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Purification and Characterization of the Antitumor Protein from Chinese Tartary Buckwheat (*Fagopyrum tataricum* Gaertn.) Water-Soluble Extracts

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A novel antitumor protein, coded as TBWSP31, was isolated from tartary buckwheat water-soluble extracts and purified by DEAE-Sepharose Fast Flow anion exchange, Sephadex G-100 gel filtration, and Sephacryl S-200 gel filtration column chromatography. TBWSP31 was identified to a homogeneous fraction by native PAGE. The antitumor effect of TBWSP31 against human mammary cancer cell Bcap37 was measured by an MTT assay. TBWSP31 showed higher antitumor activity, and time- and concentration-dependent effects were observed. SDS−PAGE analysis showed that TBWSP31 is composed of a single polypeptide with an approximate molecular weight of 57 kDa. TBWSP31 was rich in Glx (Gln+Glu), Arg, and Asx (Asp+Asn) according to amino acid analysis. Secondary structural analysis by CD spectroscopy revealed that TBWSP31 has the following: α-helix (33.9%), β-sheet (22.8%), β-turn (11.3%), and random coil (32.0%).

KEYWORDS: Tartary buckwheat; water-soluble extracts; structure analysis; antitumor activity

1. INTRODUCTION

The genus *Fagopyrum* has about 15 species distributed in different parts of the world (*1*). Among these species, only two types of buckwheat are used as food around the world: common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.) (*2*).

Tartary buckwheat grain, as an important functional food material, is commonly taken as a diet in eastern Asian countries (*3*). It is well known that tartary buckwheat contains higher rutin and vitamins B1, B2, and B6 content than common buckwheat (*4*, *5*). In China, tartary buckwheat is mainly grown in some mountainous regions, such as the Liang Shan Yi Autonomous region in the Sichuan province and Jing Zhou in the Gui Zhou province (*6*).

Buckwheat protein has high biological value due to well-balanced amino acid composition and is rich in lysine and arginine, but its digestibility is relatively low (*7*, *8*). Our previous study showed that in vitro pepsin digestibility of Chinese tartary buckwheat protein was relatively low compared to those of other edible seeds, and the four protein fractions were digested by pepsin with different modes of action according to scanning electron microscopy micrographs (*9*, *10*).

Recently, the physiological properties of buckwheat protein have also been studied. In rat feeding experiments, studies have proved that buckwheat protein extract has hypocholesterolemic (*11*−*14*), anticonstipation (*15*), and antiobesity activities (*16*). In addition, Liu et al. reported that the buckwheat protein product had a protective effect against 1, 2-dimethyhydrazine-(DMH)-induced colon carcinogenesis in rats by reducing cell proliferation (*17*). Kayashita et al. reported that consumption of the buckwheat protein extract retarded 7,12-dimetylbenz(α)-anthracene (DMBA)-induced mammary carcinogenesis in rats (*18*).

Buckwheat protein has antitumor activity, but the effective component has still not been isolated and identified. The objective of this study was to purify the antitumor protein, to investigate its physicochemical properties, and to examine its inhibitory effect on the proliferation of human breast cancer Bcap37 cells.

2. MATERIALS AND METHODS

2.1. Materials. Tartary buckwheat flour was obtained from the milling factory for minor crops in the Liang Shan region in the Sichuan province. Flour was defatted for 24 h with *n*-hexane under continuous stirring, air-dried at room temperature, and stored at 4 °C until used.

2.2. Extraction of Water-Soluble Extracts. Water-soluble protein was extracted from tartary buckwheat flour using phosphate buffer (pH 7.2, 10 mmol/L) at a ratio of 1:10 (w/v). The mixture was stirred for 60 min at room temperature and then centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was precipitated with 70−95% saturation of solid ammonium sulfate. After it was centrifuged (10,000*g* for 20 min at 4 °C), the precipitate was redissolved in the extraction buffer, dialyzed against distilled water at 4 °C, and freeze-dried.

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2.3. Isolation of the Antitumor Protein. After lyophilization, the sample was applied to a DEAE-Sepharose Fast Flow (Pharmacia) anion exchange column (1.6 cm × 50 cm). The column was pre-equilibrated with phosphate buffer (pH 7.2, 10 mmol/L) and eluted at a flow rate of 1.1 mL/min. Samples was eluted in a series as follows: 200 mL of phosphate buffer (pH 7.2, 10 mmol/L), 200 mL of 0.2 mol/L NaCl in phosphate buffer (pH 7.2, 10 mmol/L), and 200 mL of 0.5 mol/L NaCl in phosphate buffer (pH 7.2, 10 mmol/L). The fraction with the highest antitumor activity was loaded onto a Sephadex G-100 (Pharmacia) column (1.0 cm × 90 cm) and eluted at a flow rate of 0.15 mL/min with phosphate buffer (pH 7.2, 10 mmol/L). The fraction with the highest antitumor activity was further purified using a Sephacryl S-200 (Pharmacia) column (1.0 cm × 90 cm) and eluted at a flow rate of 0.15 mL/min with phosphate buffer (pH 7.2, 10 mmol/L). In each purification step, the eluate was monitored at 280 nm by UV absorbance, and fractions or individual peaks were collected, dialyzed against distilled water, and freeze-dried for assay of antitumor activity.

2.4. Native Polyacrylamide Gel Electrophoresis (Native PAGE). Native polyacrylamide gel electrophoresis (native PAGE) was carried out using the discontinuous system (7.5% separating/4% stacking gel). The sample buffer was constituted of pH 8.8 Tris-HCl, 20% (v/v) glycerol, and 0.05% (w/v) bromophenol bule. Electrophoresis was conducted at a constant current of 30 mA for about 2 h. The gels were stained in Coomassie brilliant blue R-250.

2.5. Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using the discontinuous system (12% separating/4% stacking gel) described by Laemmli (*19*). The gels were stained in Coomassie brilliant blue R-250. Molecular weights of protein subunits were calculated using the following markers: phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4kDa).

2.6. Amino Acid Analysis. The amino acid composition of the sample was determined with an automatic amino acid analyzer (Agilent 1100, USA). The samples were hydrolyzed with 6 mol/L HCl for 24 h at 110 °C in a sealed tube. Tryptophan was not determined.

2.7. Circular Dichroism Spectroscopy. Sample concentration was 0.1 mg/mL in 10 mM phosphate buffer (pH 7.2), and the sample solution was filtered through a 0.02 μm membrane prior to CD analysis.

Circular dichroism measurement was carried out at 20 °C using a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Ltd., Japan). Quartz cells with an optical path length of 0.1 cm were used. Each spectrum was recorded as the average of eight scans. The scan range was 250−190 nm, the scan speed was 50 nm/min, the data interval was 0.2 nm, the bandwidth was 1.0 nm, the sensitivity was 20 mdeg, and the response time was 0.5 s.

CD spectra were corrected for solvent contributions and expressed in terms of specific ellipticities versus wavelength. The proportions of the secondary structure fractions (α-helix, β-sheet, β-turn, and random coil) were determined using the Jasco protein secondary structure estimation program (Japan Spectroscopic Co.) based on the method of Yang and Chen (*20*, *21*). Analyses were performed in triplicate.

2.8. Cell Growth Inhibition Assay. The human breast cancer (Bcap37) cell culture was maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum at 37 °C in a maximal humidity atmosphere containing 5% CO_2.

The cell growth inhibitory effect of Bcap37 was determined using the MTT assay (*22*). Cells undergoing exponential growth were suspended in fresh medium at a concentration of 1×10^5 cells/mL and plated in 96-well plates in a volume of 100 μL/well. Cells were allowed to grow for 24 h, and serial dilutions of the sample were then added to wells in quadruplicate. The plate was incubated at 37 °C for 48 h or 72 h. After incubation, the media were removed, and 100 μL of 0.5 mg/mL aqueous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was added to each well. The plate was then incubated for 4 h under the same conditions. The media were removed, and 150 μL of DMSO was added and mixed thoroughly to dissolve the formazan crystals. The optical density was measured at 570 nm using a Labsystems Multiskan Plus plate reader. Cell Inhibition rate (I %) was calculated by the following equation:

$$\text{Inhibition rate } (I\,\%) = \frac{\text{control } A_{570\text{nm}} - \text{treated } A_{570\text{nm}}}{\text{control } A_{570\text{nm}}} \times 100$$

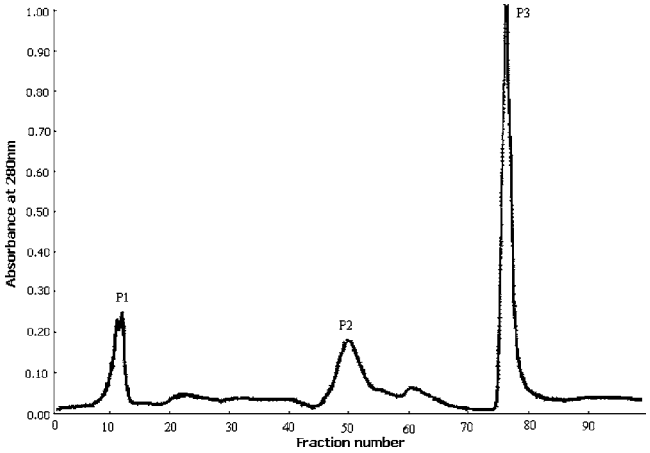


Figure 1. Anion exchange chromatography of tartary buckwheat water-soluble extracts (TBWS) on a DEAE-Sepharose FF column (1.6 × 50 cm). Eluent: 200 mL of phosphate buffer (pH 7.2, 10 mmol/L), 200 mL of 0.2 mol/L NaCl in phosphate buffer (pH 7.2, 10 mmol/L), and 200 mL of 0.5 mol/L NaCl in phosphate buffer (pH 7.2, 10 mmol/L); flow rate, 1.1 mL/min, 6 min/tube.

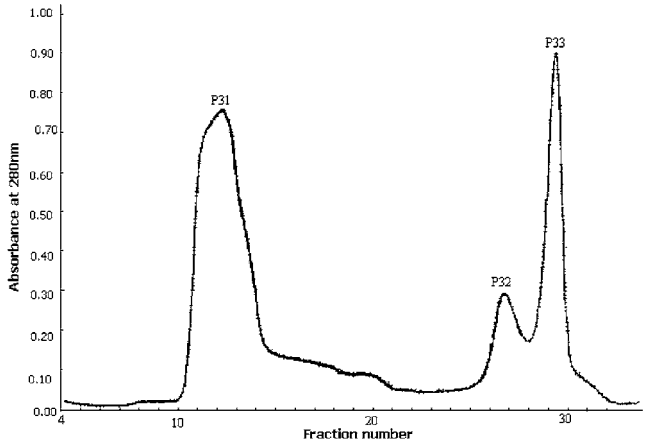


Figure 2. Chromatography of TBWSP3 on a Sephadex G-100 column (1.0 × 100 cm). Eluent: phosphate buffer (pH 7.2, 10 mmol/L); flow rate, 0.15 mL/min, 20 min/tube.

2.9 Statistical Analysis. Statistical analyses were performed using the SAS 8.1 software package. Statistical significance was determined by analysis of variance (ANOVA) ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Isolation of the Antitumor Protein. The water-soluble albumin represents the predominant Osborne fraction of Chinese tartary buckwheat seed proteins (*9*); therefore, the antitumor protein was isolated from tartary buckwheat water-soluble extracts.

The purification of the antitumor protein was accomplished by means of DEAE-Sepharose FF anion exchange chromatography, Sephadex G-100 gel filtration, and Sephacryl S-200 gel filtration. A typical elution profile using DEAE-Sepharose FF is shown in **Figure 1**. The elution profile was reproducible and showed three major peaks. Among them, the P-3 fraction indicated the highest inhibitory activity. Therefore, the P-3

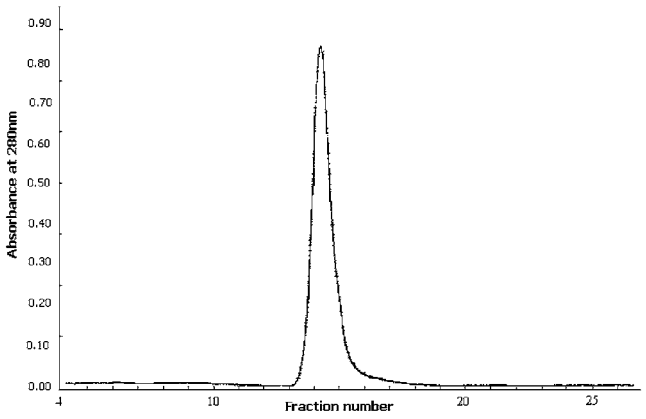


Figure 3. Chromatography of TBWSP31 on a Sephacryl S-200 column-(1.0 × 100 cm). Eluent: phosphate buffer (pH 7.2, 10 mmol/L); flow rate, 0.15 mL/min, 20 min/tube.

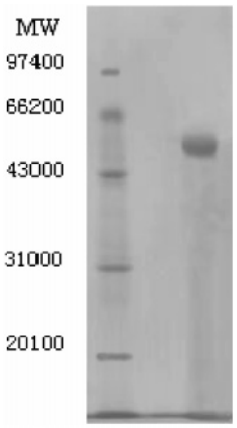
a b

Figure 4. Native PAGE of TBWSP31(a and b:TBWSP31).

fraction was collected and subjected to gel filtration chromatography on a Sephadex G-100 column for further purification. As shown in **Figure 2**, the P-3 fraction was separated into three peaks from the column, and the first fraction P-31 had the highest inhibitory activity. The P-31 fraction was applied to Sephacryl S-200 column chromatography, and a single peak was obtained, which suggested that the P-31 fraction was homogeneous (**Figure 3**). The P-31 fraction isolated from tartary buckwheat water-soluble extracts was named TBWSP31.

3.2. Electrophoretic Characterization. TBWSP31 was analyzed by native PAGE. As observed in **Figure 4**, TBWSP31 showed a single band which also indicted that the P-31 fraction was homogeneous. The subunit constitution of TBWSP31 was determined by SDS−PAGE (**Figure 5**). SDS−PAGE analysis revealed that TBWSP31 was composed of a single polypeptide with an approximate molecular weight of 57 kDa.

3.3. Amino Acid Analysis. The amino acid composition of TBWSP31 was analyzed (Table 1). TBWSP31 was rich in Glx (Gln+Glu), Arg, and Asx (Asp+Asn). Ma et al. reported that consumption of arginine inhibited the development of colon tumors in rats fed DMH (*23*, *24*). This protective effect of arginine has been considered to be due to reduced cell proliferation and nonspecific immune enhancement of the tumor-bearing host. Burns and Milner (*25*) reported that consumption of L-arginine suppressed DMBA-induced mammary carcinogenesis in rats and that its mechanism was unknown. TBWSP31 had a higher level of arginine, which may be related to the antitumor activity, but its mechanism needs to be studied further.



a b

Figure 5. SDS–PAGE of TBWSP31(**a**: molecular weight marker; **b**: TBWSP31).

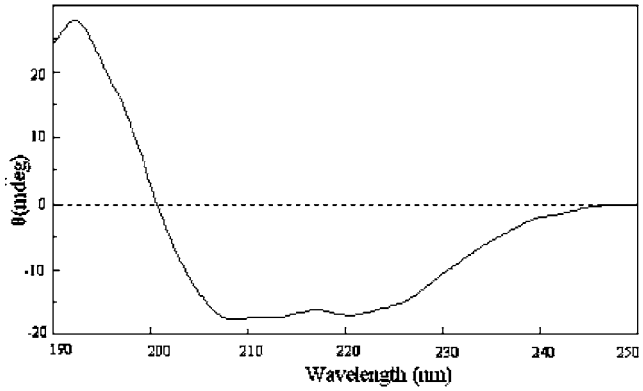


Figure 6. Far-UV circular dichroism spectra of TBWSP31.

Table 1. Amino Acid Profile of TBWSP31(g/100 g)

amino acid	TBWSP31
Asx (Asp+Asn)	10.32
Glx (Gln+Glu)	18.40
Ser	5.76
His	2.57
Gly	5.11
Thr	3.64
Ala	4.32
Arg	10.49
Tyr	3.20
Cys	1.39
Val	5.85
Met	1.59
Phe	5.75
Ile	4.48
Leu	7.17
Lys	5.25
Pro	4.35

3.4. Circular Dichroism Spectroscopy. The far-UV CD spectrum of TBWSP31 showed two negative minima at 208 and 222 nm, a strong positive band at 192 nm, and a broad shoulder that extended from 208 to about 240 nm (**Figure 6**). These features were sufficient indicators of a highly ordered structure and consistent with the general features of $\alpha + \beta$ structure. TBWSP31 contained 33.9% α-helix, 22.8% β-sheet, 11.3% β-turn, and 32.0% random coil structures. The total content of α-helix, β-sheet, and β-turn accounted for approximately 70% of the secondary structure; therefore, TBWSP31 could be considered as a protein with highly ordered and stable protein conformation.

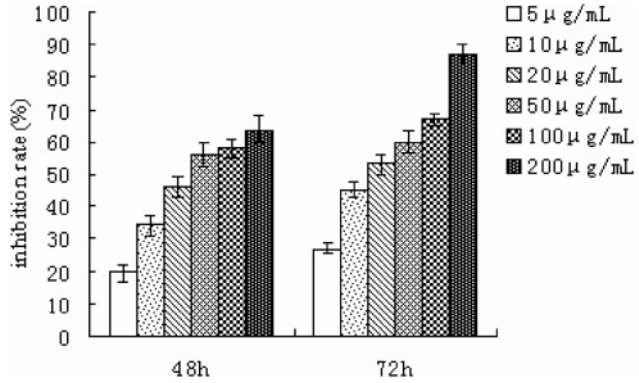


Figure 7. Inhibition effect of TBWSP31 on the proliferation of Bcap37 cells.

3.5. Cell Growth Inhibition of TBWSP31 Assessed by Colorimetric MTT-Assay. Inhibition effect of TBWSP31 on the proliferation of Bcap37 cells is shown in **Figure 7**. With the increase of the concentration of TBWSP31, the inhibition effect of TBWSP31 on the proliferation of Bcap37 cells dramatically increased ($P < 0.05$). Similarly, the inhibition rate clearly increased with the increase of incubation time ($P < 0.05$). Therefore, the inhibition effect of TBWSP31 on the proliferation of Bcap37 cells existed in a dose- and time-dependent manner. The IC_{50} values of TBWSP31 were 43.37 and 19.75 μg/mL obtained for 48 h and 72 h, respectively, analyzed by SAS software.

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