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Food Chemistry 89 (2005) 497–501
Food Chemistry
www.elsevier.com/locate/foodchem

Removal and recovery of antinutritional factors from soybean flour

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Received 20 October 2003; received in revised form 25 February 2004; accepted 25 February 2004

Abstract

An integrated process for removal purification of two antinutritional factors, namely soybean trypsin inhibitor and soybean agglutinin, from soybean flour has been developed. The process is based upon binding of soybean trypsin inhibitor and soybean agglutinin to an immobilized metal affinity chromatography medium, consisting of zinc alginate beads. Both soybean trypsin inhibitor (95%) and soybean agglutinin (94%) could be removed from an aqueous extract of soybean flour. The bound protease inhibitor and lectin could be recovered by washing the zinc alginate beads with 0.05 M imidazole solution and dissolving the beads in 0.01M EDTA, respectively. Recoveries of 89%, in the case of soybean trypsin inhibitor and 81% in case of soybean agglutinin, were possible. Both purified proteins gave single band on SDS–PAGE. Thus, soybean flour, free of these two antinutritional factors, could be obtained. Simultaneously, these two antinutritional factors were purified as value-added products.

Keywords: Antinutritional factors; Soybean flour; Soybean trypsin inhibitor; Soybean agglutinin; Immobilized metal affinity chromatography; Zinc alginate beads

1. Introduction

Legume proteins constitute an important constituent of the diet of animals. In this respect, soybean is an important legume as it has a high protein content and nutritionally balanced amino acid profile. The reasonable price and steady supply are also favourable factors in soybeans emerging as an important source of protein in animal nutrition (Olguin et al., 2003). Soybean meal, obtained after oil extraction, is also widely used as the most cost-effective feed component for many aquaculture animals (Storebakken, Refstie, & Ruyter, 2000). However, the nutritional value of soybean meal is much lower than expected, in spite of its protein content and amino acid profile of the proteins. This is largely attributed to the presence of antinutritional factors, such as protease inhibitors, lectins, phytates and tannins. Protease inhibitors are the most important antinutritional factor. Their removal has been attempted by heat-treatment with varying success (Liener, 1994; Osman, Reid, & Weber, 2002). Soybean flour also contains several haemagglutinating isolectins. Their presence in the diet is known to reduce the growth rate of young monogastric animals (Van Damme Els, Peumans, Pusztai, & Bardocz, 1997). Many efforts have been directed toward removal or decreasing the amounts of these antinutritional factors as well (Liener, 1994; Marquez & Alonso, 1999; Franco-Fraguas et al., 2003). An altogether different motivation has been to purify the protease inhibitors and lectins for their biochemical usefulness (Kumar & Gupta, 1994; Duranti, Barbiroli, Scarafoni, Tedeschi, & Morazzoni, 2003; Franco-Fraguas et al., 2003).

In the present work, successful efforts have been made to remove the protein inhibitors and lectins from soybean flour and recover these value-added products. The strategy involved the use of immobilized metal affinity chromatography (IMAC) for binding both the protease inhibitors and the soybean lectins from the aqueous extract of the soybean flour. IMAC is a well established bioseparation technique (Porath, Carlsson,

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doi:10.1016/j.foodchem.2004.02.055

Olsson, & Belfrage, 1975; Pasquinelli, Shepherd, Koepsel, Zhao, & Ataai, 2000; Gupta, Jain, & Roy, 2002) which exploits the coordinating ability of metal ions (e.g., $Cu^{++}/Zn^{++}/Ni^{++}$) toward protein surface amino acid residues, such as histidine and cysteine.

2. Materials and methods

2.1. Materials

Soybean flour (Type I, not roasted, Catalog No. S-9633), Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), trypsin (Type I, from bovine pancreas) and sodium alginate (low viscosity, Catalog No. A-2158) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Estimation of trypsin inhibitory activity

Soybean trypsin inhibitor (STI) activity was assayed as a degree of inhibition of trypsin activity using Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as substrate. One unit of STI activity is taken as the amount of STI required to completely inhibit the activity of 2 μg of trypsin (Erlanger, Kokowsky, & Cohen, 1961).

2.3. Estimation of haemagglutination activity

Haemagglutination, or soybean agglutinin activity (SBA), was measured by using glutaraldehyde-treated trypsinised red blood cells (Turner & Liener, 1975). One haemagglutination unit (HU) was defined as the reciprocal of the greatest dilution of 50 μl of lectin solution giving observable agglutination (Gade, Jack, Dahl, Schmidt, & Wold, 1981).

2.4. Estimation of protein

Protein was estimated by the dye-binding method using bovine serum albumin as the standard protein (Bradford, 1976).

2.5. Preparation of crude extract of STI and SBA

The crude extract was obtained by stirring 1 g of soybean flour in 10 ml distilled water, pH 7.0. After 2 h, the extract was centrifuged at 8000*g* for 10 min at 25 °C. The clear supernatant obtained was used for further removal/purification of STI and SBA.

2.6. Preparation of alginate beads

Alginate beads were prepared by a procedure outlined by Somers, Van't Riet, Rozie, Rombouts, and Visser (1989). Beads were formed by dropping 50 ml of 2% alginate solution through a syringe (needle size 22G) into 100 ml of a 0.1 M zinc chloride solution. The beads obtained were kept for 2 h in 0.1 M zinc chloride solution and stored in 0.006 M zinc chloride solution at 4 °C.

2.7. Equilibration of beads

One milliliter (settled volume) of beads was equilibrated with 2 ml of 0.05 M sodium acetate, pH 6.0, in 0.006 M zinc chloride at 25 °C on a shaker (100 rpm) for 10 min.

2.8. Binding of STI and SBA with alginate beads

Crude soybean extract (1 ml) and 0.05 M sodium acetate buffer, pH 6.0, containing 0.012 M zinc chloride (1 ml) was added to 1 ml of equilibrated beads. After incubation for 1 h at 25 °C with constant shaking at 100 rpm, the supernatant was separated from the beads by using a pipette. The beads were washed with 0.05 M sodium acetate buffer, pH 6.0 (containing 0.012 M zinc chloride), until no STI and SBA activities were detected in the washings. The STI and SBA activities and protein were determined in the supernatant and washings after removing zinc from the supernatant by gel filtration on a PD10 column (Sephadex G-25 columns from Amersham Pharmacia Biotech, Sweden).

The difference between the enzyme activity of the supernatant (and washings) and the activities before the addition of alginate beads represented the amount of STI and SBA bound to the beads.

2.9. Elution of STI and SBA

The bound STI activity was recovered by incubating the beads with 2 ml of 0.05 M imidazole (in 0.05 M sodium acetate buffer, pH 6.0, containing 0.006 M zinc chloride) for 18 h at 25 °C with constant shaking at 100 rpm. The eluant was collected by using a pipette and then checked for STI activity and protein after removing imidazole from the eluant by running through the PD10 column.

The SBA activity was recovered by dissolving the beads (after elution of STI) in 0.01 M EDTA (2 ml) and then precipitating alginate with 0.22 ml of 1 M $CaCl_2$ (final concentration 0.1 M) for 20 min at 25 °C. The precipitate was centrifuged at 8000*g* for 15 min. The supernatant was collected to check the SBA activity and protein after removing imidazole and EDTA by running through the PD10 column.

2.10. Polyacrylamide gel electrophoresis

SDS–PAGE of the protein samples was performed by using 10% gel (for SBA) and 15% (for STI) according to Hames (1986) using a Bio-Rad Mini Protean II elec-

trophoresis unit and standard molecular weight markers (Bio-Rad Lab. Ltd., Richmond, CA, USA).

3. Results and discussion

Fig. 1 shows the effect of varying amounts of initially added STI activity on the extent of binding of STI activity to alginate beads. The volumes of crude extract containing 976 U (0.5 ml) or 1952 U (1 ml) of STI activity gave an acceptable level of removal/binding of the activity to alginate beads. Fig. 2 shows the similar data for SBA activities. Again, use of 0.5 ml/1 ml of crude extract led to fairly high percentage (91–94%) levels of binding of the lectin activity. As 1 ml could be processed with the amount of beads, further work was done by using 1 ml of crude extract.

It has been shown earlier that STI bound to zinc alginate beads can be eluted selectively by using imidazole. Table 1 shows the purification data on STI. The data agree well with data reported earlier, obtained with crude extract of STI prepared with 0.25 M H_2SO_4 (Gupta et al., 2002). It was found that 94% of bound STI activity could be recovered by eluting with 0.05 M imidazole. The procedure also led to 12-fold purification of STI. The purified STI showed significant purification on SDS–PAGE (Figs. 3 and 4). Soybean is known to contain two main types of trypsin inhibitors, Bowman–Birk and Kunitz-types, having molecular weights of 8000 and 20,000 Da, respectively (Garcia, Torre, Marina, & Laborda, 1997). The observed molecular weight (20,000 Da) in Fig. 3 agrees well with the reported value for the Kunitz type inhibitor (Roy & Gupta, 2002). As the crosslinking of this gel is 10% where the minimum

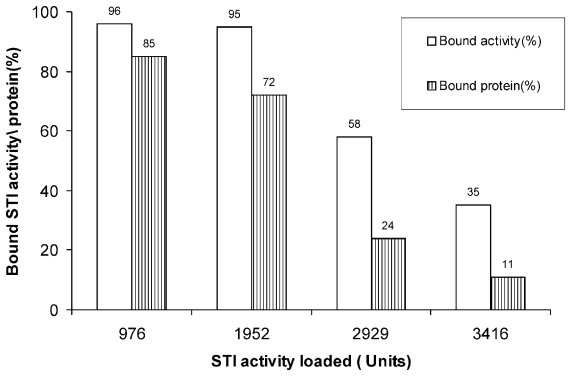

Fig. 1. Effect of varying STI load on binding of STI to alginate beads: Crude soybean extracts (containing varying STI units) and 0.05 M sodium acetate buffer, pH 6.0, containing 0.012 M zinc chloride (1 ml) were added to 1 ml of equilibrated beads. After incubation for 1 h at 25 °C with constant shaking at 100 rpm, the supernatant of each solution was separated from the beads by using pipette. The bound STI activity was determined as described in Section 2. The STI activity loaded initially was taken as 100%. The experiments were carried out in duplicate in which individual reading (in the pair) varied in a range of 2%.

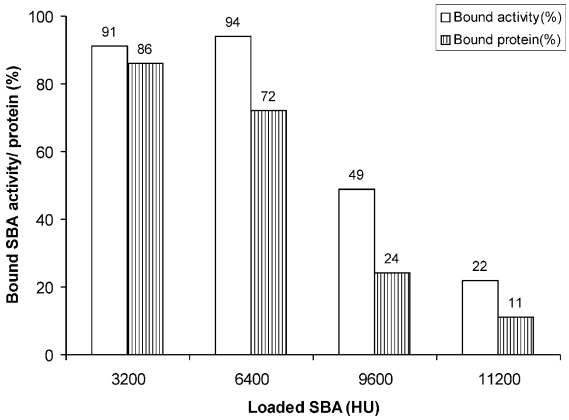

Fig. 2. Effect of varying SBA load on binding of SBA to alginate beads: Crude soybean extracts (containing varying SBA units) and 0.05 M sodium acetate buffer, pH 6.0, containing 0.012 M zinc chloride (1 ml) were added to 1 ml of equilibrated beads. After incubation for 1 h at 25 °C with constant shaking at 100 rpm, the supernatant of each solution was separated from the beads by using a pipette. The bound SBA activity was determined as described in Section 2. The SBA activity loaded initially was taken as 100%. The experiments were carried out in duplicate in which individual reading (in the pair) varied in a range of 2%.

molecular weight of the protein detectable is 14,000 Da, the band for the Bowman–Birk inhibitor is not visible, either in lane 1 (crude soybean) or in lane 3 (purified Kunitz inhibitor). The band corresponding to the Bowman–Birk inhibitor can be seen in 15% gel (Fig. 4). The band corresponds to a molecular weight of 8000 Da which agrees well with its reported value (Liener, 1994; Garcia et al., 1997). The broadness of this band is similar to that seen by others (Sessa & Wolf, 2001). Presumably it is due to presence of isoinhibitors of this type (Liener, 1994).

The beads obtained after the former steps still had bound SBA activity. This activity was bound much more strongly to zinc alginate than was STI activity. Hence SBA activity could be recovered by dissolving beads in 0.01 M EDTA and precipitating alginate by using Ca^{++}. It has been shown earlier that calcium alginate did not act as an IMAC matrix (Gupta et al., 2002). Thus, SBA activity could be recovered in the supernatant. Table 1 also gives purification data on SBA. Again, as high as 86% of the bound SBA activity could be recovered. The purified SBA (8-fold purification) also showed a single band on SDS–PAGE (Fig. 3), which agrees well with the reported molecular weight (Franco-Fraguas et al., 2003).

Thus, the supernatant obtained after initial binding (to alginate beads) step was mostly free of STI and SBA activities. This supernatant if added back to soybean flour (left after the extraction step) would be a reconstituted soybean flour free of these two antinutritional factors.

Table 1
Purification of STI and SBA on alginate beads

Steps	Activity		Protein (μg)	Specific activity (U/mg)		Yield (%)		Fold purification	
	STI (U)	SBA (HU)		STI	SBA	STI	SBA	STI	SBA
Crude extract	1952	6400	15,800	123	405	100	100	1	1
Supernatant + washing	106	360	4500	24	80	5	6	–	–
Elution of STI with imidazole	1730	250	1200	1442	–	89	4	12	
Desorption of lectin activity	58	5174	1600	–	3234	3	81	–	8

The crude soybean extract (1 ml) containing 1952 U of STI and 6400 HU of SBA, and 0.05 M sodium acetate buffer, pH 6.0, containing 0.012 M zinc chloride (1 ml) was added to 1 ml of equilibrated beads. After incubation for 1 h at 25 °C with constant shaking at 100 rpm, the supernatant was separated from the beads by using a pipette. The STI and SBA activities and protein were determined in the supernatant and washings. The bound STI and SBA activities were recovered as described in Section 2.

The above experiment was carried out in duplicate in which individual reading (in the pair) varied in a range of ±2%.

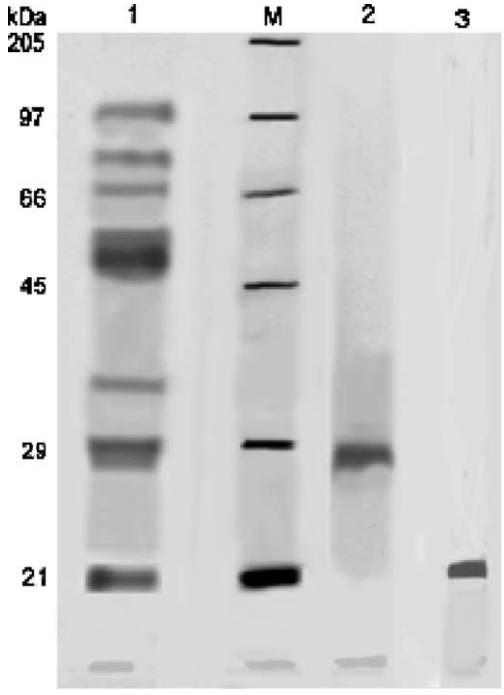

Fig. 3. SDS–PAGE of purified SBA: Lane 1 – crude extract of soybean flour (15 μg); Lane M – molecular weight marker proteins; Lane 2 – purified soybean agglutinin (25 μg); Lane 3 – purified STI (30 μg).

Thus, at the end of the treatment, not only was the soybean flour free of these two important antinutritional factors, but both lectins and protein inhibitors could be recovered at a significant level and in highly purified form.

This integration of the two aims makes economic sense. It also demonstrates the need and usefulness of applying current downstream processing techniques in the food processing area. After the whole process, a small amount of alginate, even if left in the flour, will not be harmful, as has been discussed earlier (Gupta et al., 2002). Alginate is an acceptable food ingredient and Zn^{++} can be removed by extensive dialysis. The traces of Zn^{++} are not considered harmful in food materials.

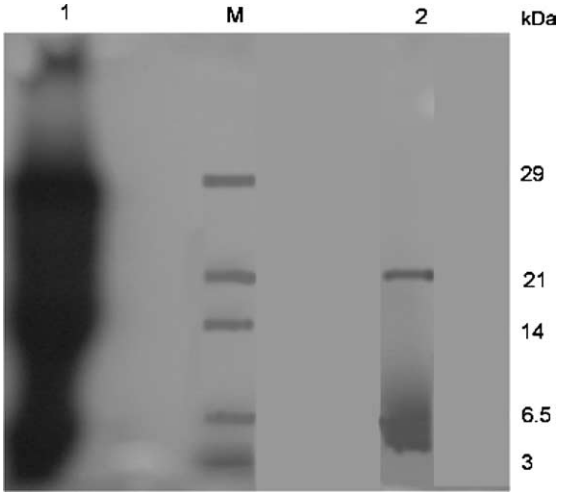

Fig. 4. SDS–PAGE of purified STI: Lane 1 – crude extract of soybean flour (15 μg); Lane M – molecular weight marker proteins; Lane 2 – purified Kunitz and Bowman–Birk soybean trypsin inhibitor (30 μg). The gel was stained with Coomassie brillant blue R-250 for 25 min and then destained with 40% methanol and 10% acetic acid.

Acknowledgements

The partial financial support provided by Department of Science and Technology, Department of Biotechnology, Council for Scientific and Industrial Research (Extramural Division and Technology Mission on Oilseeds, Pulses and Maize), National Agricultural Technology Programme, all Government of India organizations, is gratefully acknowledged.

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