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Journal of Chromatography B, 756 (2001) 249–254

JOURNAL OF CHROMATOGRAPHY B

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Determination of the IgE-binding activity of soy lecithin and refined and non-refined soybean oils

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Abstract

In the present study refined and non-refined soybean oils as well as soy lecithins were investigated for residual allergenicity and compared with extracts from native soybeans. By means of immunoblotting and EAST inhibition experiments no IgE-binding activity was detectable in refined soybean oils, which is probably due to thermal treatment during the refining. The investigated non-refined oils and soy lecithins showed a residual IgE-binding activity. In addition in the lecithin extracts a new IgE-binding structure with a molecular mass of approximately 16 kDa was detectable. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Food allergy; Immunoblotting; Enzyme allergosorbent test; Soy lecithin; Soybean oil

1. Introduction

The importance of the soybean (*Glycine max* L.) for human nutrition has increased during the last few years due to its oil and protein content [1,2]. Meanwhile soybean products are used as ingredients of many different foodstuffs. Soybean allergy is considered one of the five most common causes of food allergies among children [3,4]. Among adults the prevalence of soybean allergy has been estimated at 1% [5]. The clinical symptoms extend from atopic dermatitis [6,7] to gastrointestinal [8,9] and even anaphylactic reactions [10]. The IgE binding proteins, which are responsible for the allergenic reactions to soybeans, have been extensively investigated. Seven allergens with molecular masses between 7 and 180 kDa have already been characterized. In addition the amino acid sequences of the major allergens Gly m 1, Gly m 2, Gly m 3 and Gly m Bd 30 k have been determined [11–18,34]. The investigation of the stability towards heat and enzymatic digestion revealed a decrease in the allergenic potency [19–22]. At the same time heat treatment induced the formation of neo allergens [20]. Whilst the protein-containing soybean products have been investigated in detail [19,21,23–25], soybean oils have been examined only in one study [26], in which seven individuals with convincing histories of soybean allergy were given different amounts of soybean oil in a double-blind, placebo-controlled food challenge test. None of the seven experienced a reaction to the oils used, which included two refined and one cold-pressed oil. To our knowledge no in vitro investigations with regard to the allergenic potency of soybean oils have been carried out so far.

The aim of the present study was the examination

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00085-8

of the IgE binding activity of soy lecithin as well as of unrefined and refined soybean oil during processing. For this purpose nine sera of different patients with proven soybean allergy were used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblotting and EAST inhibition experiments.

2. Material and methods

2.1. Human sera

A total of nine sera were collected at the Division of Environmental Dermatology and Allergy, TU Munich, Germany and at the laboratory of Dr. Keeser/Professor Arndt, Hamburg, Germany. All sera were tested for specific IgE against soybean extract by enzyme allergosorbent test (EAST, Allergopharma, Reinbek, Germany). Pool-serum was collected using equal aliquots of the nine sera with EAST-classes from 1 to 4. A serum from a non-atopic individual with no history of food hypersensitivity (EAST class 0 to raw soybean extract) was used as negative control.

2.2. Soybean oils and lecithins

Soy lecithins and soybean oils were obtained from Cereol (Mannheim, Germany). The samples were declared as follows: soy lecithins (LBN 401 75/98 E4; LBN 401 73/98 E4; LBN 401 74/98 E3); non-refined soybean oils (HT 21, HT 22, HT 23); refined soybean oils (A6, A7). Soy protein isolate used as standard protein was obtained from Supro (Brussels, Belgium).

2.3. Protein extraction

Four hundred grams of the refined and non-refined soybean oils were frozen overnight with 400 ml acetone at −80°C. The protein precipitates were separated by a membrane filter (RC 58, 0.2 μm, Schleicher & Schuell, Dassel, Germany) and washed 3 times with hexane. The proteins were extracted from the membrane filter with 4 ml phosphate-buffered saline [PBS (0.01 *M* potassium phosphate buffer, pH 7.4, 0.13 *M* sodium chloride)]. The lyophilized PBS extract was dissolved in 300 μl double distilled water and used for further investigations.

Two hundred grams of the lecithins were suspended in 600 ml acetone–hexane (1+1) and centrifuged at 5000 *g* for 30 min. The residue was extracted with 5 ml PBS and filtered by a paper filter (Schleicher & Schuell). The protein contents were determined using a 1:500 dilution.

2.4. Protein determination

The protein contents of the extracts obtained as described above were determined by the method of Bradford [27] using soy protein calibration standards in a range from 40 to 400 μg/ml.

2.5. SDS–PAGE

SDS–PAGE was performed in slab gels (120, 220, and 0.5 mm) with 5% (w/v) acrylamide stacking gel and 13% (w/v) acrylamide resolving gel according to Lämmli [28]. Protein extracts dissolved in Tris–HCl/SDS sample buffer (pH 6.8) containing 5% (w/v) β-mercaptoethanol were boiled for 3 min. Electrophoresis was performed at 200 V for 0.5 h allowing samples to penetrate the stacking gel and 600 V constant voltage for 2 h at 15°C on a Multiphor II horizontal slab electrophoresis unit and MultiDrive XL power supply (Pharmacia, Uppsala, Sweden).

2.6. Immunoblotting

Proteins were electrotransferred from slab gels to a nitrocellulose membrane (0.2 μm, Schleicher & Schuell) at 0.8 mA/cm^2 for 40 min using a Nova-Blot semidry blotting apparatus (Pharmacia) according to Khyse-Andersen [29] with discontinuous buffer system as described by Vieths et al. [30]. Afterwards the membrane was dried for 30 min at room temperature. Immunostaining of IgE was performed by the method of Vieths et al. [30] slightly modified as described previously by Möller et al. [31].

In addition the separated proteins and a molecular mass (MW) marker (Pharmacia) were stained with

colloidal gold staining (Bio-Rad, Munich, Germany) according to Danscher and Noorgard [32].

2.7. EAST inhibition

EAST inhibition was performed using a modified assay, which based on the direct binding of proteins on the microtiter plates. For protein adsorption high binding MaxiSorp™-microtiter plates (Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 250 μl of soybean protein in 50 m*M* carbonate/bicarbonate buffer (pH 9.6) at a concentration of 10 μg/ml. Non-specific binding sites were blocked with 250 μl PBS containing 1% (w/v) BSA for 1 h at room temperature.

For inhibition experiments 50 μl of the pool-serum (diluted 1+1 in PBS, containing 1% (w/v) BSA and 0.5% (v/v) Tween 20) was added to 50 μl inhibitor proteins diluted in the same buffer and preincubated for 15 min. Dilution series in three to six steps of the extracts from the different soybean oils and lecithins were used as inhibitors. After washing the microtiter plates with PBS, containing 0.05% (v/v) Tween 20, 50 μl of the preincubated solutions were added, incubated for 3 h at room temperature and washed again. Finally specific IgE was measured using an EAST test kit (Allergopharma) following the manufacturer's instructions. Absorbance was measured at 405 nm [31].

3. Results

The protein contents of the different soy product extracts determined by the method of Bradford [27] are listed in Table 1. In additional the protein contents of the native soy products are also calculated. The soy lecithin extracts reveal similar contents of approximately 2.5 g/l. The extracts of the non-refined oils show protein contents of approximately 100 μg/l and the extracts obtained by the refined oils of approximately 35 μg/l.

Fig. 1 illustrates the colloidal gold stained proteins in the extracts of the investigated soybean oils and lecithins separated by SDS–PAGE followed by electroblotting on a nitrocellulose membrane. The MW marker protein bands ranged from 14 to 94 kDa. Whilst the electrophoretic pattern of the native soybean protein extract (6) shows a multitude of proteins with molecular masses between 14 and 94 kDa, in the extracts of the refined oils A6 and A7 (1 and 2) only a few protein bands are detectable. Three protein bands at 94, 85 and 67 kDa are slightly detectable, whilst proteins at 40, 25, 20 and 14 kDa are visible more distinctly. The extracts of the non-refined oils HT 21, HT 22 and HT 23 (3–5) reveal a very similar protein pattern. The protein extracts of the soy lecithins (7–9) with detectable protein bands at 45, 43 and 30 kDa are completely different from the soybean oils. In addition each lecithin shows a wide protein band between 20 and 14 kDa as well as a further one <14 kDa.

Table 1
Protein contents of the native soy products and the protein extracts

Product	Protein content	
	Extracts (mg/l)	Native products (mg/kg)
Soybean oil A6	47.1	0.0353
Soybean oil A7	44.3	0.0332
Soybean oil HT21	184.0	0.1380
Soybean oil HT22	119.7	0.0898
Soybean oil HT23	134.7	0.1010
Lecithin LBN 401 E3	207.8	2597
Lecithin LBN 401 E4 73/98	184.2	2303
Lecithin LBN 401 E4 75/98	215.1	2689

In order to characterize the allergens of the investigated extracts, 6 μg protein/cm was applied and separated by means of SDS–PAGE and immunoblotting. The immunostaining of the different extracts after incubation with pooled serum is illustrated in Fig. 2. Whilst in the native soybean extract various allergens with molecular masses between 20 and 67 kDa are detectable (6), no IgE binding capacity exists in the refined oils (1 and 2), whereas in the non-refined oils (3–5) proteins at 53 and 58 kDa are slightly provable. The lecithin extracts (7–9) reveal two allergen bands at 35 and 37 kDa, which are also detectable in the native soybean extract. In addition in each of the three lecithin extracts a further IgE binding protein (M_r 16 kDa) is distinctly visible.

The EAST inhibition experiments, applied to compare the IgE binding potency of the different oil and lecithin extracts, were carried out using the native soybean protein extract as a reference on the

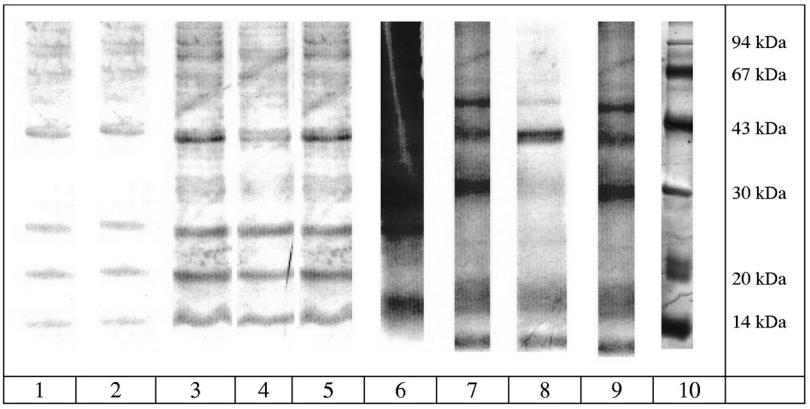

Fig. 1. SDS–PAGE-electroblotted and colloidal gold stained protein extracts. (1) A6, (2) A7, (30 HT21, (4) HT22, (50 HT23, (60 native soybean, (7) LBN 401 E4 73/98, (8) LBN 401 E4 75/98, (9) LBN 401 E3, and (10) molecular mass marker.

solid-phase and the pooled serum. To check the allergenic activity of the native soybean protein extract an additional homologous inhibition was performed. The inhibition graphs are illustrated in Fig. 3. The resulting C_{50} values of protein extracts responsible for a 50% inhibition of the IgE binding, which reflect the extracts' allergenic potencies, are listed in Table 2. Whilst the extracts of the refined oils (A6 and A7) show no inhibition, the non-refined oil extracts (HT 21–23) reach maximum inhibitions of $\leq$50%. The lecithin extracts (LBN 401 E3, E4 73/98 and E4 75/98) reveal similar C_{50} concentrations of approximately 10 mg/l. The homologous inhibition leads to a C_{50} value of 0.3 mg/l.

4. Discussion

The protein contents of 33.2 and 35.3 μg/kg in the refined soy oils determined with the method by Bradford [27] agree with the results of further studies [26,33]. A 3-fold higher protein amount was obtained in the non-refined oils. High protein contents between 2303 and 2689 mg/kg were detectable in

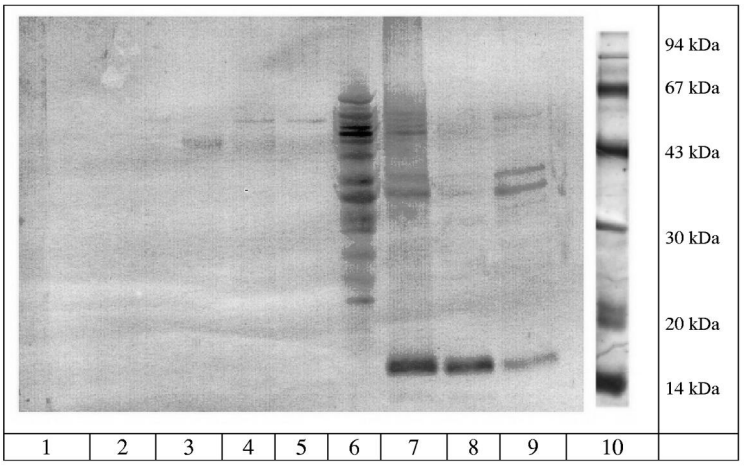

Fig. 2. Immunostaining of the protein extracts. (1) A6, (2) A7, (3) HT21, (4) HT22, (5) HT23, (6) native soybean, (7) LBN 401 E4 73/98, (80 LBN 401 E4 75/98, and (9) LBN 401 E3. The molecular mass marker (10) was colloidal gold stained.

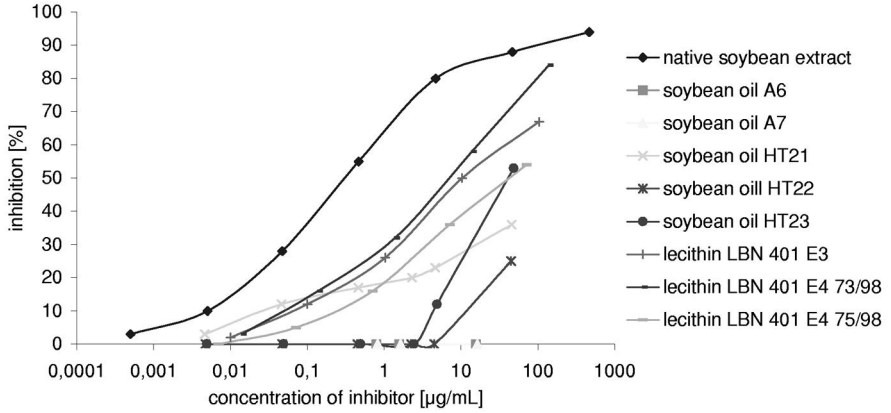

Fig. 3. EAST inhibition of the IgE binding to soybean allergen disks by soybean products. Native soybean extract was used as positive control.

the investigated soy lecithins. Müller et al. [21] determined a similar protein amount of 3100 mg/kg in a lecithin sample (Topcithin 50) from Lucas Meyer (Hamburg).

The characterization of the protein extracts by means of SDS–PAGE revealed similar protein patterns in the refined and non-refined oils, whereas in the lecithin extracts additional proteins with molecular masses between 14 and 20 kDa and <14 kDa were detectable.

While the immunoblot investigations of the refined soy oils showed no detectable proteins with IgE binding activities, allergen bands with molecular masses of approximately 53 and 58 kDa could be slightly detected in the non-refined oils. In contrast to the native soy bean extract, in the soy lecithins a further IgE binding protein with a molecular mass of 16 kDa and slight allergens <14 kDa were provable. The formation of these allergens correlate with the results of Codina et al. [20], who found neoallergens with a similar molecular mass of <14 and 16 kDa. whilst in a further study by Müller et al. [21] no neoallergens were detectable. The 16-kDa IgE binding protein can either represent a neoallergen or a degradation product of a high-molecular mass molecule containing IgE binding epitopes, which are also present on the undegraded allergen.

Table 2
C_{50} values and maximum inhibitions of the investigated soy products

Inhibitor	C_{50} (µg/ml)	Max. inhibition (%)
Native soybean	0.3	94
Soybean oil A6	–	0
Soybean oil A7	–	0
Soybean oil HT21	–	36
Soybean oil HT22	–	25
Soybean oil HT23	46.3	53
Lecithin LBN 401 E3	10.3	67
Lecithin LBN 401 E4 73/98	9.8	84
Lecithin LBN 401 E4 75/98	15.7	54

The EAST inhibition experiments verified the immunoblotting results. The protein extracts of the refined soybean oils were not able to inhibit the IgE binding activity of the native proteins, whilst the extracts of the non-refined oils caused a 53% maximum inhibition. These findings agree with the results of a study by Bush et al. [26], who determined the allergenicity of refined soybean oils in a double-blind placebo-controlled food challenge test. The investigated oils revealed no allergenic potential.

A possible explanation for the different allergenic activity of the refined and non-refined oils are the treatments applied during the refining such as the high-temperatures of approximately 250°C.

5. Conclusions

It has been demonstrated that the protein contents of the investigated soybean products strongly vary.

Whilst in the refined oils no IgE binding activities are detectable, the proteins of the non-refined oils and lecithins reveal a residual allergenic potency. In the lecithin samples the formation of a further IgE binding 16 kDa protein was provable as well.

Acknowledgements

We would like to thank Dr. K. Ragotzky, Union Deutsche Lebensmittelwerke GmbH, Germany, for kindly supplying oil and lecithin samples. Special thanks to Mrs B. Jacobsen for her technical support.

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