

Antigenic Specificity of Serum Antibodies in Mice Fed Soy Protein

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Key Words

Soy proteins · Anti-soy antibody response · Glycinin · β -Conglycinin · Immunogenicity, oral · Mice

Abstract

Background: Soybean protein is used in a number of food products but unfortunately is also a common cause of food allergy. Upon ingestion of soy protein, healthy mice like other animals and humans generate a soy-specific antibody response in the absence of signs of illness. Not much is known about the relationship between the immunogenic proteins involved in this nondeleterious antibody response and the pathological response associated with food allergy. The objective of the present study was to characterize the antigenic specificity of the soy protein-specific antibody response generated in healthy mice ingesting soy protein. **Methods:** Blood from mice fed a soy-containing diet was analyzed using ELISA and immunoblot for antibody reactivity towards various soy protein fractions and pure soy proteins/subunits. Mice bred on a soy-free diet were used as controls. **Results:** The detectable antigenic specificity of the serum antibodies of soy-consuming mice comprised glycinin and

β -conglycinin. Immunoblots with soy protein extract demonstrated antibody reactivity towards both the basic and the acidic chains of glycinin and the β -conglycinin subunits with an individual response pattern among mice. Moreover, antibody reactivity was found towards the native quaternary structure of glycinin. **Conclusions:** Mice ingesting soy protein generate an antibody response with reactivity towards glycinin and β -conglycinin. Antibody reactivity found towards the native quaternary structure of glycinin indicates an oral immunogenicity of the highly processing-resistant oligomerized glycinin.

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Introduction

Under healthy conditions, the immune system reacts towards innocuous dietary antigens by inducing antigen-specific systemic nonresponsiveness, termed oral tolerance, whereas under pathological conditions, such as allergy, it is believed that a dysregulation in the mechanisms of oral tolerance induction prevails [1]. Nevertheless, as observed in both humans and animals, ingestion of certain dietary proteins tends to provoke a weak antibody response even under normal conditions [2–4]. According to our own observations and the literature, inges-

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tion of processed soybean protein induces soy protein-reactive serum antibodies in different animals, including mice, and also in humans [5–9]. Moreover, different animals, especially calves, consuming inadequately processed soybean protein are consistently subject to hypersensitivity reactions [10]. These facts substantiate a distinct oral immunogenic capacity of soy protein under healthy conditions while soy protein is also capable of inducing an allergic response [11, 12].

The main allergens of soybeans include the major seed storage proteins 11S glycinin and 7S β -conglycinin, the oil body-associated Gly m Bd 30K (30 kD) as well as the proteins Gly m Bd 28K (26 kD) and profilin (14 kD) [13–15]. 11S glycinin and 7S β -conglycinin account each for about one third of the total seed protein [16]. Native glycinin is a hexamer with a molecular mass of 320–375 kD composed of two trimers each consisting of three nonidentical subunits formed by a $\sim$ 40-kD acidic (A) and a $\sim$ 20-kD basic (B) chain linked by a single disulfide bond. β -Conglycinin is a trimer of different combinations of the three subunits α' (71 kD), α (67 kD) and β (50 kD).

In general, not much is known about the factors that render an antigen orally immunogenic or the relationship, if any, between the antigenic immunogenicity causing a nondeleterious response and the antigenic immunogenicity causing a pathological response like food allergy. However, knowledge of the normal immune response towards dietary proteins, including identification and characterization of the proteins involved, may be advantageous for understanding food allergy and intolerance. Even so, only very few studies have focused on the antigenic specificity of the serum antibodies frequently elicited against soy protein under healthy conditions. In previous studies, we found that in mice having ingested soy protein a soy-specific antibody response was induced despite concomitant induction of oral tolerance to the soy proteins, the latter observed as a significantly suppressed specific antibody and cellular response to systemic priming with soy protein in soy-ingesting mice compared to mice bred on a soy-free diet (unpubl. results). Since the antibody response occurs in parallel with oral tolerance, it appears to be a harmless antibody response generated under healthy conditions. The present study was undertaken in order to identify and characterize the specific soy proteins involved in this non-deleterious antibody response generated in healthy mice upon ingestion of soy protein.

The results showed that the antibody response generated against soy protein was mainly reactive towards the 11S storage protein glycinin and the 7S storage protein β -conglycinin. Antibody reactivity was found towards the

native trimeric and hexameric forms of glycinin showing that native oligomerized glycinin, also known to be allergenic, is highly stable during processing and possesses an orally immunogenic capacity in soy meal.

Materials and Methods

Mice

BALB/c mice used for studying the soy protein-specific immune response were fed a standard chow for rodents containing approximately 10% defatted soybean flakes originating from oil extraction processing (Altromin 1324, Brogaarden, Gentofte, Denmark). Mice used as controls were second generation mice bred on a commercial soy-free diet (Altromin 1320, Brogaarden). Altromin 1320 was, however, found by ELISA to contain trace amounts of Kunitz soy trypsin inhibitor (KSTI, a soy protein marker, 1–2 μ g/g) occurring due to inevitable cross-contamination with soy-containing products during production. Therefore, mice were bred on a complete soy-free laboratory-produced diet based on casein as the only source of protein. These mice were used for the final studies involving immunoblot analysis. Mice were kept under standard animal housing with feed and water ad libitum. The guidelines formulated in 'The Council of Europe Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' were followed. All animal studies were approved by the Danish Animal Experiments Inspectorate. Fifty-microliter blood samples from 6- to 7-week-old mice were collected from the retroorbital plexus and immediately diluted 1:16 in phosphate-buffered saline containing 0.1% Triton X-100 (PBST; 0.01 M, pH 7.4). To obtain serum samples, blood drawn by cardiac puncture of anesthetized mice was centrifuged (10 min, 7,000 g) and the resulting serum fraction was collected. All samples were stored at -20°C until analysis.

Preparation of Soy Protein Extract

Defatted soybean flakes of the same source as used in Altromin 1324 were kindly provided by Brogaarden (Gentofte, Denmark). The soybean flakes were finely milled using a laboratory mill. Three hundred milligrams of the milled powder was suspended in 5 ml ammonium hydrogen carbonate buffer (0.1 M, pH 8.5) and placed in an ultrasound bath for 10 min followed by incubation for 30 min at room temperature while shaking. The suspension was centrifuged (15 min, 1,300 g) and the pellet was resuspended in 3 ml distilled water and centrifuged again. One more time, the pellet was resuspended in 5 ml water, incubated overnight at 4°C while shaking and then centrifuged. The supernatants from the three centrifugations were pooled and filtered (0.45- μ m sterile filter). The protein concentration was determined by amino acid analysis as described elsewhere [17].

Preparation of Acidic Supernatant

Soy protein extract prepared as described above was adjusted to pH 5.0 by slowly adding 6 M HCl and then incubated for 40 min at 4°C with stirring for the precipitate to develop [18]. After centrifugation (15 min, 1,300 g), the supernatant (termed acidic supernatant), containing mostly low-molecular-weight proteins, was collected and stored at -20°C until use. The protein concentration was determined by amino acid analysis [17].

Preparation of Glycinin Fraction

A soy protein fraction enriched in glycinin was prepared after a modified procedure of Moreira et al. [19]. Milled powder of defatted soybean flakes was suspended as 10% (w/v) in extraction buffer (0.4 M NaCl, 0.035 M KH_2PO_4 , 0.01 M β -mercaptoethanol; pH 7.6) and incubated for 1 h at room temperature. After centrifugation (30 min, 1,500 g), the precipitate was discarded and 70 ml of supernatant was dialyzed against 2 times 1 liter of dialysis buffer [0.035 M KH_2PO_4 , 0.01 M dithiothreitol (DTT); pH 6.6] at 4°C for 2 times 24 h, respectively. The dialyzed material was centrifuged (30 min, 1,500 g) and the pellet, containing a glycinin-enriched precipitate, was resuspended in 70 ml of extraction buffer. The solution, subsequently termed the glycinin fraction, was stored at -20°C until use. The protein concentration was determined by absorbance measurement at 280 nm using an extinction coefficient of 8.1 (1% solution, 1 cm light path) [20].

Gel Filtration of Glycinin Fraction

A Superdex™ 200 column (150 ml/100 cm, cutoff: 600 kD) was equilibrated with phosphate buffer (0.035 M KH_2PO_4 , 0.4 M NaCl, 0.001 M β -mercaptothiopropanoic acid, pH 7.6) and 1.5 ml of the glycinin fraction was loaded onto the column. The gel filtration was run at room temperature using a GradiFrac Apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden) with a flow of 0.5 ml/min. Two-milliliter fractions were collected and stored at -20°C until use.

Determination of KSTI and Bowman-Birk Inhibitor by ELISA

KSTI and Bowman-Birk inhibitor (BBI) in soy protein extract, acidic supernatant and glycinin fraction and also extracts of the Altromin diets were determined by sandwich ELISA assays using monoclonal antibodies produced in our laboratory. MaxiSorb microtiter plate wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with anti-KSTI or anti-BBI and subsequently, wells were incubated for 1 h at room temperature with KSTI or BBI standards or samples 2-fold serially diluted in PBST. Next, wells were incubated with biotinylated anti-KSTI or biotinylated anti-BBI in PBST followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (Dako, Glostrup, Denmark) diluted 1:5,000 in PBST. Plates were developed by incubating with a 3,3',5,5'-tetramethylbenzidine/peroxide solution and the reaction was stopped after 10 min by 2 M phosphorous acid. Optical density was measured at 450 nm with 630 nm as reference. Concentrations in samples were estimated from the standard curves using KC4 software (Kineticalc, Biotek Instruments). Assay sensitivity is 15 pg KSTI/ml and 20 pg BBI/ml, respectively.

Determination of Soy-Specific IgG1 and IgG2a Antibodies by ELISA

Blood antibodies specific for the various soy protein preparations were determined by indirect ELISA. Briefly, microtiter plate wells were coated overnight at 4°C with either soy protein extract, acidic supernatant or glycinin fraction in carbonate buffer (0.05 M, pH 9.6) at 5 µg protein/ml while β -conglycinin (kindly provided by Dr. M. Hessing, TNO, Zeist, The Netherlands), soybean agglutinin (SBA; Sigma-Aldrich, St. Louis, Mo., USA), BBI (Sigma-Aldrich), KSTI (Sigma-Aldrich) and ovomucoid (Sigma-Aldrich) were coated at 1 µg protein/ml. After washing (PBST diluted 1:10), 100 µl/well of serially diluted blood sample was added and the plate was incubated for 1 h at room temperature. Thereafter, the plate was washed and incubated 1 h further at room temperature with rabbit anti-mouse IgG1

or IgG2a (Zymed, San Francisco, Calif., USA) diluted 1:1,000 in PBST. Bound antibodies were detected by incubation with HRP-conjugated swine anti-rabbit immunoglobulin (Ig; 1:1,000 in PBST, Dako). For total Ig detection, an HRP-conjugated rabbit anti-mouse Ig (1:1,000 in PBST, Dako) was used directly after incubation with samples. Plates were developed and measured as described above. Titer values were determined as $\log_2$ to the dilution giving an absorbance of 0.2. Positive controls consisting of blood from antigen-immunized mice were included in all ELISA runs.

SDS-PAGE and Immunoblotting

SDS-PAGE of soy proteins was carried out using 10–20% tricin gradient gels (Novex, Invitrogen, Groningen, The Netherlands). Samples were mixed 1:1 with sample buffer [0.1 M Tris, 8% (w/v) SDS, 24% (v/v) glycerol, 0.025% (w/v) Coomassie blue, 0.04 M DTT; pH 6.8] and boiled for 5 min. Fifteen micrograms of protein of the soy protein extract and acidic supernatant, 20 µg of the glycinin fraction, 2 µg of KSTI and 10 µg of BBI were loaded (20 µl/well).

Before subjection to SDS-PAGE, the glycinin fraction was desalted by filtering in Millipore centrifuge tubes (cutoff: 10,000 kD; Millipore, Bedford, Mass., USA) where ammonium hydrogen carbonate (0.1 M) was added until diluted 15 times. The solution was evaporated until dryness in a vacuum centrifuge before it was redissolved in sample buffer. One milliliter of selected fractions from the gel filtration was dialyzed against 1 liter of ammonium hydrogen carbonate (0.15 M) for 24 h whereupon the solution was evaporated until dryness and redissolved in sample buffer. Electrophoresis was carried out for 1 h 20 min at 125 V (constant).

After electrophoresis, the gel was either stained with Coomassie blue or blotted onto a nitrocellulose membrane (0.45-µm pores, 10 × 8 cm) for 1 h at room temperature (0.75 mA/cm²) using CAPS buffer [10 mM CAPS in 10% (v/v) methanol, pH 11.0]. The membrane was blocked for 15 min in TBST buffer [0.05 M Tris, 0.03 M NaCl, 1% (w/v) Tween 20, pH 10.3]. The membrane was cut into strips, which were incubated overnight at room temperature with serum samples diluted 1:10 to 1:50 in TBST buffer. The strips were washed 3 times for 10 min with TBST buffer and then incubated with alkaline phosphatase-conjugated rabbit anti-mice Ig (1:5,000, Dako) for 1 h at room temperature. The strips were washed again and developed for 30 min in the dark with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrate. Selected strips with soy protein extract, glycinin fraction or molecular marker were incubated overnight at room temperature with citrate buffer (0.05 mM, pH 3.0) and then stained for approximately 30 min with freshly prepared colloidal gold sols solution [21].

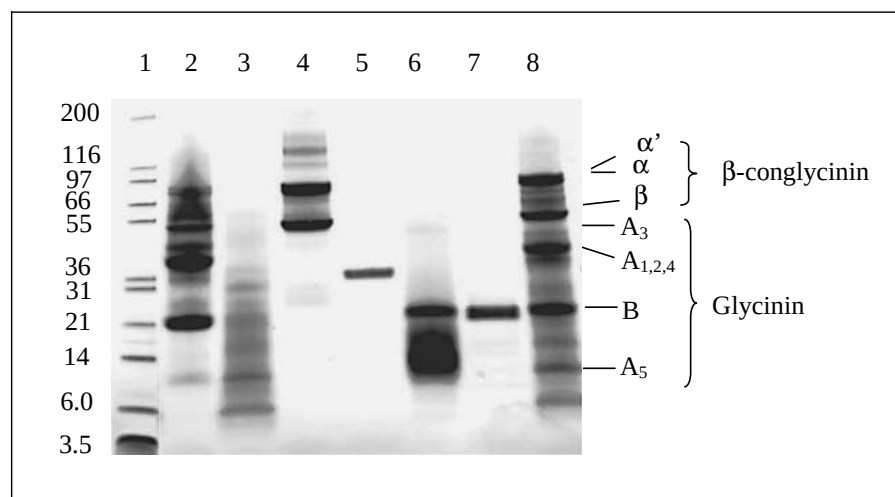
Results

SDS-PAGE of Soy Protein Preparations

In order to pin down the antigenic compounds of the orally immunogenic soy protein, soy protein was fractionated to be used for coating in ELISA. The soy protein used was of the same source as that in the diet, namely defatted soybean flakes from oil extraction processing.

Two soy protein fractions, sought to be partly complementary, were made: one enriched in glycinin (glycinin

Fig. 1. SDS-PAGE of the various soy protein preparations reduced by DTT using 10–20% tricin gradient gel stained with Coomassie blue. 1 = Molecular mass standards (kD); 2 = glycinin fraction; 3 = acidic supernatant; 4 = β -conglycinin; 5 = SBA; 6 = BBI; 7 = KSTI; 8 = soy protein extract. The marked identification of subunits is according to molecular size and thus only tentative.

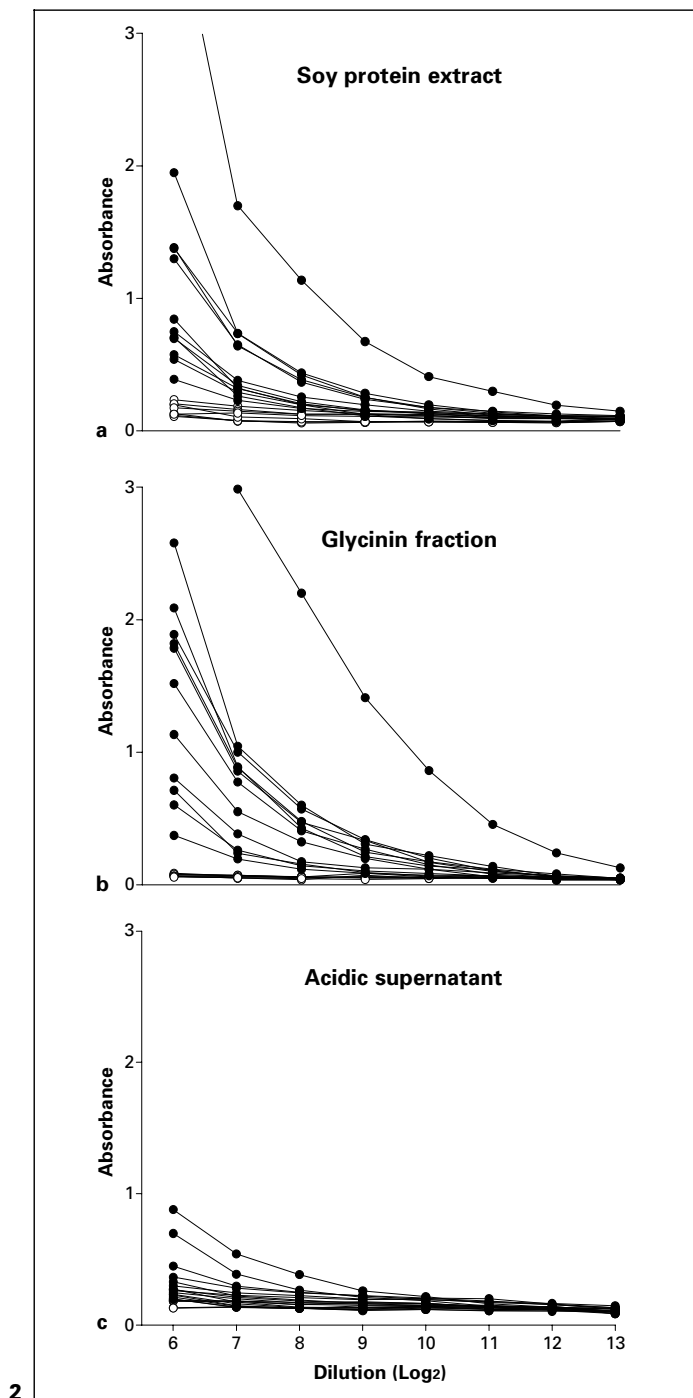


fraction) and one containing mostly low-molecular-weight proteins (acidic supernatant). The composition of these fractions and the unfractionated soy protein extract was examined by SDS-PAGE (fig. 1). The soy protein extract showed clear bands of β -conglycinin, glycinin and low-molecular-weight proteins; according to molecular size, the bands at ~ 10 , ~ 31 , ~ 38 and $40\text{--}45$ kD correspond to the various A chains (A_5 , A_4 , A_{1-2} , and A_3 , respectively) and the band at ~ 20 kD to the B chains of glycinin, whereas the 60- to 75-kD band corresponds to the α' - and α -subunits and the ~ 50 -kD band to the β -subunit of β -conglycinin. The glycinin fraction clearly showed enrichment of the glycinin A and B chains; yet it showed a certain level of β -conglycinin and other contaminants ranging between 50 and 75 kD (e.g. lipoxygenase). Nevertheless, most of the low-molecular-weight proteins were removed and by ELISA analysis, the amount of KSTI and BBI was found to be reduced approximately 250 and 10 times, respectively, as compared to the soy protein extract on a dry basis (soy protein extract: ~ 11 μg KSTI/mg protein and ~ 1 μg BBI/mg protein). In contrast to the glycinin fraction, the SDS-PAGE of the acidic supernatant revealed the presence of mostly low-molecular-weight proteins with no evidence of β -conglycinin and only a modest amount of glycinin. In this fraction, the KSTI and BBI content was increased approximately 2.5 and 3 times, respectively. The SDS-PAGE of purified soy proteins revealed a contamination of the BBI with KSTI, while β -conglycinin, KSTI and SBA were found to be quite pure.

Serum Antibody Reactivity with Soy Proteins

Blood samples from the soy-fed mice were analyzed by ELISA for antibody reactivity towards the soy protein extract, the soy protein fractions and some purified soy proteins. Blood samples from a second generation of mice fed a soy-free diet were used as control mice. When analyzing for soy protein extract-specific total Ig, a substantial level of unspecific binding blurring the specific response was seen. This background could, however, be greatly reduced by restricting detection to the IgG1 or IgG2a antibody subclass, which both were clearly present in the soy-specific antibody response (data not shown). Normally IgG1 forms the majority of the IgG subclasses and, accordingly, IgG1 showed the highest intensity to soy protein extract. We thus confined the subsequent analyses to IgG1 detection. All assays responded highly with positive controls of hyperimmune serum.

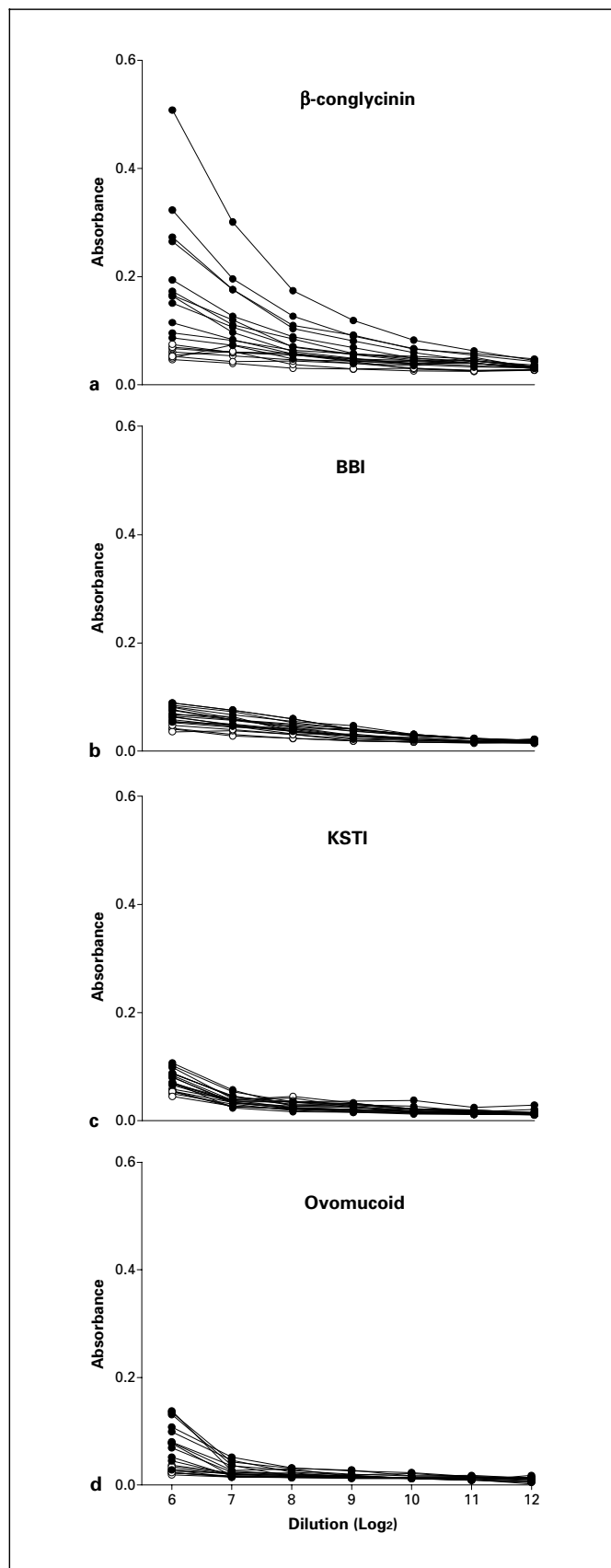
Soy protein-fed mice showed a clear IgG1 reactivity towards soy protein extract ranging from very high responders to individuals with very low or no detectable specific IgG1 (fig. 2a). Almost all individuals displayed a considerable IgG1 reactivity against the glycinin fraction (fig. 2b) reaching a higher response level than for the soy protein extract, whereas only very few mice displayed a weak reactivity against acidic supernatant (fig. 2c). The mice exhibiting a low reactivity to soy protein extract also exhibited a low reactivity to the glycinin fraction. The few mice exhibiting a weak reactivity to the acidic supernatant all had high reactivity to the glycinin fraction and the acidic supernatant reactivity may thus relate to the small amount of glycinin still present in the acidic supernatant. Titration curve patterns for total Ig reactivity against the



2

Fig. 2. ELISA titration curves of IgG1 antibodies specific for soy protein extract (a), glycinin fraction (b) and acidic supernatant (c) in blood from mice fed a soy-containing diet (●, n = 12) or control mice fed a soy-free diet (○, n = 8), the latter representing unspecific background.

Fig. 3. ELISA titration curves of IgG1 antibodies specific for β -conglycinin (a), BBI (b), KSTI (c) and ovomucoid (irrelevant control protein) (d) in blood from mice fed a soy-containing diet (●, n = 12) or control mice fed a soy-free diet (○, n = 8), the latter representing unspecific background.



3

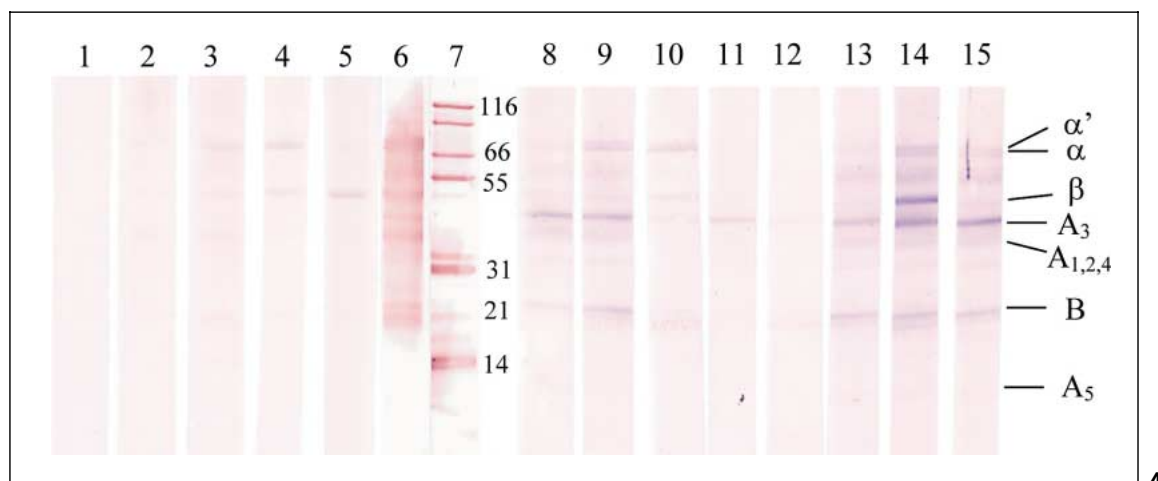
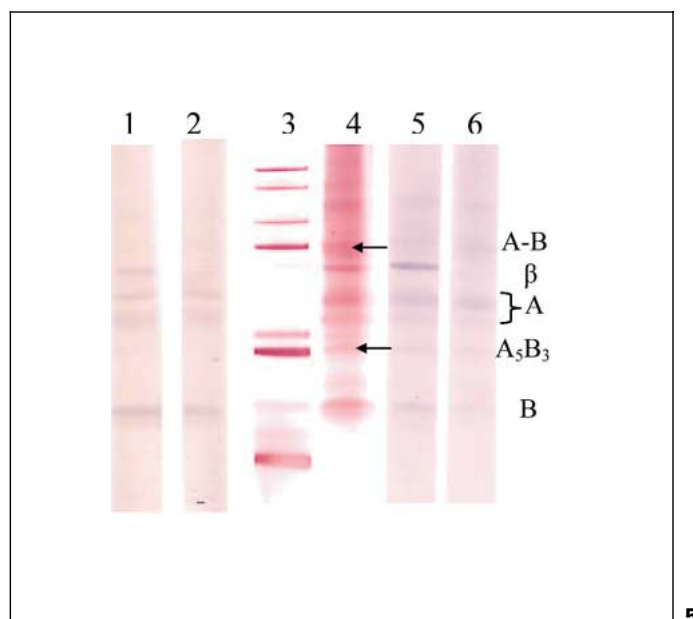


Fig. 4. Immunoblot of transferred soy protein extract incubated with representative serum samples from soy-fed mice diluted 1:20 or 1:50 (strips 2–5 and 8–15, respectively). One representative serum sample (diluted 1:20) of nine samples from control mice fed a soy-free diet appears in strip 1. Proteins from SDS-PAGE using 10–20% tricin gradient gels were blotted onto nitrocellulose membranes (1 h). Alkaline phosphatase-conjugated rabbit anti-mouse Ig was used for detection. Strip 6 and 7 are gold-stained blotted soy protein extract and molecular marker, respectively. The marked identification of subunits is according to molecular size and thus only tentative.

Fig. 5. Immunoblot of transferred glycinin fraction protein incubated with serum from 2 representative mice fed a soy-containing diet. SDS-PAGE was performed with 10–20% tricin gradient gels under reducing condition (strip 1 and 2) and under nonreducing condition partially leaving glycinin subunits intact (strip 5 and 6) and proteins were transferred to nitrocellulose membranes for 1 h as described in Materials and Methods. Strips were incubated with serum diluted 1:10. Alkaline phosphatase-conjugated rabbit anti-mouse Ig was used for detection. Gold-stained blot of molecular marker is shown in strip 3 whereas strip 4 shows gold-stained blot of soy protein extract (reduced). Bands containing unreduced subunits are indicated with an arrow.



glycinin fraction closely resembled those of the IgG1 (data not shown).

Likewise, blood samples were tested for reactivity to purified soy proteins, including β -conglycinin, BBI, KSTI (fig. 3a–c), and SBA; the titration curves for SBA resembled those of BBI and KSTI displaying no detectable reactivity (data not shown). Background binding evaluated by testing against the irrelevant egg protein ovomucoid (fig. 3d), and also other irrelevant proteins not present in the diet (data not shown) showed a slightly elevated level for soy-fed mice compared to the control mice. Nonetheless, the IgG1 reactivity of the soy-fed mice towards β -conglycinin was considerably beyond this background yet much below the response towards the soy protein extract

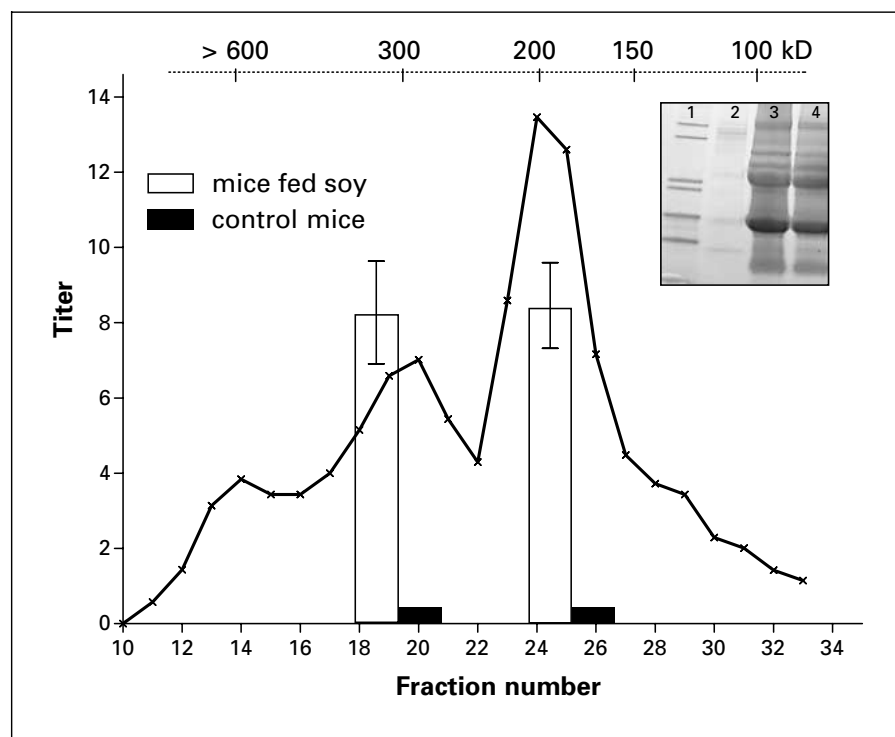
and the glycinin fraction (fig. 2). The reactivity pattern among mice to β -conglycinin did not follow that of the glycinin fraction.

Immunoblotting

To substantiate the ELISA results and to map in more detail the antigenic compounds of soy protein, immunoblotting was performed with soy protein extract and serum from mice fed a soy-containing diet ($n = 17$) and mice bred on a soy-free diet (control, $n = 9$), the latter showing no binding (fig. 4).

Seven out of the 17 soy-fed mice displayed only faint or no detectable antibody binding (represented by strip 2 in fig. 4), which is in agreement with the portion of low-

Fig. 6. Antibody reactivity of soy-fed mice towards the native structures of glycinin separated by gel filtration. Glycinin fraction was separated using a Superdex™ 200 column (elution buffer: 0.035 M KH₂PO₄, 0.4 M NaCl, 0.001 M mercaptopropionic acid, pH 7.6) and 2-ml fractions were collected. The elution curve is depicted as glycinin concentrations with a maximum of 62 µg/ml in fraction 24 using the extinction coefficient 8.1 (1%, 1 cm). Selected fractions (18 + 19 and 24 + 25, hexameric and trimeric glycinin, respectively) were used as coating antigen in ELISA and titer values for specific IgG1 reactivity were determined for blood samples from soy-fed mice (□, n = 9) and from control mice (■, n = 8) depicted as bars. Error bars indicate SD. Insert shows the SDS-PAGE of collected fractions: 1 = molecular weight marker (14, 21, 31, 36, 55, and 66 kD); 2 = fraction 14; 3 = fraction 20; 4 = fraction 24.



responding mice found in the ELISA analysis (different group of animals).

The mice displaying antibody binding showed, in general, binding to the various chains of both glycinin and β -conglycinin with a binding pattern that, however, differed somewhat among the mice. Some mice showed binding to both of the β -conglycinin bands ($\alpha + \alpha'$ and β), other mice only to one or the other chain, and yet other mice exhibited no significant binding to β -conglycinin. Nearly all of the antibody-positive mice exhibited binding to glycinin A chains with A₃ being the most evident. Likewise, binding to glycinin B chains seemed common among mice. Having the same molecular weight (~20 kD), KSTI and glycinin B chain antibody reactivity is not distinguishable. In immunoblot with the glycinin fraction, containing only very low amounts of KSTI, the ~20-kD band was however still evident for all mice confirming reactivity to the B chains (representative results in fig. 5, strip 1 and 2). Binding to a ~55-kD band observed in some individuals most probably relates to a residue of unreduced glycinin subunits.

Immunoblots using SDS-PAGE of the glycinin fraction run under nonreducing conditions leaving disulfide bond-linked A-B subunits intact were used to verify glycinin antibody reactivity. For example, 2 mice, recognizing

bands at ~20, ~38 and 40–45 kD in the blot of reduced glycinin fraction (fig. 5, strip 1 + 2), displayed additionally binding to a ~30-kD band appearing in the blot with nonreduced glycinin fraction corresponding to the subunit A₅B₃ (fig. 5, strip 5 + 6). Moreover, the decreased occurrence of glycinin B chains in the blot of nonreduced glycinin weakened the binding to the ~20-kD band accordingly, whereas the band at ~55 kD containing glycinin A-B subunits was increased in the SDS-PAGE in parallel to a slightly increased binding of antibodies to the blotted band.

Antibody Reactivity to the Native Structures of Glycinin

We further studied the presence of and antibody reactivity to the native structure of glycinin. The glycinin fraction proteins were separated by gel filtration, whereby the quaternary structures were kept intact. The eluted proteins formed two principal peaks corresponding to molecular weights of ~180 and ~320 kD, respectively (fig. 6). SDS-PAGE of fractions from each of these peaks demonstrated identical protein compositions of the two peaks (insert of fig. 6). Thus, these peaks appear to represent the trimer and hexamer form of glycinin, respectively, showing that the native form of glycinin is present in the soy

protein. The minor peak emerging at >600 kD (V_0) was ascribed to insoluble particulate material as only trace amounts of proteins were seen in SDS-PAGE fractions from this peak. Using fraction 18 + 19 and 24 + 25, representing the hexamer and trimer form of glycinin, respectively, for ELISA coating, IgG1 reactivity to either of the two conformations was examined for 9 soy-fed mice and 8 control mice (fig. 6). The results showed that the soy-fed mice had high specific antibody reactivity towards the native structures of glycinin.

Discussion

In order to study the oral immunogenicity of soy protein, we characterized in the present study the antigenic specificity of the soy-specific antibodies elicited spontaneously in mice fed a commercial diet containing soybean meal. In previous studies, we found that despite such antibody production, the mice still had normal oral tolerance induction to the soy proteins and thus the antibody response is not related to a hypersensitivity reaction (unpubl. data). In only a portion of the mice (more than one half) a detectable soy protein-specific antibody response could be found. By ELISA and immunoblot analysis, these antibodies were found to be reactive primarily towards the 11S and 7S soy globulins glycinin and β -conglycinin, respectively, both of which also constitute main allergens implicated in IgE and non-IgE-mediated hypersensitivity reactions [13–15]. No antibody reactivity towards the small soy inhibitors KSTI and BBI or to SBA was detected. Although protein conformation changes during coating for ELISA and variation in protein transfer properties in immunoblot could affect the detected antibody reactivity pattern, these data clearly indicate that glycinin and β -conglycinin constitute the major antigens of the orally immunogenic soy meal protein under investigation in the present study.

The antigenic specificity of the antibodies generated towards ingested soy protein in healthy individuals has been given only very little attention. Consistent with our findings in mice, Johnston et al. [8] found in lambs, with no adverse reactions to soy, high antibody responses to glycinin.

Resistance to processing and digestive stability affecting the mode of exposure of the food antigen to the gut immune system are factors considered important for establishing a food protein as an allergen and thus may also be involved in the general oral immunogenicity of a protein [22, 23]. Both glycinin and β -conglycinin are

inherently heat stable and resistant to proteolytic cleavage [24]. Their solubility and digestibility have been demonstrated to correlate with the extent of protein denaturation [25–27]; however, studies of variously heat-treated (with or without humid) soybean meals have shown that complete protein denaturation is rarely achieved and a nonsoluble and nondigestible residue of aggregated native compounds always remains [27, 28]. Therefore, we were interested in elucidating whether native glycinin were present in the soybean meal ingested by the mice and whether antibody reactivity of the mice was directed against native glycinin. The presence of native quaternary hexameric and trimeric conformations of glycinin in the soybean meal was demonstrated by gel filtration of a glycinin-enriched fraction prepared from soybean meal using β -mercaptoethanol and DTT to enhance solubilization of aggregates. This indicates, in accordance with the literature, an incomplete denaturation of the glycinin quaternary structure in the heat-treated soybean meal. By ELISA analysis, serum antibodies from the soy protein-fed mice showed a high reactivity towards this native oligomerized glycinin showing that these structures indeed are antigenic compounds of the soybean meal that are exposed in their intact form to the immune system. As opposed to this, the antibody reactivity against pure native β -conglycinin in ELISA was only modest. The structural stability of glycinin and β -conglycinin justifies the assumption that these molecules are stable during coating for ELISA and, thus, that the ELISA analysis using native proteins for coating reflects antibody reactivity to the native structure. Moreover, hyperimmune serum from immunized mice showed high reactivity in all of the ELISAs confirming coating efficiency. Hence, the tendency of the antibodies of soy-fed mice to react only modestly with β -conglycinin in the ELISA while showing clear bands in the immunoblot suggests that merely the subunits of β -conglycinin and not the native quaternary structure account for the antigenicity of β -conglycinin.

Glycinin and β -conglycinin differ largely in digestibility as regards kinetics, locations and extent of digestion, and aggregation of partially digested products and subunits [24], all important factors for absorption and immune response towards the proteins. Especially the higher number of intra- and intermolecular disulfide bonds in glycinin than in β -conglycinin and differences in the subunit hydrophobicity affecting the pattern of aggregation are believed to explain the differences in digestibility and thus potentially explain the distinct antigenicity observed in the present study [24, 28, 29]. It remains, however, to be elucidated if glycinin and β -conglycinin are inherently

orally immunogenic with a matrix effect simply enduing the proteins with conformational stability, or if the proteins actually acquire an immunogenic capacity only when interacting with the matrix; the latter includes formation of aggregates and interaction of protein with compounds such as saponins and protease inhibitors reducing digestibility [30] or lectins with adjuvant effect [31, 32]. In this regard, studies on the immune response towards ingested soluble native forms of glycinin or β -conglycinin as well as the insoluble aggregated residue of heat-treated soybean meal would be of interest. Likewise, studies on the capacity of other plant proteins such as peanut protein to induce an antibody response when fed as part of the normal diet would be of interest to understand oral immunogenicity of allergens.

Turning focus to the antibody binding to the different subunits and chains of glycinin and β -conglycinin, we found, although hampered somewhat by relatively low levels of soy-specific antibodies in the mice sera, that the antibody reactivity included both the basic and the acidic chains/subunits. This is consistent with studies reporting high reactivity of IgE from soy-sensitive individuals to both glycinin A and B chains [15, 33, 34] although, in other cases, mostly the A chains were found to be allergenic [14, 35]. If the overall immunogenicity of soy proteins is a shared causative factor between the mechanism of induc-

tion of the nonallergic and allergic antibody response, the identity of the antigenic and allergenic epitopes represents an interesting issue for further research.

Although serum antibodies to dietary antigens generated under healthy conditions do not appear to be of direct clinical significance, our data taken together, in addition to those of others, suggest that the same soy proteins as those known to be allergic are also involved in the nonallergic orally induced antibody response. This indicates that, at least partly, the same immunogenic properties are in action during induction of the two different immune responses. When more knowledge is obtained about the role of general oral immunogenicity conditions in the allergenic potential of a protein, characterization of nonallergic antibody responses may be a useful supportive tool in predicting the allergenic potential of a soy protein-containing product including genetically modified soy protein as well as differently processed soy.

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