Effect of Thermal and Proteolytic Processing on Glycinin, the 11S Globulin of Soy (*Glycine max*): A Study Utilizing Monoclonal and Polyclonal Antibodies

Geoffrey W. Plumb, E. N. Clare Mills,* Marcus J. Tatton, Christopher C. M. D'Ursel, Nigel Lambert, and Michael R. A. Morgan

Department of Food Molecular Biochemistry, AFRC Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K.

Glycinin-specific monoclonal antibody (Mab) IFRN 0025 and polyclonal anti-glycinin antiserum have been used in various enzyme-linked immunosorbent assay (ELISA) formats to follow the immunoreactivity of purified glycinin during thermal and proteolytic treatments. Glycinin T, a well-defined stable intermediate of glycinin trypsinolysis, exhibited 10 times the immunoreactivity of the native globulin with IFRN 0025, whereas more rigorous proteolysis destroyed Mab recognition entirely. Heating glycinin to 80 °C has no effect on immunoreactivity as determined by a two-site ELISA; when glycinin was heated further, recognition declined by about 50%. At temperatures above 92 °C reactivity increased sharply. The two-site ELISA was also used to determine glycinin immunoreactivity in a range of industrially processed soy flours and concentrates. Defatting substantially reduced glycinin immunoreactivity, suggesting this widely used procedure significantly affects glycinin conformation. In general, immunoreactivity was reduced by heat and/or protease processing, although several samples showed enhanced immunoreactivity. Furthermore, flour samples allegedly subjected to the same processing regime displayed markedly different immunoreactivities. Our data also demonstrated how ELISA format can influence detectable immunoreactivity; some flours with very high reactivities in the two-site ELISA display no activity in a direct ELISA. The data presented are discussed in view of the need for quality control of soy processing and the possibilities offered by immunological techniques.

INTRODUCTION

Soy protein has important nutritional and functional roles in food. In general, soy is processed prior to consumption, the edible oil being extracted before toasting, which destroys residual antinutritional factors such as lectins and trypsin inhibitors (Lambert and Yarwood, 1992). Processing (involving heat and protease treatment) is also used to remove the allergenic determinants which cause hypersensitivity reactions in preruminant calves and piglets fed diets containing untreated soy (Stokes *et al.*, 1987; Sissons and Pedersen, 1991). In addition to destroying unwanted components, processing of soy is used to improve food functionality properties, such as foaming, gelling, emulsifying power, and increased solubility between pH 4 and 7 (Wright and Bumstead, 1984).

As around 80% of the protein present in soy flour is in the form of seed storage globulins (this proportion rising to 90% of the protein present in an isolate), much of the effort to improve the nutritional and functional quality of soy has focused on these proteins (Wright and Bumstead, 1984). Glycinin is one of the major globulins, making up almost 60% of total globulins. Also known as 11S globulin on the basis of its sedimentation coefficient (Derbyshire *et al.*, 1976), glycinin comprises a heterogeneous mixture of polypeptides and has a structure common to other legume 11S globulins, being a hexameric oligomer ($M_r \approx$ 360 000) with a subunit molecular weight of around 60 000. There have been at least five genes identified that code for the subunits in glycinin (Nielsen *et al.*, 1989), which are synthesized as single chains. These are then post-translationally nicked to give an acidic polypeptide, A ($M_r \approx 40\,000$), and a basic polypeptide, B ($M_r \approx 20\,000$) which are disulfide bonded together to form an individual subunit.

Investigating the effects of modifying soy globulins in functional or nutritional terms has often been hampered by a lack of detailed data on the changes induced at a molecular level. Immunological methods offer a means of obtaining such information by virtue of the fact that antibodies can recognize not only the amino acid sequence of a protein but also its folded state (van Regenmortel, 1989). Such conformational epitopes (also known as discontinuous epitopes) can be disrupted by subjection to heat and proteolysis, thereby altering antibody recognition. The potential of antibodies for monitoring changes in the three-dimensional structure of a protein was recognized many years ago in the study of the structure of myoglobin with polyclonal antibodies (Crumpton, 1966).

Such antibody-based methods offer a novel means of studying food protein structure and investigating the effects processing may have on that structure. However, it is only recently that monoclonal antibody probes for food proteins such as glycinin have become available (Carter *et al.*, 1992). The characterization of one of these antibodies is reported here. The binding of anti-glycinin antibodies to glycinin modified by heat and proteolytic treatments has been investigated, together with the effects of industrial-scale treatments used to modify the properties of soy ingredients.

MATERIALS AND METHODS

Materials. Soy (*Glycine max*) meal used for preparation of purified glycinin and glycinin T, bovine serum albumin (BSA), anti-mouse IgG–horseradish peroxidase conjugate, and Tween 20 were all purchased from Sigma Chemical Co., U.K. Foetal calf serum (FCS), Optimem, and NUNC Immunoplate I microtitration plates were obtained from Gibco Europe, U.K. Substrate for ELISA assays, based on 3,3′,5,5′-tetramethylben-

* Author to whom correspondence should be addressed.

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zidine, was supplied by Cambridge Veterinary Sciences, U.K. The Titertek Multiskan MCC plate reader and the Titertek Multiplate Washer 120 were purchased from Flow Laboratories Ltd., U.K. Soy flours were obtained from a food manufacturing company and had been either heat-treated at 90 °C or subjected to a combination of protease and heat treatments. The precise details of the treatments, performed at pilot scale, were not available. Two untreated flours were provided, one "full fat" and the other defatted; all treated samples derived from defatted flours. Samples of soy flours and concentrates manufactured for use as food and feed ingredients were the kind gifts of several European companies. All materials had undergone different forms of processing to improve their allergenic/functional properties, although the precise nature of the processing was not disclosed by the manufacturers. All other reagents were of AR grade and were obtained from BDH Ltd., U.K., unless otherwise stated.

Antibody Preparations. Rabbit polyclonal antiserum $R103b_3$ was produced against degraded glycinin as described by Carter *et al.* (1992) and was found to recognize both acidic and basic polypeptides of glycinin by immunoblotting. Mab IFRN 0025 from rat was obtained as described by Carter *et al.* (1992) and used in the form of culture supernatant after growing hybridomas in either Dulbecco's modified Eagle medium supplemented with 20% FCS or Optimem containing 4% FCS.

Preparation of Glycinin and Glycinin T. Glycinin was isolated by hydroxyapatite–Ultrogel (Sigma) chromatography of a cryoprecipitated soy extract according to the method of Eldridge and Wolf (1967). The purified protein was adjudged to be >95% pure from SDS–PAGE analysis.

The purification of glycinin T was performed using the method of Plumb *et al.* (1989) for the preparation of legumin T, employing a Superose 6 HR 10/30 column (13 × 300 mm, Pharmacia-LKB, U.K.) on a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB). Glycinin T was isolated from smaller breakdown products (see Figure 2) and subsequently dialyzed exhaustively against water and lyophilized prior to analysis by inhibition ELISA.

Proteolysis of Glycinin and Gel Filtration Analysis. A sample of glycinin (5 mg/mL) was subjected to tryptic and chymotryptic digestion for 60 min at 25 °C in 50 mM Tris-HCl buffer, pH 8.2, containing 0.2 M NaCl with a sample:enzyme ratio of 40:1. After the incubation period, an aliquot (200 μL) was removed from the reaction mixture and immediately analyzed by FPLC gel filtration chromatography, employing a Superdex 75 (HR 10/30) column (13 × 300 mm, Pharmacia-LKB) equilibrated in the above Tris-HCl buffer. The flow rate was 0.4 mL/min throughout. Individual fractions were dialyzed exhaustively against distilled water and lyophilized prior to analysis by direct ELISA.

Solubilization of Protein from Soy Food Ingredients. Soy food ingredients (flours and concentrates) were extracted by stirring sample (1 g) in 10 mL of 50 mM Tris-HCl, pH 8.2, for 1.5 h at room temperature. Samples were then diluted either in PBST for analysis by the two-site ELISA or in coating buffer for analysis by the direct ELISA.

Thermal Treatment of Glycinin. Thermal treatment of glycinin was performed in two ways:

(*1*) A sample of glycinin (1 mg/mL solution) in 50 mM Tris-HCl buffer, pH 8.2, containing 0.2 M NaCl, was placed in a water bath at 100 °C for 10 min. Then the sample was removed and diluted with coating buffer to a concentration of 1 μg/mL and analyzed with the direct ELISA.

(2) A solution of glycinin (2 mg/mL) in phosphate buffer (32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4 pH 7.6, $\mu \approx 0.1$), was heated at a rate of 2 °C/min in a heating block (Ericomp Twinblock system, Laser Laboratory, U.K.). At intervals of 0.5 or 1 min, 40 μL of glycinin solution was withdrawn and immediately diluted with 0.96 mL of PBS (0.14 M NaCl, 0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.003 M KCl), pH 7.4, containing 10 g/L BSA. The immunoreactivity of the diluted glycinin was then determined using the two-site ELISA.

Enzyme-Linked Immunosorbent Assays (ELISAs). (*1*) *Direct ELISA.* Microtitration plates were coated with each sample protein at 1 μg/mL in 0.05 M sodium carbonate–bicarbonate coating buffer for 16 h at 4 °C, using the inner 60 wells only. The first two columns of 12 wells were coated with intact glycinin and the remaining 3 × 12 wells with three different glycinin fractions or samples. In this way a series of directly comparable titer curves were obtained on a single microtitration plate, and therefore antibody cross-reactivity could be calculated relative to that of intact glycinin. Plates were then washed five times in PBST (PBS supplemented with 0.5 mL/L Tween 20), dried in air, and stored at −20 °C until required. Mab IFRN 0025 culture supernatant was diluted serially in PBST and 0.2 mL per well of each dilution added in duplicate. Plates were then incubated either for 2 h at 37 °C or for 16 h at 4 °C before a further five washings with PBST. After 0.2 mL per well of anti-rat IgG labeled with horseradish peroxidase (diluted 1:1000 v/v, in PBST) was added, plates were incubated for 2 h at 37 °C. Finally, plates were washed five times with PBST before 0.2 mL per well of ELISA substrate was added and incubated for 20 min at 37 °C. The color development was stopped by the addition of 50 μL per well of 2 M H_2SO_4 and the absorbance of each well measured at 450 nm.

(*2*) *Inhibition (Competitive) ELISA.* Glycinin was used to coat microtitration plates, as described above. A serial dilution of intact glycinin or glycinin T was prepared in PBST, giving solutions ranging from 1 ng to 1 mg/mL of protein. Each sample or buffer alone was then added in triplicate (0.1 mL per well) to the coated microtitration plate. IFRN 0025 (0.1 mL per well) diluted 1:1000 (v/v) in PBST was then added to each well and the plate incubated for 3 h at 37 °C. Plates were then developed as described previously for the direct ELISA.

(*3*) *Two-Site ELISA.* A two-site (or sandwich) ELISA was developed using IFRN 0025 as the capture antibody, with the rabbit polyclonal antiserum $R103b_3$ as the detector. Microtitration plates were coated for 16 h at 4 °C with 0.2 mL per well of IFRN 0025 culture supernatant diluted 1:1000 (v/v) in coating buffer. After coating, plates were washed three times with PBST, dried in air at room temperature, and stored at −20 °C for no longer than 4 weeks. Glycinin standards were diluted in PBST from a 2 mg/mL stock solution in 50 mM Tris-HCl, pH 8.2, to give a final range of 10 μg–0.1 ng/mL. Standards, samples, or PBST alone was added in triplicate (0.2 mL per well) to coated plates which had been washed three times in PBST prior to use. Plates were then developed as described for the direct ELISA but using anti-rabbit IgG labeled with horseradish peroxidase as the second antibody. Statistical analysis of standard curves was performed using Immunofit Software (Beckman Instruments, High Wycombe, U.K.).

Protein Assays. The concentration of protein in solution was determined according to the method of Lowry *et al.* (1951) using BSA as a standard.

RESULTS

Three different ELISA formats (direct, inhibition, and two-site) were used to investigate the immunoreactivity of purified glycinin after proteolytic and heat treatment. The direct and two-site assays were then used to monitor changes in glycinin reactivity in manufactured soy food ingredients.

Proteolytic and Thermal Treatments of Purified Glycinin. Purified glycinin was proteolyzed with either trypsin or chymotrypsin, and the resulting polypeptides and peptides were separated by FPLC Superdex gel filtration (Figure 1). Similar profiles were obtained with both proteases. Substantial material eluted in the void volume (fraction 1), fraction 2 had an approximate M_r 18 000, and there were several low abundancy species (fractions 3–8) possessing M_r's < 5000. Chymotrypsinolysis produced a predominating M_r 2000 species (fraction 5). All of the above Superdex fractions were analyzed for their immunoreactivity with IFRN 0025 by direct ELISA (Figure 1 inserts). Only fraction 1, the void volume material, possessed any substantial immunoreactivity. This fraction was analyzed further by Superose 6 gel filtration (Figure 2) and found to be almost entirely composed of a M_r 280 000 species; no intact glycinin was observed. This species eluted in exactly the same place as glycinin T, a well-characterized stable intermediate of

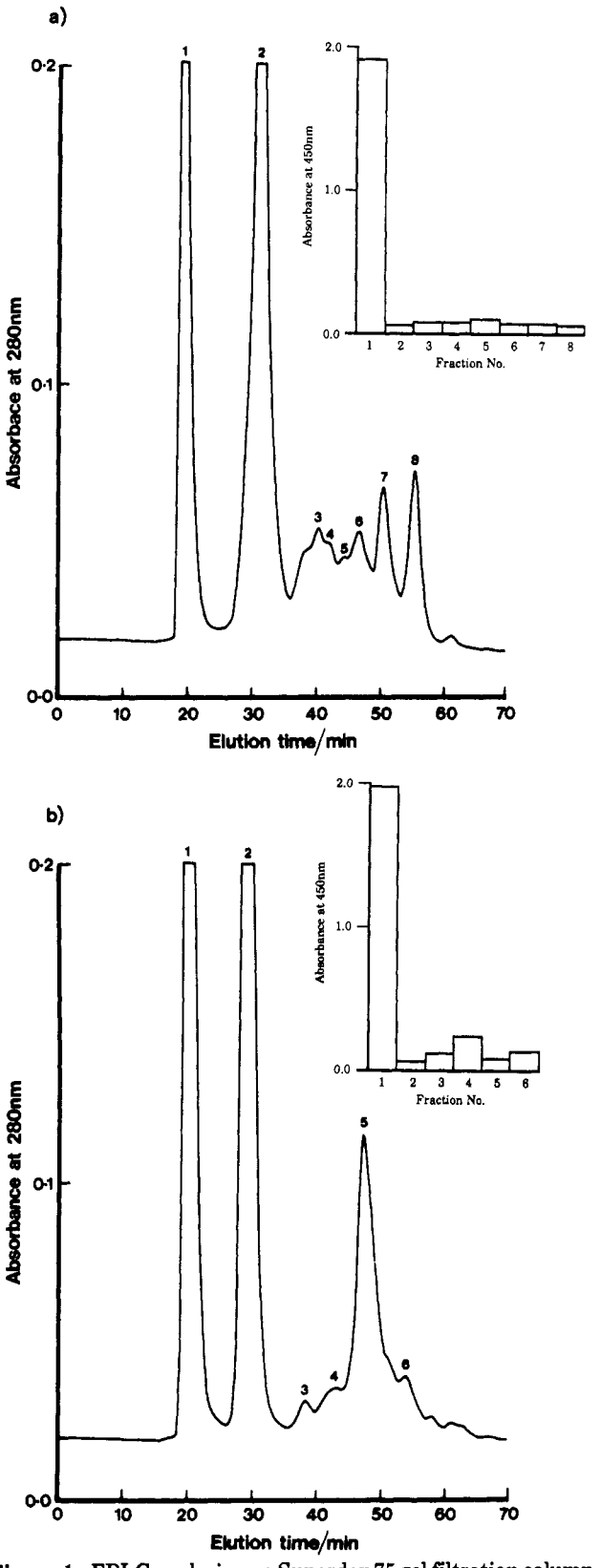

Figure 1. FPLC analysis on a Superdex 75 gel filtration column equilibrated in 50 mM Tris-HCl buffer, pH 8.2, containing 0.2 M NaCl of glycinin after 60 min of (a) trypsinolysis of (b) chymotrypsinolysis. The column was calibrated for molecular weight using the proteins bovine serum albumin ($M_r \sim 67\,000$), hen ovalbumin ($M_r \sim 45\,000$), horse heart cytochrome c ($M_r \sim 12\,500$), and bovine pancreas insulin ($M_r \sim 6000$). The inserts show the direct ELISA analysis of the respective fractions obtained, samples being prepared and analyzed as described under Materials and Methods.

glycinin trypsinolysis (Kamata and Shibasaki, 1978). SDS–PAGE analysis confirmed that fraction 1 was indeed

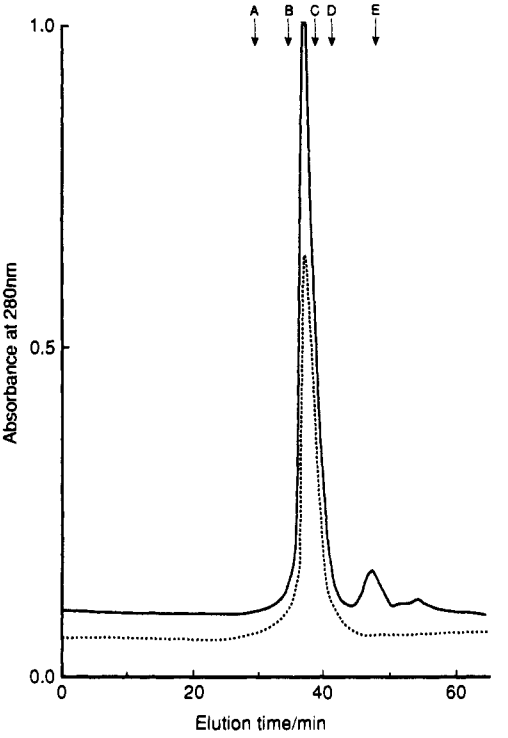

Figure 2. FPLC analysis on a Superose 6 gel filtration column, equilibrated in 50 mM Tris-HCl buffer, pH 8.2, containing 0.5 M NaCl of either (—) trypinolyzed glycinin or purified glycinin T (···). The samples were prepared and analyzed as described under Materials and Methods, and the column was calibrated for molecular weight using the following standards: A, porcine thyroglobulin, $M_r \sim 520\,000$; B, glycinin, $M_r \sim 360\,000$; C, glucose oxidose from *Aspergillus niger*, $M_r \sim 180\,000$; D, bovine serum albumin, $M_r \sim 67\,000$; E, horse heart cytochrome c, $M_r \sim 12\,500$.

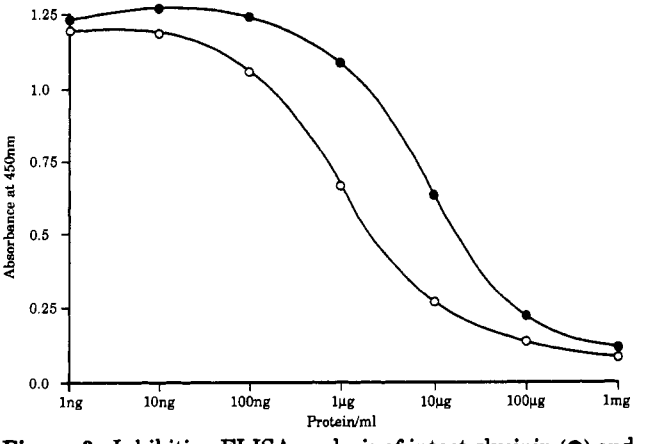

Figure 3. Inhibition ELISA analysis of intact glycinin (●) and glycinin T (○).

glycinin T (data not shown). Pure glycinin T was prepared and shown to possess 10 times the immunoreactivity of intact glycinin as determined by inhibition ELISA (Figure 3).

The effect of heat on the immunoreactivity of purified glycinin was determined. Initial studies showed that after boiling for 10 min, the reactivity of glycinin with Mab IFRN 0025 was decreased by 50% as determined by direct ELISA. In a more extensive study, purified glycinin was heated at a rate of 2 °C/min and changes in immunoreactivity were monitored throughout using a two-site ELISA. Figure 4 shows a typical standard curve obtained with this assay, which had a limit of detection of around 20 ng of glycinin/mL. Precision profile analysis indicated that the coefficient of variation was less than 5% between 20 ng and 1.5 μg glycinin/mL, this criterion being used to define the working range of the ELISA. After heating

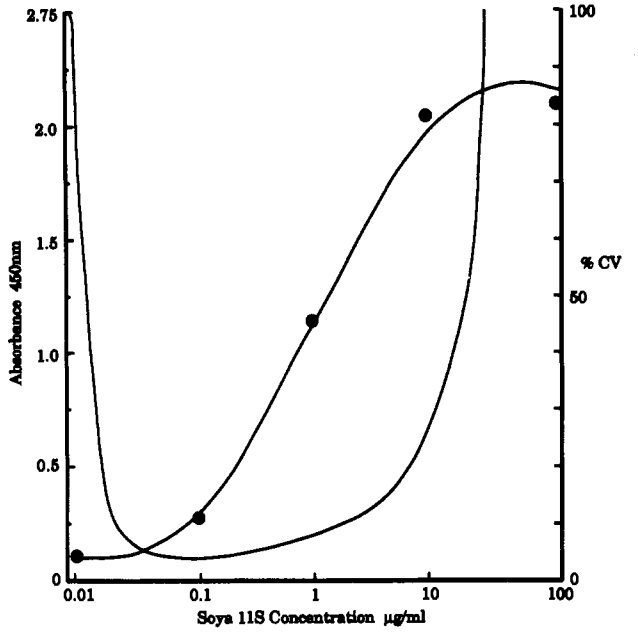

Figure 4. Standard curve (●) and precision profile (—) for a glycinin-specific two-site ELISA.

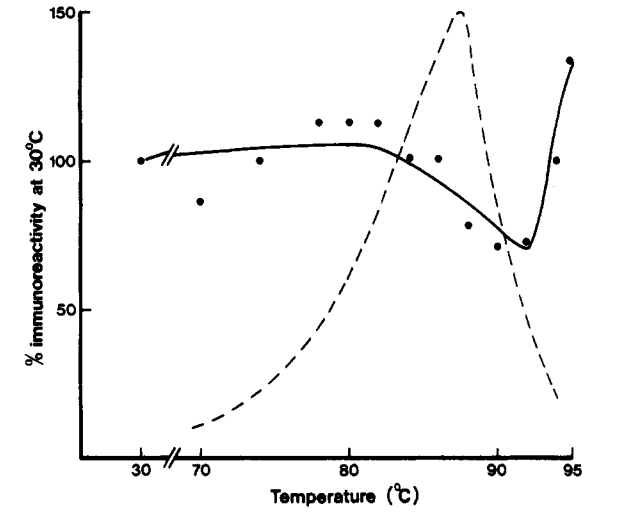

Figure 5. Effect of thermal denaturation on glycinin immunoreactivity. Immunoreactivity was determined using a two-site ELISA (—). The DSC thermogram obtained by Danilenko *et al.* (1987) under identical conditions is also shown (- - -). These results exemplify those obtained for four different experiments.

between 30 and 80 °C, glycinin immunoreactivity remained constant, but as the protein was heated further, immunoreactivity declined gradually such that at 92 °C the reaction level was around 60% of that observed at 30 °C (Figure 5). Above 92 °C, however, reactivity increased sharply and at 95 °C was 125% of that found at 30 °C. The decrease in immunoreactivity coincided with the main thermal transition observed using differential scanning calorimetry (DSC) of glycinin analyzed under identical experimental conditions (Danilenko *et al.*, 1987). There was, however, no indication of a DSC thermal transition between 92 and 95 °C coincident with increased immunoreactivity.

Thermal and Proteolytic Treatments of Soy Flours. Flours were extracted with Tris buffer and the levels of protein and immunoreactivity with IFRN 0025 (using both a direct and two-site ELISA) determined for the soluble extracts. A full-fat soy flour (U1) and a defatted flour (U2) gave differing levels of protein solubility and immunoreactivity as determined by two-site ELISA (Table 1). The soluble extract from the full-fat flour had only half the protein compared to the defatted flour, yet it possessed over 3 times the reactivity of the defatted flour on a per milligram of protein basis. In the direct ELISA, however, both extracts were recognized equally well. The soluble extracts of defatted flours subjected to apparently identical 90 °C heat treatments (H1 and H2) showed a dramatic reduction in immunoreactivity (>10-fold) in the two-site ELISA relative to U1. Sample HH, which received two 90 °C heat treatments, had a protein solubility and an immunoreactivity comparable to those of the untreated flour (U1). When a direct ELISA format was employed, H1 and H2 were both recognized as well as the untreated sample U1, while the double-heat-treated flour (HH) was not recognized.

Four defatted flours subjected to "identical" treatments of proteolysis followed by heating (PH1–PH4) had similar protein solubilities, and the resulting soluble extracts were equally as immunoreactive as U1 when assayed by direct ELISA. By two-site ELISA all had substantially reduced immunoreactivity compared to that of U1 but with a high level of variation; reactivities ranged from 3.8 to 99.4 µg/mL. Two defatted flours subjected to the same regime of heat treatment both before and after protease digestion (HPH1 and HPH2) gave very different levels of immunoreactivity in the two-site ELISA; protein solubilities were comparable. HPH1 displayed only half the immunoreactivity (on a per milliliter basis) of the untreated flour (U1), whereas HPH2 had enhanced immunoreactivity of over 10-fold. Neither sample was detected by direct ELISA.

Processed Soy Protein Ingredients. Samples of commercially available soy concentrates (C1–C3) and soy flours (F1–F5) were extracted and analyzed for glycinin immunoreactivity using the two-site ELISA and for protein solubility (Table 2).

The processed concentrates had poor protein solubilities relative to that of the untreated defatted flour (U1). The extractability of protein from concentrate C1 was much lower than for C2 and C3, yet C1 possessed 5-fold greater immunoreactivity on a per milligram of protein basis. Analysis of the treated flours (F1–F5) showed that they possessed similar protein solubilities to the concentrates C2 and C3, with the exception of F3 which had a 5-fold greater solubility. Soluble extracts of flours F1, F2, and F4 showed similar degrees of immunoreactivity on a per milligram of protein basis and were over 10-fold more immunoreactive on a per milliliter basis.

DISCUSSION

When glycinin is subjected to trypsinolysis in high concentrations (0.5 M) of salt, a "stable intermediate" is found which is referred to as glycinin T (Kamata and Shibasaki, 1978). Similarly an intermediate, glycinin C, is formed after chymotrypsinolysis. Structural analysis of these stable intermediates (Kamata and Shibasaki, 1978) has shown that the overall hexameric subunit structure of native glycinin is retained. The B-chain component of "intermediate" subunits remains intact, whereas the A-chains are reduced in size to 30 000 compared to 40 000 for native glycinin, giving glycinin T an $M_r \approx 300\,000$ as opposed to 360 000 for native glycinin (see Figure 2). The proteolytic accessibility of the A-chains and inaccessibility of the B-chains support the belief that the basic chains are buried within the interior of the protein molecule and the A-chains are more exposed (Lambert and Yarwood, 1992).

We have recently characterized a Mab (IFRN 0025) which recognizes the acidic chain of glycinin (Carter *et al.*, 1992), and from the data presented in the present paper

Table 1. Immunoreactivity of Extractable Glycinin in Treated Soy Flours[a]

soy flour	protein extracted (mg/mL)	μg of immunoreactive glycinin/mL of extract	μg of immunoreactive glycinin/mg of extracted protein	direct ELISA response[b]
untreated				
U1	63.3	1102.5	17.5	+
U2	30.3	1469.5	65	+
heat-treated				
H1	32.3	77.5	2.4	+
H2	50.3	70.4	1.4	+
HH	54.3	896.0	16.5	-
protease plus heat-treated				
PH1	37.7	3.8	0.1	+
PH2	25.1	12.6	0.5	+
PH3	29.3	79.1	2.7	+
PH4	27.6	99.4	3.6	+
heat plus protease-treated				
HPH1	49.5	490	9.9	-
HPH2	42.9	875.6	204.0	-

[a] Soluble extracts of soy flours subjected to various treatments were analyzed for immunoreactive glycinin, using two-site and direct ELISAs, and protein content. The flour treatments were as follows: U2, untreated full-fat; U1, solvent defatted flour and the starting material for all subsequent treatments; H1, H2, two different batches given a single 90 °C heat treatment; HH, double-heat-treated; PH1–PH4, four different batches given a protease followed by a heat treatment; HPH1, HPH2, two batches given heat, then a protease, followed by a second heat treatment. [b] (+) denotes the presence and (-) the absence of immunoreactivity.

Table 2. Immunoreactivity of Extractable Glycinin in Industrially Treated Soya Concentrates and Flours[a]

commercially treated soy product	protein extracted (mg/mL)	μg of immunoreactive glycinin/mL of extract	μg of immunoreactive glycinin/mg of extracted protein
treated soy concentrates			
C1	2.4	583.2	243.0
C2	9.5	478.8	50.4
C3	10.5	454.6	43.3
treated soy flours			
F1	9.5	399.0	42.0
F2	9.5	300.2	31.6
F3	59.1	4668.9	79.0
F4	10.6	439.9	41.5
F5	9.2	72.7	7.9

[a] Extracts of industrially treated soy concentrates (C1–C3) and flours (F1–F5) were analyzed for immunoreactive glycinin by a two-site ELISA and for soluble protein.

it is clear that IFRN 0025 recognizes glycinin T to a greater degree than the native globulin. This observation suggests that the epitope recognized by IFRN 0025 may be sterically obscured in the intact protein but that limited proteolysis allows the Mab greater access to its epitope. At 0.2 M salt, trypsinolysis and chymotrypsinolysis of glycinin are more extensive (Figure 1), quaternary structure is substantially destroyed, and polypeptides of 25 000 M_r and lower are produced as well as the stable intermediate. However, Mab 0025 did not recognize any of these smaller proteolytic fractions, indicating that the epitope is close to tryptic and chymotryptic cleavage sites. Thus, it is clear that the proteolysis of glycinin can have a major influence on epitope recognition, both increasing and decreasing antibody reactivity.

Heat treatment also altered glycinin reactivity, a decrease being observed between 80 and 90 °C using the two-site ELISA, indicating a reduction in epitope availability. At 90 °C, where immunoreactivity was reduced by 40%, the glycinin would be largely dissociated into component subunits (Hashizume *et al.*, 1975). The subunits would be in a denatured state, with many broken down into their component, denatured, A- and B-chains due to the vulnerability of the meta-stable interdisulfide bond linking the two chains (Staswick *et al.*, 1981). As IFRN 0025 recognizes the A-chain after SDS–PAGE and immunoblotting (Carter *et al.*, 1992), such a dissociation is unlikely to affect antibody recognition. Soluble aggregates may also be present, resulting from a coalescing of denatured subunits and/or free A- and B-chains. Thus, thermal modification of A-chain epitopes or steric hindrance due to aggregation may be responsible for the reduction in immunoreactivity. When the temperature was raised above 92 °C, glycinin immunoreactivity in the two-site ELISA was enhanced. Clearly, further changes in glycinin structure occurred such that antibody binding was increased. This contrasts with the reduction in glycinin reactivity after boiling observed with the direct ELISA. The difference probably results from conformational changes in glycinin caused by adsorption to microtitration plates in the direct format (Friguet *et al.*, 1984) masking more subtle thermally induced changes. The precise nature of these changes will probably only become clear when the immunoreactivity of IFRN 0025 with isolated soluble aggregates and subunits is investigated.

Other workers have employed immunological methods to investigate the thermal denaturation of glycinin and other 11S globulins (Catsimpoolas *et al.*, 1969; Iwabuchi and Shibasaki, 1981; Iwabuchi and Yamauchi, 1984; Quillien *et al.*, 1990). All utilized polyclonal antisera. Only Quillien *et al.* (1990) employed ELISA technology; the others used much less sensitive and less informative immunodiffusion techniques. All of the studies demonstrated that 11S globulins retain their immunoreactivity after heating, although Quillien *et al.* (1990) did show a substantial loss in immunoreactivity for pea 11S in a direct ELISA, but only after heating at 90 °C for 120 min. Thermal denaturation of soy β-conglycinin (G. W. Plumb *et al.*, 1993, unpublished results) and bovine β-lactoglobulin (Kaminogawa *et al.*, 1989) have also been shown to increase immunoreactivity with Mabs.

Mab IFRN 0025 was employed to investigate whether differences in glycinin immunoreactivity could be detected in soluble extracts of various processed soy flours and concentrates obtained from food manufacturers. Defatting flours by solvent extraction is a common process for

researchers and manufacturers alike. That more protein was extracted from the defatted flour (U1) compared with the full-fat flour (U2) is not so surprising given that the former will have a higher initial protein content. What is remarkable, however, is that immunoreactivity was far lower in the defatted flour extract despite having twice the protein concentration of the full-fat flour extract. As there is no evidence to suggest that glycinin solubility is selectively impaired by defatting, it seems logical to assume that the solvents used in the process have altered the conformation of glycinin in such a way as to reduce its immunoreactivity. This observation could have far-reaching consequences for those who utilize or study soy proteins derived from defatted flours.

Analysis of soluble extracts of heated flours showed that a single heat treatment at 90 °C reduced glycinin immunoreactivity. It therefore seems likely that the aggregation/modification of the A-chain epitope thought to underlie the reduction in pure glycinin reactivity after heating to 90 °C also occurs in heated flours. However, double heat treatment gave little overall change in soluble glycinin reactivity, possibly because it causes changes in glycinin structure similar to those responsible for the increased reactivity of pure glycinin heated above 92 °C.

Soluble extracts from four flours, allegedly treated with the same protease and heat regime (PH1–PH4) in different batches, gave widely differing immunoreactivities, as did the two flours subjected to identical heat–protease–heat treatments. The high degree of immunoreactivity observed for HPH2 may result from incomplete proteolysis, resulting in the presence of glycinin T like species. Such batch-to-batch variations indicate the problems of controlling parameters such as heating, salt concentration, pH, and effective addition of proteases at the industrial scale, all of which will affect process reproducibility. The results described in this paper, for both pure glycinin and soy samples, also emphasize how critical the choice of ELISA format is in the monitoring of such batch–batch variation. For example, some flours were not reactive using the direct ELISA (such as HH, HPH2) and yet possessed up to 10 times the reactivity of the untreated flour when analyzed using the two-site ELISA. The latter offers a quick and sensitive method for following the effects of processing on glycinin structure, avoiding the difficulties associated with a direct ELISA.

Processed soy concentrates and flours obtained from manufacturers also showed a wide variation in immunoreactivity (Table 2). All had been processed differently, and although specific details were not disclosed, it is likely that heat and/or proteolytic treatments would have been employed. Some of these (e.g., F4) were marketed as being "low in antigenicity". All except F3 had low protein solubilities relative to that of defatted flour (U1), indicative of extensive denaturation and aggregation. However, that protein which was solubilized was very immunoreactive, this being especially so for C1 which had the highest immunoreactivity per milligram of protein of any sample analyzed. Detailed compositional analysis of C1 soluble extracts would confirm whether this resulted from the presence of glycinin T like polypeptides.

Soy products are processed by manufacturers to improve their food functionality and/or their nutritional qualities. Glycinin in particular plays a central role in imparting beneficial functionality (Wright and Bumstead, 1984; Lambert and Yarwood, 1992). Nutritionally there are concerns about the "antigenicity" of soy proteins and the role glycinin may play in the etiology of the adverse reactions observed in farm animals such as preruminant calves and piglets fed soy products (Sissons and Pedersen, 1991; Tolman, 1991). There is a conventional wisdom that processing of food proteins decreases antigenicity. Our results demonstrate that while immunoreactivity (a property related to antigenicity) can indeed be decreased by processing, it may actually be increased under certain processing conditions. However, the relationship between the immunoreactivity (i.e., a measure of the recognition between antibodies and their epitopes) and the allergenicity (i.e., the ability of a substance to trigger immunologically mediated tissue inflammation and organ dysfunction of certain soy ingredients) remains to be established.

In conclusion, soy flours subjected to processing on an industrial scale can give rise to products with both increased and decreased immunoreactivity with considerable batch variation, highlighting the need for improved quality control testing. Such a need can be met by antibody-based methods of analysis such as those described in the present paper, employing specialized Mab reagents like IFRN 0025 and others specific for β-conglycinin (Plumb and co-workers, 1993, unpublished data). Elucidation of the epitope recognized by IFRN 0025 is currently in progress, information which will increase our understanding of how thermal and proteolytic treatments can modify glycinin structure. It will also be necessary to explore the relationship between protein/peptide structure and the deleterious properties of allergenicity and intolerance generated by soy as a food component. Mabs will be useful tools in this research, which may open new possibilities to plant breeders to select for improved nutritional qualities.

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