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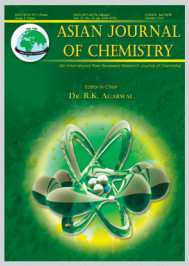

Simultaneous Extraction of Polysaccharides and Isoflavone from *Semen sojae* Preparatum and Assay for Anti-α-Glucosidase Activity

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Isoflavone from *Semen sojae preparatum* (ISSP) was extracted with 80 % ethanol by ultrasonic method. The extract was subjected to macroporous resin AB-8, eluted by 80 % ethanol and analyzed with HPLC. The main isoflavone included genistin (1076.80 μg/g), daidzein (910.22 μg/g) and genistein (1128.61 μg/g). Subsequently, polysaccharides from *Semen sojae preparatum* (PSSP) present in sediments were extracted with distilled water using ultrasonic method, the total crude polysaccharides accounted for 13.3 mg/g, which were further purified, two of them, PSSPI and PSSPII manifested molecular weight of 9500 and 7000 Da, respectively. By establishment of *in vitro* α-glucosidase inhibitor models, the inhibitory rate of ISSP to α-glucosidase was shown to be 40.6%, while both PSSPI and PSSPII exhibited anti-α-glucosidase activity of 3 %.

Key Words: *Semen sojae preparatum*, Polysaccharides, Isoflavone, Anti-α-glucosidase.

INTRODUCTION

Diabetes mellitus has become a common disease in the world. Hyperglycemia increases the risk of complications such as long-term microvascular, which leads to the development in diabetes[1,2]. For diabetic patients especially type II, postprandial hyperglycemia is more dangerous than preprandial high blood glucose. One of the effective strategies for curbing diabetic complications is to reduce the level of postprandial blood sugar. It is known from the available literature, α-glucosidase has attracted much attention for its activity to convert carbohydrate into monosaccharides in the process of intestinal absorption. Hence, inhibitors such as acarbose and voglibose are widely employed to inhibit α-glucosidase, aiming to reduce postprandial blood glucose and to protect patients from suffering type II diabetes[3].

In recent years, natural products/foods have become appealing for their inhibitory activity to α-glucosidase[4]. Of them, *Semen sojae preparatum* (Dandouchi in Chinese), a Chinese traditional medicine or healthy food prepared mainly with fermenting black soybean, sweet wormwood herb and mulberry leaf, was officially listed in the Chinese Pharmacopoeia and was employed to resolve exterior and tune lipid metabolism. Our previous study has verified its dose-dependent role in reducing blood glucose and lipids in rats, as well as alleviating diabetes mellitus[5] .

Although several studies have testified the activity of *Semen sojae preparatum*, little is known about the active ingredients therein. The objective of this study is to (i) develop an efficient method for isolating isoflavone and polysaccharides and (ii) determine their *in vitro* anti-α-glucosidase activity.

EXPERIMENTAL

Semen sojae preparatum was purchased from Anguo medical market in Hebei province of China. α-Glucosidase, genistin, daidzein and genistein were purchased from Sigma Chemical Co., *p*-nitrophenyl α-D-glucopyranoside (PNPG) and *p*-nitrophenyl (PNP) were purchased from Fluka Chemical Co.

General procedure

Isolation of isoflavone: *Semen sojae preparatum* was pulverized to 40 mesh, soaked with 80 % ethanol for 24 h, exhaustively extracted for 0.5 h at 60 ºC of total three times using ultrasonic method, the supernatant was filtrated and the extracts united. Proteins were removed with 80 % ethanol repeatedly. This resulting extract was subjected to macroporous resin AB-8, eluted by water to get rid of impurities, eluted by 80 % ethanol, finally isoflavone from *Semen sojae preparatum* (ISSP) was obtained.

Isolation of polysaccharides: The sediments were united, extracted with distilled water for 0.5 h at 70 ºC, three times by ultrasonic method. The protein was wiped off using 3 %

pepsin under the following conditions: pH 7, 60 ºC for 1 h, elevating temperature to 90 ºC for 6 min to terminate the enzyme reaction. 1.5 % active carbon was added to decolourize or eliminate the small molecules and impurities. Supernatant was filtrated, precipitated with 100 % ethanol, subsequently with acetone. The resulting precipitate was centrifuged at 4000 rpm for 10 min, dried, dialyzed against distilled water for 3 days, sank with ethanol for 12 h, crude polysaccharides from *Semen sojae preparatum* (crude PSSP) were then obtained.

Purification of polysaccharides: The crude PSSP was further purified by Sephadex G-100 gel filtration chromatography, eluted with distilled water followed by 0.2, 0.8, 1.0 and 1.2 mol/L gradient of NaCl. The flow rate was 0.6 mL/min, fractions were collected by an auto-collector. The frictions showing the same single peak were mixed, dialyzed and lyophilized to gain the purified polysaccharide.

Qualitative and quantitative analysis of ISSP: ISSP was identified by thin-layer chromatography (TLC). Genistin, daidzein, genistein standard and ISSP solution were prepared, respectively, the mobile phase was methanol-acetic acid-water (18:1:1), coloured with ethanol containing 1.5 % $AlCl_3$, observed under 254 nm ultraviolet lamp.

Preparative HPLC: Waters analytical HPLC: LC-10AT vp HPLC pump, CTO-10AS vp thermostated column compartment, SPD-10A vp detector and controller. Column: diamonsil C_{18} (250 mm × 4.6 mm, 5 μm) (DIKMA, American), mobile phase: methanol-acetic acid-water (12:1:10), isocratically eluted at a flow rate of 1.0 mL/min and 25 ºC to analyze genistin, daidzein and genistein, respectively, detection: 260 nm.

Qualitative and quantitative analysis of PSSP: The crude polysaccharide was assayed by phenol-sulphuric acid method[6]. 80 % ethanol was added, centrifuged, heated, dissolved in ethanol, added 10 % α-naphthol-ethanol, shook, added thick sulfuric acid along the inner wall of tube, a purple amaranth ring was appeared between the interface of two kinds of liquids, which proved it to be polysaccharide.

Crude PSSP sample was diluted with distilled water, added sulfate-phenol, the absorbance at 490 nm was measured immediately with the UV spectrophotometer (TU-1810PC, Beijing puxitongyong Co. China) and compared to a standard curve of prepared glucose solutions. The polysaccharide content was calculated as follows: PSSP content (%) = [(sample concentration × reaction volume × dilution multiple)/weight of *Semen sojae preparatum*] × 100.

Determination of PSSP purity and molecular weight: The PSSP was scanned from 190-550 nm by UV scanning to detect protein and nucleic acid. Molecular weight of samples was conducted by gel permeation chromatography(GPC)[7], on a combined column of TSK-GEL G3000SW(7.5 × 30), with the mobile phase of double distilled water at pH7.0 at a flow rate of 1.0 mL/min, the column was kept at 40.0 ± 0.1 ºC, RID detection.

The linear regression was calibrated by Dextrans (T-4, 9, 10, T-40, 50, 70, 110,300, 580, 700). All samples were prepared as 0.2 % (w/v) solutions and 10 μL of solution was analyzed in each run. PSSP I or PSSP II (10 mg/mL) was filtered with 0.45 μm micropore filters. Based on the calibration curve of dextran standards, the molecular weight of PSSP was calculated.

Assay for α-glucosidase inhibitory activity *in vitro*

Measurement of α-glucosidase inhibitory activity: To determine whether the extracts inhibit commercially available glycosidases, the glycosidases were assayed according to the slightly modified method[8]. In brief, α-glucosidase in sodium phosphate buffer was assayed at 37 ºC, using PNPG as a substrate at pH 6.8, the components at designated concentrations were prepared and added to the enzyme solution, then using sodium carbonate (Na_2CO_3) solution to terminate the reaction. Enzymatic activity was quantified by measuring the absorbance at 405 nm due to the hydrolysis product (PNP) from PNPG by glycosidase, monitored with the enzyme-labeling instrument (Model 550, BIO-RAD, Japan) expressed as absorbance value A. The α-glucosidase inhibitory activity of *Semen sojae preparatum* extracts was then calculated according to the formula:

$$\text{Inhibitory rate } (\%) = [(A_{originality} - A_{inhibition})/A_{originality}] \times 100$$

Determination of α-glucosidase inhibitory activity of aqueous extracts, PSSP and ISSP: A serial dilution of polysaccharides (PSSP I, PSSP II (concentration: 0.1, 5.0, 10.0, 50.0, 100.0, 500.0, 1000.0 μg/mL) and ISSP (concentration: 0.4, 0.80, 1.20, 1.60, 2.00, 2.40 2.80 mg/mL) were mixed, respectively with 0.4 mL α-glucosidase (11.6 μg/mL), 0.4 mL PNPG (3.33×10^{-5} mg/mL) and 2 mmol/L phosphate buffer (pH 6.8). The mixtures were incubated at 37 ºC for 15 min in a water bath, 1 mL Na_2CO_3 (0.1 mol/L) was then added to terminate the reaction (total volume was 4 mL) and the absorbance was monitored at 405 nm. The above experiments were repeated for 12 times, respectively.

Statistical analysis: All data were treated with SPSS10.0 statistical software and data was presented as mean ± standard error of mean (SEM). Statistical analysis was performed by analysis of *t*-test. *p*-Values equal or less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

ISSP identification by thin-layer chromatography (TLC): The value of R_f for genistin, daidzein and genistein in ISSP were 0.345, 0.455 and 0.664, respectively. They could be separated perfectly within 0.5 h (Fig. 1).

HPLC analysis of ISSP: Good separation of bioactive isolates (genistin, daidzein and genistein) was achieved by the newly developed reversed-phase HPLC method. Our efforts towards developing the present method included, an isocratic mobile phase: methanol-acetic acid-water (12:1:10), which enabled good separation of all the isolates. Retention times of genistin, daidzein and genistein are 4.87, 11.31, 17.39 min, respectively. The isoflavone was calculated according to standard curve (genistin, $y = 2.8856x + 4.3912$, $R^2 = 0.9993$; daidzein, $y = 2.6713x + 0.0955$, $R^2 = 0.9997$; genistein, $y = 5.3054x + 0.28$, $R^2 = 0.9994$), the main ISSP included genistin (1076.80 μg/g), daidzein (910.22 μg/g) and genistein (1128.61 μg/g). HPLC of ISSP was shown in Fig. 2. Isoflavones are abundantly existed in soybean in aglycone forms (*e.g.*, daidzein, genistein and glycitein) as well as in glycoside forms with glucose, 6"-O-acetylglucoside and 6"-O-malonylglucoside[9]. Isoflavone aglycones, the hydrolyzates of isoflavone glycosides by β-glucosidase, are abundantly present in soybean fermented

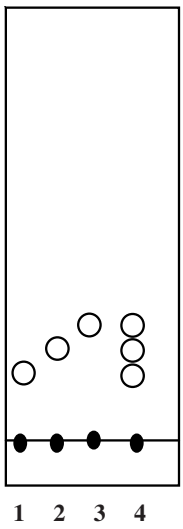

Fig. 1. TLC of ISSP (1 genistein; 2 daidzein; 3 genistin; 4 ISSP)

products[10]. The aglycones (daidzein and genistein) in *Semen sojae preparatum* were higher than that in unfermented soybean[11]. This is because *Semen sojae preparatum* was a fermented product, during fermentation process, the enzymes from microbes would hydrolyzate the glycosides to aglycones.

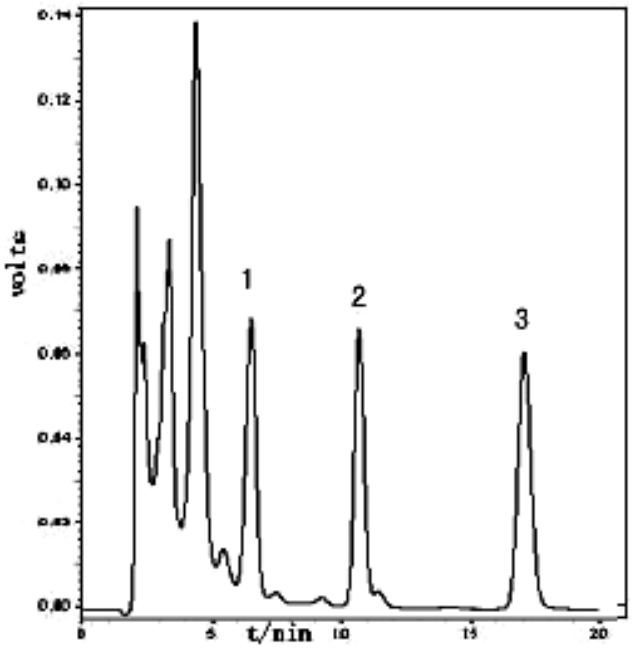

Fig. 2. HLPC of isoflavone from *Semen sojae preparatum* (ISSP)

Qualitative analysis of PSSP: The crude polysaccharides testified by phenol-sulphuric acid method showed that the absorption peak was at 490 nm, which indicated the character of polysaccharides (Fig. 3). The content of PSSP in *Semen sojae preparatum* was 13.3 mg/g.

Purification of polysaccharides: Purified by Sephadex G-100 gel filtration chromatography and eluted with gradient of NaCl, the eluting curve of PSSP was shown in Fig. 4, the same single peaks were mixed, dialyzed and lyophilized to get white purified polysaccharides, this gave SSPP I and SSPP II (Fig. 5).

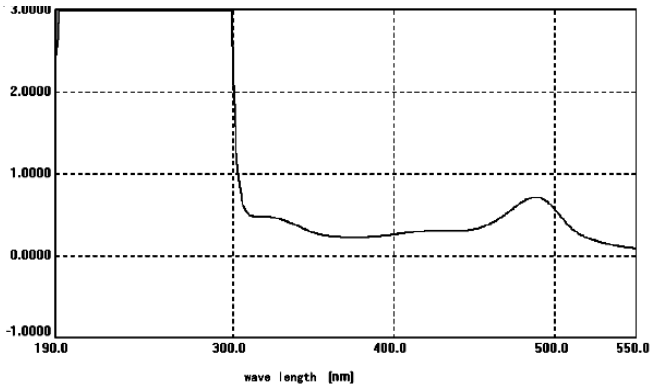

Fig. 3. Ultraviolet absorption spectrum of PSSP sample

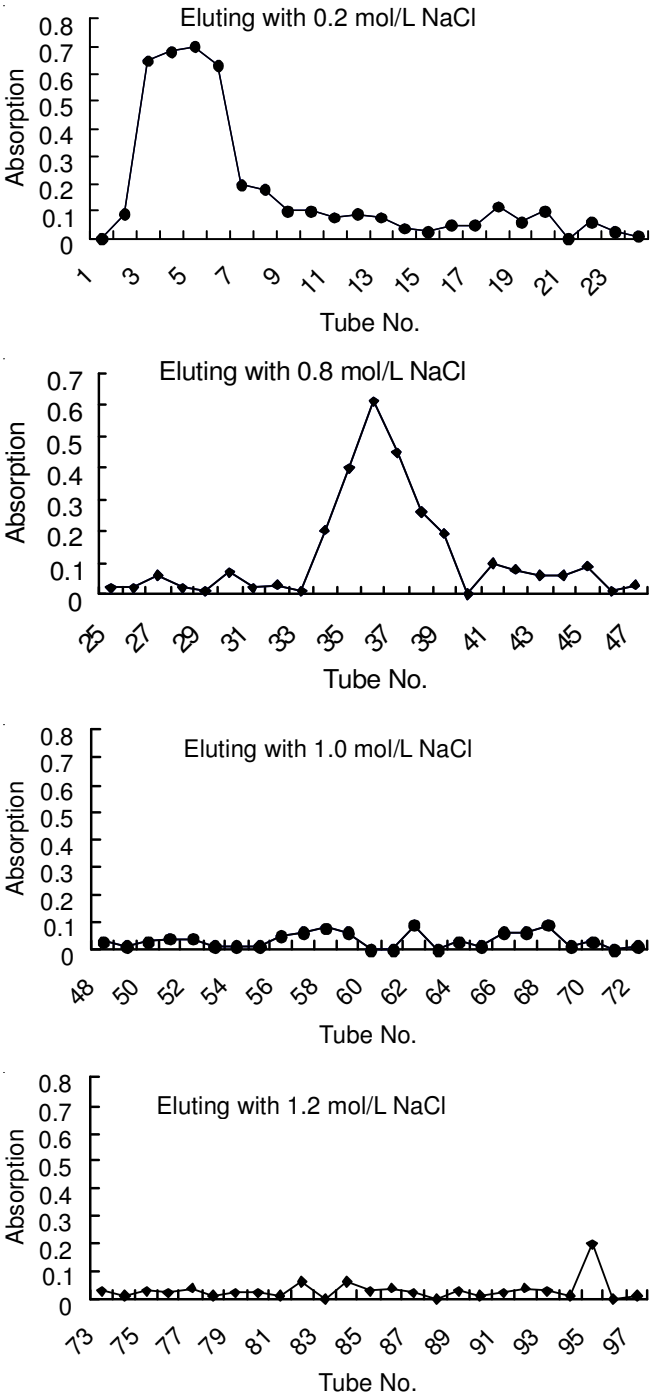

Fig. 4. Elution curve of PSSP on sephadex G-100 column (6 mL/tube)

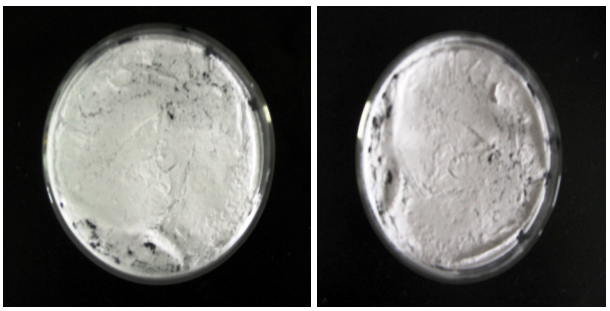

Fig. 5. SSPP I and SSPP II sample

Determination of SSPP molecular weight: No absorption peak was observed in 260 and 280 nm, which indicated that SSPP I or SPSS II contained no nucleic acid and protein. SSPP I or SPSS II was symmetric figure, respectively. The formula was lg Mw = $0.0035t^3 - 0.0806t^2 + 0.3986t + 4.3659$ (Mw: molecular weight, t: eluting time). Based on the calibration curve of dextran standards, the molecular weight of SPSS I and SPSS II were 9500, 7000 Da, respectively.

Anti-α-glucosidase activity of polysaccharides: Based on pre-experiment, in this study, polysaccharides from *Semen sojae preparatum* (SSPP I and SSPP II) with concentrations ranging from 0.4-4000.0 (μg/mL) were assigned to investigate the anti-α-glucosidase activities. Both SSPP I and SSPP II showed little anti-α-glucosidase activity *in vitro*. Among these samples, PSSP I of 4000.00 μg/mL showed only 3.25 % inhibitory rate (Fig. 6), hence, the concentrations of SSPP I or SSPP II were elevated to 10 mg/mL, however, the absorbance value did not show evident change with the increase of concentrations (data not shown). Considering the weak anti-α-glucosidase activities, structure analysis was not conducted.

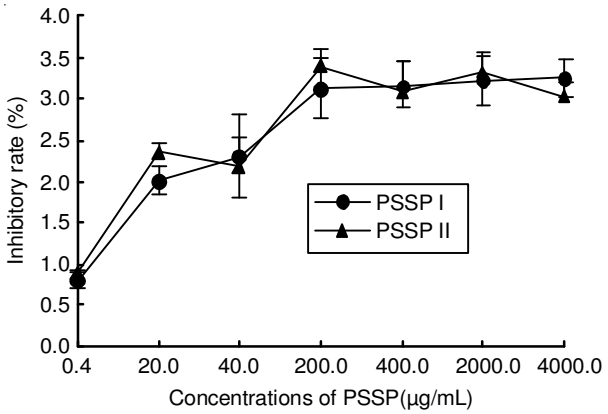

Fig. 6. Anti-α-glucosidase of *Semen sojae preparatum* polysaccharide. Enzyme solutions were treated with polysaccharide of designated concentrations, the mixtures of enzyme and polysaccharide were kept at 37 ºC for 15 min

Anti-α-glucosidase activity of ISSP: ISSP of different concentrations showed anti-α-glucosidase activities *in vitro* (Fig. 7). Among these samples, 2.80 mg/mL ISSP demonstrated the highest anti-α-glucosidase activity with 40.6 % inhibitory rate. At the same concentration, the genstein showed a little higher inhibitory rate in anti-α-glucosidase activity compared with ISSP. Aglycones (daidzein and genistein), the main active ingredients in *Semen sojae preparatum* were highly bioactive due to their effective absorption into the human body[12]. To determine whether saponins or other components in *Semen sojae preparatum* bear the same function, in-depth study is required.

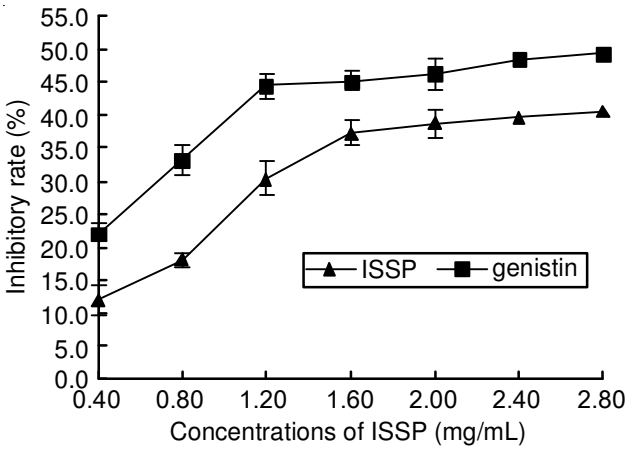

Fig. 7. Anti-α-glucosidase activity of *Semen sojae preparatum* ISSP. Enzyme solutions were treated with ISSP of designated concentrations, the mixture of enzyme and ISSP was kept at 37 ºC for 15 min

Conclusion

The ISSP was extracted with 80 % ethanol by ultrasonic method, subjected to macroporous resin AB-8, eluted by 80 % ethanol. Subsequently, the PSSP in the sediments were extracted with distilled water using ultrasonic method. The main isoflavone in *Semen sojae preparatum* was genistin, daidzein and genistein, the contents were 1076.80, 910.22 and1128.61 μg/g, respectively and the total crude polysaccharides was about 13.3 mg/g. This study confirmed the inhibition of *Semen sojae preparatum* on α-glucosidase and isoflavone is the effective ingredient, which was effective for patients suffering from non-insulin-dependent diabetic mellitus. By contrast, polysaccharides therein contributed limited inhibition on α-glucosidase. These findings support the viewpoint that *Semen sojae preparatum* imposes protective effect on the onset of type 2 diabetes.

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