

A Soybean G2 Glycinin Allergen

2. Epitope Mapping and Three-Dimensional Modeling

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Key Words

Soybean · Glycinin · Food allergens · Epitopes

Abstract

Background: Multiple allergens have been documented in soybean extracts. IgE from individuals allergic to soybeans, but not to peanut, has been shown by immunoblot analysis to bind to proteins with a molecular weight of approximately 22 kD. These findings suggested that this unique protein fraction from soybean might be responsible, in part, for soybean allergic reactivity. The objective of the present study was to characterize specific B cell epitopes, to determine if any amino acid was critical to IgE binding and to model the 22-kD G2 soybean allergen to the three-dimensional (3-D) phaseolin molecule. **Methods:** B cell epitopes were identified using SPOTs peptide analysis. Structural orientation of the IgE-binding regions was mapped to the 3-D phaseolin molecule using molecular modeling of the protein tertiary structure. **Results:** Eleven linear epitopes, representing 15 amino acid peptide sequences, bound to IgE in the glycinin molecule. These epitopes were predicted to be distributed asymmetrically on the surface of G2 trimers.

Conclusions: Only 1 epitope could be rendered non-IgE binding by alanine substitutions in the peptide. The non-random distribution of the IgE binding sites provides new insight into their organization in trimers in 11S complexes of the G2 glycinin allergen.

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Introduction

Approximately 20% of the population in industrial countries believe they suffer from food allergies. The allergic reaction is antigen/allergen specific, and the structure of the allergen is involved in both the sensitization process and the elicitation of allergic symptoms. Therefore, one of the answers to the question of why a protein becomes an allergen must be related to its structural characteristics.

The three-dimensional (3-D) structure of allergens is important in understanding the molecular basis of allergenicity. In the past few years, the primary structures of a large number of different allergens have been reported. In a few cases, the 3-D structures have also been determined using X-ray diffraction and NMR. Four of these are from plants: birch pollen Bet v 1 [1] and Bet v 2 [2], mouse ear

cross Ara t [3] and timothy grass pollen Phl p 2 [4]. Structural information in relation to B cell and T cell epitopes that can be derived from these structures will be necessary to design effective, safe recombinant derivatives of allergenic proteins for specific immunotherapy. In addition to exhibiting the expected functional properties, recombinant proteins produced in this manner must be stable in the subcellular location where they are accumulated.

A number of allergens have been described in soybean extracts that have both cross-reactive and unique IgE-binding properties. Several of these relate to the proteins described in this paper. Eigenmann et al. [5] used antigen affinity chromatography with peanut-specific IgE to remove cross-reactive antibodies from soybean extracts, and identified two unique allergenic fractions from soybean extracts that had apparent molecular weights of 40 and 21 kD. Herian et al. [6], who used IgE from individuals allergic to soybeans but not to peanut, showed that IgEs bound strongly to proteins with a molecular weight of approximately 20 kD. Helm et al. [7] extended these observations. By using serum IgEs from 7 clinical soybean-sensitive individuals, they demonstrated that the 22-kD proteins bound to these proteins were the basic chains from subunits of the 11S seed storage proteins.

The 11S storage proteins of soybean are called glycinins. When isolated from seeds, these 350-kD globulins are hexamers. To date, five genes have been identified that encode glycinin subunits [8]. The primary structures of the glycinin subunits are homologous to one another, and the subunits assemble randomly to form hexamers that are accumulated in massive amounts in the storage vacuoles of seed cotyledons [9]. A Danish study attempted to determine the relative allergenic activity among glycinin polypeptides, and the authors concluded that no specific chain was entirely responsible for allergic reactions to soybean proteins [10, 11]. Zeece et al. [12] used serum from soybean-sensitive individuals and identified an acidic polypeptide chain designated soybean glycinin G1. These authors were able to narrow the region of IgE binding to a 15-kD peptide that corresponded to amino acid residues 192–306. The glycinin G1 acidic subunit, when compared to the G2 acidic chain, had stronger IgE binding, even though the sequence homology was quite similar.

In this report, the glycinin G2 subunit [3] was characterized using B cell epitope mapping. Thus, 11 linear epitopes were identified that bound to IgE in the glycinin G2 molecule. Four immunodominant epitopes were identified among them. Alanine was systematically substituted for each amino acid in all of the epitopes in an effort to identify those residues that were necessary for IgE bind-

ing. Mutations of this type successfully eliminated IgE binding for only 1 of the epitopes. 3-D modeling of glycinin was undertaken to determine the spatial arrangement of the epitopes using this information. The 3-D model was developed using the structure of the 7S phaseolin protein from the French bean. Interestingly, the epitopes of G2 glycinin were all located on the exterior of the molecule, and predominantly on one surface of glycinin trimers.

Materials and Methods

Human Sera

Human sera used in this study were from 7 individuals with convincing histories of soybean allergy. The characteristics of the sera can be found in the report of the first part of this study [7].

B Cell Epitope Mapping

The predicted amino acid sequence of the complete G2 soybean glycinin was used to generate individual peptides for IgE-binding studies. The SPOTs method of individual peptide synthesis on a cellulose membrane containing free hydroxyl groups using Fmoc amino acids was used according to the manufacturer's instructions (Genosys Biotechnologies, The Woodlands, Tex., USA). Synthesis of each peptide was initiated upon esterification of an Fmoc amino acid to the cellulose membrane. After washing, all residual amino functions on the membrane were blocked by acetylation to render the cellulose membrane unreactive during subsequent steps. Each additional Fmoc amino acid was esterified to the previous one by this same process. After addition of the last amino acid in the peptide chain, the amino acid side chains were deprotected using a mixture of dichloromethane/trifluoroacetic acid/triisobutylsilane (1/1/0.05), followed by treatment with dichloromethane, dimethyl formamide and washing with methanol. Following synthesis on the membrane, the peptides were blocked using Tris-buffered saline with 0.05% Tween 20 (TBST) in 1% BSA, and analyzed for IgE binding using serum from soybean-sensitive individuals. The serum from 7 individuals with a clinical sensitivity to soybean was diluted 1/10 (v/v) with TBST in 1% BSA and incubated with the nitrocellulose membrane for at least 12 h at 4°C or for 2 h at room temperature. The membrane was subsequently washed and incubated with ¹²⁵I-radiolabeled anti-IgE (Sanofi Diagnostics, Chaska, Minn., USA) overnight at 4°C. After the membrane was washed and dried, it was exposed to X-Omat or BioMax X-ray film (Eastman Kodak Company, Rochester, N.Y., USA) at –80°C for 18–48 h.

Mutations of B Cell Epitopes

A mutational analysis was performed to identify changes that would render each epitope non-IgE binding. For this purpose, alanine was systematically substituted for each amino acid in peptides that corresponded to each epitope using the SPOTs method. The capacity of the mutant peptide to bind IgE was then compared with that for unmodified peptides (nonsubstituted epitope) to determine the relative IgE binding as determined by autoradiographic analysis following IgE ¹²⁵I-radiolabeled immunoblot analysis as described above.

Fig. 1. Regional localization of IgE binding to the amino acid sequence of G2. A SPOTs membrane representing individual 15-mers offset by 8 amino acids of G2 was incubated with pooled serum from soybean-sensitive individuals and used to identify 6 IgE-binding regions. The upward-pointing arrow indicates the N-terminal sequence of the basic polypeptide of G2. The peptide representing amino acids 62–72 (shown in bold print) bound peanut-specific IgE in region 2.

Region 1	2	
<u>MAKLVL</u> SLCF LLFSGCFALR EOAQ QNECQI QKLNALKPDN RIESEGGFIE TWNPNNK PFO		60
<u>GAGVAL</u> SRCT LNRNALRRPS VTNGPOEIVY OOGNGIFGMI FPGCPSTYOE PQESQQRGRS		120
	3	
QRPQDRHQKV HRFREGDLIA VPGVAWWMYN NEDTPVAVS IIDTNSLENO LDMPRRFYLL		180
<u>AGNQEOEFLK</u> YOOQOOGGSO SOKGKQOEFE ENEGSNILSG FAPEFLKEAF GVMNQIVRNL		240
	4	
QGENEEEDSG AIIVTVKGGLR VTAPAMRKPO QEEDDDDEEE QPQCVETDKG CQRQSKRSRN		300
	5	
GIDETICTMR LRQNIQNSS PDIYNPQAGS ITTATSLDFP ALWLLKLSAO YGSLRKNAME		360
<u>↑VPHYTLNANS</u> IYALNGRAL VQVVCNNGER VFDGELQEGG VLIIVQNFAV AAKSQSDNFE		420
6YVSFKTNDRP SIGNLAGANS LLNALPEEVI QHTFNLKSOO AROVKNNNPF SFLVPPQESQ		480
RRAVA		

Homology-Based Model Alignment of 7S and 11S Proteins

Based on previous computer modeling experiments, the core structures of 11S and 7S proteins are regarded as being similar [13]. Molecular replacement predictions also demonstrated that the β -barrel and helical regions of glycinin and phaseolin could also be superimposed [Nielsen, unpubl. observations]. Thus, the 2.2-ampere resolution of the 3-D structure of phaseolin, the major storage protein in the cotyledons of the French bean (*Phaseolus vulgaris*), provides a means of interpreting the structure of additional members of the vicilins or 7S class of legume storage proteins [13]. This canonical model provided a means of interpreting the structure of the 11S (legumin) family of storage proteins, for which no X-ray diffraction data was available.

Molecular modeling and computations were performed using the RasMol and Swiss-PDB viewer programs [14, 15]. The crystal structure of the phaseolin chain from *P. vulgaris* was used as a template for homology-based modeling. Although the model was energy minimized by the program, the primary structures of G2 and phaseolin were additionally aligned according to Lawrence et al. [13]. Based on this information, the alignment provided by Lawrence et al. [13] and the phaseolin space-filled model were used to determine the relationship of the glycinin G2 protein to phaseolin.

Results

IgE-Binding Characteristics of G2

The Genosys SPOTs method was used to determine the peptide sequences in glycinin G2 that bound IgE. Peptides corresponding to the entire amino acid sequence of G2 proglycinin were prepared as 15-mer chains that were

offset by 8 amino acids. Using this approach, 6 large regions that bound IgE were identified at amino acid sequence positions 1–23, 57–111, 169–215, 249–271, 329–383 and 449–471 (fig. 1). A duplicate set of 15-mers was analyzed with peanut-specific IgE for identification of specific and cross-reactive IgE-binding regions. Region 1 was completely soybean specific, with no detectable binding by peanut serum IgE. Region 2 amino acids 62–72 specifically bound serum IgE from peanut-sensitive individuals (fig. 1). Regions 3–6, identified with serum from soybean-sensitive individuals, shared soybean and peanut IgE-binding peptides (fig. 1).

To further analyze the IgE-binding regions, peptides representing 15 amino acids offset by 2 amino acids were synthesized for each of the 6 IgE-binding regions. This permitted the minimal linear amino acid sequences required for IgE binding to be identified. In this manner, individual IgE-binding epitopes within the larger IgE-binding regions of the G2 molecule were identified. As indicated in figure 2, there were 11 linear soybean-specific epitopes in these regions. The epitopes were distributed throughout the entire molecule, and no amino acid sequence motif was shared among any of the epitopes. Four of the 11 epitopes were located within the 22-kD C-terminal portion of the protein that corresponded to the basic chain of glycinin G2. Earlier, the basic chain had been demonstrated to bind IgEs from patients allergic to soybean products [7].

Fig. 2. IgE-binding epitopes in glycinin G2. **a** Identification of epitopes within the 6 IgE-binding regions. Each IgE-binding region was mapped by preparing 15-mers offset by 2 amino acids and incubating the SPOTs membrane with pooled serum from soybean-sensitive individuals. An example is shown for region 1 (amino acids 1–23). The predominant IgE-binding sequence was then identified as the epitope. When a definite break in the intensity of the spots representing 15-mers could be identified in a region, a new epitope was identified. **b** Identification of the 11 epitopes showing their respective amino acid position in the G2 subunit.

a Region 1: AA1 – 23
MAKLVL~~SLCFL~~LFSGCFALREQA
MAKLVL~~SLCFL~~LFSG
KLVLSLCFLLFSGCF
VLSLCFLLFSGCFAL
SLCFLLFSGCFALRE
CFLLFSGCFALREQA

b

<u>AA 3 – 17</u> KLVLSLCFLLFSGCF	<u>AA 83 – 97</u> NGPQEIIYIQQNGIF	<u>AA 90 – 104</u> QQNGIFGMIFPGCP	<u>AA 177 – 191</u> RFYLAGNQEQEFLKY
<u>AA – 189 – 203</u> LKYQQQQGGSQSQK	<u>AA 255 – 269</u> VKGGLRVTA ~~PAM~~ RKP	<u>AA 337 – 351</u> LDFPALWLLKLSAQY	<u>AA 345 – 359</u> LKL ~~SAQY~~ GS ~~LR~~ KNAM
<u>AA 358 – 372</u> MEVPHYTLNANSIIY	<u>AA 367 – 381</u> NANSIIYALNGRALV	<u>AA 469 – 475</u> QHTFNLKSQ ~~QAR~~ QVK	

Fig. 3. Individual patient serum IgE recognition of the 11 G2 epitopes. A SPOTs membrane representing the 11 epitopes was incubated with individual serum IgE to determine the specific epitopes recognized by each individual's serum IgE. The control used was high-titer serum from an atopic individual with no known food allergies.

PATIENT #	EPITOPES										
	1	2	3	4	5	6	7	8	9	10	11
1		+	+		+		+	+	+	+	+
2	+			+				+			
3	+	+		+	+			+			+
4		+	+		+			+			
5	+	+		+				+			+
6	+	+		+	+			+	+		+
7		+		+				+			+
Control Total	4	6	2	5	4	0	1	7	2	1	5

Frequency of Individual Serum IgE Binding to G2 Epitopes

To determine the frequency of binding to the 11 epitopes among individual patients, replicate patterns of the synthesized epitopes were reacted with serum IgE from each soybean-sensitive individual. The results obtained are shown in figure 3. The intensity of IgE binding and epitope recognition with pooled serum IgE from 7 individuals sensitive to soybeans varied substantially. Because epitopes 2, 4, 8 and 11 reacted with IgE from serum from the majority of patients, these were considered to be immunodominant. No IgE binding could be identified by

Fig. 4. Alignment of peanut allergens and the soybean G2 allergen with the phaseolin molecule. The phaseolin and soybean allergen G2 were aligned according to the model alignment of 7S and 11S sequences according to Lawrence et al. [13]. The peanut allergens were added by manual intervention to align the match of the Pro/Gly motif conserved regions for the 7S and 11S sequences.

Fig. 5. RasMol fit of the glycinin G2 to the published structure of phaseolin. The primary structures of G2 and phaseolin were first aligned by amino acid sequence. The various epitopes of the G2 glycinin subunit were then identified in the 7S molecule and colored. Region 2 = green; region 3 = yellow; region 4 = blue; region 5 = red; region 6 = purple. The regions were then superimposed on the trimer model in a space-filled design (**a**, **b**). Region 3 is situated on the outside of the opposite side of the molecule.

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-----NNPFYFSSRR-FSTRYGNQNGRIKVLQRF
MGRARVPLLLGLILFLASLSASFATSLREEESQDNPFYFNSDNSWTLFTQNYGHIRVLQRF
MA-----LVLRGCTFLESGA-A--LREQAQNECQIQKLNALKPDNRIESEGGFI---ETWN
-----IS--FRQQPEENACQFQRLNAQRPDNRIESEGGYI---ETWN

QRSRQFQNLQNHRIWQIEAKPNTLVLPKHADADNIIIVIOQSQATVTVANGN-NRKSFNLDEGH-
QQSKRLQNLQEDYRLVEFRSKPETLLLPQADAELLVVRSGSAILVLVKPDDRREYFFLTSDNP
PNNKPFQC-AGVALSRCTLNRLNARLPSTY-NPQSLIQGNPQSLIQGSTYQEPQ----
INNQEFEC-AGVALSRVLRLNARLRPFYSNAPQEIFIQQGRGYFGLIFPGCFRHYEEPH----

-----ALRIFSGFISYILNRHQNQLRVAKISMFPVNTPGQ
IF-----SDNQKIPAGTIFTLVNFPQKEDLRRIQLAMPVNNPQI
--ESQQRGRSQRPDQRHQK-----VHRFREGDLIAVP-GVAMWYNNEDTFVVAVSIIDTNSLENQL
--TQGRRSQSQRPPRLQGEDQSQQQRDSNQLVHRFDEGDLIAVPTGVAFWLYNDHDTDVVAVSLDTNNNNQL

---IEDFFPASSRDQS-----
---H-DFFLSSTEAQQ-----
DQMFR-FFVLRGCTFLESGA-----SGGKQEEEEENEG
DQFPR-RFNLAGNTEQEFLRYQQQSQRSSRLPSPYSPQSQRQEEREFSFRFQJSRRER---AGQEEE-NEG

-SYLQGSRNTLEAAF-NAEFNEIRVLLLEENAGGEQEERGQRRWSTRSSENNEGVIVKVSKE-----
-SYLQFSKHILEASF-NSKFEENQVLFEELGQQEGV-----IVNIDSE-----
SNILSGFAPEFLKEAF-GVMQIVANLQGENEEEDSGA-----IVTFGDLVTAFADREQQEEEDDDDEEQPQCVETDK
GNIFSGPTFFLQFOVDDRQIVQNLGCTESEEECA-----ITVRGGLRLSPDRRR-ADEEEEDFDEVEYDEED

HVEELTKHAKSVSKNGSEEE-----GDITNPINLRCEPDLSNNFGKLFEVKPD--KKNPQLQDLQMLTVCVEIKEGALMLP
QIKELSKHAKSSSKSLSKQD-----NTIGNEF-----GNLTERTON--SLN-----VLISSEMEGALFVP
GCQRQSKRSNGIDETICTNRLRWIGQNSSPDIYNPQAGSIT-----TATSCPFALMLCPFALML-----PLSQTONLSNAPP
KRGGRSGRGRNGIETICTASALLNIGGRSPDIYNPQAGSLK-----TANGLNLLILRLG-----LSAEYGNLYRNALFVA

HFNKAMVIVVVNKGTDGNLELVAVRKEQQQRGRREEEEDDEEEEGSNREVRRYTARLKEGDVFFIMPAAHFVAINASSEE-LMLLG
HYYSKAIIVLVNNEGAHVLPQPGNKETLEYE-----SYRAELSKDOVFFIPAAFFVAIKATSN-VNFTGF
HYTNNAHSITVACPCPQVVNCGER-----VFDGELQEGGVLIQPNFAVAAKSQSDNFYVSF
HYNTNAHSIIYRLRGRAHVQVVDSDNGNR-----VYDEELQEFJVLVVQNFVAVACKSQSENFEYVAF

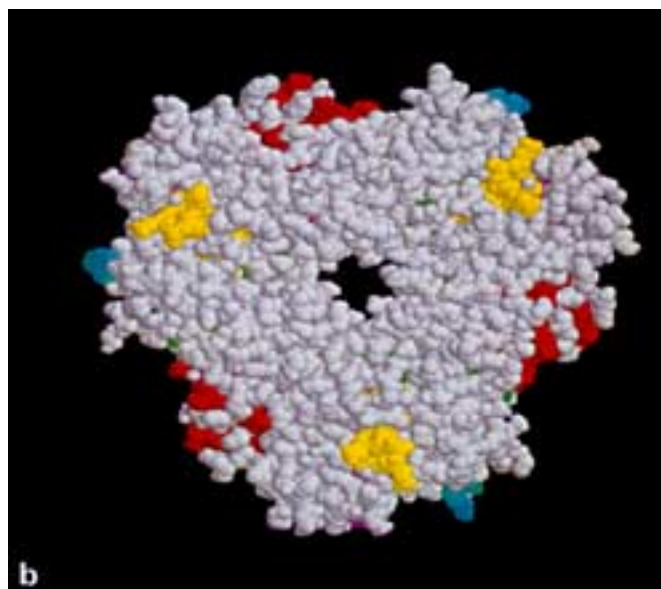
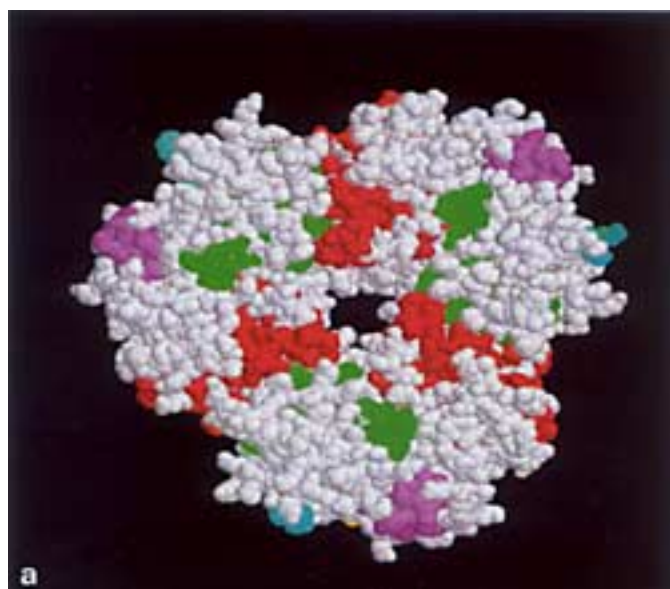
GINAENNHRIFLAGEKDNVIQIE-----KQAKDLAFPGSGQVEKLIKNQKESNFVSAR
GINANNNRLLAGKTDMVISSIGRALDGKDVLGLTFSGSGDEVMKLINKQSGSYFVDANHHQQEQQKGRKGAFVY
KTNDRPSIGN-LAGA-NSLLNALP-----EEMDRTFRSGQAGQQNNPFSLV----PPQESQRA-----VA
KYDSRPSIAN-LAGE-NSVIDNLP-----EEVVANSYGLQREQARQLKNNNPFFKFFV----PPSQQSRA-----VA

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Ara h 1 epitopes
Phaseolin
G2 epitope
Ara h 3 epitopes

P/Q residue pair globally conserved across 11S and 7S families

4



5

the high-titer IgE serum used as a control (data not shown).

Alanine was systematically substituted for each amino acid in the various epitopes to identify those residues important for IgE binding. Only epitope 2 could be rendered non-IgE binding, and this resulted when alanine was substituted at positions 7, 10 and 13–15 (data not shown). There was no detectable reduction of IgE binding following alanine substitutions in the other epitopes.

Location of IgE-Binding Regions on the 3-D Model of G2

The protein sequences for glycinin G2 and β -phaseolin were extracted from GenBank protein databases and aligned using the globally conserved residue pairs first identified by Lawrence et al. [13]. Vicilin, Ara h 1 [16] and the legumin Ara h 3 [17], previously identified as peanut allergens, were selected for comparison with glycinin G2. The amino acid sequence alignments and the identification for peanut Ara h 1 and 3, glycinin G2 and β -phaseolin are shown in figure 4. Although amino acid sequence homology could be identified between epitopes of glycinin G2 and epitopes of Ara h 3, the primary structure of epitopes 1, 2 and 3 from glycinin G2 do not share IgE binding sequences with the corresponding amino acid sequence in the peanut allergen. Taking into consideration the IgE binding specificity revealed by serum from peanut- and soybean-sensitive individuals, respectively, these three epitopes represent soybean-specific IgE-binding epitopes.

An homology-based model of the tertiary structure of glycinin G2 was generated to predict the 3-D location of epitopes in the molecule. To construct this energy-minimized model, the RasMol and Swiss viewer programs [14, 15] were used to align the structure of G2 with that of β -phaseolin. Figure 5 shows the trimer formed from β -phaseolin subunits. For modeling purposes, the residues in G2 glycinin that were predicted to be present at the homologous positions in the molecule were superimposed onto each phaseolin subunit. Five of the 6 IgE-binding regions in glycinin G2 are located on the surface of the trimer model. Four of the 5 IgE-binding regions were located on one surface and edge of the trimer. Only a single region was found on the opposite surface, and it was located near the periphery of the trimer. The sixth region in G2 is located at the NH₂-terminal of the G2 subunit. The NH₂-terminal end of the molecule is missing from the electron density map because it is a region of high mobility on the surface of the molecule.

Discussion

Recognition of allergens by IgE and by receptors on Th2 lymphocytes is a critical requirement for allergenicity. Th2 cells recognize allergens as processed linear peptides in an association with MHC class II molecules on antigen-presenting cells. On the other hand, IgE antibodies bind to the surface of allergen molecules, and therefore the B cell epitopes are often conformational, and dependent on the 3-D structure. Thus, allergen/antigenic epitopes fall into two distinct groups. They are either conformational, in which case they depend upon a 3-D structure, or alternatively, they are sequential, or they depend on linear amino acid sequences.

In this study, epitopes of the linear type are described. Because of their linear nature, we sought to determine their physical location in the molecule using a 3-D model for glycinin that was based on the 3-D structure of phaseolin [13]. Two considerations justify this approach. First, structural predictions that are based on a comparison of primary structures of the two proteins reveal that the 7S and 11S seed storage proteins share amino acid sequences that are predicted to have homologous secondary structures [18]. Second, crystallographic determinations recently reported by Adachi et al. [19] suggest that the structure of the glycinin subunit resembles that of phaseolin, except that the two β -barrels that form the core of the molecules are offset slightly with respect to one another in glycinins.

The results from the structural modeling experiments show that each of the 6 regions bearing epitopes in G2 are located on the surface of the subunits. This can be seen in the trimer structure shown in figure 5. The epitopes are distributed unequally between the two large surfaces of the trimers. The nonrandom distribution of the IgE binding sites provides new insight into the organization of IgE-binding epitopes in the trimers in 11S complexes. Mature glycinin is isolated from seeds as a hexamer that consists of two trimers that are appressed against one another. The two trimers probably interact with one another via the amino acid sequences in which epitopes are not located. These appressed amino acid sequences in the structure would not be accessible to IgE.

Our present study showed a diverse recognition of G2 glycinin epitopes by serum IgE from soybean-sensitive individuals. In our analysis of IgE binding to 15-mers of G2, we identified two IgE-binding regions, regions 3 and 4, that are located within the 15-kD peptide fragment identified by Zeece et al. [12]. In both studies, differences in protein recognition may be related to the individual

serum used to determine IgE-binding regions. Using this structure, each of the 11 linear epitopes that were identified by IgE binding was also located in the model in the 6 distinct regions in the subunits. The IgE-binding regions and epitopes were distributed asymmetrically on the surface of the G2 trimers. These studies further demonstrate the need not only to determine which protein(s) provoke a soybean allergic state, but also to further identify the epitopes in these allergens. Structural modeling of the G2 glycinin to known 3-D structures provides valuable information toward an understanding of the molecular basis of allergenicity. Structural information in relation to B and T cell epitopes provides rational strategies for the design

of recombinant derivatives of allergenic molecules. Information like that presented above may assist in the construction of recombinant nonallergenic soybean proteins that retain native structure and can be used both for immunotherapy and the potential production of hypoallergenic soybean plants.

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