Technical Research of 7S and 11S Soy Protein Fractionation in Laboratory-scale

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Abstract: Combining with our laboratory's isolation techniques (application of Mucor pusillus), this article compared different separation methods of 11S and 7S fractions in laboratory-scale. The principles of most separation techniques in early period were mainly focus on "alkali extraction and acid precipitation" and "cold precipitation" effects, and then other physical or biological technologies were continuously supplemented for the purpose of improving the yield and purity of protein components. Among them, Thanh's method was the first to realize the contemporary separation technique of soy protein components. Nagano's method and Wu's method were the classical methods quoted for many times. And the Deak's method got the best isolation efficiency by using Ca^{2+} as a precipitating agent.

Key words: Soy protein; Separation techniques; 7S rich fraction; 11S rich fraction; Yield; Purity.

中图分类号: TS201.2 **文献标识码**: A **文章编号**: 1000-9841(2010)02-0325-07

实验室规模分离大豆蛋白 7S 和 11S 组分技术研究进展

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摘 要:该文围绕目前实验室制备 11S 和 7S 组分的分离技术成果,结合作者所在实验室的分离技术(微小毛霉法),比较了各种分离方法的差异。早期分离提取技术利用的原理多为"碱溶酸提"和"冷沉"作用,之后不断辅以其它物理或生物技术进行改进,以提高各蛋白组分的回收率和纯度。其中,Thanh 法首次完整地提出了大豆蛋白的组分分离方法,而 Nango 法和 Wu 法的引用次数较多,Deak 法采用的 Ca^{2+} 沉淀的方法效果则是实验室分离方法中的最优方法。

关键词:大豆蛋白;分离技术; 7S 富集蛋白;11S 富集蛋白;产量;纯度

1 Introduction

Soy protein, an important vegetable protein, is widely used in food industry. Two major components of soybean protein are Glycinin (11S) and β-conglycinin (7S), which account for approximately 40% and 30% of total soybean protein, respectively[1-3].

β-conglycinin is a glycoprotein containing 3.8% mannose and 1.2% glucosamine with a molecular weight of 126 to 171 kDa. It is composed of α′, α, and β subunits, which make up seven heterogeneity proteins including B_0 (α′ ββ), B_1 (αββ), B_2 (α′ αβ), B_3 (α′α′ β), B_4 (ααα′), B_5 (ααα) and B_6 (βββ) with hydrophobic and hydrogen bonding[4]. Glycinin is a heterogeneous protein having a molecular weight of 340 to 375 kDa[5-7]. Main structure of the 11S is a hexameric protein composed of several subunits, each subunit consists of an acidic (A) and basic (B) polypeptide chain connected by a disulphide linkage(An—S-S—Bn)[8].

In order to make a thorough research on functional or physiological activity of soy protein components, the demand for raw materials should be resolved firstly. Therefore, the isolation techniques of 7S and 11S components in laboratory-scale have been extensively studied. This paper summarized the laboratory-scales methods for separation 7S and 11S fractions, and analyzed and discussed the differences of these methods in separating method, product yield and product purity.

2 Laboratory-scale Methods for separation of 7S and 11S fraction

Detailed understanding of chemical or biological

Received: 2009-12-21

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activity studies of 7S and 11S globulin are the main purpose of laboratory-scale separation. So in order to meet the requirements for the different use of isolates, the additives and the technical routes which used in the separation process are different. For instance, for the purpose of studying the physiological activity of 7S and 11S globulin, the Tris-HCl buffer was chosen for keeping the nature protein structure. Adding the reducing agent such as 2-mercaptoethanol (2-ME) or sodium bisulfite (SBS) in the extracting process to break disulfide bonds between the protein components was just for further improve the purity of isolates[9]. In order to improve the product yield, the inorganic salt is added for the "salting-out" effect breaking the electric double layer of protein and promoting the protein precipitation[10].

The research of 7S and 11S soy globulin separation technique was started in 1960s. Wolf and others utilized the "cold precipitation" effects of 11S to get the crude 11S faction, and then prepared a relatively pure 11S globulin according to the classic method of ammonium sulfate salting-out in 1962[11]; however, this method could not be used for separation of 7S globulin at the same time[12].

Soy storage protein is a kind of alkali-soluble protein. Thus, it can be extracted from the defatted soybean with alkaline solution. The pI of 11S globulin is pH 6.4, while the pI of 7S globulin is pH 4.8-4.9[13-14]. With utilize the principle of different soybean protein components could precipitate in its isoelectric, Thanh and other[15-16] directly separated 11S globulin and 7S globulin by adjusting the pH. That is the so-called "alkali extraction and acid precipitation" method.

2.1 Common classical and improved methods

2.1.1 Method of Thanh Thanh and others[16] achieved the contemporary separation technique of soy protein foremost. For the sake of stabilizing the reaction system, keeping the nature state of protein and improving the product purity, the protein was extracted with the Tris-HCl buffer with 2-ME. This method is divided into two steps: defatted soybean meal was extracted with 20 volumes 0.03 mol · L^{-1} Tris buffer (pH 8.0) containing 0.01 mol · L^{-1} 2-ME at room temperature for 1 h. After insoluble fraction was removed by centrifugation, the extract was adjusted to pH 6.4. The 11S globulin is collected by centrifugating at 2 ~ 5℃. The supernatant was adjusted to pH 4.8 with HCl, and 7S glycinin-rich fraction was obtained as the precipitated curd. The prepared 11S globulin was washed with pH 6.4 0.03 mol · L^{-1} Tris buffer, and then dispersed in the standard buffer (protein content, 2%-3%). NaOH solution was added with stirring until the protein was dissolved (pH 7.8). The protein solution was kept at 3-5℃ overnight. A trace of precipitate was removed by centrifugation and the supernatant was just the 11S-rich fraction. Meanwhile, 2 mol · L^{-1} NaOH was added to the 7S globulin precipitate while stirring until the protein dissolves (pH 7.6), then adjust to pH 6.2 with 2 mol · L^{-1} HCl, after the precipitate was removed by centrifugation, the supernatant was just the 7S-rich fraction.

Due to the first time to realize the contemporary separation technique of soy protein, this method became the classical method and the primary method of extraction and separation of soybean protein components in the laboratory-scale at present.

2.1.2 Methods of Nagano 7S and 11S globulin are both subunit compounds and it is impossible to separate them completely during the separate process. So taking the elimination of cross- contamination between two proteins as the starting point, Nagano and others[17] developed a three-step fractionation, discarded the intermediate protein fraction, and improved the purity of 7S fraction. In addition, the method of Nagano modified the method of Thanh from the following three aspects: (1) Deionized water was replaced by the Tris buffer as the extracted solution, and the pH was adjusted to 7.5; (2) 980 mg · L^{-1} SBS was took the place of 2-ME as the reducing agent; (3) 0.25 mol · L^{-1} NaCl was added to the supernatant after the 11S rich fraction was separated with the purpose of increasing the precipitation rate of 7S rich fraction. It is also a widely used method for laboratory-scale preparation of soy protein components at the moment.

2.1.3 Method of Wu and the modified methods On the basis of Nagano method, Wu[18] modified the separation methods of 7S and 11S fraction, and carried out the pilot-plant fractionation successfully (described in the latter). It was also developed a wide range of application method for separation. The only difference with the Nagano's method was the dissimilitude of

centrifugal speed in three steps (Table 1).

As the Wu-based method, Rickert[19] studied the separation methods of soy protein in bench-scale and pilot-plant-scale in 2004. The influences of pH, alcohol, temperature of reaction and flake-to-water ratios to separating effects were studied, and the parameters and optimized separation processes were determined. Meanwhile, on the basis of Nagao and Thanh, Chun Liu[20] researched the optimal method for separation of soy protein from the extracting solution, pH values, temperature, flour/Tris-HCl ratio and the $NaHSO_3$ concentration, and the parameters were also determined according to the final yield and purity of each protein components. The optimized processes of each method are shown in Table1.

2.2 Separation methods with CO_2 as a volatile electrolyte

Traditional soybean-protein-purification techniques of 7S and 11S components often involve the use of inorganic acids to adjust the pH and precipitate the protein. However, when using acidic solutions such as HCl and H_2SO_4[21], the phenomenon of local excess concentrations of precipitant can occur. And that phenomenon may lead to many problems, such as coprecipitation of other unwanted species, irreversible damage to protein structure, formation of many small highly solvated particles and so on[22-24]. As a kind of volatile acid, carbon dioxide can be used to acidification of the solution and can be carried out as the electrolyte for soy protein separation technology. At the mean time, the use of carbon dioxide is preferable over conventional acids because no alkali is required for neutralization, which occurs simply by release of pressure. It is further simplify the separation technique and reduce the content of ash.

Russell Thiering[25] developed a 3-steps protein separation method with CO_2 as the acidifier in 1999. In order to enhance the protein yield, intermediate-precipitate fraction was recovered to initial slurry. The whole fraction process was achieved by regulating the extracting protein solution concentration, pressure, reaction temperature, adding volume of CO_2 and acidity of solution. It was found that, the degree of acidification strengthened as the pressure increased, precipitation of the glycinin fraction was most efficient from highly concentrated soy protein solutions at pH 6.1 and production of β-conglycinin-rich precipitates was best from dilute solutions at pH 4.8.

Marijana Golubovic[26] employed CO_2 as a volatile acidifier to prepare the pure 11S component in 2005 as well, and his method is afterward modified by Russell[25]. The differences between them were the initial solution and pH for precipitation: Deionized water was used in Russell while Marijana adopted 30 mmol · L^{-1} Tris buffer (pH 8.0) with 10 mmol · L^{-1} 2-mercaptoethanol as the extract solution; the pH for precipitate 11S rich fraction was 6.1 for Russell method, while it was 6.4 for Mraijana's method. However, this method was only extracted 11S rich fraction without carrying out the separation research of 7S fraction.

2.3 Separation methods of Ca^{2+} and Mg^{2+} replace Na^+ as precipitating agent

In the study of separation 7S and 11S fraction, some salt materials were added as the precipitating agent before the step of precipitation 7S globulin. The "salting-out" effect was used to destruct surface hydration layer and promote the precipitation of 7S. The Na^+ was used as the precipitating agent mostly, while Ca^{2+} or Mg^{2+} were still used in some researches.

Koshiyama[27] used 250 mmol · L^{-1} of $CaCl_2$ to purify the supernatant remaining after cold precipitation of a glycinin-rich fraction, but yields and purities of this crude β-conglycinin fraction were not determined. Saio and Watanabe[28] added 10 mM of $CaCl_2$ to extraction buffer and defatted soybean meal to first extract a 7S-rich supernatant and the residue was redissolved and centrifuged to obtain an 11S-rich fraction. The purities on ultracentrifugal basis were about 60%.

Until 2006, on the basis of Wu method, Deak[29] studied the separation effects of Ca^{2+} unceasingly. But the calcium salt was not effective in fractionating soy proteins by merely adding it to the fractionation procedure developed by Wu and others[18]. Then he changed the pH of precipitate intermediate fraction to pH 6.4, and the separation effects was better than others and the dilution step was no longer employed to obtain the β-conglycinin-rich fraction.

Meanwhile, Deak further modified the extract method for protein solution, brought forward a two-step fractionation, and studied the best concentration of SO_2 and $CaCl_2$. The optimal process was as below: after the protein extracted supernatant was collected as the

method of Wu[18], 5 mol · L^{-1} SO_2 and 5 mol · L^{-1} $CaCl_2$ were added to the solution and pH was adjusted to 6.4 with 2 mol · L^{-1} HCl. The resulting slurry was stored at 4℃ for 12 to 16 h and centrifuged at 14 000 r · min^{-1} and 4℃ for 30 min. A 11S-rich fraction was obtained as the precipitated curd, neutralized, and freeze-drying. The supernatant was adjusted to pH 4.8 with HCl, stirred for 1 h, and then centrifuged at 14 000 r · min^{-1} and 4℃ for 30 min. A 7S-rich fraction was obtained as the precipitated curd. Furthermore, Deak carried out the components fraction research in 4℃ (referred to as D4C) and 25℃ (referred to as DRT), discovering the yield and purity of isolated protein were significantly different in the different temperature[30]. Generally, the yield and purity of protein components produced by this method are better than others.

Teng[31] made a comprehensive comparison of the protein separate efficiency by using Ca^{2+} or Mg^{2+}, which is based on the two-step fractionation of Deak[29]. 0.5 mmol · L^{-1} Mg^{2+} could achieve an effective protein separation by using Mg^{2+} as a precipitating agent. 10 mmol · L^{-1} SBS and 5 mmol · L^{-1} $MgCl_2$ were added to the protein extracting solution, the resulting slurry was adjusted to pH 6.4 and stirred for 1 h. The 11S-rich fraction was obtained as the precipitated curd after being centrifugalize at 3 000 r · min^{-1} for 15 min, while the supernatant was then adjusted to pH 4.8 with 2 mol · L^{-1} HCl and stirred for 1 h. After the third centrifugation at 3 000 r · min^{-1} for 15 min, the precipitated phase was collected as the 7S-rich fraction. Both fractions were redispersed in deionized water and lyophilized.

2.4 Application of enzymes in components separation

Protein aggregation has been observed when phytate in soybean was hydrolyzed by phytase under certain conditions, and the aggregate is mostly consists of glycinin. Based on this characteristic, Tsutomu Saito and others[32] developed a novel method for separating β-conglycinin and glycinin by a phytase treatment. The protein extract which was extracted in pH 7.5 was adjusted to pH 6.0 with 2 mol · L^{-1} HCl. Phytase was added to this soymilk (1000FYT/100 g of protein in the defatted milk). After that, the resulting mixture was kept for 1 h at 40℃. The solution was then centrifuged to separate the precipitate at room temperature. The precipitate collected by centrifugation was dispersed in a 4-fold volume of distilled water and neutralized to pH 7.0 with 2 mol · L^{-1} NaOH (the glycinin fraction). The supernatant was adjusted to pH 5.0 with 2 mol · L^{-1} HCl and centrifuged. The precipitate was dissolved in a 4-fold volume of distilled water and neutralized to pH 7.0 with 2 mol · L^{-1} NaOH (the β-conglycinin fraction).

Mucor pusillus is commonly used in milk curd[33-34]. It can precipitate 47% protein by using in the precipitation of soy protein, and the aggregate is mostly consists of glycinin[35]. So our lab developed a technology for separating soybean protein components by using Mucor pusillus. The extract protein solution was adjusted to pH 5.6 after centrifugation, adding rennase to 15U and incubating at 60℃ for 40 min. The insoluble 11S fraction was obtained by centrifugation at 3600r · min^{-1} for 10 min. The supernatant was adjusted to pH 4.5 (2 mol · L^{-1} HCl), and then centrifuged again at 3000r · min^{-1} for 5min. The 7S globulin was obtained as sediment.

3 Comparison of separation effects on various separation methods

According to the available separation techniques, it seems that most methods were based on the principles of "alkali extraction and acid precipitation." and "cold precipitation". However, those fractionations were obviously different in some details, such as separation steps, pH value, precipitating agent, which also lead to variation of the resultant products of 7S and 11S. Subsequently, the effects of various separation techniques on the yield and purity of the product are compared. With the different ways to express the isolation efficiency in various articles, in order to facilitate the comparison, this article take the yield, protein content and purity of 7S fractions or 11S fraction as the index. All the numbers were quoted from the original articles or convert from the original data in the articles.

Table 2 presents the comparation of the classical and modified methods. Thanh's method is a 2-step fractionation. The yield of the 7S rich fraction and 11S rich fraction were the highest in five methods, and the purity of 7S rich fraction was relatively higher. However, Iwabuchi[36] repeated Thanh's method, and repor-

ted that there are about 6% β-conglycinin, and 15% other components in 11S rich fraction beside 79% glycinin, while 52% β-conglycinin, 3% glycinin, and 45% other protein components are in 7S rich fraction. The subsequent researches which commented the isolation efficiency of Thanh's method were almost followed the results of Iwabuchi[36].

On the basis of Thanh's method, Nagano developed a new method of 3-step fractionation, which discarded the intermediate protein fraction and improved the purity of 7S globulin. However, the yield of 7S rich fraction was only 6%, which was one third less than Thanh's method because of removing intermediate fraction. The purity of 7S and 11S fractions were greatly improved to more than 90%.

Wu, Ricket and Liu modified the separating conditions and parameters base on the methods of Thanh and Nagano. The yield and purity of each fraction increased markedly. In Liu's method, the yields of 7S rich fraction and 11S rich fraction were 14.4% and 10.7%, respectively; meanwhile, the purity of 7S globulin was exceeded 95.5% (Table 2).

Beside the above classical methods and modified methods in laboratory-scale separation method of 7S and 11S globulin, the other separation techniques by using Ca^{2+}, Mg^{2+}, enzyme and CO_2 also produce different isolation efficiency, the results produced by these methods were summarized in Table 3.

Table 1　Operating Process for Several Laboratroy – sale Methods in Separating 7S and 11S Fracation[a]

Operating process		Nagano	Wu	Ricket	Liu
Stage 1 extraction	Material	DSF ,H20	○	○	○
	additive	–	–	–	0.03 mol · L^{-1} Tris – HCl
	RML	1∶15	○	1∶10	○
	pH	7.5	○	8.5	8.5
	Extract time	1h	○	○	2h
	Temp.	Room Temp.	○	45℃	45℃
	Centrifuge	9 000 r · min^{-1} ×30 min	14 300 r · min^{-1} ×30 min	Didn't show	○
Stage 2 Separation	Step 1: 11S fraction	Sup1.	○	○	○
		Additive	0.98 g · L^{-1} SBS	○	○	0.01 mol · L^{-1} SBS
		pH	6.4	○	○	○
		Time	overnight	○	–	○
		Temp.	4℃	○	○	○
		Centrifuge	6 500 r · min^{-1} ×30min	7 500 r · min^{-1} ×20 min	○	○
	Step 2: Intermeidiate fraction	Material	Sup2.	○	○	○
		Additive	0.25 mol · L^{-1} NaCl	○	○	○
		pH	5.0	○	○	5.5
		React time	1h	○	○	0.5h
		Temp.	4℃	○	○	○
		Centrifuge	9 000 r · min^{-1} ×30min	14 300 r · min^{-1} ×30 min	○	○
	Step 3: 7S fraction	Material	Sup3.	○	○	○
		Additive	2-fold H_20	○	3-fold H_20	○
		pH	4.8	○	○	○
		Temp.	4℃	○	○	○
		Centrifuge	6 500 r · min^{-1} ×20min	7 500 r · min^{-1} ×20 min	○	○

[a] Nagano, Wu, Ricket, and Liu represents the different methods named by the inventers, respectively; Liu's method extracted protein solution twice and each time was 1 h; "○" represents the requirement process or condition is identical with the Nagano's method. "-" represents the process or requirement is not exist; DSF, defatted soybean flake; RML, ratio of material to liquid.

Table 2　Comparison of separation effects on classical and modified laboratory – scale preparation methods[a]

Methods	11S rich fraction			7S rich fraction		
	Yield/%	Protein content/%	Purity/%	Yield/%	Proteincontent/%	Purity/%
Thanh	15.3	92.0	78.0	18.7	91.0	86.0
Nagano	12.0	–	>90.0	6.0	–	>90.0
Wu	12.9	94.7	95.7	9.8	96.7	77.6
Ricket	11.29	96.7	86.0	9.51	94.04	66.0
Liu	14.4	96.3	92.5	10.7	93.3	95.5

[a] Thanh, Nagano, Wu, Ricket, and Liu represent the different methods named by the inventers.

Table 3 Comparison of separation effects on other laboratory – scale preparation methods[a]

Methods		11S rich fraction			7S rich fraction		
		Yield /%	Protein content /%	Purity /%	Yield /%	Protein content /%	Purity /%
Application of Ca^{2+}	Modification	12.9	91.8	90.1	15.2	75.8	100
	Deak	15.5	96	85.0	23.6	90.0	81.0
	D4C	15.5	98.9	81.0	23.1	90.0	85.6
	DRT	15.7	96.6	71.0	23.3	91.2	78.6
Applicationof Mg^{2+}		22.4	–	88.9	16.2	–	81.4
Application of enzyme	Phytase	18.0	88.9	>80.0	16	78.6	>80
	Mucor pusillus	19.8	93.4	93.0	9.5	95.1	88.4
Application of CO_2	Russell	28.0	–	95.0	4.8	–	80.0
	Marijanal	34.0	85.0	98.0	–	–	–

[a]Deak, Russell, and Marijanal represent the different methods named by the inventers.

Deak modified Wu's method Ca^{2+} as a precipitating agent firstly in 2006. The pH value of precipitating intermediate protein fraction was changed to 6.4, and the purity of 7S rich fraction was almost 100%. At the mean time, the author developed a 2-step separation technique by using Ca^{2+}, this method gave the highest yield of 7S rich fraction in all the current laboratory-scale separation methods. Deak further studied the effect of reaction temperature on separation and found low temperature (4℃) could increase the protein content and purity of each fraction due to the effect of "cold precipitation". Comparatively, when Mg^{2+} was used as the precipitating agents, the yield and purity of 11S rich fraction was relatively high, up to 22.4% and 80%, respectively.

By the application of phytase, the yield of 11S rich fraction and 7S rich fraction were higher than that of the methods illustrated in Table 2. Meanwhile, the cooling process and a reducing agent are not needed in this method, so that the processing is simplified and the consumption of resource is saved. As to using the Mucor pusillus, the yield of 11S rich fraction was relatively high. 11S is a stable hexagonal structure formed by the corresponding interaction between six acidic subunits and six basic subunits. Although the pI of glycinin is 6.4, the pI of each subunit is different the pH values nearby the pI of glycinin probable have a role of precipitating 11S glycinin. The separation method of using Mucor pusillus was carried out at pH 6.0. Though it is worked for the separate effect, it is hard to conclude whether it is the role of pH value or the enzyme. Hence, in order to explain the mechanism, further studies of applying Mucor pusillus in protein separation technique should be carried on.

Another particular method for separating soy protein is using CO_2 as the volatile electrolyte. Using the pressure as a well-controlling process parameter, CO_2 could acidification the pH of the solution according the different requirement. Thus the protein components could separation successful in their own pI. The results of Russell's way showed this method was fit for the separation of 11S rich fraction, whose yield and purity were 28% and 95%, respectively. As to the 7S rich fraction, although the yield was lower, the purity was up to 80%. Thus, it is feasible for using CO_2 as the volatile electrolyte in the protein separation technique because the yield and purity of product can be guaranteed. Marijana further used CO_2 as a volatile electrolyte to separate and purify 11S glycinin and obtained a high yield and purity of 11S glycinin (34% and 97.98%, respectively). The yield and purity of 11S globulin in this method reached the highest in all the laboratory-scale methods. Nevertheless, the author did not separate the 7S globulin.

4 Discussion

The mainly purpose of separation in laboratory-scale was to do researches, so it requires the raw maintains its nature structure as soon as possible. Meanwhile, how to simplify the separation steps under the premise of ensure the product yield is also one of the reasons to keep the continuous improvement of the current laboratory isolation methods. This article was mostly from the perspective of raw materials preparation to introduce the separation methods, and the other techniques were required to make a further special physiological activity research of protein components, such as reversed-phase high performance liquid[37], chromatography Fourier transform infrared (FTIR) method[38] and so on. In addition, besides the labora-

tory-scale of separation 7S and 11S globulin, there were many separate methods in pilot-plant-scale which will be discussed in another paper.

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