Tropical Journal of Pharmaceutical Research November 2018; 17 (11): 2177-2182
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i11.10

Original Research Article

Structure characterization and antioxidant properties of proteins extracted from the larva of *Bombyx mori* L.

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Sent for review: 25 May 2018 *Revised accepted: 20 October 2018*

Abstract

Purpose: *To explore the structural characteristics and antioxidant properties of proteins extracted by salt extraction and ammonium sulfate precipitation (SEAP) from the larvae of Bombyx mori L. (PLB) that died due to infection with Beauveria bassiana (Bals.) Vuill.*
Methods: *The extraction yield of PLB was measured, and protein patterns were examined by SDS-PAGE. The amino acid composition of PLB was analyzed using an automatic amino acid analyzer while the structural characteristics of PLB were analyzed by Fourier transform infrared (FT-IR) and UV techniques. In addition, the antioxidant properties of PLB were investigated in vitro.*
Results: *The extraction yield of PLB was 2.16 ± 0.04 %. PLB protein was mainly present in 10 – 20 and 25 – 35 kDa fractions. PLB consisted of 15 types of amino acids. Glu (12.45 ± 0.06), Ala (6.87 ± 0.11) and Val (4.55 ± 0.11) contents of PLB were significantly ($p < 0.05$) higher relative to that of other amino acids. PLB exhibited the FT-IR and UV spectra characteristics of proteins. In addition, PLB exhibited significant antioxidant effects ($p < 0.05$).*
Conclusion: *The results indicate that PLB exhibits significant antioxidant effects and may be suitable for development into antioxidant drugs.*

Keywords: *Bombyx mori L., Proteins, Structural characteristics, Antioxidant, Beauveria bassiana*

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INTRODUCTION

Jiangcan (in Chinese), is the dried larva of *Bombyx mori* L. (silkworm of 4 - 5 instars) that have died from infections caused by *Beauveria bassiana* (Bals.) Vuill [1]. *Jiangcan*, a known traditional Chinese medicine (TCM), has been used to treat convulsions, cough, asthma, headaches, tonsillitis, purpura and other diseases in China for thousands of years [2,3]. In recent years, besides its traditional usages, it has been reported that extracts/compounds isolated from *Jiangcan* possess various pharmacological activities, including antioxidant effects, anticoagulant effects, antitumor effects, etc [4,5]. Previous studies have also reported that *Jiangcan* contains abundant proteins, peptides, flavonoids, polysaccharides, etc. [6].

Currently, there is increasing evidence have proved that macro-molecular compounds in TCMs, including protein, peptide and polysaccharide, possess important bio-activities, such as antioxidant, antitumor, and immunomodulatory effects [7,8,9]. As an animal TCM, proteins are the characteristic bioactive substances of *Jiangcan* [10,11]. However, no report has systemically investigated the characteristics and bioactivities of proteins from *Jiangcan* so far. In the present study, the characteristics and antioxidant activities of PLB were investigated, and the results will prove useful in developing the clinical use of proteins extracted from *Jiangcan*.

EXPERIMENTAL

Materials

Jiangcan was purchased from Chengdu *Min-Jiang-Yuan* Pharmaceutical Co., Ltd (Chengdu, China). A specimen was stored at College of Pharmacy, Chengdu Traditional Chinese Medicine (Chengdu, China). Pepsin (1:3000) and trypsin (1:250) were purchased from Amresco Inc. (USA). DPPH (1,1-diphenyl-2-picrylhydrazyl), EDTA-2Na, ascorbic acid, 1,2,3-trihydroxy-benzene, ferrozine and $ABTS^{+}$ were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All reagents used in the experiment were of analytical grade.

Extraction of proteins

Salt extraction and ammonium sulfate precipitation

Jiangcan was powdered and defatted with petroleum ether (w/v = 1:5) according to the previous reported method [12]. PLB were obtained as reported by Chen *et al* [13]. Briefly, defatted *Jiangcan* was mixed with 30 mM phosphate buffer (pH 8.0 30 mM), extracted on ice bath for 1 h using ultrasonic extraction, and centrifuged at 5000 rpm for 30 min. The soluble proteins in the supernatant were precipitated by saturated ammonium sulfate ($(NH4)_2SO_4$) at 4 °C overnight and centrifuged at 5000 rpm for 30 min. The obtained precipitates were pooled, re-dissolved in PBS, and dialyzed at 4 °C for 24 h against distilled water using 30-kDa dialysis membranes and lyophilized to obtain the PLB sample.

Determination of PLB content and yield

The protein content of PLB was determined using the BCA protein assay reagent. Protein extraction yield (Y) was calculated as Eq 1.

$$Y\ (\%) = \{(M_1.M_2)/M_0\}100 \ldots\ldots\ldots (1)$$

where M_1 represents protein content (%); M_2 represents the protein weight (g) obtained by different extraction methods and M_0 is the weight of defatted *Jiangcan* (g).

Assessment to protein patterns

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out according to the method previously reported by Laemmli [14]. The PLB was separated by SDS-PAGE with a 12 % acrylamide separating gel and a 4 % stacking gel (Bio-Rad Laboratories, Mini- Protean 3 Cell). A pre-stained protein maker (10 - 180 kDa, Thermo Fisher Scientific Inc, Waltham, Ma, USA) was used as standard.

Determination of amino acid composition of PLB

The amino acid composition of PLB was determined by using an automatic amino acid analyzer (Hitachi, L-8900A, Tokyo, Japan). To prepare samples, PLB was sealed in a tube and hydrolyzed with 6 M HCl at 110 °C for 24 h, and then centrifuged at 10000 rpm for 15 min, and supernatants were filtered through a 0.22 μm membrane.

Structure characterization of PLB

The structural features of PLB were determined by analyzing Fourier transform infrared (FT-IR) and UV-Vis spectra. PLB powder and KBr powders were thoroughly mixed and pressed into a 1-mm pellet. The spectrum was measured using a TENSOR 37 FT-IR spectrophotometer (Bruker, Ettlingen, Germany) between 500 - 4000 cm^{-1}. Samples of PLB were prepared in PBS (pH 8.0, 30 mM) and then measured by TU-1901 ultraviolet visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing China) at wavelengths of 190 - 600 nm. The PBS was used as the blank control.

Determination of *in vitro* antioxidant activity

The *in vitro* antioxidant activities of PLB were evaluated according to the reported methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{+}$) and superoxide anion radical scavenging and Fe^{2+} chelating assays [7]. A series concentrations of PLB (0.063, 0.125, 0.25, 0.5, 1 and 2 mg/mL) was prepared with PBS (pH 8.0, 30 mM). DPPH, $ABTS^{+}$ and superoxide anion radical scavenging and Fe^{2+} chelating

activities were measured at 517, 734, 320 and 562 nm, respectively, by UV spectrophotometry. Vitamin C was used as a positive control in experiments of DPPH, $ABTS^{+}$ and superoxide anion radical scavenging activity, and ethylenediamine tetraacetic acid disodium salt (EDTA-2Na) was used in the experiment of Fe^{2+} chelating assay. The scavenging rates were calculated by using the following Eq2, and the IC_{50} value was calculated to evaluate the scavenging superoxide anion radical abilities of the different samples.

$$S\,(\%) = \left(1 - \frac{A_2 - A_1}{A_0}\right) 100 \quad \text{..... (2)}$$

where A_0: the absorbance of negative control (deionized water instead of sample solution); A_1: the absorbance of the sample background (without ferrous chloride solution); A_2: the absorbance of the tested sample.

Statistical analysis

All tests were conducted in triplicate and the data are presented as mean ± SD. IC_{50} values were analyzed using SPSS software (SPSS for Windows 19.0, SPSS Inc., USA).

RESULTS

Content and extraction yield of PLB

The protein content and extraction yields of PLB are presented in Table 1. The protein content in PLB was found to be 71.99 ± 1.49 %, and the extraction yield was 2.16 ± 0.04 %.

Protein patterns

The SDS-PAGE information of PLB in *Jiangcan* is shown in Figure 1. It can be seen that the majority of the proteins in PLB had molecular weight below 35 kDa, and were mostly present in the 10 - 20 and 25 - 35 kDa fractions.

Amino acid composition of PLB

The amino acid composition of PLB in *Jiangcan* is described in Table 1. It was clearly observed that PLB from *Jiangcan* was composed of 15 amino acids, and the total amino acid (TAA) content of PLB was 57.76 ± 0.63 g/100 g. In addition, the Glu (12.45 ± 0.06 g/100 g), Ala (6.87 ± 0.110.06 g/100 g) and Val content (4.55 ± 0.110.06 g/100 g) in PLB was significantly higher relative to that of other amino acid.

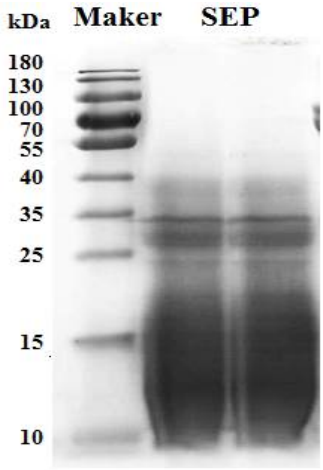

Figure 1: Protein patterns of PLB in *Jiangcan*

Table 1: Amino acid content of PLB

Amino acid	PLB (g/100 g)
Asp	3.96 ± 0.07
Thr	3.77 ± 0.07
Ser	3.36 ± 0.08
Glu	12.45 ± 0.06
Gly	3.33 ± 0.09
Ala	6.87 ± 0.11
Val	4.55 ± 0.11
Met	1.42 ± 0.09
Ile	1.88 ± 0.09
Leu	2.86 ± 0.10
Tyr	1.16 ± 0.06
Phe	1.55 ± 0.07
Lys	2.14 ± 0.05
His	3.65 ± 0.08
Arg	3.80 ± 0.06
Total amino acid	57.76 ± 0.63

FT-IR and UV–vis spectra

The infrared spectrogram of PLB was shown in Figure 2. It showed that spectra of PLB contained a broad weaker peak at 3295.07 cm^{-1}, which is characteristic of N-H stretching vibrations. Amide I, II and III vibrations were also clearly observed in PLB (1654.67, 1556.98 and 1310.68 cm^{-1}), as shown in Figure 2. Furthermore, the strong absorption band of PLB (1654.67) was due to the presence of α – helix.

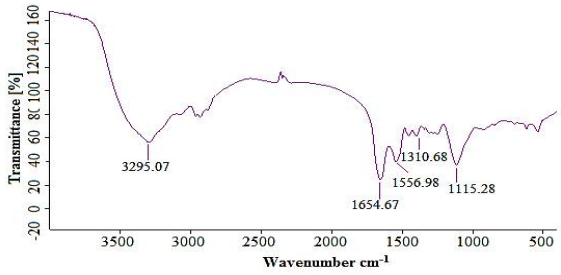

Figure 2: FT-IR spectrum of PLB from *Jiangcan*

The UV–Vis absorption spectrum of PLB in *Jiangcan* is presented in Figure 3. PLB had characteristic absorption at 283 nm, which was mainly due to the presence of phenylalanine, tyrosine and tryptophan.

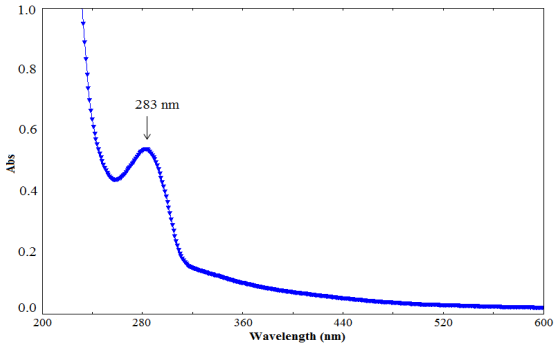

Figure 3: UV-vis spectrum of PLB from *Jiangcan*

Antioxidant activity

The results of the DPPH, $ABTS^+$, superoxide anion radical scavenging activity and Fe^{2+} chelating activity assays are presented in Figure 4 and Table 2. It can be found that the DPPH, $ABTS^+$, superoxide anion radical scavenging and Fe^{2+} chelating rates were gradually enhanced with the increasing concentrations of PLB (from 0.063 to 2 mg/mL). PLB exhibited significant scavenging properties against DPPH-radicals with IC_{50} values of 1.266 mg/mL. In addition, PLB showed strong concentration-dependent $ABTS^+$ radical scavenging effects with an IC_{50} value of 0.854 mg/mL. Furthermore, PLB exhibited moderate scavenging effects on superoxide anion radicals with an IC_{50} value of 2.419 mg/mL. PLB possessed weak Fe^{2+} chelating activities with IC_{50} values of 5.804 mg/mL.

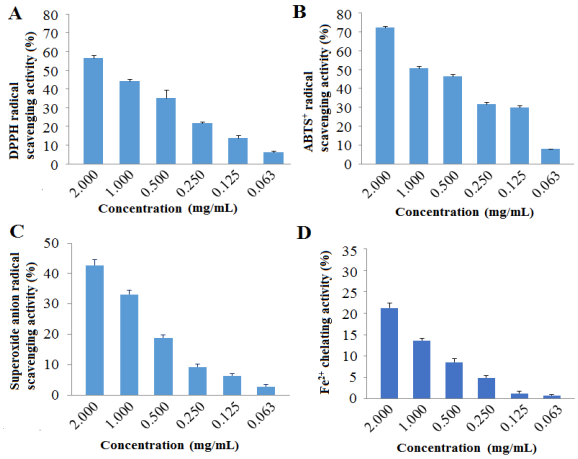

Figure 4: Antioxidant activities of PLB extracted from *Jiangcan*. (A) DPPH radical scavenging assay, (B) $ABTS^+$ radical scavenging assay, (C) superoxide anion radical scavenging assay, (D) Fe^{2+} chelating assay

Table 2: IC_{50} values (mg/mL) for *in vitro* antioxidant activity of PLB from *Jiangcan*

Method	PLB	Vitamin C	EDTA-2Na
DPPH	1.266	0.013	---
$ABTS^+$	0.854	0.030	---
Superoxide	2.419	0.018	---
Fe^{2+}	5.804	---	0.011

DPPH means DPPH radical scavenging activity; $ABTS^+$ means $ABTS^+$ radical scavenging activity; Superoxide means Superoxide anion radical scavenging activity; Fe^{2+} means Fe^{2+} chelating activity.

DISCUSSION

Results of SDS-PAGE revealed that SEAP method was suitable for extracting proteins below 35 kDa. N-H stretching vibrations have been reported in protein, including amide A and B (> 3300 and > 3170 cm^{-1}) [15]. In general, the amide band from 1700 - 1200 cm^{-1} is often used to analyze protein structural characteristics; this includes the amide I (-1650 cm^{-1}) and amide II (-1550 cm^{-1}) and amide III (1400 - 1200 cm^{-1}) [15,156]. FT-IR peaks positions at 1600 - 1639, 1640 - 1650, 1651 - 1660 and 1661 - 1700 cm^{-1} are considered to represent β - sheet, random coil, α - helix and β - turn features in proteins. Peak positions in the amide II and III regions can reflect the intensity and number of hydrogen bonds in the protein and thereby reflect the regularity of the protein structure [17].

DPPH radical can be scavenged by donating hydrogen or electrons to an antioxidant to form a stable diamagnetic molecule [18]. The $ABTS^+$ radical is a stable blue-green cationic radical and shows the specific absorbance at 734 nm [19]. When antioxidants encounter $ABTS^+$, it can donate electrons or hydrogen atoms to the radical cation and eventually lead to discoloration [20]. Superoxide anion radical is one of the precursors of singlet oxygen and hydroxyl radicals [21].

The antioxidant effect of iron-chelating is related to the formation of cross bridges between the carboxyl group in uronic acid and divalent ions. The complex formation can be disrupted by chelating agents and then the red color decreases [22]. Collectively, these results show that PLB from *Jiangcan* possesses potential radical scavenging properties *in vitro*, especially for the DPPH and $ABTS^+$ radicals assays.

Moreover, PLB had strong antioxidant activity, which might be correlated to its molecular weight, amino acid composition and structural characteristics.

CONCLUSION

The results of this study indicate that PLB from *Jiangcan* are potential antioxidant agents and provide a scientific basis for the clinical use of proteins from *Jiangcan*.

DECLARATIONS

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 81773906), National standardization project of traditional Chinese Medicine of China (No. 2YB2H-Y-SC-41) and Science and Technology Development Foundation of Chengdu University of Traditional Chinese Medicine (no. ZRQN1731).

Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. In addition, all authors read and approved the manuscript for publication. For preparation of the paper, Liang Li and Chun-Jie Wu conceived and designed the study; Mei-Bian Hu, Jiao-Long Wang and Yu-Jie Liu done the experiments; Xing Yuan and Jiang-Hua Li analyzed the data; Liang Li and Mei-Bian Hu wrote the manuscript.

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