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Short communication

Ion exchange chromatographic conditions for obtaining individual subunits of soybean β-conglycinin

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ABSTRACT

Soybean β-conglycinin is a complex protein possessing health-promoting properties. β-Conglycinin is a trimeric glycoprotein. Little information related to methods for separation of the individual chains forming β-conglycinin has been so far published and it is of great interest. As a consequence, less data on the bioactivities of α, α′ and β subunits of this glycoprotein have been published. The present research aimed to find out new alternative chromatographic conditions to obtain β-conglycinin subunits that are free of contaminating proteins. In the present short communication, we propose the use of a two-step ion exchange chromatographic protocol to achieve this goal. Firstly, β subunit was separated by means of anionic exchange fast protein liquid chromatography. Secondly, α and α′ chains were separated from each other by cationic exchange. Our data indicated the feasibility of proposed fractionation protocol to separate soybean β-conglycinin α and α′ subunits from other contaminating proteins and to obtain enough amounts of the three individual chains forming this glycoprotein for further characterization and application. The procedure may be easily up-scaled.

1. Introduction

Soybean β-conglycinin has been described as a functional food ingredient and it is available commercially (Fuji Oil Co. Ltd.) in Asian countries such as Japan. This protein is one of the most abundant storage soybean proteins constituting between 24.7 and 45.3% of total proteins [1]. β-Conglycinin is a trimeric protein composed of three subunits: α (MW, 57–76 kDa), α′ (MW, 57–83 kDa) and β (MW, 42–53 kDa) [2] assembled by hydrophobic forces and hydrogen bridges that may be differently combined (ααα′, ααβ, αββ, αα′β, αββ or α′ββ) [3]. The percentage of each subunit varies among genotypes: α from 10.4 to 20.8%, α′ from 8.1 to 20.7% and β from 4.5 to 12.9% of total proteins [1] and mutant cultivars with homotrimmers have been also described [4]. p*I* values of 4.9, 5.2 and 5.7 have been reported for α′, α and β chains, respectively [5]. During the synthesis of β-conglycinin in soybean plant, it undergoes post-translational modifications including proteolysis and glycosylation. The latter post-translational process causes variation in glycosylation pattern of the polypeptides chains; for example, α and α′ subunits contain two N-linked high mannose glycans whereas β subunits generally contain only a single chain [5].

Few studies have established association between structure and activity of β-conglycinin subunits [6,7]. The role of each subunit in key biological activities of this protein such as immunoreactivity is a matter of controversy and partially unknown [8,9].

The study of the structure and effect of a particular protein subunit requires suitable purification techniques to obtain enough amounts of it. Few works on the isolation of β-conglycinin subunits have been previously carried out. An Immobilized Metal Affinity Chromatography (IMAC) method under dissociation conditions for obtaining soybean β-conglycinin α′ subunit was proposed by Duranti et al. [6]. However, α and β subunits were not separated by these authors. More recently, Zheng et al. [10] proposed a protocol to purify soybean β-conglycinin α, α′ and β subunits by combination of anion exchange chromatography and IMAC under 6 M urea. The method developed by these authors allowed obtaining high amounts of fractions rich in soybean β-conglycinin. Therefore, their results have a huge practical significance. However, data on SDS-PAGE with Coomassie brilliant blue staining given by these authors indicated the presence of contaminating proteins in soybean β-conglycinin α and α′ fractions separated by IMAC while they obtained only one obvious band by the analysis of β-conglycinin β fraction obtained by ion exchange chromatography. Chemical and biochemical characterization and application of the individual chains require the removal of other protein contaminants and this was the goal of the present research. To achieve this goal and taking into account the feasibility of the results previously published by others [6,10], a new alternative purification

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Table 1
Gradient conditions for fast protein liquid chromatography (FPLC).

Column	Mono Q HR 5/5		Mono S HR 5/5	
Step	Time (min)	% Phase B	Time (min)	% Phase B
1	0	0	0	0
2	20	60	20	16
3	2	100	1	100
4	5	100	4	100
5	1	0	1	0

method based on the combination of anion and cation exchange chromatographic principles was tested. Ion exchange chromatography separates biomolecules based on differences in their charge and it is considered a highly selective chromatographic technique because it is able to resolve proteins that differ only by a single charged group. The separation was carried out in fast protein liquid chromatography (FPLC) to prepare large scale batches of purified products. On the other hand, the use of this technology has the advantage of being easily up-scaled for industrial use.

2. Materials and methods

2.1. Chemicals

All the chemicals were of analytical grade. Soy protein isolate was purchased by Manuel Riesgo Company (Manuel Riesgo, S.A. Madrid, Spain). Sodium dihydrogen phosphate monohydrate and anhydrous disodium hydrogen phosphate (Merck, Darmstadt, Germany), and Tris and urea (Sigma–Aldrich, St. Louis, MO, USA) were employed for the buffer preparations. Sodium chloride and ammonium bicarbonate were provided by Panreac (Barcelona, Spain).

2.2. Subunit isolation

Soybean β-conglycinin was isolated from commercial soy protein isolate by isoelectric precipitation following the protocol described by Nagano et al. [11]. β-Conglycinin subunits were separated by ion exchange fast protein liquid chromatography (FPLC) in two sequential steps. FPLC system consisted of an LKB binary gradient 500 pump equipped with a manual injector and a Pharmacia LKB UV-MII detector (Pharmacia-Biotech, Uppsala, Sweden). In the first step, mobile phases were as follows: phase A, 20 mM Tris–HCl buffer (pH 8.5) with 6 M urea; and phase B, 1 M NaCl in phase A. β-Conglycinin was diluted at 5 mg protein/mL in phase A and injected (1 mL) onto a MonoQ HR 5/5 anion exchange column (10 μm particle size, Pharmacia-Biotech). Elution was carried out under gradient conditions (Table 1) and the detection was performed at a wavelength of 280 nm. Peaks were collected, mixed and concentrated by means of Centricon YM-30 system (Millipore, Bedford, MA, USA) provided with a 30,000 Da cut-off membrane. The composition of the fractions collected was established by means of SDS-PAGE performed in 12% or 4–12% polyacrylamide gels under reducing conditions following manufacturer's instructions (Invitrogen, Barcelona, Spain). Protein bands were visualized by silver staining [12]. In this first step, β subunit was separated from α subunits.

Peaks containing α and α′ subunits were mixed and injected (1 mL) onto a MonoS HR 5/5 cationic exchange column (10 μm particle size, Pharmacia-Biotech). Mobile phases were as follows: phase A, 20 mM phosphate buffer (pH 6.8) with 6 M urea; and phase B, 1 M NaCl in phase A. Gradient conditions are shown in Table 1 and detection followed at 280 nm wavelength. Urea was eliminated by diafiltration with NH_4HCO_3 buffer (pH 7.8) in Centricon YM-30 system (Millipore). Protein was quantified by the BCA™ assay using bovine serum albumin (BSA) as standard following manual's instructions (Pierce Chemical Co., Rockford, IL, USA). Composition of the collected fractions was identified by SDS-PAGE and silver staining as was described above.

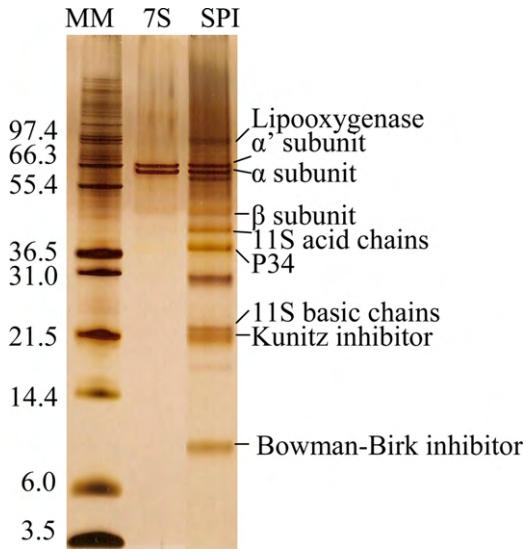

Fig. 1. SDS-PAGE 12% silver stained of soy protein isolate (SPI) and β-conglycinin (7S). Molecular weight markers (MMs). Amount of protein injected on to the gel was of 30 μg for SPI and 5 μg for β-conglycinin.

3. Results and discussion

β-Conglycinin isolation process was followed by SDS-PAGE (Fig. 1). Two clear bands corresponding to α and α′ subunits and a faint band for β subunit were detected by the application of this approach. Data agreed with those previously reported by others [1] who have detected β subunit as a minor constituent of soybean β-conglycinin.

As can be observed in Fig. 2, the ion exchange FPLC method is a feasible procedure for the separation of β-conglycinin subunits. The β subunit showed a retention time of 11 min while α and α′ eluted together at 17–19 min (Fig. 2A) in anion exchange chromatography. Alfa and α′ subunits were separated by cationic exchange chromatography showing retention times of 1.6 and 23.7 min, respectively (Fig. 2B). As it is shown in Fig. 3, only one band was detected for α and α′ subunit by SDS-PAGE with silver staining while in the fraction corresponding to β subunit (lane 4, Fig. 3) was detected a very intense protein band accompanied by other highly fainted bands indicating the presence of traces of contaminating proteins. Since the contaminants present different molecular weights than soybean β subunit, they may be easily removed from the protein fraction by employing low cost and simple methods like such as ultrafiltration and or/size exclusion chromatography. Both methods may be also easily up-scaled for industry use. The molecular weights (~68,000 Da for α′, ~66,000 Da for α and ~49,000 for β) of the bands here assigned as β-conglycinin subunits (Figs. 1 and 3) were similar to those described in the literature [1–3].

The glycoprotein subunits were dissociated from 6 M urea prior to the chromatographic separation. Ion exchange chromatography separated β-conglycinin subunits based on their net surface charge. The absorption of the molecules in the solid support is driven by the ionic interaction between moieties, and binding capacities are generally quite high. The strength of the interaction is determined by the number of the charges on the molecules and solid support. The ion with the greatest size and charge has the highest affinity. The ionic interaction was disrupted by the increase of salt concentration in both chromatographic steps.

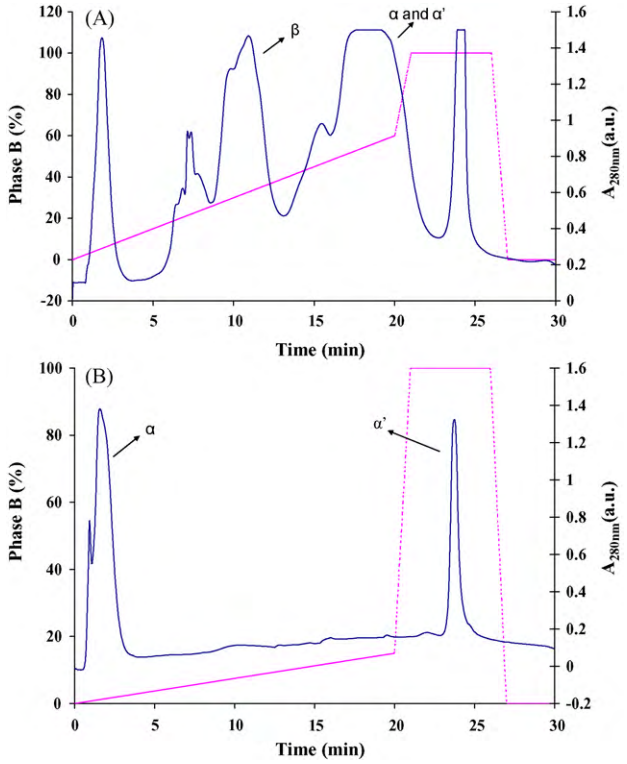

Fig. 2. Ionic chromatographic profiles of β-conglycinin (A) obtained by anionic exchange chromatography and α subunits (B) obtained by cationic exchange chromatography.

Under anion exchange conditions, negatively charged molecules are attracted to positively charged solid support (quaternary amino functional groups). At pH 8 the three subunits were negatively charged and they were bound to the ion exchange support (Fig. 2A). The strength of the ionic bonds between the charged groups on the subunits and the ion exchange can be explained on the basis of the p*I* of the subunits to be separated. In agreement, under these conditions β subunit (p*I* = 5.7) showed the weakest affinity among the samples. The strength of the ionic interaction is also determined by the size of the interacting molecule. Therefore, the biomolecule possessing less negative charge at pH 8 and smaller size was less retained (β chain). Results agreed with those described by Zheng et al. [10]. On the other hand, α and α′ subunits possessing close p*I* values and molecular weights presented similar affinity for the charged groups of the ion exchange support. Chromatographic resolution is also affected by the concentration of the compounds to be separated. According to the SDS-PAGE data (Fig. 1) β chain was in lower concentration than the rest of the protein subunits which were in very similar concentration.

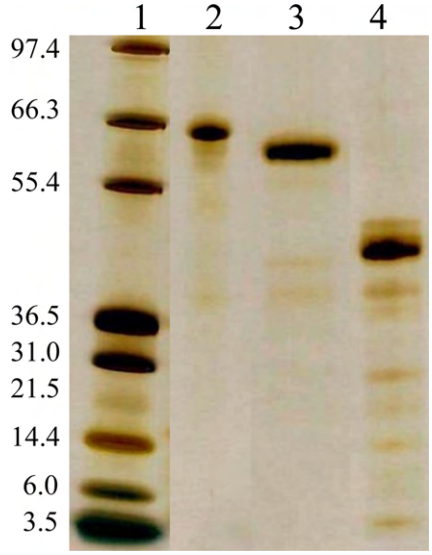

Fig. 3. SDS-PAGE 4–12% silver-stained. 1: Molecular weight markers; 2: α′ subunit; 3: α subunit; and 4: β subunit. Sample amounts ranged from 10 to 20 μg protein.

In the cation exchange chromatography, positively charged molecules are attracted to a negatively charged solid support (sulfonic acid functional groups). On our specific conditions α chain was weakly bound to the ion exchange chromatographic support (Fig. 2 B). Very low salt concentration was required for removing the ion bonds between the biomolecule and the charged groups of the ion exchange support while 1 M NaCl was needed to dissociate the ionic interaction between α′ and the charged groups of the Mono S column. Differences in molecular mass and p*I* may explain the separation of these two subunits by employing cationic exchange chromatographic conditions. In addition, the apparent better resolution found by cationic separation compared to the anionic chromatography may be influenced by the number of molecules to be separated that was smaller in the cation step than in the anion step. The 7S sample injected in the first step was a mixture comprising the three basic subunits of this glycoprotein and other contaminating proteins while in the second step the mixture to be separated contained only α and α′ subunits.

Chromatographic parameters such as particle size determine the chromatographic resolution of the columns. Mono Q and Mono S, the gel supports employed for the separation of β conglycinin subunits, are strong anion and cation exchangers based on beaded hydrophilic resin with charged groups and one of the narrowest particle size distributions available (10 μm) called monobeads (http://www.gelifesciences.co.jp/tech_support/manual/pdf/52162300.pdf) that may explain the high chromatographic resolution achieved in each case.

To our knowledge, only few papers on β-conglycinin subunit isolation have been published [5–6,10]. In all cases urea is used as a denaturing agent in different concentrations (from 6 to 8 M). Thanh and Shibasaki [5] purified β-conglycinin subunits by ionic exchange chromatography with different buffers used in this study. Duranti et al. [6] isolated α′ subunit based on MAC with Zn^{2+} obtaining a yield of 13% (w/w) starting from β-conglycinin, but they did not continue with α and β subunit isolation. Very recently, Zhen et al. [10] combined anionic exchange chromatography with MAC to isolate in the first separation β (with a yield of 25% (w/w) from β-conglycinin), and in the second one, α and α′ subunits. β-Conglycinin subunit purification has been also carried out by SDS-PAGE [7]. These authors purified only α subunit by preparative SDS-PAGE but more work is necessary in order to release the polypeptide from polyacrylamide gel. In our opinion, among all theses methods the combination of anionic exchange chromatography and MAC proposed by Zhen et al. [10] is the best option to be upscaled for industrial use. However, according to the data given by them, the fractionation method may be improved for obtaining α and α′ subunits that are free of contaminating proteins and high yields of β subunit.

4. Conclusions

Ion exchange chromatography in two steps is an adequate and convenient approach for obtaining individual polypeptide chains of β-conglycinin. The proposed method allowed obtaining high purity α, α′ and β subunits and may be up-scaled for industrial use, so the technique here proposed is simple and useful for future research.

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