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Extraction of proteins and preliminary characterization of physicochemical properties in *Toona sinensis* fruit

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Genet. Mol. Res. 16 (1): gmr16019177
Received September 6, 2016
Accepted December 20, 2016
Published February 8, 2017
DOI http://dx.doi.org/10.4238/gmr16019177

ABSTRACT. We investigated the extraction of *Toona sinensis* fruit proteins and preliminarily characterized their physicochemical properties. The results showed that optimal extraction occurred under conditions of pH 10.5, a duration of 40 min, a liquid-to-solid ratio of 25:1, and a temperature of 40°C by an orthogonal design using *T. sinensis* fruit protein as the index and single factor. The total nitrogen content was 13.8 g/100 g and included 17 different amino acids. The glutamate level was highest at 35.37%, followed by arginine at 15.31%. The isoelectric point of *T. sinensis* fruit protein was between 6.8 and 10.0 with a typical absorption peak by infrared chromatography. Three protein bands were analyzed using SDS-polyacrylamide gel electrophoresis, with relative molecular weights of 55, 51, and 22 kDa. This study provides a theoretical basis for the comprehensive utilization of *T. sinensis* fruit by further investigating the biological activity of its proteins.

Key words: Toona sinensis fruit; Extraction technology; Total nitrogen content; Amino acids; Isoelectric point; SDS-PAGE

INTRODUCTION

Toona sinensis fruit, a kind of Meliaceae, was recorded in "Materia Medica of the Tang Dynasty" (Xing and Chen, 2010), and is distributed widely in China. It is a medicine and food product, and its root, tree bark, bud, leaf, and fruit are usually used as medicine. As a traditional Chinese medicinal material, *T. sinensis* fruit is efficacious at dispelling wind and cold, analgesia, and is mainly used in the treatment of wind chill, exopathy, stomach ache, rheumatic joint pain, chest pain, anal fistula, chronic gastritis, headache, and migraine (Xing and Chen, 2010; He and Lin 2013).

The active components in *T. sinensis* fruit include phenols, tannins, alkaloids, saponins, steroids, terpenes, and volatile oil (Chen et al., 2000; Wang et al., 2006; Chen et al., 2008). The proteins from this plant possess specific physiological functions, which have attracted the attention of nutrilogical scientists. These proteins may reduce cholesterol levels (Higashi et al., 2001; Lee and Jung, 2002), prevent cancer (Rowlands et al., 2001), improve cardiovascular and kidney diseases (Jenkins et al., 2001; Appel, 2003), and support nutrition in patients with liver cirrhosis (Tang et al., 1996).

Few studies have been performed on *T. sinensis* fruit proteins, and the extraction technology used for health products, foods, and medicines was under the exploring stage in these fields. This study aimed to investigate the extraction of proteins from *T. sinensis* fruit, and to preliminarily characterize the physicochemical properties of these proteins. In order to comprehensively develop resources and investigate the nutrition and functional properties of these plant proteins, this study has important practical significance and broad market potential.

MATERIAL AND METHODS

Equipment and reagents

A Mettler Toledo electronic balance (EL204), European quality Household life grinder (DSY-9002), and thermostatic water container (HH600-2B) were obtained from Shanghai Binlon Instrument Co., Ltd. An air-blower-driven Drying Closet (101-1AB) was obtained from Tianjin Taisite Instrument Co., Ltd. An ultraviolet spectrophotometer (UV-800A) was obtained from Shanghai Metash instruments Co., Ltd. The pH meter (pHS-3C) was obtained from Shanghai REX Instrument Factory. The automatic Kieltec Distilling system azotometer (K9860) was obtained from Jinan Hanon Instruments Co., Ltd. A HITACHI high speed AA analyzer (8900), and fourier transform infrared spectrometer (Vector22 FT-IR) were obtained from Bruker Corporation. A double-sided vertical electrophoresis tank (JY-SCZ2) was obtained from Beijing JUNYI Electrophoresis Co., Ltd., and the electrophoresis apparatus (DYY-6C) was obtained from Beijing LiuYi Instrument Factory.

T. sinensis fruit was brought from JiNan ShengKe Technology Development Co., Ltd. and identified as the fruit of *T. sinensis* (A. Juss.) Roem. by Dr. Xu from The Pharmacognosy Department of WeiFang Medical University. Coomassie brilliant blue G250 (Amresco 0615), an unstained protein molecular weight marker (Thermo Scientific), and standard reagents for analyzing amino acids (AAs) were obtained from Wako Pure Chemical Industries, Ltd.

Extracting proteins from *T. sinensis* fruit

T. sinensis fruits were ground, defatted in 95% ethanol, and vacuum filtered. Distilled

water was added to tubes containing appropriate filter residues, and the pH was adjusted to alkaline. The extraction process was conducted in a heated water bath. After centrifugation, the supernatant was discarded, and distilled water was added to adjust the pH to neutral. The precipitant was freeze-dried and the proteins were obtained.

Measurement of protein contents

Protein concentrations were measured as previously described (Wang et al., 2008).

Preparation of test solution

Bovine serum albumin (BSA) standard solution was prepared by dissolving 10.0 mg BSA in distilled water with a constant volume of 100 mL.

Coomassie brilliant blue G250 was prepared by dissolving 10 mg Coomassie brilliant blue G250 in 90% ethanol (5.0 mL) with 85% (W/V) phosphoric acid (10.0 mL) and a constant volume of 100 mL. Coomassie brilliant blue G250 was stored in a brown volumetric flask.

Standard curve

The BSA standard solutions (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mL) were added to separate tubes and distilled water was added to make each solution up to 1.0 mL. Coomassie brilliant blue G250 (5 mL) was mixed and incubated for 5 min. The absorbance was determined at 595 nm. The standard curve was generated using concentrations of BSA standard solution (mg/mL) as the abscissa and the absorbance as the ordinate (Figure 1).

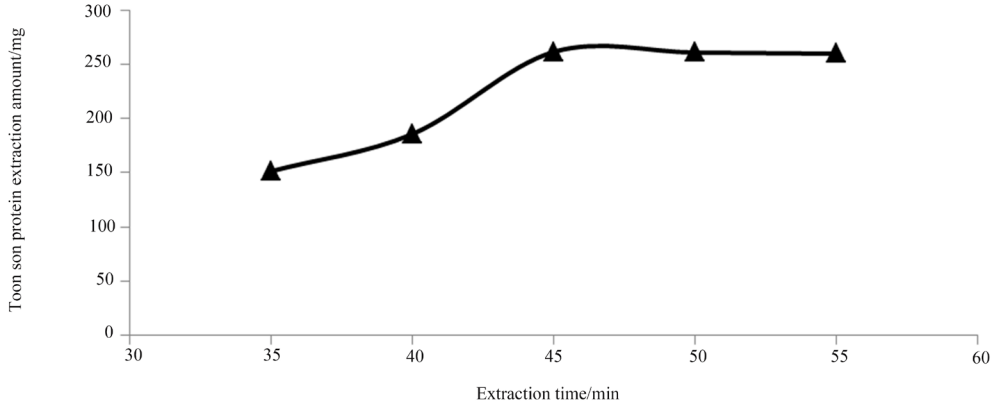

Figure 1. Effect of extraction time on extraction amount.

Measurement of protein content

The protein content in *T. sinensis* fruit was calculated by measuring the absorbance against the standard curve. Each experiment was repeated three times.

Extraction technology: *Single factor exploration*

Extraction time

The following conditions were used: weighted 5-g filter residues with a liquid-to-solid ratio of 25:1 and pH 10.0 at 40°C. The amount of extracted protein was measured at different times (Figure 2).

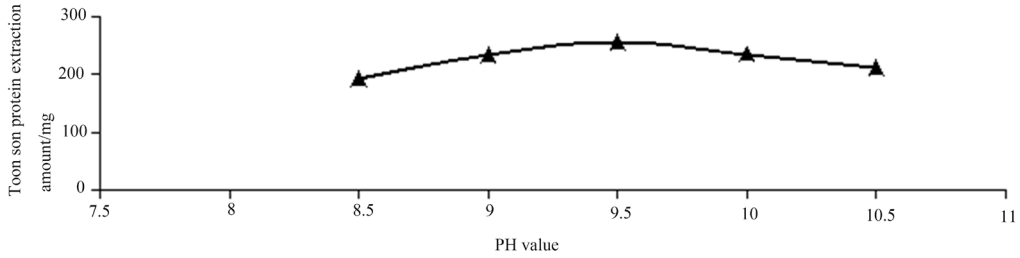

Figure 2. Effect of extraction pH on extraction amount.

Extraction pH

Under the above conditions, the amount of extracted protein was measured at different pH levels (Figure 3).

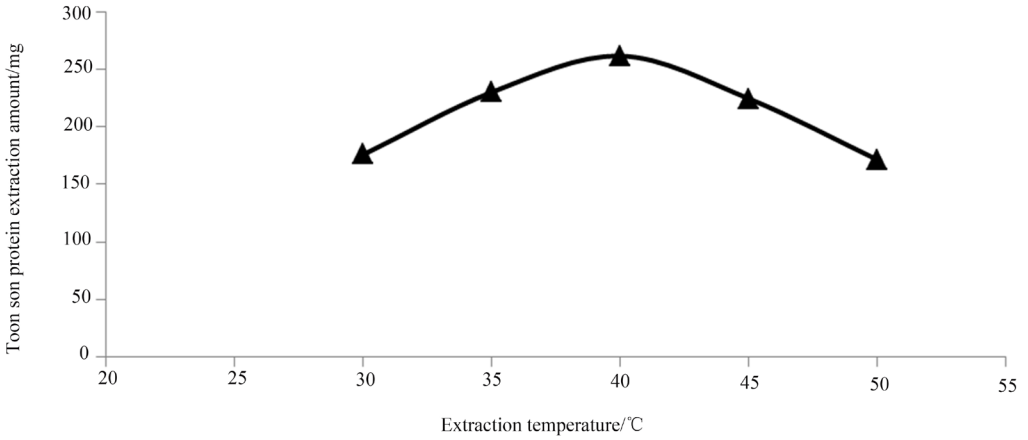

Figure 3. Effect of extraction temperature on extraction amount.

Extraction temperature

Using the conditions described above, the amount of extracted protein obtained at different temperatures was measured (Figure 4).

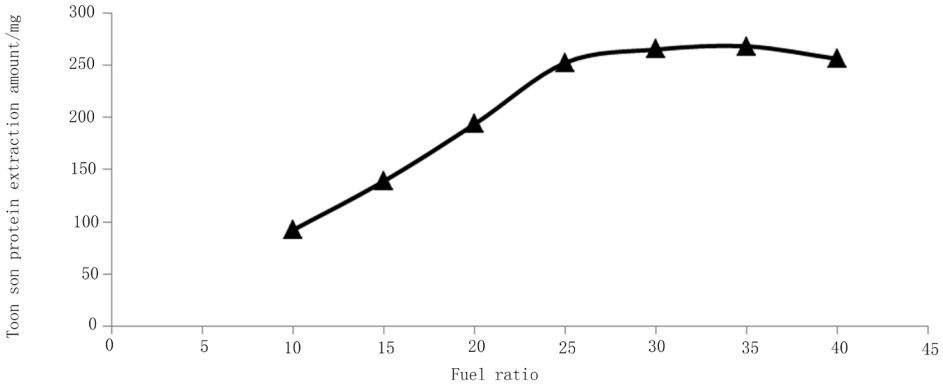

Figure 4. Effect of the liquid-to-solid ratio on extraction amount.

Liquid-to-solid ratio

Under the above conditions, the amount of extracted protein obtained under different liquid-to-solid ratios was measured (Figure 5).

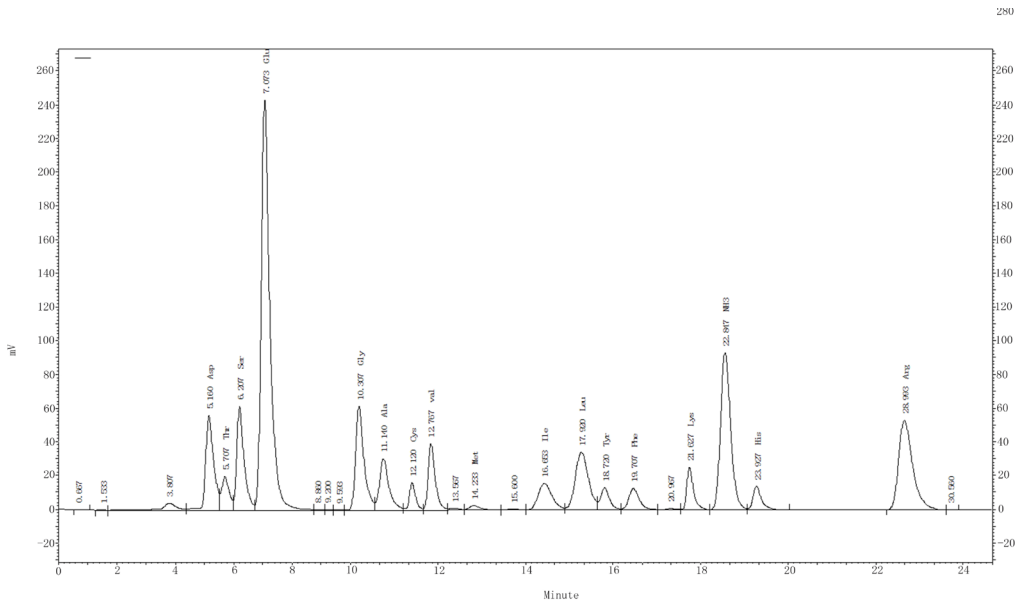

Figure 5. Chromatogram of hydrolyzed amino acid content in protein from *Toona sinensis* fruit.

Orthogonal design

The extraction time, pH, temperature, and liquid-to-solid ratio were selected based on the results of the single factor exploration experiments. An $L_9(3^4)$ orthogonal table was used to confirm the extraction technology (Tables 1, 2, 3).

Table 1. Factor levels of extraction conditions for *Toona sinensis* proteins.

Level	A	B	C	D
	pH	Time	Liquid-to-solid ratio	Temperature
1	8.5	40	20:1	35
2	9.5	45	25:1	40
3	10.5	50	30:1	45

Table 2. $L_9(3^4)$ orthogonal test.

No.	A	B	C	D	mg
1	1	1	1	1	193.7
2	1	2	2	2	246.7
3	1	3	3	3	227.6
4	2	1	2	3	258.8
5	2	2	3	1	207.7
6	2	3	1	2	190.3
7	3	1	3	2	237.8
8	3	2	1	3	183.2
9	3	3	2	1	253.0
K1	668	690.3	567.0	654.4	
K2	656.8	637.6	758.5	674.8	
K3	674	670.9	673.1	669.6	
R	5.73	17.57	63.77	6.8	

Table 3. Variance analysis.

Factors	Sum of squares	ν	F	P
pH	50.81	2	0.68	
Time	473.62	2	6.32	
Liquid-to-solid ratio	6122.63	2	81.73	*
Temperature	74.92	2	1	
Errors	50.81	2		

$F_{0.05}(1, 2) = 19$.

Preliminary characterization of the physicochemical properties of T. sinensis fruit

To determine the total nitrogen content in proteins from *T. sinensis* fruit, we weighed three 28-mg protein samples and measured the total nitrogen content according to Chinese Pharmacopoeia (2010, IX L) using the micro-Kjeldahl method.

The AA composition of *T. sinensis* fruit proteins was analyzed as follows: protein samples were pretreatment and 0.02816 g samples of each protein were placed in a tube for hydrolysis. To these, 6 N HCL (15 mL) was added and the samples was then vacuum degassed for 30 min, nitrogenized, sealed, and placed in the dry oven at 110°C for hydrolysis for 22 h. After cooling, the digested samples were transferred into a 50-mL volumetric flask and made up to a constant volume with ultrapure water. Next, 2 mL of the digested sample was transferred to a 5-mL bottle and placed in a vacuum drying chamber at 50°C. After drying, 1 mL ultrapure water was added and the samples were dried again. HCL (1 mL, 0.02 N) was added to dissolve the samples, which were then filtered through a 0.22-μm film.

The following chromatography conditions were used: 2622 # PHion exchange chromatograph (4.5 x 60 mm); sample size (20 μL); wavelengths of AAs were 570 nm except for proline (440 nm); the flow velocity of ninhydrin and loading buffer was 0.35 and 0.4 mL/min, respectively; the temperature of the derivative reactor was 57°C; and the standard concentration of AA was 2 nmol/20 μL with a single point correction (Table 4 and Figure 6).

Table 4. Hydrolysis amino acid content in *Toona sinensis* fruit proteins.

AA	Content (%)	AA	Content (%)
ASP	5.57	ILE	2.48
THR	1.55	LEU	5.32
SER	3.94	TYR	1.90
GLU	27.05	PHE	1.94
GLY	3.33	LYS	1.85
ALA	2.25	NH3	3.10
CYS	1.52	HIS	1.49
VAL	2.58	ARG	11.71
MET	0.78	PRO	1.23
Sum (%)	76.48		

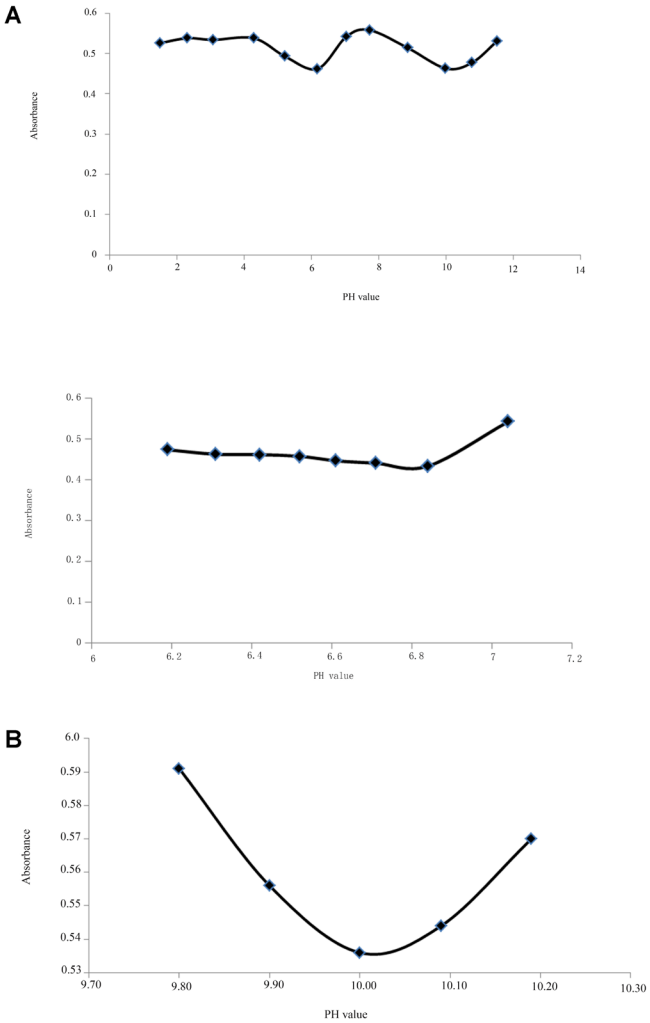

Figure 6. A Measuring the isoelectric point of proteins at pH 6-8 in *Toona sinensis* fruit.

In addition, we detected the isoelectric point of the proteins in *T. sinensis* fruit.

The pH of the protein extracts was adjusted between 2 and 13 with 12 points in total using 0.2 M NaOH and 0.2 M HCl (Huang et al., 2008). The pH values used to obtain minimum and the maximum measurable absorbance were explored and eight points around the maximum were set with spacings of 0.4 to confirm the isoelectric point.

The infrared spectrum was used to analyze the proteins in *T. sinensis* fruit. Polysaccharide samples were mixed with dried KBr powder, fully ground, and scanned at the wave band of 4000-400 cm^{-1}.

SDS-PAGE was used to determine the molecular weight of the proteins in *T. sinensis* fruit. According to Laemmli (Hamisi and Likewude, 1994), the SDS-PAGE conditions included 5% stacking gel, 12% separation gel, and 200-mA current for 2.5 h.

RESULTS

BSA standard curve

The absorbance increased as the BSA concentration increasing from 0 to 0.9 mg/mL, which presented a linear relationship. The equation was as follows: $y = 7.7931x + 0.0152$, $r = 0.9994$.

Extraction technology: Single factor exploration

Extraction time

As shown in Figure 1, the protein content increased as the extraction time increased within the range 0-45 min. After 45 min, the protein content tended to be stable. Thus, the optimal extraction time was determined to be 45 min.

Extraction pH

The results showed that the protein content increased with pH from 8.5 to 9.5. When the pH exceeded 9.5, the protein content decreased gradually. The reason for this might be the increased negative charges of the protein in alkaline solution, which influence the interactions between protein and solution, and protein and protein, and hence, the solubility (Figure 2).

Extraction temperature

The results showed that the protein content increased as the temperature increased from 0 to 40°C. When the temperature exceeded 40°C, the protein content decreased gradually. Thus, the optimal extraction temperature was 40°C as shown in Figure 3.

Liquid-to-solid ratio

As shown in Figure 4, the protein content increased as the liquid-to-solid ratio increased. The maximum protein content was obtained when the liquid-to-solid ratio was 25:1. Combining the extraction amount and the costs, the optimal liquid-to-solid ratio was 25:1.

Orthogonal test

As shown in Tables 1, 2, and 3, using the *T. sinensis* fruit protein as the index, the liquid-to-solid ratio had the most influence followed by the extraction time, temperature, and pH (C > B > D > A). The optimal condition was $A_3B_1C_2D_{2,}$ (pH 10.5, 40 min, 40°C, liquid:solid = 25:1).

Verification of technology

Using the optimal condition of $A_3B_1C_2D_2$, three samples of proteins from *T. sinensis* fruit were selected. Their weights were 255.9, 260.5, and 264.7 mg, with an average of 260.4 mg, standard deviation (S) of 4.40, relative standard deviation (RSD) of 1.69% (N = 3), which indicated the stability of the $A_3B_1C_2D_2$ technology.

Preliminary characterization of the physicochemical properties of T. sinensis fruit proteins

The automatic Kieltec Distilling system azotometer was used to measure total nitrogen content in *T. sinensis* fruit protein, and their total nitrogen content was 13.8 g/100 g, 13.9 g/100 g, and 13.7 g/100 g, with an average of 13.8 g/100 g.

According to GB/T18246-2000 analysis, the AA composition of *T. sinensis* fruit proteins is shown in Table 4 and Figure 5. There were 17 different AAs in *T. sinensis* fruit proteins. The glutamate level was the highest at 35.37%, followed by arginine at 15.31%, and methionine was the lowest at 1.02%.

The isoelectric points of proteins in *T. sinensis* fruit were measured and are shown in Figure 6A and B. The results revealed that there were two clear wave crests between pH 1.0 and 11.0 when the protein content was measured by Coomassie brilliant blue staining, which indicated that there were two different proteins with different isoelectric points, and their pH values were 6.0 and 10.0, respectively. The absorbance of the supernatant was low, indicating that the quantity of precipitate was high at this pH. Figure 6B shows that the absorbance was lowest, and the precipitation was highest, at pH 10.0, which indicated that pH 10.0 was an isoelectric point.

Next, the infrared spectrum was used to analyze the proteins in *T. sinensis* fruit. The strong and wide absorption peak at 3395.79 cm^{-1} represented the stretching vibration of the N-H bond; 2933.19 cm^{-1} was fermi resonance; 1655.68 cm^{-1} was the stretching vibration of the C=0 bond; 1536.10 cm^{-1} was the bending and stretching vibration of the N-H and C-N bonds; 1322.25, 1281.50, and 1238.61 cm^{-1} were the bending vibrations of C-N and N-H; 594.82 cm^{-1} was the out of plane of C = 0 bending vibration. The results are shown in Figure 7.

In addition, we also analyzed the molecular weights of the proteins from *T. sinensis* fruit. The proteins were mainly concentrated between 66.2 and 18.4 kDa, including three bands at 66.2-45.0 and 25.0-18.4 kDa, which were 55, 51, and 22 kDa, respectively, as shown in Figure 8.

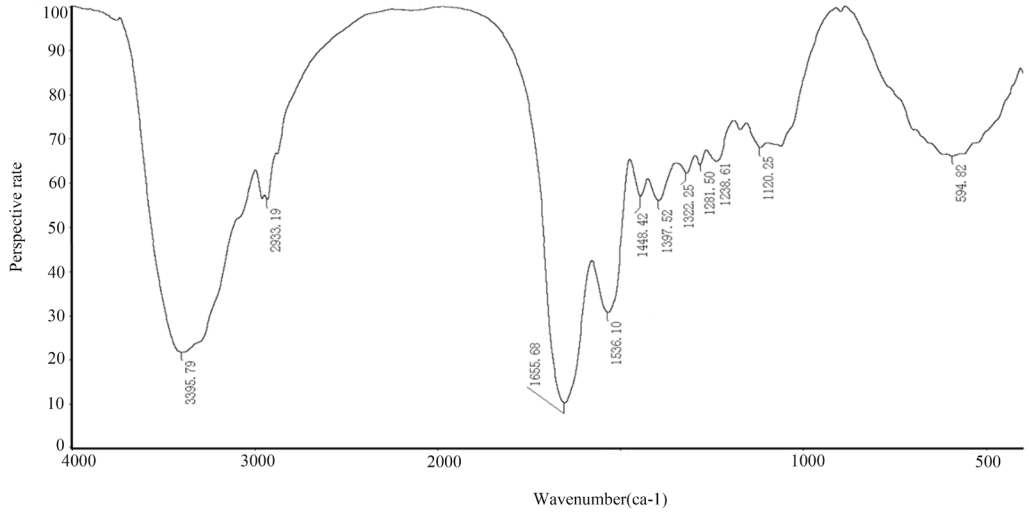

Figure 7. B Measuring the isoelectric point of proteins at pH 9-10 in *Toona sinensis* fruit.

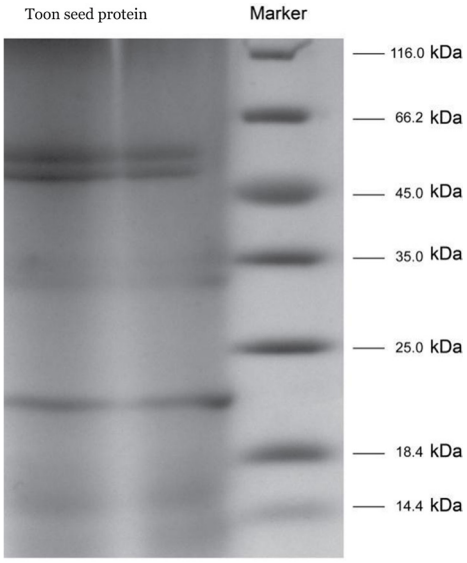

Figure 8. Infrared spectrum analyzing the proteins in *Toona sinensis* fruit.

DISCUSSION

As a component of traditional Chinese medicine, *T. sinensis* fruit has many medical effects, due to the presence of many active compounds, including phenols, tannins, alkaloids, saponins, steroids, terpenes, and volatile oil.

Although there have been some studies on its effects, few studies on *T. sinensis* fruit proteins, and the extraction technology used for health products, foods, and medicines was

under the exploring stage in these fields. This study was performed to investigate the extraction of *T. sinensis* fruit proteins, and to preliminarily characterize the physicochemical properties of the proteins.

In our study, we optimized the extraction technology of proteins in *T. sinensis* fruit. The results showed that the optimal pH was 10.5, extraction time was 40 min, liquid-to-solid ratio was 25:1, and the temperature was 40°C. Under these optimal conditions, the concentration of extracted protein was 260.4 mg. The total nitrogen content in *T. sinensis* fruit proteins was 13.8 g/100 g, and included 17 different AAs. The content of Glu and Arg was 50.68% of the total, which indicated that this protein has potential uses as well as developmental prospects. The isoelectric point of *T. sinensis* fruit proteins was between 6.8 and 10.0. The results of SDS-PAGE revealed three protein bands at 55, 51, and 22 kDa. Our investigation of the proteins of *T. sinensis* fruit provided the evidence and references for further researches.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Supported by the National Natural Science Foundation of China (Grand #81274049).

REFERENCES

Appel LJ (2003). The effects of protein intake on blood pressure and cardiovascular disease. *Curr. Opin. Lipidol.* 14: 55-59. http://dx.doi.org/10.1097/00041433-200302000-00010

Chen TS, Luo ZP, Cui HA, et al. (2000). Preliminary study of chemical constituents from leaves of Toona sinensis. *Shanxi For. Sci. Technol.* 20: 1-2.

Chen YL, Yuan ZP and Li CL (2008). Studies on chemical constituents and pharmacological effects toon. *J. Changzhi Medical College.* 22: 315-317.

He ZY and Lin L (2013). Toon chemical constituents and pharmacological research progress. *Gansu Zhongyi Xueyuan Xuebao* 30: 64-66.

Higashi K, Abata S, Iwamoto N, Ogura M, et al. (2001). Effects of soy protein on levels of remnant-like particles cholesterol and vitamin E in healthy men. *J. Nutr. Sci. Vitaminol.* 47: 283-288. http://dx.doi.org/10.3177/jnsv.47.283

Huang J, Xiong H, Liang LI, et al. (2008). Enzymolysis of residue of rice and determination of its protein isoelectric point. *Sci. Technol. Food Ind.* 37: 174-176.

Hamisi BD and Likewude D (1994). Gel electrophoresis of proteins: a practical approach[M] Beijing Science Press 19221.

Jenkins DJ, Kendall CW, Vidgen E, Augustin LS, et al. (2001). High-protein diets in hyperlipidemia: effect of wheat gluten on serum lipids, uric acid, and renal function. *Am. J. Clin. Nutr.* 74: 57-63.

Lee YS and Jung EH (2002). Dietary Soy Protein and Calcium Reduce Serum Lipid and Cholesterol in Rats Fed Fat - Enriched Diets. *J. Food Sci. Nutr.* 7: 367-372.

Rowlands JC, He L, Hakkak R, Ronis MJ, et al. (2001). Soy and whey proteins downregulate DMBA-induced liver and mammary gland CYP1 expression in female rats. *J. Nutr.* 131: 3281-3287.

Tang ZT, Jin XH, Xie JL, Bo JB, et al. (1996). Study on protein energy malnutrition cirrhosis after hepatitis. *Chin. J. Dig.* 16: 346-348.

Wang ML, Tu BK and He D (2006). Chemical constituents of Toon progress. *Hubei For. Sci. Technol.* 140: 38-40.

Wang WP, Guo SY, Li L, Wang ML, et al. (2008). Coomassie brilliant blue method for the determination of protein polysaccharides. *Food Res. Dev.* 29: 1150-117.

Xing SS and Chen C (2010). *Advances in Chemical Constituents and Toon Journal of Anhui Agricultural Sciences* 38: 8978-8979.