 ml of initial radical solution. The absorption was measured at the given wavelength after 1 h incubation at room temperature. Three samples of each concentration of the investigated extracts were measured and the mean value and the standard deviation (SD) were calculated.

The radical scavenging activity is a linear function of the concentration of the extracts so for evaluation of antioxidant scavenge capacity we can use SC_{50}, which we define as amount of cocoon, the extract of which reduces the absorbance (the amount of free radical) by half. Smaller amount of SC_{50} means higher antioxidant activity.

NIR spectral measurements and data analysis

Diffuse reflection spectra from the intact cocoon surfaces were obtained with a scanning spectrophotometer NIRQuest 512 (Ocean Optics) in the range 900-1700 nm. For each cocoon, nondestructive measurements were made at 3-4 different points on its surface and then averaged.

The NIR spectral data of intact cocoons were used for development of calibration equations for estimation of antioxidant activity of the water extracts of cocoons. The spectral data were processed by software program Pirouette 4.5 (Infometrix, Inc., Woodinville, WA, USA). Partial Least Square Regression (PLS) was used for quantitative analysis using spectral data transformed as second derivatives. The calibration equations for each parameter were validated with leave-one-out cross validation. Leave-one-out method is recommended when a few samples are used to build the calibration equations. The leave-one-out cross-validation procedure works by omitting one observation, recalculating the equation using the remaining data, and then predicting the omitted observation. This routine is repeated until each observation in the data set is used once as validation data. Finally, the generated validation errors are combined into a standard error of cross-validation SECV.

The prediction capacity of each calibration equation was evaluated using R – multiple correlation coefficients between reference values and NIR spectra, SEC – standard error of calibration, SECV – standard errors of cross validation and the ratio performance deviation (RPD) parameter, which can be defined as the relationship between the standard deviation of the values of the investigated parameter, determined by chemical method (SD) and the standard error of cross-validation SECV in the NIR model. The RPD evaluated the prediction errors considering the standard deviation of the reference data and thus enabled comparison between models for constituents with different variation ranges. The RPD values showed levels of prediction accuracy as follows: RPD between 2.0 and 2.5 indicates

good prediction; RPD between 2.5 and 3.0 indicates very good prediction; and RPD >3 indicates excellent prediction.

RESULTS AND DISCUSSION

The absorption spectrum in the UV region of one of the extracts (the rest have the same behavior) and the spectrum of a solution of pure sericin are shown on Fig.1. In all spectra, two characteristic peaks around 210 nm and 280 nm are observed due to the presence of sericin. No absorption is observed in the spectra of the extracts at wavelengths above 450 nm and in the sericin spectrum above 300 nm. The observed difference in the spectra is the low intensity band between 250-450 nm, seen in the extracts spectra, which can be due to dissolved non-sericin components.

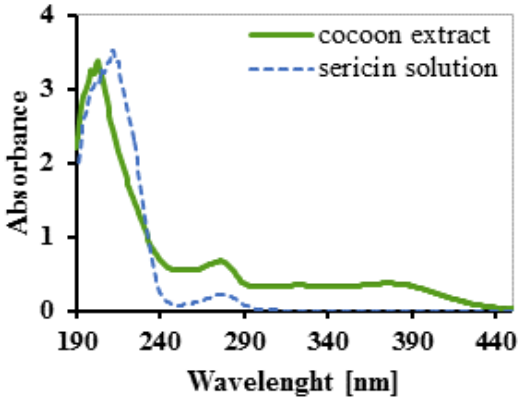

Fig. 1. UV absorption spectra of one of the cocoon extracts and of pure sericin solution.

Table 1. Average values and standard deviations (SD) for SC_{50}[mg/ml] for all measured cocoons and for pure sericin solution. The smaller the SC_{50} the higher is the AOA.

Methods	SC_{50}(US)	SC_{50}(60°C)	SC_{50}(US):SC_{50}(60°C)	SC_{50}(sericin)
$SC_{50(ABTS)}$	0.59± 0.16	0.32± 0.13	1:2	0.029± 0.003
$SC_{50(DPPH)}$	3.71± 1.69	1.93± 0.21	1:2	0.053± 0.005
$SC_{50(ABTS)}$:$SC_{50(DPPH)}$	1:6	1:6		1:2

The extracts exhibit radical scavenging properties in both ABTS and DPPH model systems. The distribution of values, obtained by both methods, for individual cocoons can be seen on the x-axis in Figs. 2 and 3, respectively. The relatively big SC_{50} differences are due to the individual characteristics of the cocoons and lead to higher standard deviation of the average values.

In Table 1 we summarize the average values for SC_{50} for two types of extractions and for pure sericin, obtained by ABTS and DPPH methods. On average, the incubation at 60°C increases twice the yield of substances with antioxidant properties. The ratios between SC_{50} from both radical tests per each method of extraction are approximately equal (1:6), but are quite different from the same ratio for pure sericin (1:2), which shows that AOA of our extracts is not only due to the sericin, but there is contribution from dissolved non-sericin components. The difference in the ratios shows that predominant components in the extracts exhibit greater SET activity.

We observe variations in obtained NIR spectra of intact cocoons, which could be related to differences in their chemical composition. It can be expected that differences in the amount of sericin affect cocoons spectra, as sericin is one of the two major components in cocoon shells. This allows us to investigate the correlation between the NIR spectra and the antioxidant activity of the extracts of the cocoon shells.

The results from NIR spectral data analysis described in the previous paragraph are presented in Table 2. We show statistical parameters from the calibration procedure for quantitative determination of antioxidant activity of the extracts of the cocoons.

The obtained multiple correlation coefficients R between reference values and NIR spectra were bigger than 0.96 and the ratio performance deviation RPD parameter bigger than 3, which showed excellent prediction abilities of the model. Graphical illustrations of the accuracy of determination of the tested parameters are presented in Figs. 2 and 3. The similar graphs are obtained for extracts after incubation at 60°C. Very high correlation between AOA of the investigated extracts and measured NIR spectra of cocoons and low error of estimation confirm the possibilities for fast and nondestructive evaluation of the properties of silkworm cocoons by using NIR spectroscopy.

Table 2. Statistical data of the calibration equations for NIRS for each method of extraction and radical model. R is multiple correlation coefficient, SEC – standard error of calibration, SECV – standard error of cross validation, RPD – relationship between the standard deviation of the chemical method (SD) and the standard error of cross-validation SECV in the NIR model.

Radical – extraction method	SECV	Rcv	SEC	Rcal	RPD
DPPH – US	0.464	0.961	0.458	0.962	3.62
DPPH – 60°C	0.060	0.962	0.060	0.968	3.61
ABTS – US	0.038	0.972	0.038	0.973	4.15
ABTS – 60°C	0.035	0.968	0.034	0.968	3.96

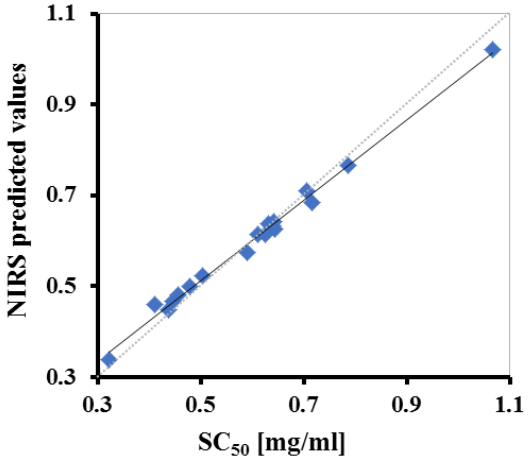

Fig. 2. Scatter plots of laboratory measured and near-infrared spectroscopy (NIRS) predicted values of SC_{50} determined using the ABTS model system for ultrasound extraction.

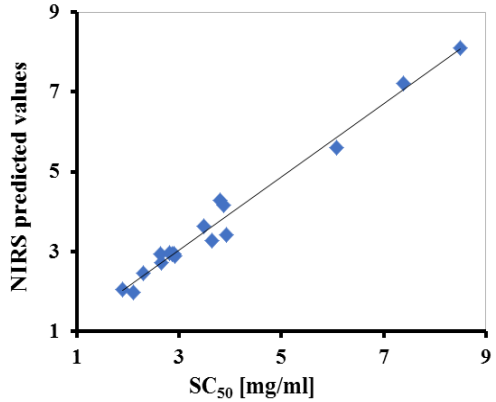

Fig. 3. Scatter plots of laboratory measured and near-infrared spectroscopy (NIRS) predicted values of SC_{50} determined using the DPPH model system for ultrasound extraction.

CONCLUSION

The extract of Daizo cocoons exhibits radical-scavenging properties in both ABTS and DPPH model systems. Incubation at 60°C increases twice the yield of substances with antioxidant properties. Comparison with measurements of the standard sericin at the same conditions shows that the AOA of the obtained extracts is not only due to sericin, but to other extracted components which contribute to the observed biological effect.

Very good correlation between AO of the investigated extracts and measured NIR spectral characteristics of intact cocoons was found – obtained errors of estimation are low and correlation coefficients are higher than 0.96. This shows the potential of the NIR spectroscopy method for estimation of the quality of the cocoons.

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АНТИОКСИДАНТНИ СВОЙСТВА НА ЕКСТРАКТИ ОТ ПАШКУЛИ НА КОПРИНЕНИ БУБИ Daizo И ОПРЕДЕЛЯНЕТО ИМ ЧРЕЗ СПЕКТРИТЕ НА ПАШКУЛИТЕ В БЛИЗКАТА ИНФРАЧЕРВЕНА ОБЛАСТ

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(Резюме)

Целта на това изследване е да се определят антиоксидантните свойства на водоразтворими екстракти, получени от пашкули на копринена буба (*Bombyx mori*), порода Daizo и зависимостта на тези свойства от спектрите на пашкулите, измерени в близката инфрачервена област (NIR). Екстракцията е проведена на два етапа: ултразвукова екстракция за 30 минути и последваща инкубация при температура 60 °C за 1 час. Антиоксидантната активност (АОА) е определяна след всеки етап на екстракция чрез ABTS и DPPH тестове. Установено е, че получените екстракти проявяват радикал-улавящи свойства и по двата използвани метода. Инкубацията при 60 °C увеличава два пъти добива на вещества с антиоксидантни свойства. Измерването на стандарта серицин при същите условия показва, че АОА на получените екстракти се дължи не само на серицина, а и на извлечените заедно с него други компоненти, притежаващи антиоксидантни свойства. Сравнението между АОА на екстрактите със спектрите на пашкулите, от които са получени, в NIR област показва добра корелация между тези свойства и спектралните им характеристики. Получените уравнения за определянето на АОА показват много малки грешки на определяне и корелационни коефициенти по-големи от 0,96.