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Optimization of low-abundance protein extraction and abundant protein removal from defatted soybean meal*

Ming-mei LIU[†1,2], Bin QI[3], Zheng-xu LIU[1], Jin-shun ZHAN[1], Kang ZHAN[1], Guo-qi ZHAO[†‡1]

([1]*College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China*)

([2]*Huai'an Bioengineering Branch of Jiangsu Union Technical Institute, Huai'an 223200, China*)

([3]*Key Laboratory of Food and Biotechnology, Changshu Institute of Technology, Suzhou 215500, China*)

[†]E-mail: liummzhou@163.com; gqzhao@yzu.edu.cn

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Abstract: The aim of this study was to optimize the conditions for the extraction of low-abundance proteins (LAPs) and the removal of abundant proteins (APs; β-conglycinin and glycinin) from soybean meal. Single factor and orthogonal experiments were designed to determine the effects of four factors (isopropanol concentration, total extraction time, ultrasonic power, and ultrasonic time) on protein concentration in isopropanol extracts. Proteins in the isopropanol supernatant and the cold acetone precipitate of isopropanol were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). The results showed that the optimal conditions were 50% isopropanol, ultrasonic pretreatment for 15 min at 350 W, and a total extraction time of 1 h. Under these conditions, the protein concentration in the isopropanol extracts reached 0.8081 g/L. Many LAPs were detected, including β-amylase, soybean agglutinin, soybean trypsin inhibitor, fumarylacetoacetase-like, phospholipase D alpha 1-like, oleosin, and even some unknown soybean proteins. The soybean APs (β-conglycinin and glycinin) were not found. The method may be useful for discovering new soybean proteins and extracting enough LAPs of soybean to allow further studies of their physiological effects on animals without the influence of APs.

Key words: Protein extraction; Abundant protein; Low-abundance protein; Defatted soybean meal; MALDI-TOF-MS
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1 Introduction

Soybean proteins have complex compositions, which result in the diversity of their classification and function. According to their abundance in soybeans, they can be classified as abundant proteins (APs) or low-abundance proteins (LAPs). As soybeans are the main source of plant protein, the extraction and physiological functions of soybean proteins have been widely studied. Most such studies have focused on soybean meal (Tibaldi *et al*., 2006; Wang *et al*., 2006; Saki *et al*., 2012), soybean protein isolates (SPIs) (Jankowski *et al*., 2009; Cornish *et al*., 2011), and the soybean APs (β-conglycinin and glycinin) (Deak *et al*., 2006; Adams *et al*., 2008; Sun *et al*., 2008). The extraction of most LAPs is beyond the scope of traditional techniques (Deak *et al*., 2006), and they are very difficult to separate from APs. New methods are needed to facilitate studies of their extraction and physiological functions on animals and humans.

With the rapid development of proteomics techniques, the separation and identification of soybean LAPs have received increasing attention. Treatment with trichloroacetic acid (TCA)/acetone, verified as an efficient and reliable method for the separation and identification of soybean proteins, has been used in

‡ Corresponding author

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ORCID: Ming-mei LIU, http://orcid.org/0000-0001-7075-1457

the characterization of soybean storage proteins (Natarajan *et al*., 2006a) and for the analysis of allergen and anti-nutritional proteins in wild and cultivated soybean seeds (Natarajan *et al*., 2006b). Natarajan *et al*. (2009) found that isopropanol treatment resulted in the depletion of APs and was better than TCA for preferentially enriching soybean LAPs. On the other hand, many studies have confirmed that ultrasound treatment facilitates the disintegration of particles. High-power ultrasound was reported as a powerful method to enhance the extraction of intracellular compounds from plant materials, including the extraction of oil, sugar, and protein from soy flakes (Li *et al*., 2004; Karki *et al*., 2009a; 2009b; Bu *et al*., 2012).

To obtain enough LAPs for further studies of their physiological functions on animals without the influence of APs, we carried out single factor (isopropanol concentration, ultrasonic power, ultrasonic time, and the total extraction time) and orthogonal experiments to optimize the conditions for LAP extraction and AP removal. The concentration and identity of the proteins in the isopropanol extracts were determined by Coomassie brilliant blue R-250 (CBB) kit, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS).

2 Materials and methods

2.1 Materials and chemicals

Defatted soybean meal (crude protein, 55%) was obtained from Jilin Fengzheng Soybean Food Limited Company (China). A Coomassie brilliant blue kit was purchased from the Nanjing Jiancheng Bioengineering Institute (China). Ammonium bicarbonate, acetonitrile, and trifluoroacetic acid (TFA) were all of chromatographic grade (Sigma, USA). Trypsin (Promega, USA) was of sequencing grade. A 170-kD protein molecular mass marker (Thermo, USA) was used in electrophoretic analyses. Isopropanol and other reagents were of analytical grade (Sinopharm, China). Ultrapure water was self-made (Thermo, USA) and used for making all solutions.

2.2 Single factor and orthogonal experimental design

First, without ultrasound treatment, the effects of isopropanol concentration and total extraction time on the extraction of soybean proteins were determined. Then, under the conditions of 40% isopropanol, a 1-h total extraction time, and ultrasound treatment for 20 min, the effect of ultrasonic power was investigated. The fourth experiment was conducted using 40% isopropanol, a 1-h total extraction time and ultrasound pretreatment at 400 W, to confirm the influence of ultrasonic time on soybean protein extraction.

To further determine the optimal conditions for LAP extraction and AP removal from defatted soybean meal, orthogonal experiments were designed based on the results of the above experiments (Table 1), and the proteins in extracts were identified by SDS-PAGE and MS.

Table 1 $L_9(3^4)$ orthogonal design

Level	Factor			
	A (%)	*B* (W)	*C* (min)	*D* (min)
1	40	350	5	40
2	45	400	10	60
3	50	450	15	90

A: isopropanol concentration; *B*: ultrasonic power; *C*: ultrasonic time; *D*: total extraction time

2.3 Protein extraction from defatted soybean meal

The defatted soybean meal powder (500 mg) was homogenized with 5 ml of isopropanol solutions of known concentrations (35%, 40%, 45%, 50%, and 55% (v/v)), and then extractions were carried out for certain total time (0.5, 1, 2, and 3 h; ultrasound treatment and vibrating water-bath in sequence). The ultrasound treatment included ultrasonication at variable power (0, 200, 300, 400, or 500 W) for 10 min, or for a variable time (0, 5, 10, 15, or 20 min) at 400 W. Finally, the supernatant of the extracts was obtained after centrifugation at 12000 r/min for 15 min at 4 °C, and the protein concentration in the supernatants was determined immediately using the Coomassie brilliant blue kit.

The supernatant was isolated and placed in a clean 20-ml tube, and then double the volume of cold acetone was added to the supernatant before incubation at −20 °C overnight. Next day, the samples were centrifuged for 10 min at 12000 r/min at 4 °C and the pellet was dried using a freeze dryer.

2.4 Identification of proteins in isopropanol extracts

2.4.1 SDS-PAGE

According to Natarajan *et al*. (2009) and Krishnan *et al*. (2009b), the proteins in isopropanol extracts and cold acetone precipitates can be

separated by SDS-PAGE on a 12% resolving gel at 100 V using a Mini-PROTEAN Tetra System (Bio-Bad, USA) and visualized by staining for about 1 h with CBB and fading overnight.

2.4.2 Trypsin digestion and mass spectrometry

Trypsin digestion and MS were performed using the method of Liu *et al*. (2016). After being excised and washed twice with ultrapure water and three times with 25 mmol/L ammonium bicarbonate in 50% acetonitrile for destaining, the protein bands of the CBB-stained gel were dehydrated with 200 μl of acetonitrile. The protein band pieces were then re-swollen in 10.0 ng/ml trypsin for trypsin digestion. The digestion was performed overnight at 37 °C after incubation at 4 °C for 30 min. Next day, the supernatant was extracted three times in 50% acetonitrile containing 0.1% trifluoracetic acid (TFA) and dried using a freeze dryer.

All samples were analyzed using a 5800 matrix-assisted laser desorption/ionization-time of flight (MALDI TOF)/TOF analyzer (Applied Biosystems, Framingham, MA, USA). Mass spectra (*m*/*z* 800–4000) were acquired in positive ion reflector mode, and the 20 most intense ions were selected for subsequent MS/MS sequencing analysis in 2 kV modes. Protein identification was performed by searching MS/MS spectra, and the peptide mass was obtained from National Center for Biotechnology Information (NCBI) protein databases using the search engine Matrix Science (http://www.matrixscience.com).

2.5 Statistical analyses

SPSS 16.0 statistical software was used for data analysis. Data were analyzed by one-way analysis of variance (ANOVA).

3 Results and discussion

3.1 Effects of single factors on soybean protein extraction

There were significant differences in the protein concentrations of extracts obtained using five different isopropanol concentrations (35%, 40%, 45%, 50%, and 55%) ($P<0.01$; Fig. 1a). Krishnan (2004) found that isopropanol could facilitate the enrichment of LAPs and the removal of APs in soybean, but Natarajan *et al*. (2009) found that high isopropanol concentrations (exceeding 50%) resulted in a sharp decrease in both APs and LAPs identified by SDS-PAGE. This may be why in our study we found a highly significant ($P<0.01$) decrease in protein concentrations with increasing isopropanol concentrations.

In a kinetic study, the expression of dissolution was found to be analogous to the surface diffusion control of crystal growth (Tang and Nancollas, 2002), implying that dissolution continues until equilibrium is reached. Before reaching equilibrium, the soybean protein dissolved in the extraction solution increased with increasing extraction time. In this study, the protein concentration after 1 h of extraction was significantly higher than that after 0.5, 2, or 3 h ($P<0.01$). There were no significant differences between protein concentrations after 0.5, 2, and 3 h of extraction ($P>0.05$). So, we considered 1 h as the optimal total extraction time for protein extraction from defatted soybean meal (Fig. 1b).

It has been reported that while LAPs have been observed in 30%, 40%, and 50% isopropanol extracts by 1D-PAGE analysis, there was a decrease in both APs and LAPs at higher isopropanol concentrations (Natarajan *et al*., 2009). So, it is impossible to separate the maximum number of LAPs (without APs) in defatted soybean meal by changing only the isopropanol concentration. Karki *et al*. (2009b) found that ultrasound did not modify the peptide profile, regardless of the ultrasound conditions used, and after ultrasound for 120 s the SPI yield was increased by 13% at low power and 34% at high power. The particle size decreased nearly 10-fold following ultrasonic treatment at high amplitude for 120 s, resulting in the highest increase in total protein yield (46%), compared to samples with no ultrasonic treatment (Karki *et al*., 2009a). In our study, ultrasound treatment improved the protein concentration in the isopropanol extracts, which showed a parabolic tendency in response to increasing ultrasonic power or duration (Figs. 1c and 1d). When the ultrasonic power exceeded 400 W, the protein concentration was higher in isopropanol extracts than in those without ultrasonic pretreatment ($P<0.01$), but there was no difference between 400 and 500 W ($P>0.05$). The protein concentration was higher in extracts with ultrasound treatment for 10 or 20 min than in those without ultrasonic pretreatment ($P<0.01$).

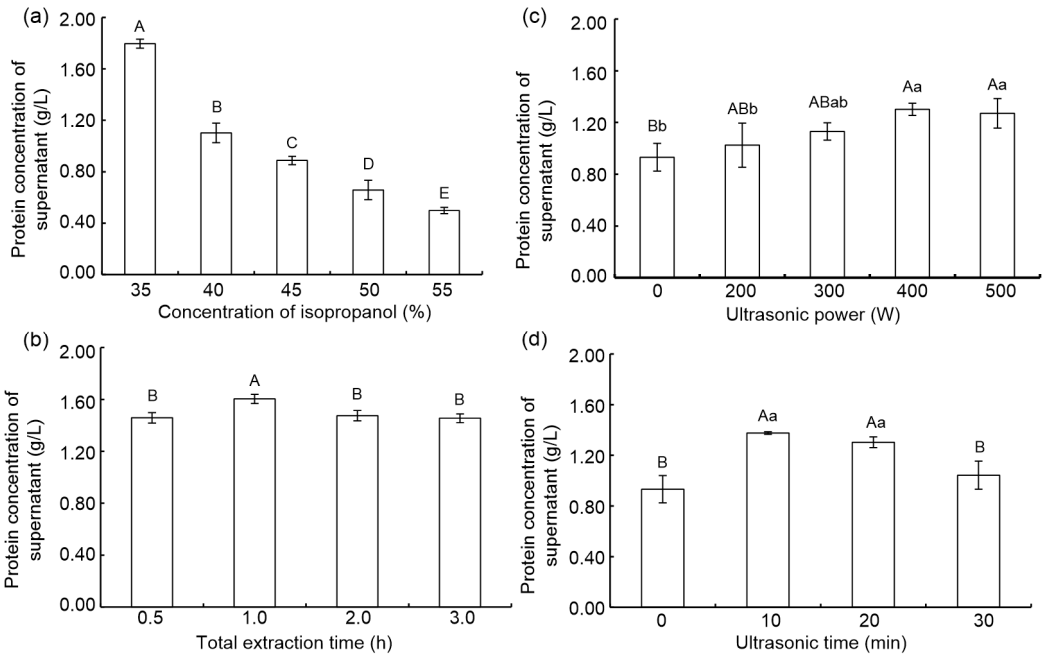

Fig. 1 Effects of isopropanol concentration (a), total extraction time (b), ultrasonic power (c), and ultrasonic time (d) on protein extraction from defatted soybean meal

Data are expressed as mean±standard deviation (n=6). Columns labeled with different capital letters indicate a significant difference at the 0.01 level (P<0.01), and columns labeled with different lowercase letters indicate a significant difference at the 0.05 level (P<0.05)

3.2 Orthogonal experiment for the optimization of LAP extraction and AP removal

We confirmed that the concentration of proteins extracted by isopropanol from defatted soybean meal was higher at low isopropanol concentrations (35%, 40%, and 45%) than at the higher isopropanol concentration (50%) (Fig. 1a). This was confirmed by the lower protein concentration under conditions 3 (5 min (ultrasonic time), 450 W (ultrasonic power), 50% (isopropanol concentration), 90 min (total extraction time)), 5 (10 min, 400 W, 50%, 40 min), and 7 (15 min, 350 W, 50%, 60 min) compared to those under the other conditions (Fig. 2). In addition, the protein concentration under condition 7 increased by 9.54% and 8.93% compared with those under conditions 3 and 5, respectively, indicating that ultrasonic power, ultrasonic time, and total extraction time play important roles in soybean protein extraction. In our study, we also found that with 45% isopropanol the protein concentration increased by 12.98% under the conditions of ultrasound treatment for 15 min at 450 W with 40 min of total extraction time compared to that of ultrasound treatment for 10 min at 350 W with 90 min of total extraction time (Fig. 2). This not only confirmed that the four factors we investigated all affect soybean protein extraction, but also showed that ultrasound treatment had a greater influence on protein extraction than the total extraction time.

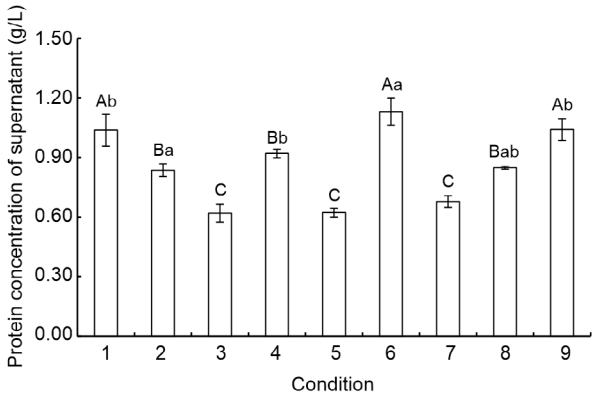

Fig. 2 Effects of different conditions on the soybean protein concentration of isopropanol extracts

1: 5 min, 350 W, 40%, 40 min; 2: 5 min, 400 W, 45%, 60 min; 3: 5 min, 450 W, 50%, 90 min; 4: 10 min, 350 W, 45%, 90 min; 5: 10 min, 400 W, 50%, 40 min; 6: 10 min, 450 W, 40%, 60 min; 7: 15 min, 350 W, 50%, 60 min; 8: 15 min, 400 W, 40%, 90 min; 9: 15 min, 450 W, 45%, 40 min. Data are expressed as mean± standard deviation (n=6). Columns labeled with different capital and lowercase letters indicate a significant difference at P<0.01 and P<0.05, respectively

To confirm the optimal conditions for LAP extraction and AP removal, the proteins in the isopropanol extracts under nine different conditions were identified by SDS-PAGE (Fig. 3) and MS (Table 2). There were more protein bands or the staining intensity of protein bands was stronger at lower isopropanol concentrations irrespective of the ultrasonic time, ultrasonic power, and total extraction time (Fig. 3). The staining intensity of protein bands in lane 7 (condition 7) was stronger than those in lanes 3 and 5, which was in accord with their protein content in isopropanol extracts (Fig. 2).

β-Amylase, the major enzyme of starch breakdown in leaves (Fulton *et al*., 2008), soybean trypsin inhibitor (STI) and soybean agglutinin (SBA), accounting for 6% and 5%–7% of soybean proteins, respectively (Rackis *et al*., 1986; Bajpai *et al*., 2005), have been shown to be the important anti-nutritional factors of soybean (Fasina *et al*., 2006; Zang *et al*., 2006; Hart *et al*., 2010). β-Conglycinin is composed of three subunits, α', α, and β, which are potential food allergens (Krishnan *et al*., 2009a). In our study, the proteins in SDS-PAGE gels were excised and identified by MS (Table 2). β-Amylase, kunitz trypsin inhibitor, and SBA were the top three proteins in the nine isopropanol extracts. The α subunit of β-conglycinin and many other LAPs, such as fumarylacetoacetase-like, dehydrin, oleosin 16 kDa-like, and iron-superoxide dismutase, were also detected in defatted soybean meal under some conditions (Table 2). The α subunit of β-conglycinin was not detected under conditions 3, 5, or 7.

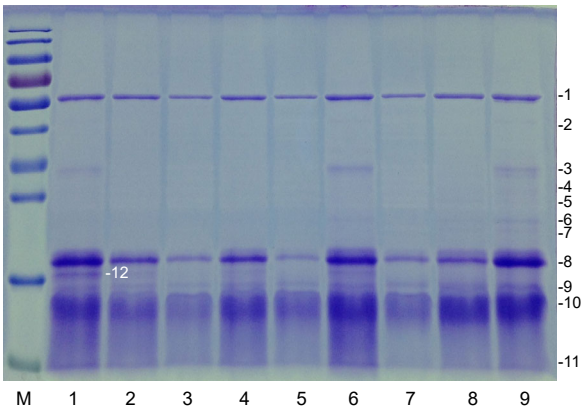

Fig. 3 1-D electrophoretogram of isopropanol extracts under different conditions

M, marker, indicates 170, 130, 100, 70, 55, 40, 35, 25, 15, and 10 kD; Lanes 1–9 were the conditions 1–9 as shown in Fig. 2

3.3 Determination of LAP extraction and AP removal

The preferable orthogonal experimental conditions (conditions 3, 5, and 7) were inconsistent with those identified in our previous study (Liu *et al*., 2016) of the effects of ultrasonic treatment on LAP extraction (10 min, 400 W, 50%, 60 min). So, to determine the optimal conditions for LAP extraction and AP removal, a comparative analysis was carried out between the orthogonal experiment (under conditions 3, 5, and 7) and the ultrasonic treatment experiment (under optimal conditions). The results are shown in Figs. 4 and 5, and Table 3. The protein concentration

Table 2 Proteins in the isopropanol extracts identified by MS

Band No.	Protein accession	Protein mass	Protein score	Protein description [species]
1	gi|62122635	56406	1280	β-Amylase [*Glycine max*]
2	gi|356555724	46192	141	Fumarylacetoacetase-like [*Glycine max*]
3	gi|6729836	27555	231	Chain A, soybean agglutinin complexed with 2,6-pentasaccharide
4	gi|543177317	27512	110	Iron-superoxide dismutase [*Phaseolus vulgaris*]
5	gi|37495455	23720	217	Dehydrin [*Glycine max*]
6	gi|9967357	63184	144	α Subunit of β-conglycinin [*Glycine max*]
7	gi|9967357	63184	121	α Subunit of β-conglycinin [*Glycine max*]
8	gi|157838208	19853	516	Chain B, complex porcine pancreatic trypsin soybean trypsin inhibitor, orthorhombic crystal form
9	gi|356515553	17534	80	Oleosin 16 kDa-like [*Glycine max*]
10	gi|13375351	16124	74	Truncated kunitz trypsin inhibitor [*Glycine max*]
11	gi|145495350	17407	143	Hypothetical protein [*Paramecium tetraurelia*]
12	gi|157838208	19853	179	Chain B, complex porcine pancreatic trypsin soybean trypsin inhibitor, orthorhombic crystal form

of up to 0.8081 g/L under condition 7 was higher than that under the optimal conditions from the ultrasonic treatment experiment (10 min, 400 W, 50%, 60 min) (P<0.05), and condition 5 (P<0.05) and condition 3 (P<0.01) from the orthogonal experiment. This result confirmed the important role of the ultrasonic treatment and total extraction time in LAP extraction (Fig. 4).

Cold acetone precipitation of isopropanol extracts was performed by SDS-PAGE and the proteins were identified by MS (Fig. 5, Table 3). There were no differences in the number of protein bands but some differences in the intensity of their staining among the four conditions, indicating different protein contents but the presence of the same protein varieties. This result was consistent with the protein concentrations of isopropanol extracts (Fig. 4). Some LAPs were detected in the cold acetone precipitates, such as phospholipase D, β-amylase, oleosin 1-like, uncharacterized and even an unknown protein (Table 3).

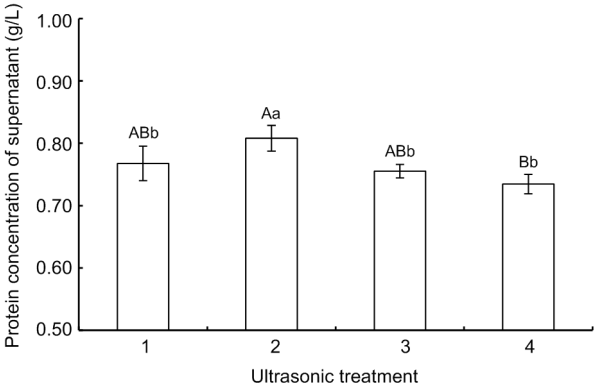

Fig. 4 Effects of various conditions on the soybean protein concentration of extracts

1: 10 min, 400 W, 50%, 60 min; 2: 15 min, 350 W, 50%, 60 min (condition 7); 3: 10 min, 400 W, 50%, 40 min (condition 5); 4: 5 min, 450 W, 50%, 90 min (condition 3). Data are expressed as mean±standard deviation (n=6). Columns labeled with different capital and lowercase letters indicate a significant difference at P<0.01 and P<0.05, respectively

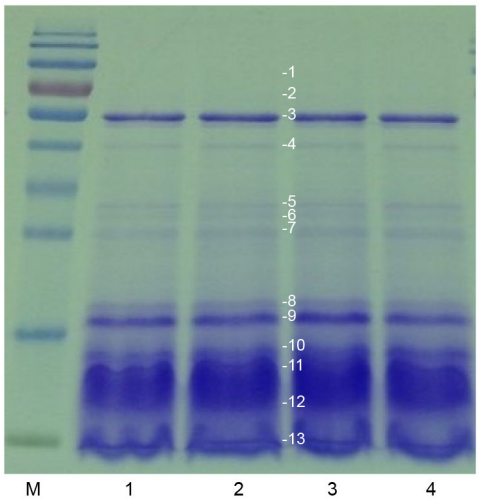

Fig. 5 1-D electrophoretogram of cold acetone precipitation under four different conditions

M, marker, indicates 170, 130, 100, 70, 55, 40, 35, 25, 15, and 10 kD; Lanes 1–4 were the conditions as shown in Fig. 4

Table 3 Proteins in cold acetone precipitates identified by MS

Band No.	Protein accession	Protein mass	Protein score	Best protein description [species]
1	gi|571503799	92477	144	Phospholipase D alpha 1-like isoform X2 [*Glycine max*]
2	gi|62122633	56391	267	β-Amylase [*Glycine max*]
3	gi|62122635	56406	725	β-Amylase [*Glycine max*]
4	gi|63259123	56457	353	β-Amylase [*Glycine max*]
5	gi|363806904	29349	153	Uncharacterized protein LOC100799849 [*Glycine max*]
6	gi|255638626	17463	131	16.5 kDa oleosin [*Glycine max*]
7	gi|255638626	36632	147	Unknown [*Glycine max*]
8	gi|351720944	22718	290	Uncharacterized protein LOC100305855 precursor [*Glycine max*]
9	gi|157838209	19853	450	Chain B, complex porcine pancreatic soybean trypsin inhibitor, tetragonal crystal form
10	gi|571459448	22698	285	Oleosin 1-like [*Glycine max*]
11	gi|571459448	22698	132	Oleosin 1-like [*Glycine max*]
12	gi|356556690	15180	98	Uncharacterized protein LOC100800510 [*Glycine max*]
13	gi|351726299	17463	176	16.5 kDa oleosin [*Glycine max*]

4 Conclusions

The results of this study show that: (1) isopropanol contributed to the removal of APs, and ultrasonic treatment and total extraction time played an important role in enriching the LAPs of soybean; (2) the effect of ultrasonic treatment on LAP enrichment was higher than that of total extraction time; (3) the optimal conditions were 50% isopropanol, ultrasonic treatment for 15 min at 350 W for a total extraction time of 1 h. This method may be useful for preparing sufficient soybean LAPs to study their physiological effects on animals without the influence of APs.

Acknowledgements

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Compliance with ethics guidelines

Ming-mei LIU, Bin QI, Zheng-xu LIU, Jin-shun ZHAN, Kang ZHAN, and Guo-qi ZHAO declare that they have no conflicts of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：低温脱脂大豆粕的低丰度蛋白提取和高丰度蛋白去除条件的优化

目　的：优化低温脱脂大豆粕中的低丰度蛋白提取和高丰度蛋白去除条件，为进一步探讨大豆低丰度蛋白对动物的生理功能影响提供试验材料。

创新点：结合提取时间和异丙醇浓度，将超声波（超声时间、功率）用于辅助异丙醇去除大豆高丰度蛋白和富集低丰度蛋白的研究。

方　法：在单因素试验基础上，设计了超声时间、超声功率、提取时间和异丙醇浓度等四因素三水平的正交试验。并通过测定提取液中的蛋白浓度和通过聚丙烯酰胺凝胶电泳（SDS-PAGE）、质谱分析（MS）等蛋白质组学手段鉴定提取液或丙酮沉淀物中的蛋白质含量，实现了大豆高丰度蛋白去除和低丰度蛋白提取条件的优化。

结　论：50%异丙醇，350 W 超声 15 min，提取 1 h 是大豆低丰度蛋白提取的最佳条件。提取物含多种低丰度蛋白（油质蛋白、大豆未知蛋白、磷酸酯 D 和无特征大豆蛋白），且无大豆高丰度蛋白（大豆球蛋白和 β-伴大豆球蛋白）。

关键词：蛋白提取；高丰度蛋白；低丰度蛋白；低温脱脂豆粕；质谱