

MAPPING OF IgE BINDING REGIONS IN THE MAJOR RAT URINARY PROTEIN, α_{2u} -GLOBULIN, USING OVERLAPPING PEPTIDES

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ABSTRACT

Exposure to laboratory animals poses a hazard for development of occupational allergy. Identification of antigenic determinants of allergenic proteins may be valuable for immunotherapeutic purposes. Overlapping peptides of the major allergen in rat urine, Rat n 1.02, corresponding to the protein α_{2u} -globulin were synthesised on solid support and screened simultaneously to locate IgE binding linear epitopes using a simple modified ELISA procedure. Thirty-nine peptides were synthesised, each 8 amino acids long with 4 amino acids overlaps. Sera from fifteen rat-sensitized subjects were analyzed and as controls sera from 7 non-rat-sensitized individuals were used. In general low binding and a great individual variation between sera from rat allergic individuals were seen. Some peptides were more frequently recognized by IgE antibodies in sera from rat allergics. These peptides were mainly clustered towards the N-terminal and C-terminal parts of the protein. Taken together our data suggest the existence of linear IgE binding epitopes in the rat urine allergen, Rat n 1.02. However, the role of these sequences in the allergic reaction needs further investigation.

INTRODUCTION

Work with laboratory animals involves risk of developing allergy. Approximately 20–30% of persons handling animals get allergic symptoms like rhinitis, conjunctivitis, urticaria and asthma (1). Laboratory animal allergy is thus classified as an occupational disease. Besides being among the most frequently used laboratory animals, rats are also becoming increasingly popular as pets. Certain proteins in urine of fertile male rats have been shown to be very allergenic (2). To understand the allergenic mechanisms at the

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molecular level, localization of IgE-binding domains of the major allergens in rat urine is essential. Here, we describe attempts to define B cell epitopes in the major protein allergen in rat urine, Rat n 1.02 also called α_{2u} -globulin with the accession number P02761 in the SWISS-PROT Protein Sequence Database. The name α_{2u} -globulin originates from the observation that the protein's electrophoretic mobility is similar to that of serum α_2 -globulin (3, 4), and the letter u stands for urine. Further, α_{2u} -globulin is a transport protein for hydrophobic molecules and it belongs to the lipocalin family (5).

B cells typically recognize surface-accessible determinants, generally composed of hydrophilic amino acids of both sequential and non-sequential conformation. Sequential epitopes are usually from 5 to 8 residues long (6-8). To locate continuous epitopes within α_{2u} -globulin, overlapping octapeptides spanning the whole protein sequence were synthesized using the Multipin Peptide Synthesis System (9). This solid-phase method utilizes stepwise elongation of the peptide chain from C-terminus to N-terminus. The reactivity of antibodies, in sera from rat allergic individuals, with these synthetic peptides was analyzed.

Alternative peptide mapping methods commonly used, particularly for purified proteins and proteins with unknown sequence, are enzymatic digestion and chemical cleavage. According to this, both proteins corresponding to the isoallergens Rat n 1.01 and Rat n 1.02 were enzymatically cleaved with Endoproteinase Lys-C and the derived peptide fragments were compared.

Furthermore, using the BLAST program (10) the amino acid sequence of the α_{2u} -globulin was searched for homology to other proteins and 59 homologies were found.

MATERIALS AND METHODS

Patient sera

Sera from 15 rat-allergic patients, all with immunoblot reactivity to the rat urinary protein α_{2u} -globulin, were studied. A pool of sera from seven non-allergic persons was used as control. Additionally three non-allergic sera were studied individually. The serum samples were taken with informed consent of the subjects and approved by the local ethics committee.

Allergen-specific IgE analysis

Purified proteins corresponding to the allergens Rat n 1.02 (α_{2u} -globulin) and Rat n 1.01 (prealbumin) (11) were tested in the ELISA system. Microtitre plates (Maxisorp™, Nunc, Roskilde, Denmark) were coated overnight at room temperature with 5 μ g/ml of the respective protein. The plates were washed and two different dilutions, 1:25 and 1:50, of individual patient serum were added followed by incubation overnight at room temperature. The diluent was TBS-T (0.1% Tween 20) containing 1% BSA. After

washing the secondary antibody, alkaline phosphatase conjugated goat anti-human IgE (Biosource, Camarillo, CA, USA), diluted 1:500, was added and incubated for three hours at room temperature. The plates were then washed before the substrate p-nitrophenyl phosphate (pNPP) (Sigma, Diagnostics, St. Louis, MO, USA) was added. The optical density was read at 405 nm in a SPECTRAmax™ 250 (Molecular Devices, Sunnyvale, CA, USA) plate reader.

Western blotting

Each serum used in the Multipin system was studied with immunoblotting as described (12). Briefly, concentrated rat urinary proteins (13) were separated by SDS-PAGE under non-reducing conditions followed by electrophoretic transfer to a PVDF membrane, 0.2µm, (BioRad, Hercules, CA, USA). The membrane was then cut into strips and blocked. Subsequently each strip was probed overnight at 37°C with individual sera diluted 1:13. Thereafter, the strips were washed followed by incubation for 4 hours at 37°C in alkaline phosphatase conjugated goat anti-human IgE (Biosource) diluted 1:2000. The strips were then washed and the bound antibodies were visualized using BCIP/NBT (BioRad) as substrate (14). The stained strips were scanned in a StudioStar scanner (Agfa-Gevaert, Leverkusen, Germany) using the program QuantiScan (Biosoft, Cambridge, U.K.).

Peptide synthesis

Octapeptides, overlapping four amino acids, corresponding to the α_{2u} -globulin sequence (Fig. 1) were synthesized on derivatised polyethylene rods arranged in the format and spacing of the wells of a microtitre plate using the Multipin™ Peptide Synthesis Kit (Chiron Technologies, Clayton, Australia). Peptide synthesis was performed according to the instructions of the manufacturer. Briefly, Fmoc-amino acid active esters were coupled by repetitive cycles to the pins; one residue per cycle from the C-terminus to the N-terminus of the peptide chain. The synthesis was finished by capping the Fmoc-deprotected terminal amino group using acetylation followed by removal of side chain protecting groups with trifluoroacetic acid (TFA). In each coupling solution the amino acid concentration was 60 mM. Coupling was performed overnight. All solvents used were of pro analysi or peptide synthesis grade. The peptides were satisfactorily synthesised as indicated by the absorbance values in the ELISA assay for the positive and negative control peptides, respectively.

ELISA screening

The set of peptides was scanned for sequential epitopes in an ELISA system. In the study we focused mainly on the allergenicity and hence the IgE-antibodies. In all steps to follow EIA/RIA plates (flat bottom, high binding) (Costar, Cambridge, MA, USA) were used.

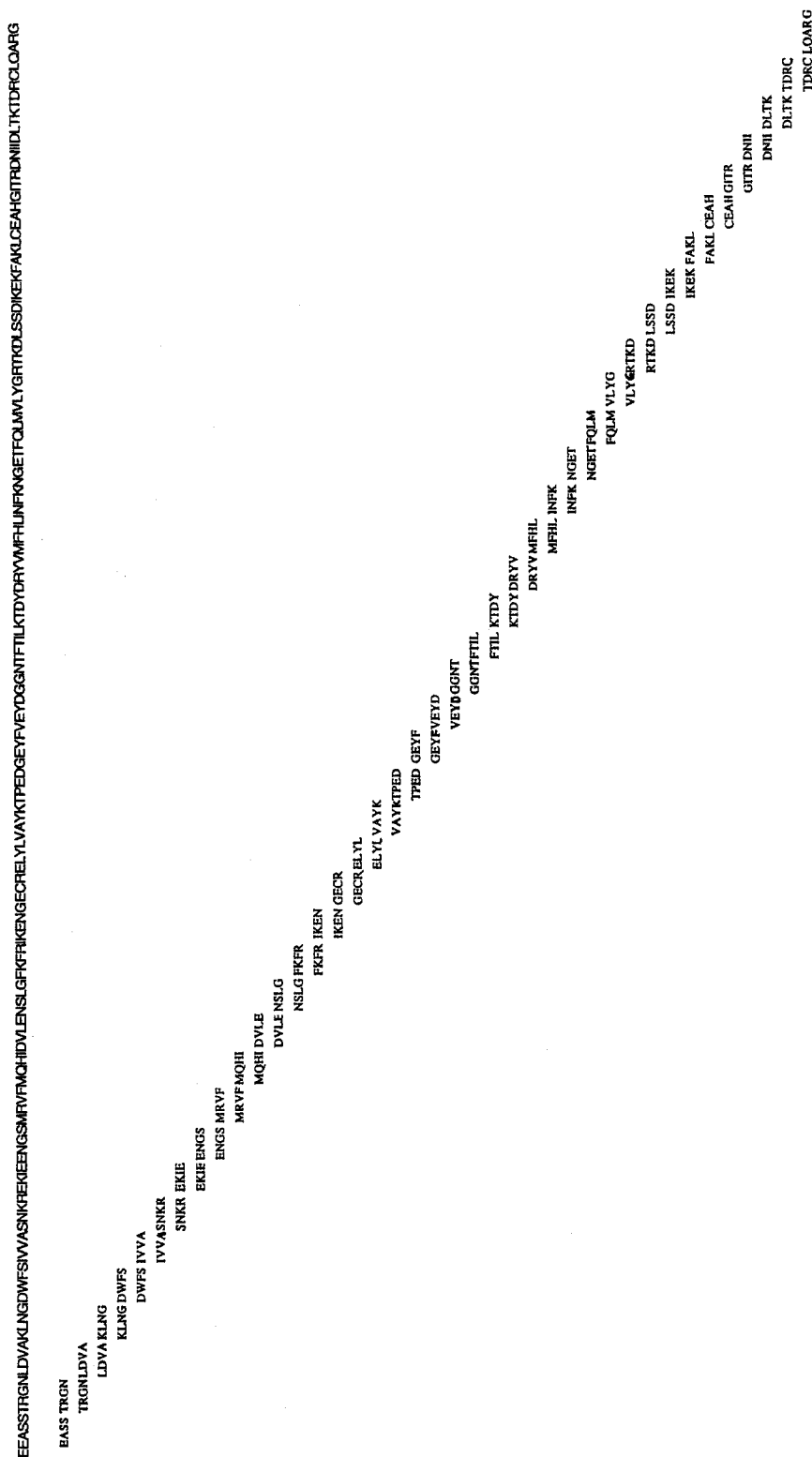


FIGURE 1
The synthetic octapeptides spanning the α_{2u} -globulin protein sequence from N-terminus to C-terminus with four amino acids overlap.

The ELISA procedure was as follows. The pins with the peptides were blocked for one hour at room temperature by agitation in a pre-coating buffer, containing 2% w/v bovine serum albumin (BSA), fraction V (Boehringer-Mannheim, Mannheim, Germany), 0.1% v/v Tween-20 (Merck, Darmstadt, Germany), and 0.1% w/v sodium azide in 0.01 M phosphate buffered saline (PBS) pH 7.2. Excess pre-coat buffer was flicked off and the pins were incubated with gentle shaking overnight at 4°C in a 50-fold dilution of patient sera in pre-coat buffer. Subsequently the pins were brought to room temperature by incubation for additionally one hour with the diluted test sera. After four washings in PBS, each 10 min., the pins were incubated for three hours on a plate shaker at room temperature with a secondary alkaline phosphatase conjugated antibody, goat anti-human IgE-AP (Biosource) diluted 1:500 in conjugate diluent, containing 1% w/v BSA, 1% w/v ovalbumin and 0.1% v/v Tween-20 in PBS. After washing in PBS 4 X 10 min by agitation at room temperature, the pins were immersed in freshly prepared p-nitrophenyl phosphate, pNPP, (Sigma) substrate solution and incubated while protected from light with aluminium foil. After 90 minutes the optical density was read at 405 nm in a V_{max} ELISA plate reader (Molecular Devices).

The covalently linked peptides on the pins could be repeatedly tested by removing the bound antibodies after each assay by a disruption procedure. Briefly, the pins were sonicated at 65°C for 30 minutes in disruption buffer containing 0.1 M phosphate buffer, 1% w/v SDS and 0.1% v/v mercaptoethanol followed by rinsing twice in warm high-quality water at about 60°C. Additional sonication for two minutes in warm ultra pure water was performed followed by immersing the pins in boiling methanol for two minutes. Thereafter the pin device was dried for thirty minutes in a desiccator.

IgG screening was done in analogy to the IgE screening except for the following modifications: 1) the sera were diluted 1:1000 in precoat buffer; 2) the secondary antibody, alkaline phosphatase conjugated goat anti-human IgG (Mabtech AB, Stockholm, Sweden) was diluted 1:1000 in conjugate buffer; 3) the pins were incubated for 1 hour with the conjugate and 4) the absorbance was read after 60 minutes at 405 nm. For some sera the IgE binding values were under or only slightly above the conjugate values. This might indicate binding of the IgE conjugate to the peptide pins due to charge effects or saturation. Therefore a pool of seven non-allergics was used for background subtraction in both the IgE and the IgG assays even though the IgG response for each peptide was well above the IgG conjugate level. The absorbance value for each pin of the pooled control sera was subtracted from the corresponding pin values of the allergic sera in all readings. In this way the net allergy associated antibody reactivity with the peptides could be compared.

The serum samples used were not repeatedly thawed and frozen since this decreased the antibody reactivities.

Inhibition studies

Concentrated rat urinary protein extract (13) was used to inhibit the binding of the antibodies to the peptides on the pins. The procedure was as follow: a Maxisorp plate was coated with concentrated rat urinary proteins, 10 µg/ml, overnight at room temperature. The plate was then washed followed by incubation overnight at room temperature with patient serum diluted 1:50 in precoat buffer. Thereafter the serum was transferred to a new ELISA-plate and the preblocked pins were immersed and incubated overnight at 4°C with gentle shaking. Subsequent washings and incubations with conjugate and substrate were as described above except that the incubation time with the conjugate was two hours.

Enzymatic cleavage

The purified proteins corresponding to the allergens Rat n 1.01 and Rat n 1.02 were treated with dithiothreitol (Sigma) and iodoacetamide (Sigma) for reduction and alkylation of the cysteines. Then followed SDS-PAGE and Coomassie blue staining without fixing. The stained protein bands were cut out from the gel and digested using the enzyme endoproteinase Lys-C (15, 16). Isolated peptides were sequence analyzed on a PE-Applied Biosystems model 494A instrument (Applied Biosystem, Foster City, CA, USA) following the manufacturer's instruction.

Computer analysis

B-cell epitope prediction

Chou-Fasman prediction of B-cell epitopes (17) was done using the Wisconsin package, version 8.1 (Genetics Computer Group, Madison, Wisconsin, USA).

BLAST

The amino acid sequence of α_{2u} -globulin was searched for homology to other proteins using the BLAST program (Basic Local Alignment Search Tool) (10). All available sequence databases for proteins and DNA are explored by this search engine. The BLAST program is designed to distinguish real matches from random background hits by use of well-defined statistical methods (18, 19).

PeptideMass

Theoretical enzymatic cleavage of α_{2u} -globulin was performed with Lys-C using the program PeptideMass (20) available on the molecular biology World Wide Web server ExPASy tools menu.

Molecular mass determination

Molecular masses of isolated peptides were determined by mass spectrometry on a BIFLEX MALDI-TOF (Bruker Franzen Analytik GmbH, Bremen, Germany) according

to the manufacturers recommendation. Matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid.

RESULTS

Western blotting and IgE ELISA

Reactivity profiles of IgE-binding to concentrated rat urinary proteins were obtained with sera from rat allergic subjects after Western blotting (Fig. 2). Individual response differences were seen but all sera displayed IgE binding to the major protein, α_{2u} -globulin. In contrast, very low IgE binding to α_{2u} -globulin was seen with control sera both in Western blotting and in the specific ELISA. Scanning of the α_{2u} -globulin band in individually incubated membrane strips, stained after incubation with patient sera, revealed poor correlation to the corresponding ELISA values in the allergen specific IgE analysis with the native protein (α_{2u} -globulin coated assay) (not shown).

IgE binding to overlapping peptides

To investigate if the IgE antibodies preferentially bound to certain regions of α_{2u} -globulin, the reactivity of IgE in sera from rat-allergic individuals was assayed using overlapping synthetic peptides representing the α_{2u} -globulin amino acid sequence. The net absorbance value for the IgE-binding activity with each peptide was obtained by subtracting the background values for the individual peptides seen with the control serum pool. The specificity of the IgE antibody reactivity with the peptides was assessed by the inhibition of the antibody reactivity of selected sera using a rat urinary protein extract (not shown). The degree of antibody binding to each peptide varied for the individual sera. Generally low reactivities were obtained and the peptides showing the highest IgE binding varied between the individual sera.

With an arbitrary cut-off of OD 0.05, to account for the variability in the assay, some peptides stood out as more frequently recognized by IgE antibodies (Fig. 3). Thus peptides 4, 7, 11, 15, 24, 32, 36 and 38 showed reactivity with 5 to 7 of the 15 sera. By these criteria peptides 13, 22 and 34 were not recognized by any of the sera.

Also the IgG binding to the overlapping peptides was analyzed for a few sera from both atopic and non-atopic individuals. As with IgE, the peptides recognized by IgG antibodies varied considerably between different donors and no specific pattern could be seen (not shown). In general the IgG reactivities were higher than the IgE reactivities, but there appeared to be no correlation between IgG- and IgE-binding reactivities with individual peptides. For some atopic sera, the IgE reactivity with some of the peptides was higher than that of IgG, but no specific allergenic sequences could be distinguished (not shown).

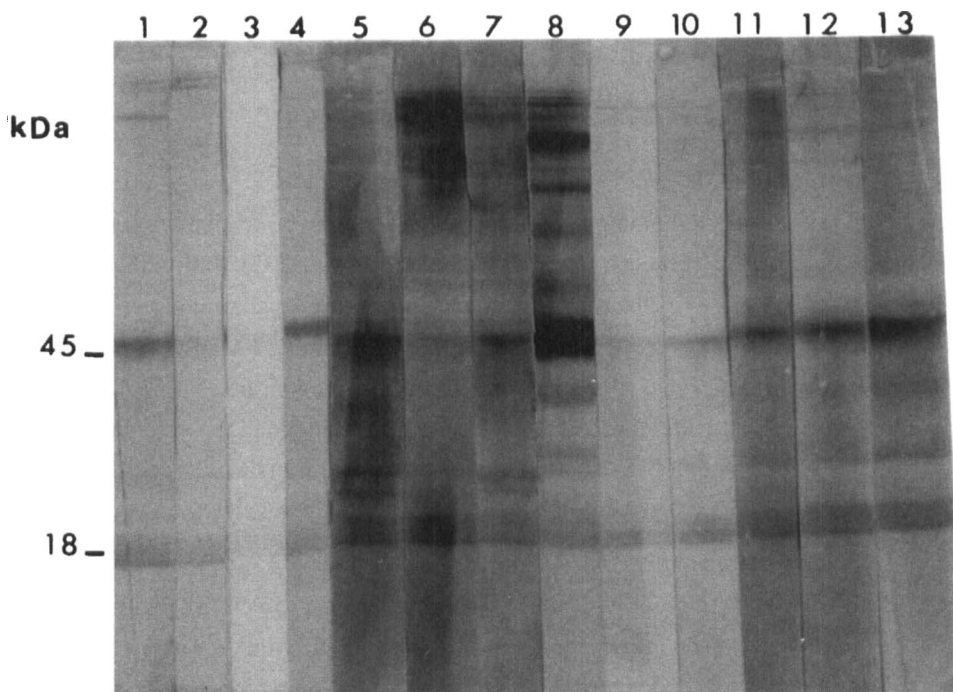


FIGURE 2

Concentrated rat urinary proteins having bound specific patient IgE after separation under non-reducing conditions by SDS-PAGE, electroblotted to a PVDF membrane and visualized by alkaline phosphatase staining. Sample volume was 300 μ l. The protein α_2 -globulin is seen in the 18 kDa region. Lane 3 is probed with serum from a nonallergic patient and used as control.

B-cell epitope prediction

The Chou-Fasman prediction model indicated that the most probable epitope region in the protein sequence was between amino acids 50 and 102. According to the criteria used above two out of the seven peptides with highest recognition frequency namely peptides no. 11 and 15 corresponding to the amino acids 61-68 and 77-84, respectively, were located within this region. However, peptide 13 with no reactivity to any of the allergic sera, using the same criteria for IgE binding, was also located within this predicted region (Fig. 4).

BLAST

Using the BLAST program 59 alignments were found with homology to α_2 -globulin. For most of the sequences the homology was limited, sometimes not more than 25%. Bos d 2, the predominant allergen of bovine dander, bovine dander major allergen precursor, showed 27% sequence homology. This protein and α_2 -globulin are members of the

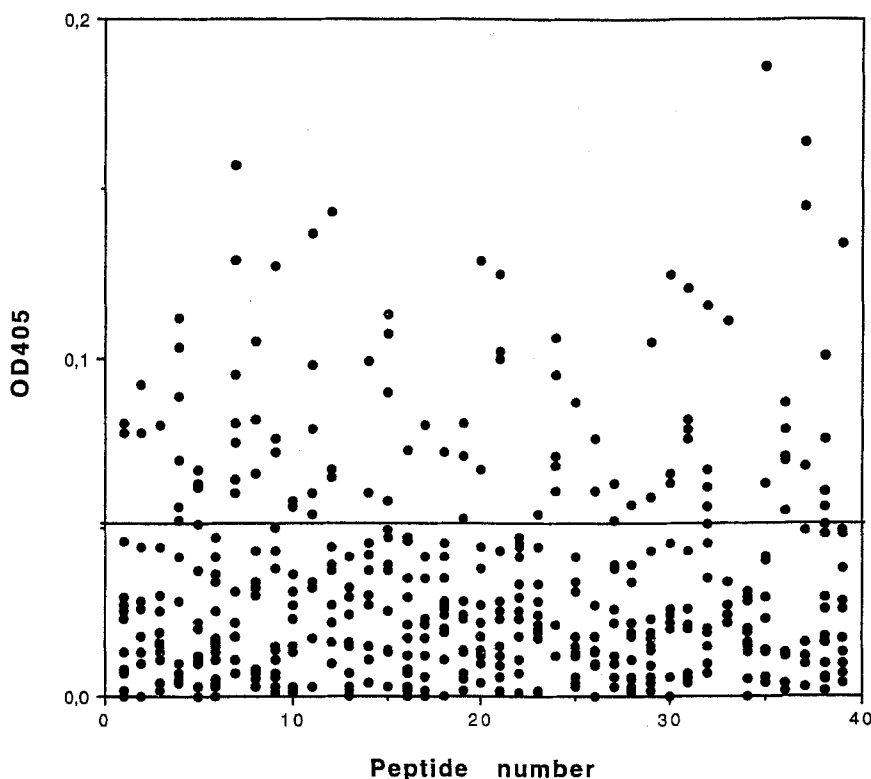


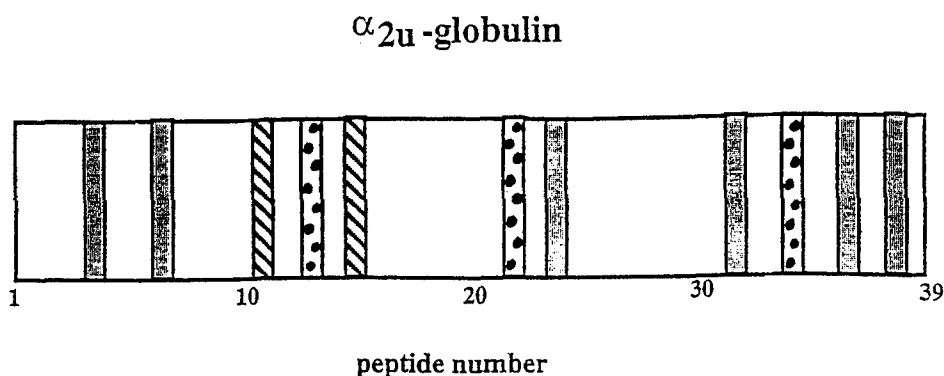
FIGURE 3

Obtained values of OD at 405 nm in the analysis of octapeptides having bound specific patient IgE. The horizontal line indicates an arbitrary cut-off at OD 0.05 used to account for the variability in the assay. A few peptides gave values above this limit and thus stood out as more frequently recognized by IgE antibodies.

lipocalin family (21, 22). Other allergens were recognized as well, e.g. horse allergen (Equ c 1) and minor dog allergen (Can f 2) with 49 and 30% identity, respectively. These proteins are also members of the lipocalin family (23, 24).

Enzymatic cleavage

Peptide mapping by enzymatic digestion and chemical cleavage are used for proteins with unknown sequence. Both proteins corresponding to the allergens Rat n 1.01 and Rat n 1.02 were enzymatically cleaved with Endoproteinase Lys-C and the derived peptide fragments were compared. Significantly different mapping chromatograms for the two allergens were obtained (Fig. 5). Sequence analysis of peptides from these two allergens was in accordance with earlier studies (11) thereby clearly verifying our findings of



Octapeptides, overlapping four amino acids, corresponding to the α_{2u} globulin were tested for IgE binding reactivities using sera from rat allergic individuals.

-  = peptides showing IgE binding reactivities
-  = peptides showing IgE binding reactivities and predicted to be B-cell epitopes according to the Chou-Fasman prediction
-  = peptides showing no IgE binding reactivities

FIGURE 4

Octapeptides, overlapping four amino acids, corresponding to the α_{2u} -globulin were tested for IgE binding reactivities using sera from rat allergic individuals.

isoforms of α_{2u} -globulin. Rat n 1.02 was also theoretically cleaved with Lys-C using the program PeptideMass in the molecular biology World Wide Web server ExPASy under Tools menu (20). The program PeptideMass confirmed the obtained sequences of the experimental peptides.

DISCUSSION

Overlapping octapeptides derived from the parent protein α_{2u} -globulin were synthesized by the multipin peptide synthesis system and used to identify IgE-binding epitopes. However, by this method antibodies reactive with conformational epitopes within an antigen are not detected. Even though 90% or more of surface associated B-cell epitopes may be assembled from non-contiguous parts of macromolecules (25) and linear peptides only represent a part of a more complex discontinuous epitope it is relevant to determine

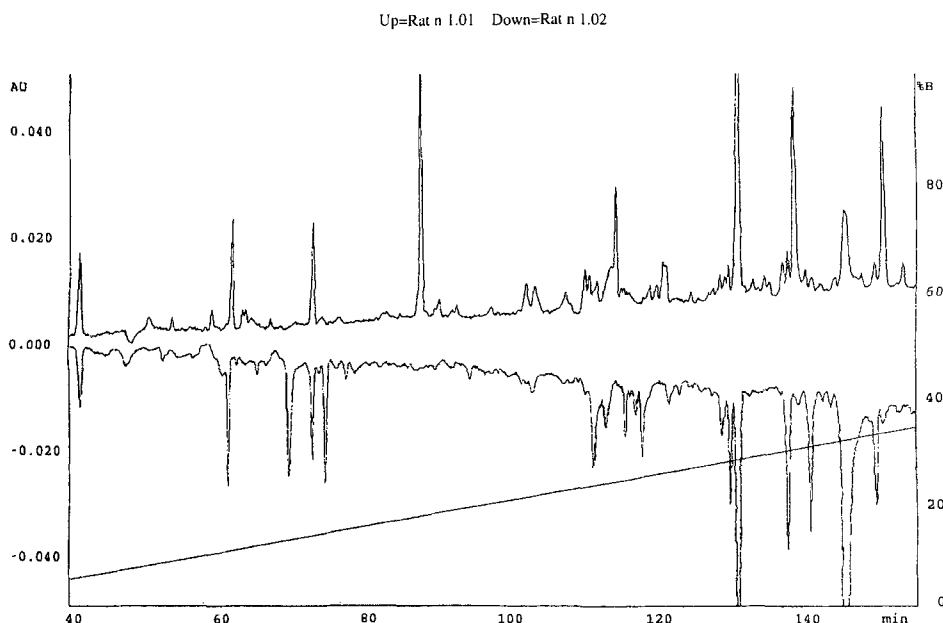


FIGURE 5

Peptide maps of Rat n 1.01 and Rat n 1.02 after separation by reversed phase chromatography using the SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden). The maps display close similarity but a few peptides were different, e.g. at 70, 75, 90 and 140 minutes.

them, since a linear fragment may have characteristic conformational features resembling those of the closely related structure in the parent protein. Furthermore, a suitable conformation could arise in the peptide during binding to the antigen binding site of the antibody (26).

No exclusively specific linear IgE binding regions were found in the $\alpha_2\mu$ -globulin sequence. Rather many of the peptides were frequently recognized by patient IgE-antibodies. However, a few separate peptides were recognized more often than others. These peptides seemed to be clustered towards both the C-terminal (peptides 32, 36 and 38) and the N-terminal (peptides 4, 7 and 11) parts of the protein. This is consistent with reports of B-cell epitopes tending to be located in flexible and protruding regions such as the terminal parts of the protein (27).

The antibodies bound selectively ensuring that the observed response is directed against the sequence synthesized and not to small amounts of peptide by-products which unavoidably are formed during peptide synthesis (9).

The IgE binding capacity of the synthetic peptides was generally relatively low. Low peptide reactivity has also been reported by others using both non-cleavable solid support bound peptides (25), cleavable peptides coupled to CNBr-activated Sepharose-4B (28-30) as well as recombinant fusion peptides (31). The weak reactions with the octapeptides seen in the ELISA assay could be due to low affinity mimotopes or that the peptides only comprised a portion of the correct epitope (8). Mimotopes resemble the antigenic regions of the native protein where both shape and charge of the complementary conformation of the antibody's binding site and the molecular surface of the binding peptide is maintained (32, 33). Neighbouring but non-contact residues, which not actively are involved in the antibody binding, may contribute to the conformation of the antigen required for binding to a particular antibody (9).

Each individual patient's IgE was found to be fine-tuned to react with different subsets of allergens as illustrated in Western blotting (Fig. 2), (12). The poor correlation between the scanning results of the $\alpha_2\mu$ -globulin band for respective sera and the corresponding ELISA values could be explained by differences in the two assay systems used. In the immunoblotting system the binding strength of the antigen to the membrane is influenced by the detergent used during the blocking, washing and incubation steps. The detergent could cause destabilization of the binding of the antigen to the membrane and thereby loss of protein (34). Furthermore, varying grades of denaturation exist in the two systems. Even though no SDS is used in the ELISA system, denaturation of the native protein can occur during attachment to the microtiter plate plastic surface (35, 36). As a consequence antibodies specific for the native protein might fail to recognize the denaturated protein or bind to a lesser extent.

The ELISA results indicate that IgE antibodies show better reactivity with the parent protein than with the octapeptides. This could be ascribed to the linear peptides which may represent only a small part of a more complex discontinuous epitope. Another aspect is the common heterospecificity of the antibodies due the polyspecific paratope of the antibody. Furthermore, the full length protein contains several antigenic determinants as compared to the peptides to which the antibody can bind.

The low IgE absorbance values obtained could be due to the blocking of the epitopes by IgG antibodies, because of their high abundance compared to the IgE molecules.

Data from the IgG analysis are more difficult to interpret and no significant differences were seen between non-allergic and allergic subjects. Thus, peptide specific IgG antibodies are most probably present in both non-allergic and allergic subjects.

Classical methods used for peptide mapping, i.e. enzymatic digestion and chemical cleavage are less reliable and controllable compared to the synthetic approach described here. Analysis of the cleavage process could sometimes be difficult to interpret due to

inefficient cutting, incomplete reduction or alkylation of the protein and also oxidation processes giving artifactual protein modifications (37). Nevertheless, these methods are important for peptide mapping when the sequence of the protein is unknown. Accordingly, peptide fragments were compared as derived from enzymatic cleavage of Rat n 1.02 with Endoproteinase Lys-C both experimentally and theoretically using the program PeptideMass. The obtained results indicated a good concordance between these two comparisons. Lys-C treatment of Rat n 1.01 and Rat n 1.02 revealed two different peptide maps. Subsequent sequencing of these peptides clearly confirmed our earlier findings regarding the close relatedness of these two allergens (11).

Successful and complete prediction of the antigenicity of a protein is hard to achieve with currently available methods (26). Accordingly, relatively poor prediction of B-cell epitopes was obtained as compared to our experimental results.

In the BLAST search, 59 proteins with amino acid sequence homologies to α_2 -globulin were found. Among these proteins several other allergens were recognized, e.g. bovine major dander allergen (Bos d 2), horse allergen (Equ c 1), dog allergen (Can f 2) and mouse major urinary protein. This suggests allergenicity as a common property for a group of proteins within the lipocalin family. Proteins from many mammalian species and also proteins from, e.g. frogs and chicken were represented among these homologies.

The three-dimensional structure of the lipocalin family demonstrates a conserved folding pattern (38), showing three characteristic sequence motifs corresponding to the three largest structurally conserved regions (39). Motifs 1 and 2 lie close to each other in the three-dimensional structure and they are also near the surface (40) and thereby most likely constitute a binding site. One of the more frequently recognized IgE binding peptides, no. 4, was completely located within motif 1, whereas peptide no. 7 was only partly located within this motif. Peptide no. 24 and 32 were located entirely within motif 2 and motif 3, respectively. Peptide no. 22 on the other hand with no IgE reactivity was partly located in motif 2. In addition, the magnitude of responses to the peptides corresponding to these three motifs was relatively low in most individuals.

Also common in the tertiary structure of the lipocalin family is an internal ligand binding site (39, 41). This ligand binding pocket is highly hydrophobic. In α_2 -globulin the pocket is formed by the amino acids 59,73,75,109,122,137 and 139 (using the numbering in the SWISS-PROT Protein Sequence Database) (42). However, further studies are required to define if the IgE binding peptides found in this study could be located close to this pocket in the tertiary structure.

One approach in allergy treatment is repeated subcutaneous injections of increasing low-doses of allergens resulting in reduced severity of type I reactions. This effect is probably caused by a shift towards IgG production. The IgG antibodies bind to the

allergen forming a complex which could be removed by phagocytosis and as a result the allergen is no longer available for the IgE antibodies on the mast-cell membrane. In this context, identification of IgE-epitopes common for rat allergics could be useful for development of immunotherapy. However, this study could not identify any peptides suitable for immunotherapeutic use.

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