

Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin

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Ovalbumin is a major allergen in hen egg white that causes IgE-mediated food allergic reactions in children. In this study, the immunodominant IgE-binding epitopes of ovalbumin were mapped using arrays of overlapping peptides synthesized on activated cellulose membranes. Pooled human sera from 18 patients with egg allergy were used to probe the membrane. Five distinct regions were found to contain dominant allergic IgE epitopes, these being L38T49, D95A102, E191V200, V243E248 and G251N260. The critical amino acids involved in IgE antibody binding were also determined. These epitopes were composed primarily of hydrophobic amino acids, followed by polar and charged residues and being comprised of β -sheet and β -turn structures. One epitope, D95A102, consisted of a single α -helix. These results provide useful information on the functional role of amino acid residues to evaluate the structure–function relationships and structural properties of allergic epitopes in ovalbumin. They also provide a strategic approach for engineering ovalbumin to reduce its allergenicity.

Keywords: food allergy/IgE epitope mapping/ovalbumin/peptide synthesis/SPOTs assay

Introduction

Food allergy is a major concern for human health and there is therefore an urgent issue to identify and characterize the sensitizing potential of food proteins and to find a way to prevent such hypersensitivity (Taylor and Hefle, 2001). IgE-mediated allergic reactions (type I) are known to play an important role in food allergic reactions (Bruunzeel-Koomen *et al.*, 1995). Hen's egg white is a major cause of the type I allergic reactions in humans, particularly in children under 3 years old (Sampson and Ho, 1997). Ovalbumin is a major allergen in hen egg white consisting of 385 amino acids with a molecular weight of 45 kDa, comprising 58% (w/w) of the whole egg white (Nisbet *et al.*, 1981; Langeland, 1983; Bernhisel-Broadbent *et al.*, 1994). The allergenic properties of proteins present in hen egg white have been characterized previously (Holen and Elsayed, 1990; Mine and Zhang, 2002). IgE-binding epitopes of allergens play an important role in the disease process (Yunginger and Jones, 1987). It is important to determine the primary structure of the major allergens in egg white, the IgE-binding sites of these allergens and the frequency of recognition of the IgE-binding epitopes. Characterization of these allergens will provide a better

understanding of the human immune response mechanism involved in food hypersensitivity reactions.

It has been reported that treatment of ovalbumin with carboxymethylation, 6 M urea or heating at 95°C had no significant effect on its binding capacity to human IgE derived from egg-allergic patients' sera (Mine and Zhang, 2002). This indicates that anti-ovalbumin IgE antibodies from egg-allergic patients recognize mainly sequential epitopes. The IgE class of antibodies for ovalbumin also have been shown to exist in sera from patients with allergy (May *et al.*, 1977). Epitope mapping of specific regions in ovalbumin have been done by several groups using enzyme-digested fragments and synthetic peptides (Elsayed and Stavseng, 1994; Masuda *et al.*, 2000). The studies on IgE antibody responses to ovalbumin have been shown earlier (Elsayed *et al.*, 1991; Kahlert *et al.*, 1992). Protein structure also plays a critical role in determining the immunodominant IgE-binding epitopes and there seems to be a link between the allergen structure and the IgE-binding epitopes (Shimojo *et al.*, 1994; Sen *et al.*, 2002).

Although a few IgE epitopes have been identified earlier, there are no obvious sequence motifs shared by these epitopes. In the present study, we have determined the entire mapping of the IgE-binding epitopes in the primary sequence of ovalbumin, and the critical amino acids within each of the IgE-binding epitopes of ovalbumin that are important to IgE binding were identified. Interestingly, the substitution of a single amino acid within each of the peptides led to either reduction or loss of IgE binding. In some cases, substitution led to an increase in IgE-binding activity. This information is important to design diagnostic and therapeutic approaches to reduce the risk of the food hypersensitivity caused by ovalbumin.

Materials and methods

SPOTs assay and peptide synthesis

The individual peptides were synthesized on a derivatized cellulose membrane using Fmoc amino acid active esters (SPOTs synthesis) according to the manufacturer's instructions (Sigma Genosys Biotechnologies, Woodlands, TX). The synthesis of peptides using the SPOTs technique was designed based on the primary amino acid sequence of ovalbumin (Nisbet *et al.*, 1981). Synthesis of each peptide began by esterifying a Fmoc amino acid to the cellulose membrane derivatized with a dimer of a β -alanine-NH₂ group and was monitored at certain stages using colour development of bromophenol blue. In short, the primary sequence was synthesized so that it contained synthetic peptides that were 12 amino acids in length and offset by two amino acids. Coupling reactions were followed by acetylation with acetic anhydride (4%, v/v) in *N,N*-dimethylformamide to render peptides unreactive during the subsequent steps. After acetylation, Fmoc protective groups were removed by the addition of piperidine to render nascent peptides reactive. The

remaining amino acids were added by this same process of coupling, blocking and deprotection until the expected desired peptide was generated. After addition of the last amino acid in the peptide, the amino acid side chains were deprotected using a solution of dichloromethane–trifluoroacetic acid–triisobutylsilane (1:1:0.05, v/v/v) and washed with methanol. Membranes containing the synthetic peptides were either probed immediately or stored at -20°C until needed (Frank and Overwin, 1996).

IgE-binding assay

The cellulose membranes containing the synthesized peptides were washed with Tris-buffered saline (TBS) and blocked overnight with the blocking buffer (Sigma-Genosys Biotechnologies) with 5% (w/v) sucrose. After blocking, the membranes were washed with TBST (50 mM Tris, pH 8.0, 136 mM NaCl, 2.7 mM KCl and 0.05% Tween-20) for 10 min and incubated with three pooled sera, each consisting of six egg-allergic patients' sera, for 4 h at 1:20 dilution at room temperature. The membranes were washed with TBST and incubated with monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma, St. Louis, MO; 1:2000 dilution) at room temperature for 2 h. After incubation, the membranes were washed with TBST and the bound antibodies were detected using the chemiluminescent substrate CDP-Star (Boehringer Ingelheim, Mannheim, Germany) and enhancer Nitro-Block II (Tropix, Bedford, MA) both diluted to 1:100 with 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5. The membrane was visualized under a Molecular Light Imager (EG & G Berthold, Bad Wildbad, Germany). The quantified signal of each spot was obtained using Win Light software (EG & G Berthold) and the value was expressed as relative percentage of signal intensity.

Analysis of the critical amino acids

The amino acids critical to each ovalbumin IgE-binding site were identified by substitution of a single amino acid at each position in the epitope by synthesizing multiple peptides. Peptides were synthesized on a SPOTs membrane with each amino acid sequentially replaced with either alanine (A), glycine (G) or glutamine (Q) and probed as described in the previous section.

Regeneration of SPOTs membrane

The membrane was regenerated according to the manufacturer's instructions (Sigma-Genosys Biotechnologies) by washing with regeneration buffer A [8 M urea, 1% (w/v) sodium dodecyl sulphate, 0.1% (v/v) β -mercaptoethanol], followed by washing with regeneration buffer B [50% (v/v) ethanol, 10% (v/v) acetic acid]. The membrane was then washed with methanol and dried and stored at -30°C . Regenerated membrane was used for evaluating human IgE-binding activity with different pooled sera from egg-allergic patients.

Human serum

Human serum was collected from 18 patients exhibiting allergies to egg white. The RAST scores were determined according to the procedures of the manufacturer's kit (Phadebas RAST kit; Pharmacia Diagnostics, Tokyo, Japan). Table I gives a brief summary of each patient's clinical history. The serum samples were kindly provided by Dr A.Urisu (Department of Pediatrics, Fujita Health University, Japan) and Dr Morikawa (Department of Pediatrics, Gunma University, Japan) and stored at -80°C until use. The serum samples were pooled into three groups consisting of six patients' sera each for the experiments. A pool of three non-allergic individual's

Table I. Clinical history of egg white allergic patients' sera

Group	Patient	Sex	Age (years)	IHR	IgE RAST (PRU/ml)
1	MN	M	2	AD, BA, UN	10.7
	YK	F	1	AD, UN	>17.5
	NK	M	1	AH, UN	>17.5
	ES	F	2	AD, BA, VM	2.2
	NK	M	2	AD, WZ	1.0
	KK	F	3	AH, UN	4.7
2	AH	M	4	ERY, VM, UN	8.0
	ST	F	2	AD, AR, BA	2.2
	HK	M	1	AD, UN	>17.5
	MI	F	3	AD, BA, ERY	10.5
	IY	M	1	AD, BA, UN	14.6
	RN	F	4	AH, BA, UN	>17.5
3	SO	M	2	AD, ERY,	3.6
	MF	M	3	AR, UN	>17.5
	MS	M	1	AH, BA	>17.5
	MN	F	2	AD, BA	15.1
	KN	M	5	AD, UN	1.0
	RT	F	4	AD, ERY, AR	7.5

IHR, immediate hypersensitivity reaction; AD, atopic dermatitis; AH, anaphylaxis; AR, allergic rhinitis; BA, bronchial asthma; ERY, erythema; UN, urticaria; VM, vomiting; WZ, wheezing.

sera was also used as a control. Sera from food hypersensitivity with positive double-blind, placebo-controlled food challenges (DBPCFC) to egg white were used in this study.

Statistical analysis

Data were analysed by using analysis of variance (ANOVA-SPSS version 7.5 for Windows; SPSS, Chicago, IL) and the level of significance was defined at $P < 0.05$.

Results

Identification of the immunodominant allergic IgE epitopes in ovalbumin

One hundred and eighty-seven overlapping peptides were synthesized on cellulose membranes to determine which regions of the primary sequence of ovalbumin protein were recognized by allergic patients' sera. Each synthesized peptide consisted of 12 amino acids and was offset by two amino acids from the previous peptide. This approach led to the epitope mapping of the IgE-binding region of the entire ovalbumin sequence. Prior to testing the membrane with egg-allergic patients' sera, it was incubated with non-allergic control sera to evaluate non-specific antibody binding (data not shown). Figure 1a shows a typical SPOTs membrane probed with the pooled serum of group 1. Spot numbers 20, 119–122 and 126 showed strong signals, while numbers 46–48 and 95–96 showed lower intensity. Two groups of pooled sera were used in this manner and the average spot intensities are summarized in Figure 1b. Five prominent epitope regions were identified. The IgE-binding regions were represented by amino acid residues 38–49, 95–102, 191–200, 243–248 and 251–260. Epitopes 1–5 were designated as L38T49, D95A102, E191V200, V243E248 and G251N260, respectively. Figure 2 corresponds to the entire primary sequence of the ovalbumin allergen where the epitopes can be seen to be distributed along the entire length of the sequence that is recognized by the sera obtained from patients with egg hypersensitivity.

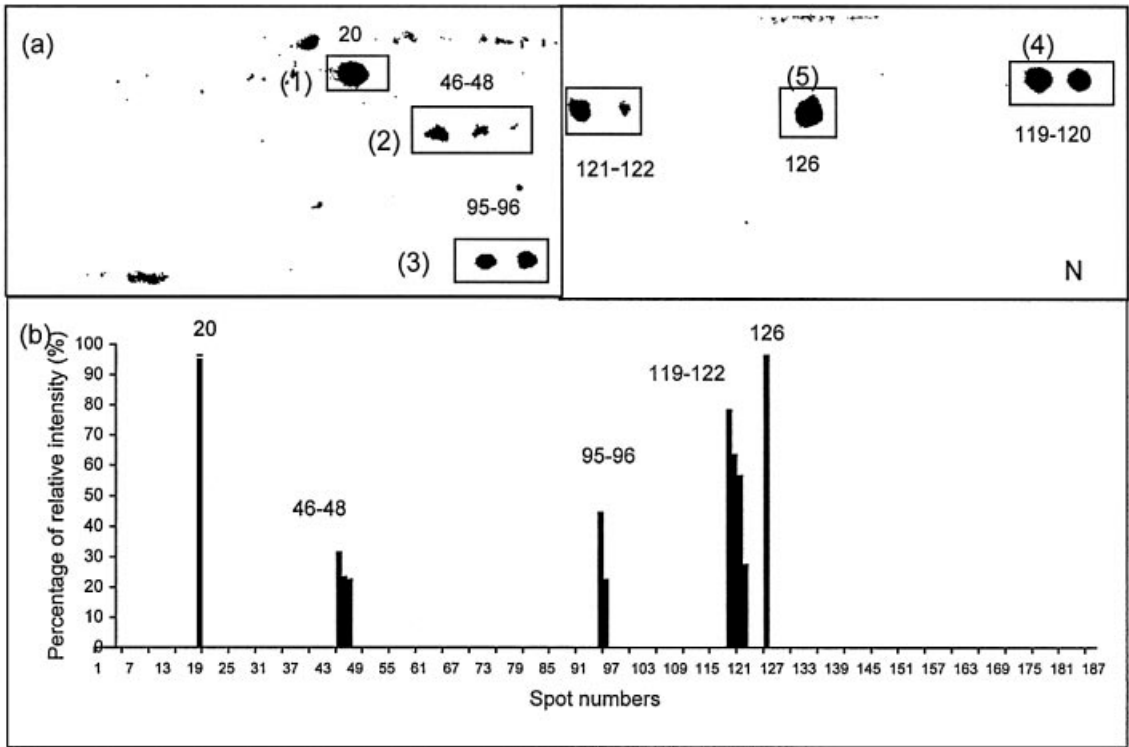


Fig. 1. SPOTs membranes showing the positive spots for IgE-binding regions of ovalbumin. Synthetic overlapping peptides, corresponding to the sequence of ovalbumin, were synthesized on cellulose membranes and bound with sera from the pool of egg-allergic patients. Five distinct regions were detected and numbered 1–5. The negative control spot composed of a single glycine is indicated in the bottom right-hand corner of the second membrane (a). Mean spot signals from three pooled sera are plotted for each peptide (b).

Critical amino acids to IgE binding

The amino acids that were critical for IgE binding to ovalbumin were determined by synthesizing multiple peptides with a single amino acid mutation at each IgE-binding site. Each amino acid in each of the five epitopes was sequentially replaced by alanine (A), glycine (G) or glutamine (Q) depending on the amino acid composition of the epitope and re-probed with human sera from egg-allergic patients. Figure 3 represents an immunoblot strip containing the wild-type and mutant peptides for each IgE epitope after being probed with pooled sera group 1. Two other serum pools exhibited a similar pattern of spots and the average signals of three groups are shown in Figure 3b. Binding to epitopes, L38T49, D95A102, E191V200 and V243E248 were strictly regulated by human IgE antibodies, since a single mutation caused complete loss of IgE binding and no visible spots were detected except with the signal of A102G. The epitope G251N260 showed a different pattern from the others. In some cases (G251A, L252A and E253A), the amino acid substitutions were found to significantly increase binding, while in other cases (L255A, E256A, S257A and I258A), they were found to significantly reduce or completely eliminate IgE binding to the epitope. Amino acids within each of the IgE epitopes of ovalbumin that are critical to IgE antibody binding were identified in this manner and are summarized in Table II. The amino acids of all five epitopes were then classified as either hydrophobic, polar or charged residues and enumerated (Figure 4), and the properties of those amino acids considered to be critical were examined.

1 GSIGAASMEF CFDVFKELKV HHANENIFYC PIAIMSALAM VYLGAKDSTR
51 TQINKVVRFD KLPFGDSIE AQCGTSVNVH SSLRDILNQI TKPNDVYSSFS
101 LASRLYAEER YPILPEYLQC VKELYRGGLE PINFQTAADQ ARELINSWVE
151 SQTNGIIRNV LQPSSVDSQT AMVLVNAIVF KGLWEKTFKD EDTQAMPFRV
201 TEQESKPVQM MYQIGLFRVA SMASEKMKIL ELPFASGTMS MLVLLPDEVS
251 GLEQLESIIN FEKLTEWTSS NVMEERKIKV YLPRMKMEEK YNLTSVLMAM
301 GITDVFSSSA NLSGISSAES LKISQAVHAA HAEINEAGTE VVGSAAEAGVD
351 AASVSEEFRA DHPFLFCIKH IATNAVLFFG RCVSP

Fig. 2. Amino acid sequence of ovalbumin. Amino acid residue sequence in bold and underlined corresponds to the sequence of the five IgE peptides identified. The numbers on the left of the figure indicate the position of the amino acid sequence in the native ovalbumin.

Table II. Amino acids critical in each ovalbumin IgE epitope

No.	Amino acid sequence	Position
1	LAMVYLGA KDST	38–49
2	DVYSFSLA	95–102
3	EDTQAMPFRV	191–200
4	VLLPDE	243–248
5	GLEQLESI IN	251–260

Bold letters correspond to the critical amino acid in each epitope.

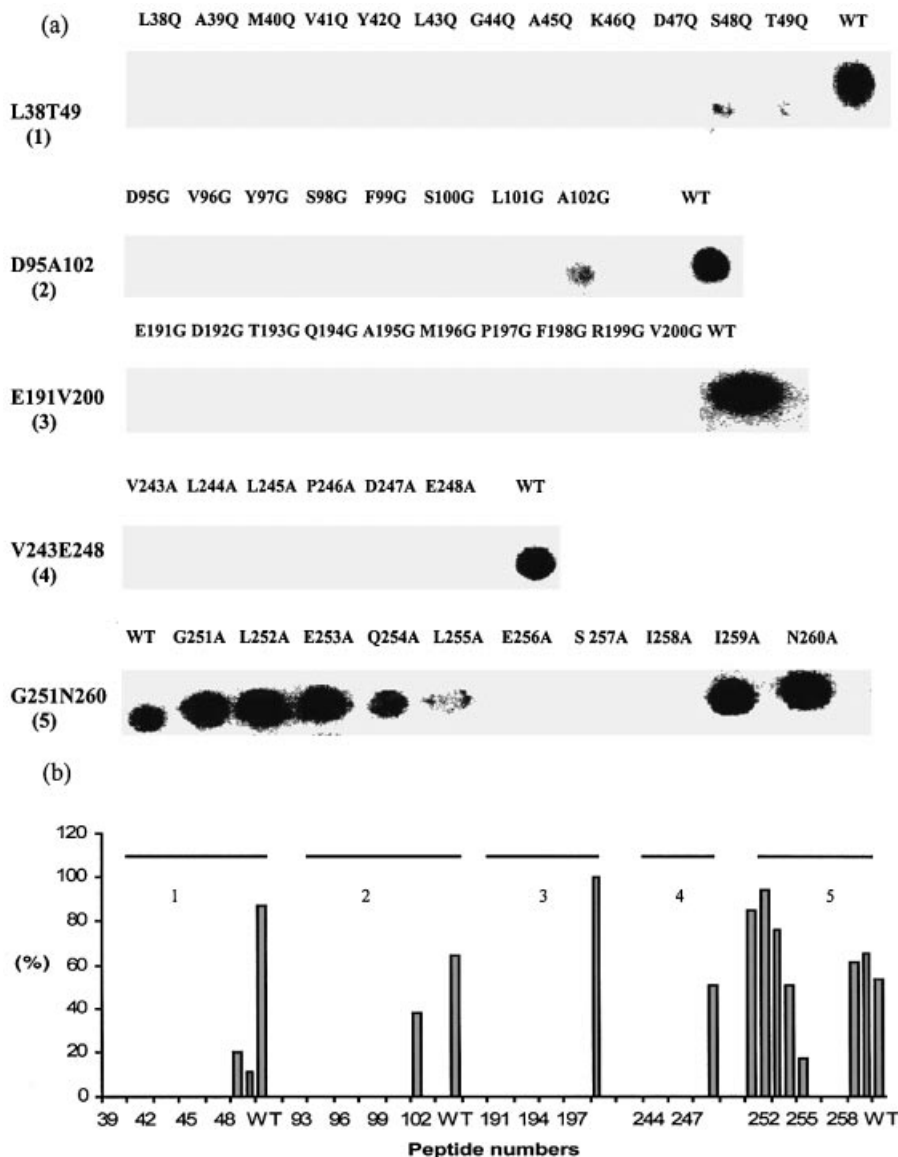


Fig. 3. Critical amino acid analysis of ovalbumin IgE epitopes (a). Binding of human sera to the epitopes was characterized by synthesizing each epitope with each amino acid sequentially substituted with alanine (A), glycine (G) or glutamine (Q). The letters on the left of the number correspond to the one-letter code for the residue normally occurring at that position and the letters on the right represent the substituted alanine, glycine or glutamine residues at each position. WT indicates the wild-type peptide with no amino acid substitutions (b). Mean spot signals are shown plotted for each peptide.

Discussion

To understand the complexity of food hypersensitivity reactions, it is important to identify the IgE-binding epitopes in food allergens because allergen-specific IgE plays a critical role in the aetiology of allergic disease. Due to the increase in egg allergy, control and suppression of allergic reactions are an important health issue. Various studies have concentrated on the hypersensitivity reactions caused by egg white (Sampson and Cook, 1997) and immediate type 1 allergic reactions within the intestinal tract (Reiman *et al.*, 1985). Ovalbumin, being one of the major allergens in patients with hen egg allergy (Bernhisel-Broadbent *et al.*, 1994), was investigated for its IgE-binding epitopes. Although there have been several earlier reports of IgE-binding sites to some regions of intact ovalbumin (Elsayed and Stavseng, 1994; Honma *et al.*, 1994;

Shimojo *et al.*, 1994) or its peptides (Elsayed *et al.*, 1988; Johnsen and Elsayed, 1990; Elsayed, 1992; Renz *et al.*, 1993), the IgE epitope mapping for the entire primary sequence of ovalbumin has not been completed. In this study, we mapped the IgE epitopes for the entire ovalbumin sequence by testing serum IgE binding to synthetic overlapping peptides.

Five distinct IgE-recognition sites were identified in the primary sequence of the ovalbumin protein by the pooled sera obtained from 18 patients with egg hypersensitivity and these immunodominant epitopes seem to be distributed throughout the ovalbumin primary sequence. There have been several reports on IgE-binding sites of ovalbumin using synthetic peptides or chemical cleavage by means of western blots and 2D-PAGE quantitative precipitation inhibition assay, including amino acids 1–10 (Elsayed *et al.*, 1988), 323–339 (Johnsen and Elsayed, 1990), 34–46, 47–55 (Elsayed and Stavseng, 1994),

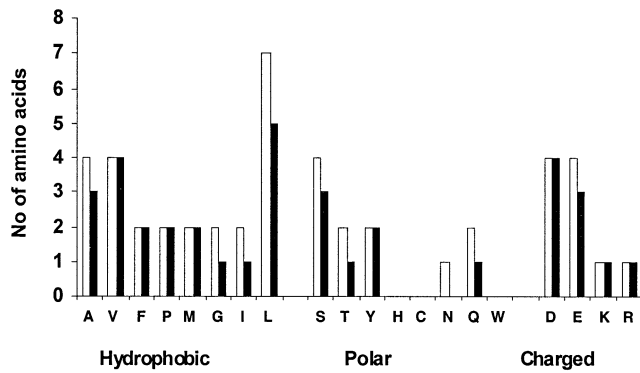


Fig. 4. Classification of amino acids critical to IgE binding. The type of each amino acid within the ovalbumin epitopes that were critical to IgE binding was analysed. The open boxes represent the total number of amino acid residues found in the immunodominant epitopes and the closed boxes represent the number of amino acids which were found to be critical.

41–172 and 301–385 (Kahlert *et al.*, 1992). Furthermore, detailed IgE epitope mapping in the C-terminal region comprising residues 347–385 of ovalbumin was carried out using immunoblot and histamine release assays (Honma *et al.*, 1996). The amino acid residues 347–366, 347–385, 357–366, 357–376 and 367–385 were identified as allergenic epitopes of ovalbumin (Honma *et al.*, 1996). The present sequential IgE epitope mapping results seem to be different from earlier results. We could not identify any IgE-binding sites in the N- or C-terminal regions where previous workers have reported them in the sequence of 1–19 and 347–385. The differences in these results may be attributed to a difference in the patient populations employed in each study. Elsayed and Stavseng (Elsayed and Stavseng, 1994) selected three of 16 patients' sera due to their total IgE and RAST class, and pooled sera were used. Furthermore, earlier studies (Elsayed *et al.*, 1988; Kahlert *et al.*, 1992) did not provide sufficient clinical information about the patients' serum used. Three patients who showed RAST scores for egg white class 3 were used for C-terminal IgE-binding site mapping (Honma *et al.*, 1996). Therefore, the general applicability of the previous work may limit comparison to the present results which were obtained from three pooled groups of six patients' sera each, all having egg allergy.

It is well known that ~40–60% of egg-hypersensitive patients outgrow the allergy (becoming clinically tolerant) with age (Bock and Atkins, 1990). The outgrowing of hypersensitivity is observed even in individuals maintaining high IgE antibodies and a positive skin response to egg white. More recently, it has been shown that six epitopes in cow's milk proteins (α s1-casein, α s2-casein and κ -casein) were not recognized by IgE antibodies from any patients with transient milk allergy, but showed binding by the majority of the patients with persistent allergy (Vila Sexto *et al.*, 2001; Järvinen *et al.*, 2002). This might reflect different epitope recognition of antigens between patients with persistent egg allergy and those who have outgrown their egg allergy. Although no studies have been done comparing the allergens or IgE-binding epitopes for individuals with persistent egg allergy versus those who have outgrown their egg allergy, serum from both patients with respect to levels of egg-specific IgE and the epitopes on egg allergens may be different. This could offer a possible explanation about some of the differences in epitopes that were mapped in this study and previous studies where this

matter was not paid any attention. IgE-binding epitopes in ovalbumin could be different between patients with active egg allergy and those who have outgrown egg allergy. This is of great interest in predicting the persistence of egg allergy. Recently, a newer radio-allergo-sorbent test (CAP-RAST) system has been employed and its values correlated with the probability of positive DBPCFC (Sampson and Ho, 1997; Sampson, 2001). In this study, the Phadebas RAST test, as well as earlier work, was employed for screening human sera from egg allergy, and oral challenge testing was conducted. However, studies comparing Phadebas RAST IgE concentrations with CAP-RAST IgE were not done. Further investigation using a large number of patients' sera with sufficient characterization of the egg-allergic individuals could provide more reliable information to address these conflicts.

The substitution of a single amino acid was accomplished by synthesizing multiple peptides with a single amino acid mutation in each to determine the critical amino acids involved in IgE binding. We observed that the substitution of a single amino acid in some cases led to the loss of IgE-binding activity, in some cases decreased the activity and in a few cases increased the IgE-binding activity. The IgE epitopes L38T49, D95A102, E191V200 and V243E248 showed strictly regulated epitope structure because single amino acid substitution throughout the sequence, except A102, caused a complete loss of IgE-binding activity. In contrast, only three amino acids in the G251N260 epitope (10 amino acids in length) were identified as critical, indicating that this IgE epitope antibody binding is less stringent than others. Some substitutions, however, were found to enhance human IgE binding. The substitution of G251, L252 and E253 with alanine (A) in epitope G251N260 produced noticeably darker spots (Figure 3) than with the native signals, indicating an increase in human IgE binding. This apparent increase in IgE binding may be due to an increase in the similarity of peptide secondary structure, which mimics its original structure in the entire ovalbumin molecule. This suggests that the synthesized epitope (peptide) structure is not always completely identical to that in the native protein structure. This should be studied in detail in the future to address the structural homology between synthesized peptides and native protein, and how each may relate to allergic reactions.

The amino acids of the epitopes were classified as hydrophobic, polar or charged (Figure 4). There were a total of 46 amino acids in all of the sequential IgE epitopes. Hydrophobic residues were found to be the most frequently occurring (25/46), followed by polar residues (11/46) and then charged residues (10/46). Of these polar residues, aspartic acid (D), glutamic acid (E), lysine (K) and arginine (R) appeared to be more important. Most of the critical residues belonged to the hydrophobic group, followed by polar and charged residues. In fact, relative epitope hydrophobicities, as determined by the method of Kyte and Doolittle (Kyte and Doolittle, 1982), identified all five IgE epitopes as relatively hydrophobic regions of the protein (Figure 5). Therefore, the ovalbumin IgE epitopes identified in this study tend to consist mainly of hydrophobic residues, but charged residues (D, E, K and R) seem to have an important role.

Figure 6 shows the location of each of the IgE sequential epitopes obtained in this study with respect to the tertiary structure of ovalbumin. As expected, four of the five epitopes in the three-dimensional structure are exposed to the protein surface, except epitope E191V200, and their major secondary

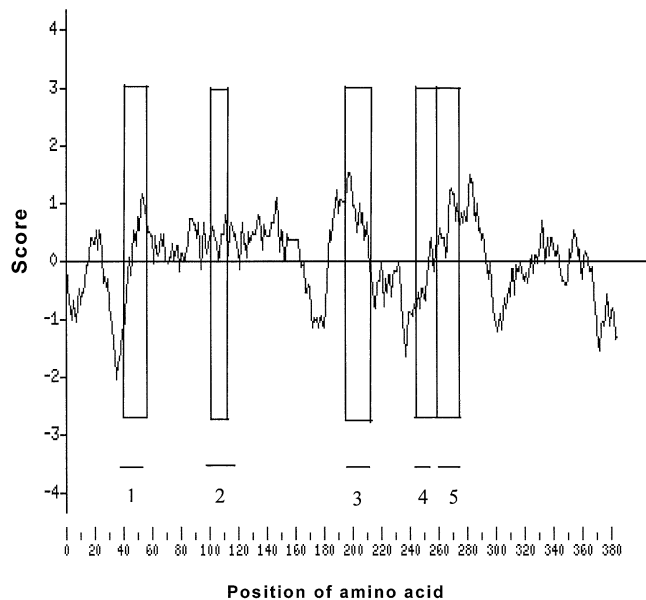


Fig. 5. Hydropathy profile of ovalbumin. A hydropathy plot (Kyte and Doolittle, 1982) representing the location of the different immunodominant epitopes is indicated. Solid lines indicate the regions of the epitope location corresponding to the positions of the amino acids. Numbers 1–5 refer to epitopes L38T49, D95A102, E191V200, V243E248 and G251N260, respectively.

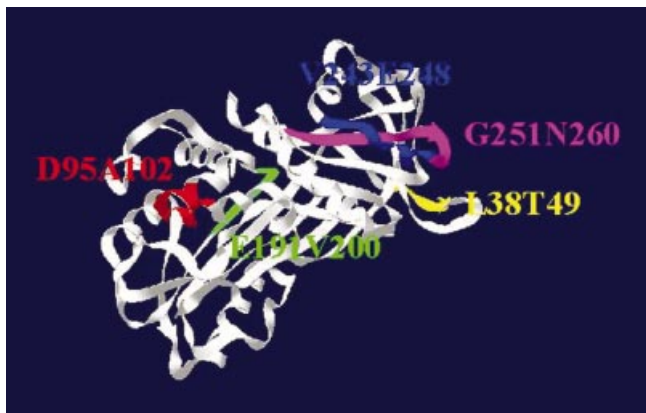


Fig. 6. Localization of the immunodominant IgE epitopes identified in the tertiary structure of ovalbumin. The picture represents the location of the epitopes on the ribbon diagram tertiary structure of ovalbumin. The five epitopes are represented in five different colours on the ribbon structure. The structure was drawn by Dep View/Swiss-Pdb Viewer version 3.7 from Glaxo Smithkline (Geneva, Switzerland).

structure consists of a β -sheet and a β -turn. L38T49 is part of a pair of α -helix A (39–44) and β -turn (45–48). E191V200 is made up of β -sheet s3A (191–196) plus random (197–200), V243E248 of β -turn (type II) (243–248) and G251N260 of β -sheet s3B (251–255) plus β -turn (type I) (256–258). One epitope, D95A102, is composed of a single α -helix D (94–104) (Stein *et al.*, 1991). These results indicate that major structural components of IgE epitopes in ovalbumin are β -sheet and β -turn structures.

Acknowledgements

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