

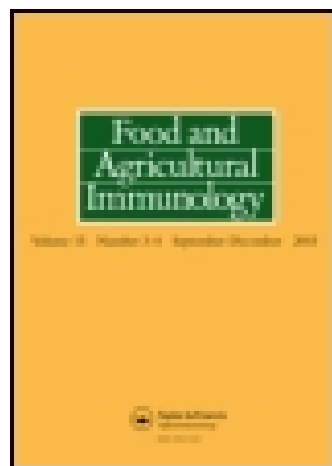
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Antigenic and Allergenic Properties of Acidic and Basic Peptide Chains from Glycinin

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The acidic and basic peptide chains from the soyabean storage protein glycinin were isolated and tested for antigenic and allergenic properties. All acidic chains reacted similarly with antibodies from rabbits raised against native glycinin, indicating a very homogeneous structure. The basic chains did not react with the antibodies in this trial. The total reaction towards the isolated chains accounted for 25% of the total reaction towards native glycinin, suggesting a relatively large proportion of conformational epitopes on the intact molecule. Similar studies using sera from soya-allergic patients showed the same reaction patterns, which indicates that no specific chain is entirely responsible for the observed adverse reactions towards soya protein.

Introduction

It has long been known that soyabeans and other vegetable proteins can cause adverse reactions in man, especially when consumed at an early age (Ratner & Crawford, 1955; Cook, 1960; Mortimer, 1961; Fries, 1966, 1971; Mendoza *et al.*, 1970; Halpern *et al.*, 1973; Halpin *et al.*, 1977; Gruskay, 1982). The specific allergens in the protein fraction from soyabeans have not been determined precisely, but several crude protein fractions have been claimed responsible for these unwanted reactions (Moroz & Yang, 1980; Shibaski *et al.*, 1980; Burks *et al.*, 1988a, b). The 2S fraction seems to contain several major allergens (Moroz & Yang, 1980; Shibaski *et al.*, 1980), but this fraction is seldom contained in commercial preparations of soya protein. The main storage proteins, glycinin and β -conglycinin, have also been shown to carry antigenic epitopes (Shibaski *et al.*, 1980; Burks *et al.*, 1988a, b).

The antigenic determinants (epitopes) on proteins are those specific areas which can be recognized by antibodies. Epitopes can be categorized in two separate groups. Conformational epitopes depend on at least a partially 'native' 3D structure, while sequential epitopes are still able to be recognized by their corresponding antibodies after disruption of the native protein structure. In this case, it is the linear amino-acid sequence that is being recognized by the antibodies. When analysing proteins for

antigenic or allergenic epitopes in more detail, fragmentation of the protein is very often necessary. Hereby, most of the conformational structure will be lost, and consequently sequential epitopes are the most likely to be preserved and recognized by the majority of applied analytical methods.

In this study we have attempted to characterize the antigenic and allergenic properties of glycinin, the main storage protein in the soyabean. The isolated subunit chains were investigated as a first attempt to identify the specific antigenic and allergenic epitopes on the molecule.

MATERIALS AND METHODS

Patients

Sera were obtained from 10 atopic patients, 11–36 years of age, who showed positive RAST values against soya. All patients had a well-documented history of adverse reactions to soya, and some had reactions against other legumes as well. Whether this indicates a cross-reaction or a true multi-allergic reaction was not proven. Bernhisel-Broadbent & Sampson (1989) have recently shown that cross-reactions are not very frequent within the legume family. A pool of sera from several hundred non-allergic individuals were included as a negative control, to ensure that no observed reaction was caused by non-specific binding of IgE.

Purification of Glycinin

Glycinin was prepared essentially as described by Moreira *et al.*, 1979. Commercially defatted soya flour was extracted with buffer A (0.035 M-KH₂PO₄, pH 7.6 containing 0.4 M-NaCl, 0.02% NaN₃ and 0.01 M-β-mercaptoethanol) by stirring for 1 h at room temperature. The suspension was centrifuged at 15 000 g for 30 min at 4°C and dialysed against two shifts of 10 l of 0.035 M-KH₂PO₄, pH 6.6 containing 0.02% NaN₃ and 0.01 M-β-mercaptoethanol. To remove glycoproteins the preparation was affinity-purified by passing it down a 35×1 cm column of Con-A sepharose, equilibrated and eluted with PBS (phosphate buffered saline: 0.4 M-NaCl, 0.032 M-K₂HPO₄, 0.0026 M-KH₂PO₄, 0.0015 M-NaN₃, pH 7.6). As a final separation of glycinin from other contaminating molecules the preparation was passed over a 90×2.5 cm sephadex G-200 gel filtration column, equilibrated and eluted with buffer A.

Purification of Acidic Subunits from Glycinin

Following the procedure of Moreira *et al.* (1979) glycinin dissolved in buffer A was dialysed against two shifts of 10 l dialysis buffer (0.1 M-(NH₄)HCO₃, pH 8.1) at 4°C. Guanidinium hydrochloride was added up to 6 M and the pH adjusted to 8.5 before addition of dithiotreitol up to 10 mM. The reaction mixture was stirred for 1 h at room temperature. The reaction was stopped by the addition of iodoacetamide to 40 mM and incubation for 1 h. The material was dialysed against two shifts of 10 l dialysis buffer at 4°C and freeze-dried. The reduced and carboxymethylated glycinin was redissolved in 20 ml 0.1 M-K₂HPO₄/KH₂PO₄, pH 6.6, containing 0.02 M-β-mercaptoethanol and 6 M-urea and separated on a 40×2.5 cm DEAE-sephadex column equilibrated with the same buffer. Elution was effected with a linear gradient from 0 to 0.4 M-NaCl in the same buffer. Fractions were dialysed against two shifts of 10 l dialysis buffer at 4°C and freeze-dried.

This procedure resulted in the purification of subunits A_{1a}, A_{1b}, A₂, A₃ and A₄, defined using the nomenclature of Nielsen (1984).

Preparation of Rabbit Antisera

Three rabbits were each immunized with 0.56 mg purified glycinin dissolved in 50% (v/v) Freund's Complete Adjuvant in PBS. Injection with the same amount of antigen was repeated every three weeks, and rabbits were bled initially after six weeks and then one week after the following injections. After coagulation, the serum samples were centrifuged and immunoglobulins purified as described by McKinney & Parkinson (1987).

ELISA Assays

A direct ELISA (enzyme-linked immunosorbent assay) was carried out to measure the total antigenicity of different samples. A dilution of antigen, either native glycinin or isolated subunits, from 2000 to 1 ng/ml in carbonate buffer (0.015 M-Na₂CO₃, 0.035 M-NaHCO₃, pH 9.6) was coated on microtitre plates (4-39454, 96F, Tecnunc, Denmark) for 1 h at room temperature. Rabbit antiserum raised against native glycinin was used for detection. Diluted at 1:2000 in PBST (phosphate buffered saline with Triton: 0.5 M-NaCl, 0.0027 M-KCl, 0.0015 M-KH₂PO₄, 0.0065 M-Na₂HPO₄, 1% Triton X-100, pH 7.6), the antiserum was incubated on the plates for 1 h. After thorough rinsing, detection was carried out using a 1:2000 dilution of peroxidase labelled pig anti-rabbit antiserum (P217, DAKO Ltd, Denmark) with orthophenylenediamine (2 µg/ml in citrate-phosphate buffer: 0.038 M-citric acid, 0.067 M-Na₂HPO₄, pH 5.0) as substrate. Between each incubation the plates were washed four times in PBST.

A capture ELISA was used to evaluate the effect on antigenicity that coating of the antigen to the microtitre plates could create. Rabbit anti-glycinin antiserum was coated on the plate for 1 h at room temperature after dilution at 1:2000 in carbonate buffer. Antigen samples, native glycinin or isolated subunits were applied to the plates as a dilution in PBST and incubated for 1 h at room temperature. The amount of bound antigen was detected by using the same rabbit anti-glycinin antiserum, but this time biotin-labelled (Wilchek & Bayer, 1988) and diluted 1:2000 in PBST. Detection with peroxidase-labelled avidin (P347, DAKO) was performed after dilution to 1:10 000.

A competitive ELISA was carried out after initial coating of 2 µg/ml antigen in carbonate buffer on the microtitre plates. The competing sample antigen was incubated overnight with the antiserum, and these complexes were then incubated on the plates coated with antigen. Detection of bound antiserum was carried out using peroxidase-labelled pig anti-rabbit antiserum.

Reaction with patient sera were carried out essentially as described in the direct ELISA assay. Native glycinin or isolated subunits were coated on microtitre plates after dilution to 2 µg/ml in carbonate buffer. After incubation for 1 h at room temperature, patient serum samples diluted 1:100 in PBST were applied to the plates and incubated for 1 h at room temperature. Detection was carried out with rabbit anti-human IgG (A090, DAKO) or rabbit anti-human IgE (A094, DAKO) antiserum, diluted 1:2000 in PBST and subsequently with either peroxidase-labelled pig anti-rabbit antiserum (IgG) or biotin-labelled pig anti-rabbit antiserum (IgE) (E353, DAKO) diluted 1:2000 followed by peroxidase-labelled avidin diluted 1:10 000.

RESULTS AND DISCUSSION

Isolation of Subunits

The acidic subunits isolated by the method of Moreira *et al.* (1979) were identified using SDS-PAGE as A_{1a}, A_{1b}, A₂, A₃ and A₄ using the nomenclature of Nielsen (1984).

These subunits can be placed in two separate groups based on their homologous primary structure.

The general structure of a glycinin protomer is a dimer, made up of one acidic and one basic peptide chain covalently bound by one disulphide bond. With the exception of A₄, there is a substantial homology between the acidic subunit chains. Including the basic chains, two homology groups can be formed. A_{1a}B₂, A_{1b}B_{1b} and A₂B_{1a} comprises one group and A₃B₄ and A₄A₅B₃ makes up the other. Homology is 85–90% within groups and approximately 50% between groups. A₄ is somewhat different from the rest of the acidic subunits in a number of ways. A₄ in the assembled molecule is not covalently attached to any basic chain. In the protomer, A₄A₅B₃, A₅ and B₃ are covalently bound and non-covalently associated with A₄. In addition the primary structure of A₄ is homologous with A₂ from residue 100 onwards (Nielsen, 1984).

Antigenicity of Isolated Subunits

The isolated subunits all showed approximately the same degree of reaction with rabbit-anti-glycinin antiserum. As shown in Figure 1 the dilution curves are very similar with the exception of that for subunit A_{1b} which reacts slightly more than the other subunits, but not as much as unfractionated glycinin. The reaction using biotin-labelled subunits as antigens in an equivalent analysis is illustrated in Figure 2. In this assay the dilution curve of A₄ was different, and had a much steeper slope. This might

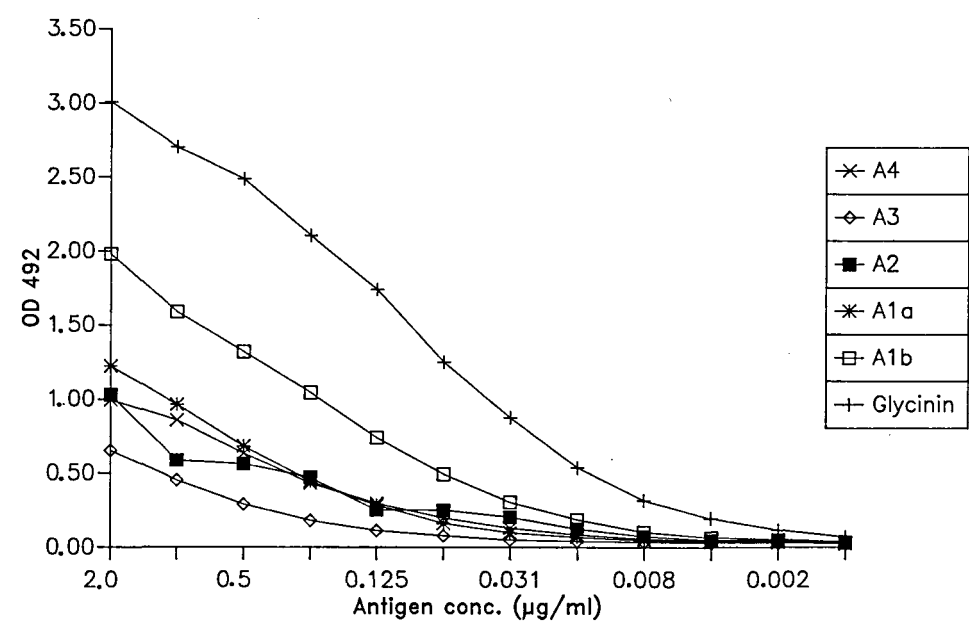


FIG. 1. Direct ELISA using acidic subunits as coating antigen. The isolated acidic subunits and native glycinin were coated on the microtitre plates and detected with rabbit anti-glycinin antiserum and peroxidase-labelled pig anti-rabbit antiserum.

TABLE 1. Antigenic homologies (% inhibition) as estimated from competitive ELISA

Competing antigen	Coating antigen					
	Glycinin	A1b	A1a	A2	A3	A4
Glycinin	60	62	67	71	67	76
A1b	0	85	78	83	80	65
A1a	0	83	77	82	72	53
A2	0	85	80	86	77	57
A3	6	76	74	78	81	81
A4	0	68	73	73	82	93

Isolated acidic subunits, glycinin and BSA were coated on microtitre plates as well as preincubated with rabbit anti-glycinin antiserum. The complexes formed were transferred to the microtitre plates for final incubation. Detection was carried out using peroxidase-labelled pig anti-rabbit antiserum. Percentage inhibition is calculated by comparison with the inhibition showed by buffer.

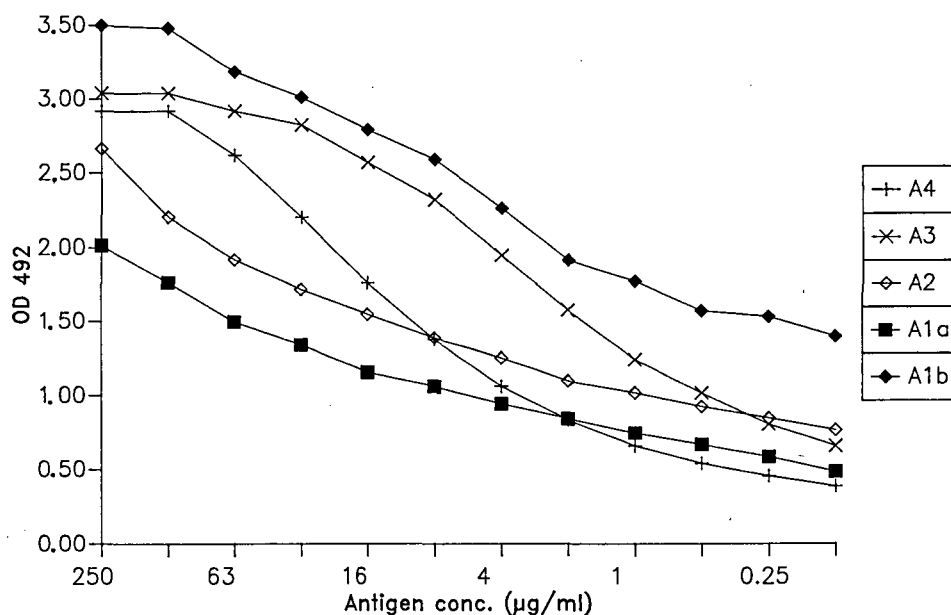


FIG. 2. Capture ELISA for detection of biotin-labelled acidic subunits. Rabbit anti-glycinin antiserum, diluted 1:2000, was coated on the microtitre plate and dilutions of biotin-labelled acidic subunits were added. Detection was carried out with peroxidase-labelled avidin.

indicate a different affinity between the subunit and the applied antiserum. The reaction of A_{1b} was higher in this assay, but did not differ significantly from the other subunits. These results indicate that no specific subunit contains major epitopes, but A₄ may be slightly different from the others concerning epitope structure. This

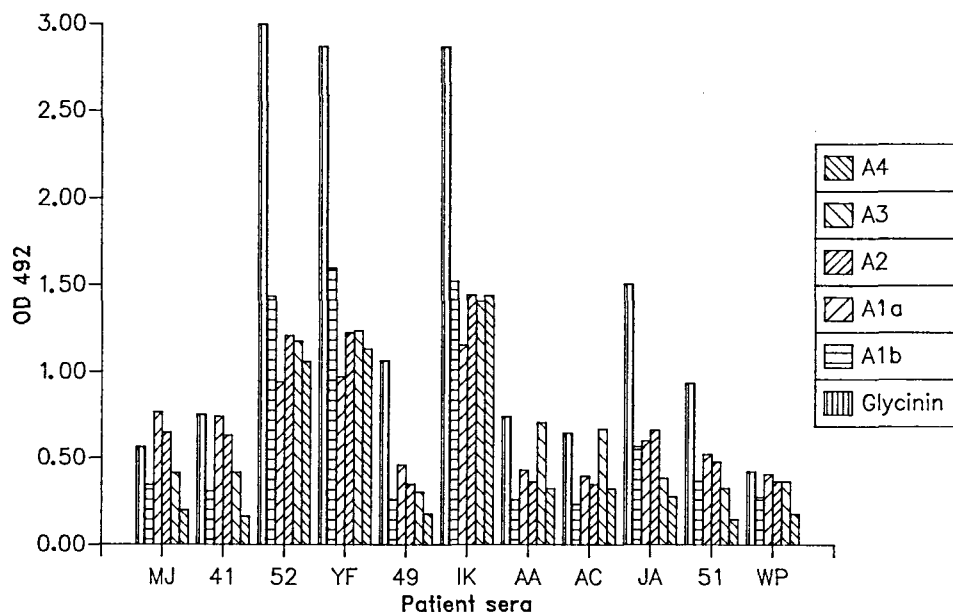


FIG. 3. Direct ELISA for detection of patient sera IgG. The isolated acidic subunits and glycinin were coated on microtitre plates. Patients sera, diluted 1:100, were added and incubated with all fractions. Detection was carried out using rabbit anti-human IgG antiserum and peroxidase-labelled pig anti-rabbit antiserum. Groups on the x-axis represent the individual patient sera, while the columns each represent the coated antigen.

correlates well with the slightly different structure of A_4 as compared with the other subunits.

A control for non-specific binding was performed using BSA as antigen and no reaction was detected in the ELISA assays (results not shown).

Analyses using a competitive ELISA assay to evaluate the degree of homology between the individual acidic subunits indicate very high degree of homology. As shown in Table 1 all acidic subunits are able to compete with each other for binding to the antiserum. Using the same level between bound and free antigen a level of inhibition around 80% is detected, but in a few cases a somewhat smaller inhibition is observed. This is the case with subunit A_4 . Using this subunit as coating antigen, A_4 itself is able to inhibit the binding nearly to completion (93%), but native glycinin, A_{1b} , A_{1a} and A_2 only show inhibition in the range 53–76%. As shown in Figure 2, the antigenic structure of A_4 seems to be slightly different from the other subunits and from native glycinin as well. Subunit A_3 inhibits the binding to A_4 by 81% and this is significantly higher than for the other subunits. As mentioned later, the homology between subunits is high for A_4 and A_3 and somewhat lower between A_4 and the rest. Native glycinin, on the other hand, is only capable of inhibiting the binding to itself by 60% and to the other subunits in the range 62–76%, binding significantly different from that of the isolated subunits. This suggests that several epitopes co-exist on the

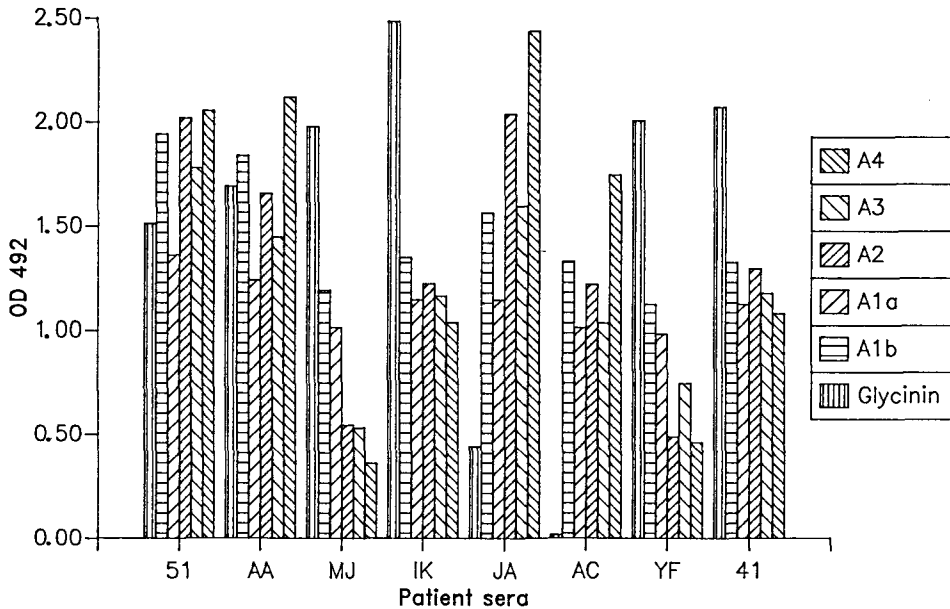


FIG. 4. Direct ELISA detecting patient sera IgE. The isolated acidic subunits and glycinin were coated on microtitre plates. Patient sera, diluted 1:100, were added and incubated with all fractions. Detection was carried out using rabbit anti-human IgE antiserum followed by biotin-labelled pig anti-rabbit antiserum and peroxidase-labelled avidin. Groups on the x-axis represent the individual patient sera, while the columns each represent the coated antigen.

surface of the intact molecule. It therefore requires a higher competing epitope concentration to inhibit the binding than is the case with the isolated subunits, where a lower number of epitopes facilitates the specific inhibition. Again, the degree of inhibition showed by native glycinin is highest with A₄ as coating antigen. This suggests that A₄ carries fewer epitopes than the other subunits. A₄ probably contains one or more epitopes common to the other acidic subunits, indicated by the inhibition potential of this fraction. But the fact that A₄ is less efficient in inhibiting the other subunits, indicates that they do contain at least one additional epitope, probably located within the first 100 residues of their primary structure.

No single subunit could significantly displace the binding between native glycinin and the antiserum as shown in Table 1. This indicates that the isolated subunits do not contain the total antigenic repertoire of glycinin. Quite a large fraction must be considered as conformational epitopes, which are disrupted during the separation of individual subunits.

The fraction containing the basic subunits did not show any antigenic reaction with the rabbit anti-glycinin antiserum either in the direct ELISA or with the biotin-labelled serum in the capture ELISA, nor as competitor (results not shown). This confirms the hypothesis of Nielsen *et al.* (1988) that the basic subunits are located in the interior of the assembled molecule. For that reason, they will not be able to interact with the immune system of the affected animal.

Allergenicity of Isolated Subunits

The capability of patient sera to react with the individual subunits has been investigated. The level of IgG binding between the different sera varies very much (see Figure 3). Six sera bind native glycinin preferably and no sera samples seem to be directed specifically towards a single subunit. All subunits bind to the applied sera in very much the same way, indicating a homologous binding reaction.

Quite the opposite effect is observed when analysing for IgE binding (see Figure 4). In four sera samples the binding to subunit A₄ is the most pronounced and in only four sera samples the binding to native glycinin is more pronounced than the binding to the isolated subunits. One serum did not bind native glycinin at all, but still showed significant binding to all subunits. Excluding subunit A₄, all subunits reacted similarly.

These observations lead to some very interesting suggestions. It seems that IgG binding preferably identifies the native molecule, while IgE binding tends to be directed towards fragmented parts of the molecule. A possible explanation is that IgE antibodies can only be stimulated to form when a certain degree of antigenic processing (fragmentation) of the intact glycinin complex has taken place. This conclusion awaits further investigations.

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