

Françoise Maynard^a
Rolf Jost^a
Jean-Michel Wal^b

^a Nestlé Research Centre, Nestec Ltd.,
Lausanne, Switzerland;

^b INRA-CEA, Laboratoire d'Immuno-
allergie Alimentaire, Service de
Pharmacologie et d'Immunologie,
CE SACLAY, Gif-sur-Yvette, France

Human IgE Binding Capacity of Tryptic Peptides from Bovine α -Lactalbumin

Key Words

Bovine α -lactalbumin
Tryptic peptides
Allergenicity
Specific human IgE binding

Abstract

The specific IgE binding capacity of native bovine α -lactalbumin (α -La), a globular whey protein, and tryptic peptides was investigated using 19 sera from patients with cow's milk protein allergy. The specific anti-bovine α -La IgE titers ranged from 0.6 to 125 IU/ml. Highly purified tryptic peptides from native and disulfide-bond-reduced α -La were obtained by reverse phase chromatography. By ELISA technique using immobilized native protein or peptides, 11 of the 19 sera reacted exclusively with intact protein while 8 of them also presented a specific IgE response to different tryptic peptides. Polyclonal IgE population specificity was not related to anti-bovine α -La IgE levels. Sequence (17G-K58) and larger peptides sharing this sequence were most strongly and frequently recognized. Competitive ELISA inhibition tests confirmed this IgE-specific response and gave also clear evidence for IgE binding to smaller peptides corresponding to sequences (6C-R10):S-S:(115L-L123) and (109A-L123). IgE binding to native α -La and large peptides confirmed the importance of conformational epitope(s). However, in some sera reduced and S-alkylated peptide (59I-K94) exhibited a similar or higher IgE binding capacity than the native corresponding fragment, suggesting the existence of sequential epitope(s) exposed through protein denaturation. Moreover, IgE binding sequences were also located within hydrophobic regions of α -La and/or within parts with high sequence homology to human α -La.

Introduction

Cow's milk allergy is a common food allergy in infants, the disease prevalence ranging from 0.5 to 7.5% in all children [1, 2]. The allergenicity of cow's milk is related to protein constituents and the most prominent allergens correspond to α - and β -caseins, β -lactoglobulin and α -lactalbumin (α -La).

α -La is a major protein of whey. It is a monomeric globular protein of 123 amino acid residues with 14.4 kD molec-

ular weight and has four disulfide bridges [3, 4]. The complete amino acid sequence of bovine α -La [4] shows extensive homology with human α -La since 74% of the residues are identical and another 6% chemically similar [5]. However, despite this great similarity, bovine α -La has been identified as one of the allergens of cow's milk [6]. On the basis of their amino acid sequences [7] and three-dimensional structures [8, 9], α -La and hen's egg white lysozyme are closely homologous, having evolved by divergence from a common ancestral gene [10, 11].

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Correspondence to: Dr. F. Maynard
Nestlé Research Centre, Nestec Ltd.,
Vers-chez-les-Blanc
CH-1000 Lausanne 26 (Switzerland)
Tel. (+41/21) 785 87 25, Fax (+41/21) 785 85 54

Compared to β -lactoglobulin which, because of its absence in human milk, is often considered as a major milk allergen, only a few reports deal with the antigenicity and allergenicity of peptides from bovine α -La.

Using radioimmunoassay, Hopp and Woods [12] investigated the antigenic activity of some peptides from a peptic digest of bovine α -La with rabbit antisera. The largest amount of immunoreactivity was observed for peptide (60–80):S-S:(91–96), but other peptic fragments (26–31):S-S:(105–117), (4–9):S-S:(119–123) and (10–18) were also antigenic.

To our knowledge, the only study performed with human sera from cow's milk allergic patients has been reported by Adams et al. [13]. According to the prediction of IgG binding sites [14–16], they synthesized the fragment (5–18) and showed by RAST® inhibition that this synthetic peptide from bovine α -La exhibited an IgE binding capacity.

The aim of the present study was to investigate *in vitro* the allergenicity of peptides isolated from tryptic digests of native and disulfide-bond-reduced bovine α -La. The IgE binding capacity of each purified peptide was tested by direct and by competitive ELISA inhibition using human sera from patients with allergy to cow's milk proteins and particularly to α -La.

Materials and Methods

Materials

Bovine α -La (type I) and trypsin (EC. 3.4.21.4, bovine pancreas, 12,200 U BAEE/mg) treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma (St. Louis, Mo., USA). Human serum albumin was obtained from the 'Centre National de Transfusion Sanguine' (Les Ulis, France).

HPLC grade water and acetonitrile (Licrosolv) were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), phenylisothiocyanate (PITC) and amino acid standard were from Pierce (Kontron Instruments, Lausanne, Switzerland). All other reagents were of analytical grade.

HPLC was performed with a HP1050 system (Hewlett Packard, Basel, Switzerland) consisting of an autosampler, a quaternary pump fitted to an on-line degasser, a column heater and a diode array detector. The FPLC system (Pharmacia, Dübendorf, Switzerland) was composed of a LCC-500 gradient controller equipped with two P500 pumps, a 214 nm wavelength detector linked to a chart recorder and a Frac 100 collector. Mass spectrometry analyses were performed on a Finnigan TSQ 700 mass spectrometer equipped with thermospray interface.

The ELISA equipment consisted of an automatic microplate washer 120, an automatic dispenser (Autodrop) and a spectrophotometer (Multiskan MC) from Flow Laboratories (Helsinki, Finland). Microtiter plates (96F immunoplates I with certificate) were purchased from Nunc (Denmark).

Methods

Reduction and S-Carboxymethylamidation of α -La. Reduction and S-carboxymethylamidation of bovine α -La disulfide bridges were performed according to a method derived from Swenson et al. [17]. α -La was dissolved in 1.44 M Tris HCl buffer, pH 8.6, in the presence of 3 M final concentration guanidine HCl, 0.13 M EDTA and 2- β -mercaptoethanol. After 4 h incubation in the dark at room temperature, 1.44 M iodoacetamide was used for blocking of free thiols. The reduced and S-carboxymethylamidated α -La (α -La R) was dialyzed against distilled water and lyophilized.

Digestion of Native and Reduced Bovine α -La. Native α -La (α -La N) and α -La R were digested by TPCK-treated trypsin at 55°C in 1 mM CaCl_2 , 0.02% NaN_3 , pH 7.7, using an enzyme:substrate ratio of 1:100 (w/w) under constant stirring. After 30 (α -La R) or 45 min (α -La N) hydrolysis, the reaction was stopped by inactivation of trypsin 10 min at 80°C. The two hydrolysates were then lyophilized.

Reverse-Phase HPLC Analyses. The tryptic hydrolysates of α -La N and α -La R were characterized in reverse-phase (RP)-HPLC on a C18 Nucleosil 300–5/ET 250 column (Macherey Nagel, Oensingen, Switzerland). Operating conditions were as follows: column temperature, 40°C; flow rate, 1 ml/min; solvent A, 0.1% (v/v) TFA in water; solvent B, 60% (v/v) acetonitrile in solvent A. Elution was performed with a linear gradient established from 0 to 35% (v/v) solvent B in 15 min, 35–65% (v/v) B in 25 min and 65–100% (v/v) B in 10 min. Absorbance was recorded both at 214 and 280 nm.

Collection and Purification of Peptides. The peptides from α -La N and α -La R tryptic hydrolysates were collected after separation by RP-FPLC on a C18 Pharmacia Pep RPC HR 5/5 column (Pharmacia). Elution was performed by an acetonitrile gradient from 100% solvent A [0.1% (v/v) TFA in water] to 35% (v/v) solvent B [60% (v/v) acetonitrile in solvent A] in 15 min, 35–55% (v/v) B in 20 min and 55–100% (v/v) B in 3 min. The column was at ambient temperature, the flow rate was 1 ml/min and absorbance was recorded at 214 nm. Collected peptides were dried in a Speed-Vac concentrator and each peptide was further purified using the same column and solvents but with varying gradients.

Peptide Identification. After acid hydrolysis under vacuum in the presence of 6 N HCl for 24 h at 110°C in a Pico-Tag station (Waters, Schlieren, Switzerland), amino acids were derivatized with PITC according to the method of Bidlingmeyer et al. [18], and quantified by RP-HPLC on a C18 Pico-Tag column (Waters). The carboxymethylcysteine content was quantified after 1 h hydrolysis at 150°C in 6 N HCl [19] to prevent the carboxymethylcysteine conversion into cysteic acid. The results of amino acid analyses were sufficient for assignment of the tryptic peptides within the known sequence of bovine α -La. However, mass spectrometry was also used to confirm the identity of some peptides.

Human Sera. Serum samples were provided by different hospitals in France and were obtained from patients presenting clinical symptoms of type I (immediate) allergy to milk proteins. Nineteen different sera were preselected according to their RAST-positive response to milk proteins and particularly to bovine α -La. The children/adult repartition among these sera was as follows: 3 were obtained from 2- to 3-year-old children, 10 from 5- to 12-year-old children and 6 from adults, i.e. more than 15 years. In this study, there was no serum from infants less than 2 years old.

Specific IgE to the main milk proteins (total caseins, α -La, β -lactoglobulin and bovine serum albumin (BSA)) were determined according to Wal et al. [20]. Eight of 19 sera of which (e.g. S7) had specific anti-bovine α -La IgE and also anti-total caseins, anti- β -lactoglo-

bulin and anti-BSA IgE. Five of 19 sera were IgE sensitive to α -La plus two others of these milk proteins (e.g. S5). Four of 19 sera reacted to α -La and another milk protein, either total caseins, β -lactoglobulin or BSA (e.g. S2, S3, S6, S8). Two of 19 sera were only IgE sensitive to α -La (e.g. S4).

ELISA. Enzyme immunoassays (EIA) of specific human IgE were used as described [20]. Briefly, microtiter plates were coated with 100 μ l/well of a 5 μ g/ml solution of each purified protein (α -La N) or tryptic peptides in the coating buffer (0.05 M sodium carbonate buffer, pH 9, 0.01% NaN₃). After incubation overnight at ambient temperature, plates were extensively washed with a 10 mM potassium phosphate buffer, pH 7.4, 0.05% Tween 20. Plates were then saturated with 250 μ l EIA buffer (0.1 M potassium phosphate buffer, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% human serum albumin and 0.01% sodium azide) per well, sealed and stored at 4°C until use. Plates were washed before use. For direct ELISA of human specific IgE, 50 μ l of individual human serum at appropriate dilution were dispensed per well. After incubation overnight at room temperature, plates were washed. Then, 50 μ l of a solution of mouse monoclonal antibody (mAb) anti-human IgE (clone BS17) labelled with acetylcholinesterase (AChE) were added per well. Following an incubation overnight with mAb-AChE, plates were washed and 200 μ l of Ellman's reagent, prepared according to Ellman et al. [21] and used as enzyme substrate, were dispensed into each well. Absorbance was measured at 414 nm.

Six sera from individuals not allergic to cow's milk were used as negative controls. Moreover, nonspecific binding was tested in α -La- and peptide-positive sera using a synthetic 20-amino-acid-residues non- α -La-related peptide as immobilized antigen.

For specific human IgE, quantification as IU (i.e. 2.4 ng/ml) was made by comparison with dose-response curves obtained with a total IgE assay performed with known standards of human IgE (Pharmacia Prist test kit), as described by Grassi et al. [22]. The corresponding immunometric assays of total and specific IgE were performed under identical conditions. An anti-human IgE mAb (clone LE27), complementary to the BS17-AChE tracer, was used as capture antibody for the total IgE assay, instead of the immobilized, purified allergen preparation used for specific IgE determination [20].

The background (i.e. nonspecific binding) was defined as the mean value obtained on specific IgE determinations in 6 different control sera from non-allergic individuals. The interassays standard deviation was calculated on these 6 values and the detection limit of specific anti- α -La and anti-peptide IgE was defined as the background plus 3 times standard deviation.

To confirm direct determinations, human sera with specific IgE to α -La and peptides were also tested by competitive ELISA inhibition, when sufficient volumes of sera were available. The inhibitors at variable concentration (30 μ l in EIA buffer) were preincubated 18 h with human sera (30 μ l at appropriate dilution in EIA buffer) at ambient temperature. Inhibitors were used at concentrations ranging from 2 to 20 nmol/ml for α -La and 1 nmol to 1 μ mol/ml for peptides. Quantification of peptides was made according to their amino acid composition analyses. Then, 50 μ l of the mixture were transferred into the allergen/antigen-coated wells. The amount of tracer bound in the absence of competitors (Bo) was determined using an incubation mixture containing 30 μ l of EIA buffer instead of sample and 30 μ l of different human sera. All other steps of the competitive inhibition were performed as reported above. The results of competitive inhibition were expressed as percent inhibition, calculated as $(Bo-B/Bo \times 100)$ where B and Bo represent the absorbance measured on the bound fraction in the presence or absence of competitors, respectively.

In all competitive ELISA inhibition tests, specific inhibition was measured by comparison with nonspecific background observed with a synthetic α -La-unrelated peptide used as negative control. At the highest concentration (1 μ mol/ml), the nonspecific background was always lower than 15% (12–15%). Inhibition was thus considered as significant when it was $\geq 25\%$.

Results

Tryptic Peptides of Native and Reduced, S-Alkylated α -La

Despite S-S bond reduction, no change was observed in the elution time (46 min) of the α -La R versus α -La N in RP-HPLC (results not shown). As shown by RP-HPLC peptide profiles of α -La N and α -La R tryptic hydrolysates (fig. 1), intact protein was no longer detectable in the two digests. The complete tryptic hydrolysis of α -La N was resolved into 9 main peaks, T1–T9 (fig. 1b). All these peaks corresponded to homogeneous peptides except T1 which was separated into T1a and T1b during repurification on RP-FPLC.

The sequences of peptides from α -La N were identified from their amino acid composition (table 1) as follows: T1a $\rightarrow$ (14D-K16), T1b $\rightarrow$ (11E-K13), T2 $\rightarrow$ (1E-K5), T3 $\rightarrow$ (95I-K98), T4 $\rightarrow$ (104W-K108), T5 $\rightarrow$ (99V-K108), T6 $\rightarrow$ (6C-R10):S-S:(115L-L123), T7 $\rightarrow$ (59I-K93), T8 $\rightarrow$ (17G-K58):S-S:(109A-K114) and T9 $\rightarrow$ (17G-K58):S-S:(109A-L123):S-S:(6C-R10) (fig. 2). This set of peptides covered the entire amino acid sequence of bovine α -La [4].

Because of the large size of these peptides and due to the presence of intrachain S-S bridges, smaller sequences were prepared via reduction and S-carboxymethylamidation of bovine α -La disulfide bonds followed by tryptic hydrolysis (fig. 2). As expected, this treatment only affected the disulfide-bond-containing peptides T6, T7, T8 and T9 identified above which were missing in the α -La R digest (fig. 1c). Peptides T1a, T1b, T2, T3, T4 and T5 were not modified. The identity of these last peptides T1–T5 in the α -La R hydrolysate was confirmed by RP-HPLC as above. Moreover, the α -La R digestion by trypsin induced the formation of new specific fragments R1–R6 (fig. 1c), which were all found as homogeneous peptides during repurification. Peptides R2, R5 and R6 were identified by their amino acid composition (table 2) as sequences (115L-L123), (109A-L123) and (17G-K58), respectively, of α -La R (fig. 2). Sequences (59I-K94) and (59I-K93) were assigned to peptides R3 and R4 according to both amino acid analyses and mass spectrometry determinations (table 2). Despite repurification, no sequence from α -La R could be assigned to peptide R1. The bovine α -La amino acid sequence

Table 1. Amino acid composition and molecular weight of tryptic peptides from native α -La

Amino acids residue/mol ^a	peptide T1a	peptide T1b	peptide T2	peptide T3	peptide T4	peptide T5	peptide T6	peptide T7	peptide T8	peptide T9
Asx	1.03 (1)			0.92 (1)		1.05 (1)	1.06 (1)	11.04 (11)	5.48 (6)	6.48 (7)
Glx		1.19 (1)	2.08 (2)				2.41 (3)	1.46 (1)	5.71 (6)	9.01 (9)
CMC ^b										
Ser								2.80 (3)	3.42 (4)	4 (4)
Gly						1.19 (1)			4.88 (5)	5.24 (5)
His					0.92 (1)	1.07 (1)		1.03 (1)	1.28 (1)	1.41 (1)
Arg							0.92 (1)			1.13 (1)
Thr			1.06 (1)					1.25 (1)	4.62 (5)	4.79 (5)
Ala					1.12 (1)	1.15 (1)			1.89 (2)	2.22 (2)
Pro								1.02 (1)	1.27 (1)	1.23 (1)
Tyr						1 (1)			2.62 (3)	2.79 (3)
Val						1.15 (1)	0.86 (1)	1 (1)	2.56 (3)	3.65 (4)
Met								0.99 (1)		
Ile				0.97 (1)		1.07 (1)		3.37 (4)	1.54 (2)	1.77 (2)
Leu	1.25 (1)	0.89 (1)	1.04 (1)	1.02 (1)	0.79 (1)	1.08 (1)	2.70 (3)	2.17 (2)	2.78 (3)	5.55 (6)
Phe							1.20 (1)	1.03 (1)	1.91 (2)	2.63 (3)
Lys	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	2.73 (3)	2 (2)	2.47 (3)
MW, Da ^c	374.4	388.44	617.67	487.55	653.73	1,200.31	1,798	4,041.2	5,304.46	7,084.5
MW by MS ^d	374	388	617.5	486	709.4	1,200.3	1,798	ND	ND	ND
Sequence assigned	(14–16)	(11–13)	(1–5)	(95–98)	(104–108)	(99–108)	(6–10)- (115–123)	(59–93)	(17–58)- (109–114)	T6+T8

ND = Not determined.

^a Theoretical values are in parentheses.^b Carboxymethylamidocysteine quantified after 1 h of hydrolysis in 6 N HCl at 150°C.^c Calculated molecular weight according to theoretical sequence.^d Molecular weight determined by mass spectrometry.

was not entirely covered by the reduced tryptic peptides since the pentapeptide (6C-R10) was not identified among R2–R6.

IgE Binding Capacity of Tryptic α -La Peptides in Direct ELISA

In the 19 sera of patients allergic to cow's milk protein, the specific anti- α -La N IgE titers ranged from 0.6 to 125 IU/ml. The IgE immunoreactivity of peptides T5–T9, R2, R3, R5 and R6 was first investigated by direct ELISA. Table 3 shows the human IgE binding capacity of these immobilized peptides. In each case, specific IgE response was quantified as IU/ml as described in the Methods. The detection limit for specific IgE directed against α -La or peptides varied slightly depending on the immobilized antigen. It was determined as described in the Methods from the non-specific binding measured with negative control sera, and could be roughly estimated to 0.1 IU/ml when determinations were performed in sera diluted 1/16.

Of the 19 human sera preselected and tested, only 8 gave a positive and significant IgE response to immobilized peptides. All the others, whatever their IgE anti- α -La N titers were, only reacted with immobilized α -La N. In all sera, the polyclonal IgE populations were reactive to more than one peptide, e.g. three to nine depending on the serum tested. All the native and reduced peptides tested exhibited an IgE binding capacity at least in some sera.

The IgE responses to the different tryptic peptides were either in the same order of magnitude as the specific IgE response to α -La N (half of sera) or much lower (table 3).

The most frequently recognized peptides in direct ELISA were peptides T9 (T8+T6; 8/19), T8 (7/19), R6 and T7 (5/19), R3 (3/19), T5, T6 and R5 (2/19). No significant signal was observed with allergic patient sera on human serum albumin or synthetic 20-residues α -La-unrelated peptide-coated microplates.

The highest IgE binding capacities were observed with peptides T9, T8 and R6 (176-K58). For one serum, S3, spe-

Table 2. Amino acid composition and molecular weight of tryptic peptides from reduced and S-alkylated α -La

Amino acids residue/mol ^a	peptide R2	peptide R3	peptide R4	peptide R5	peptide R6
Asx	1.3 (1)	11.06 (11)	9.09 (11)	1.36 (1)	6.18 (6)
Glx	2.37 (2)	1 (1)	2.17 (1)	3.19 (3)	5.04 (5)
CMC ^b	1 (1)	3.47 (4)	3.04 (4)	1.98 (2)	0.67 (1)
Ser		2.69 (3)	3 (3)	1.04 (1)	2.78 (3)
Gly					5 (5)
His		0.73 (1)	0.76 (1)		0.80 (1)
Arg					
Thr		0.96 (1)	1.20 (1)		4.71 (5)
Ala				1 (1)	1.09 (1)
Pro		0.98 (1)	1.21 (1)		1.06 (1)
Tyr					2.03 (3)
Val		1.05 (1)	0.93 (1)		2.27 (3)
Met		0.77 (1)	0.57 (1)		
Ile		3.41 (4)	2.62 (4)		1.38 (2)
Leu	3 (3)	2.19 (2)	2.50 (2)	4.09 (4)	1.78 (2)
Phe		0.89 (1)	0.73 (1)		1.35 (2)
Lys	0.41 (1)	2.09 (4)	2.04 (3)	1.70 (2)	0.34 (1)
MW, Da ^c	1,223.35	4,477.65	4,349.48	1,931.13	4,732.8
MW by MS ^d	1,204	4,400.5	4,272.6	1,893	ND
Sequence assigned	(115–123)	(59–93/94)	(59–93)	(109–123)	(17–58)

ND = Not determined.

^a Theoretical values are in parentheses.

^b Carboxymethylamidocysteine quantified after 1 h of hydrolysis in 6 N HCl at 150°C.

^c Calculated molecular weight according to theoretical sequence.

^d Molecular weight determined by mass spectrometry.

cific IgE immunoreactivity against peptide R6 (17G-K58) was similar to those directed to the larger peptides T9 and T8 that contained this sequence (fig. 2). These three peptides appeared to be the most immunoreactive portion of the molecule and to be or to contain within their sequences major epitopes. Particularly, they are the only ones recognized by sera S2, S3 and S4. It is worth noting that using direct ELISA for which peptides are directly immobilized onto solid phase, shorter peptides T6 (6C-R10):S-S:(115L-L123) and R5 (109A-L123) were not recognized by the IgE of these sera although they share common sequences with peptides T9 and T8. This could be related either to the absence of epitope(s) located in their sequences or to structural modification or steric hindrance for IgE binding due to solid phase-peptide interactions. Moreover, the reduced immobilized sequence (59I-K94; peptide R3) exhibited an IgE binding capacity in the same order of magnitude as corresponding native immobilized peptide T7 that contains two intact disulfide bridges. This result could be due either to the presence of sequential epitope or to a conformational modification through solid phase interactions.

All other sera presented IgE reactivity to several peptides representing different segments of bovine α -La but only the serum S7 was sensitive to all the peptides tested.

Competitive ELISA Inhibition of IgE Binding

Competitive ELISA inhibition tests were performed using tryptic peptides and/or proteins as competitors.

Table 3. Specific IgE response to tryptic peptides in direct ELISA

Serum code	α -La N ^a	α -La R ^a	Specific IgE							
			native tryptic peptides ^b					reduced tryptic peptides ^b		
			peptide T5 (99–108)	peptide T6 (6–10)–(115–123)	peptide T7 (59–93)	peptide T8 (17–58)–(109–114)	peptide T9 T6+T8	peptide R3 (59–94)	peptide R5 (109–123)	peptide R6 (17–58)
S1	13	1.3	ND	ND	7.7 (76.9)	4.6 (46.15)	6.15 (61.5)	7.7 (77)	ND	ND
S2	14	2.4	ND	ND	ND	25.7 (>100)	22.85 (>100)	ND	ND	28.5 (>100)
S3	5	2	ND	ND	ND	64 (>100)	60 (>100)	ND	ND	60 (>100)
S4	32	31	ND	ND	ND	53 (54.8)	21.9 (22.6)	ND	ND	46.9 (48.4)
S5	1.3	0.8	ND	ND	7.7 (12.5)	7.7 (12.5)	7.7 (12.5)	ND	ND	ND
S6	0.75	0.4	ND	ND	33 (62.5)	40 (75)	46.7 (87.5)	ND	ND	40 (75)
S7	33	2.2	6 (90.9)	5.45 (81.8)	1.8 (27.3)	1.4 (20.45)	2.1 (31.8)	1.5 (22.7)	1.8 (27.3)	1.8 (27.3)
S8	12	0.22	0.85 (45.45)	0.85 (45.45)	2.5 (>100)	ND	1.6 (90.9)	2 (>100)	2.5 (>100)	ND
Positive/total tested	19/19	19/19	2/19	2/19	5/19	7/19	8/19	3/19	2/19	5/19

^a Specific IgE (IU/ml)

^b Percent of the response to α -La N and α -La R (in parentheses).

Detection limit: 0.1 IU/ml = average of nonspecific binding plus 3 $\times$ standard deviation. ND = Not detectable, i.e. <0.1 IU/ml.

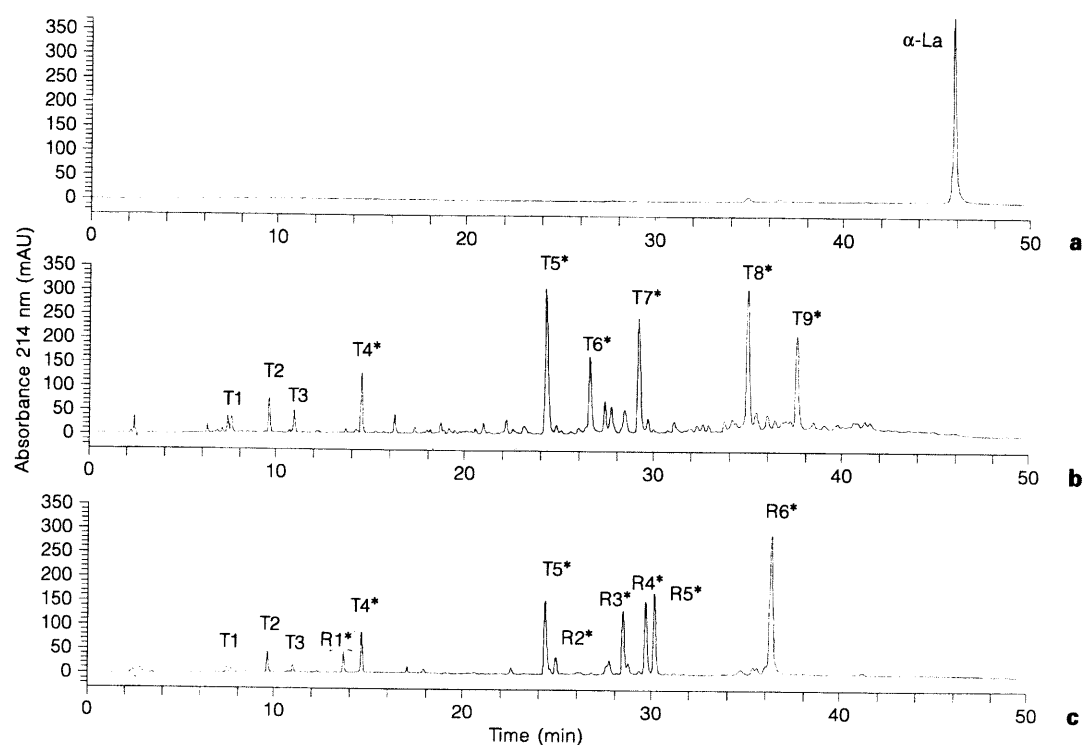


Fig. 1. RP-HPLC analyses of α -La N (a) and tryptic hydrolysates of α -La N (b) and α -La R (c). C18 Nucleosil 300–5/ET 250 column; temperature, 40°C; flow, 1 ml/min; detection, 214 nm; solvent A, 0.1% (v/v) TFA in water; solvent B, 60% (v/v) acetonitrile in solvent A. Elution was performed with a linear gradient established as follows: 0–35% solvent B in 15 min, 35–65% solvent B in 25 min and 65–100% B in 10 min. Asterisks denote peaks detected at 280 nm.

In a first series of experiments, the ability of increasing concentrations of peptides in solution to inhibit the IgE binding to immobilized α -La N was tested. In agreement with previous results, a quite complete inhibition was obtained when using low concentrations of α -La N (2–20 nmol/ml) or large peptides as T9 or T8 (5–100 nmol/ml) as inhibitors. No inhibition was observed with smaller peptides in this concentration range. Figure 3 shows the inhibition curves obtained with serum S4 as an example. Similar patterns were obtained with other sera.

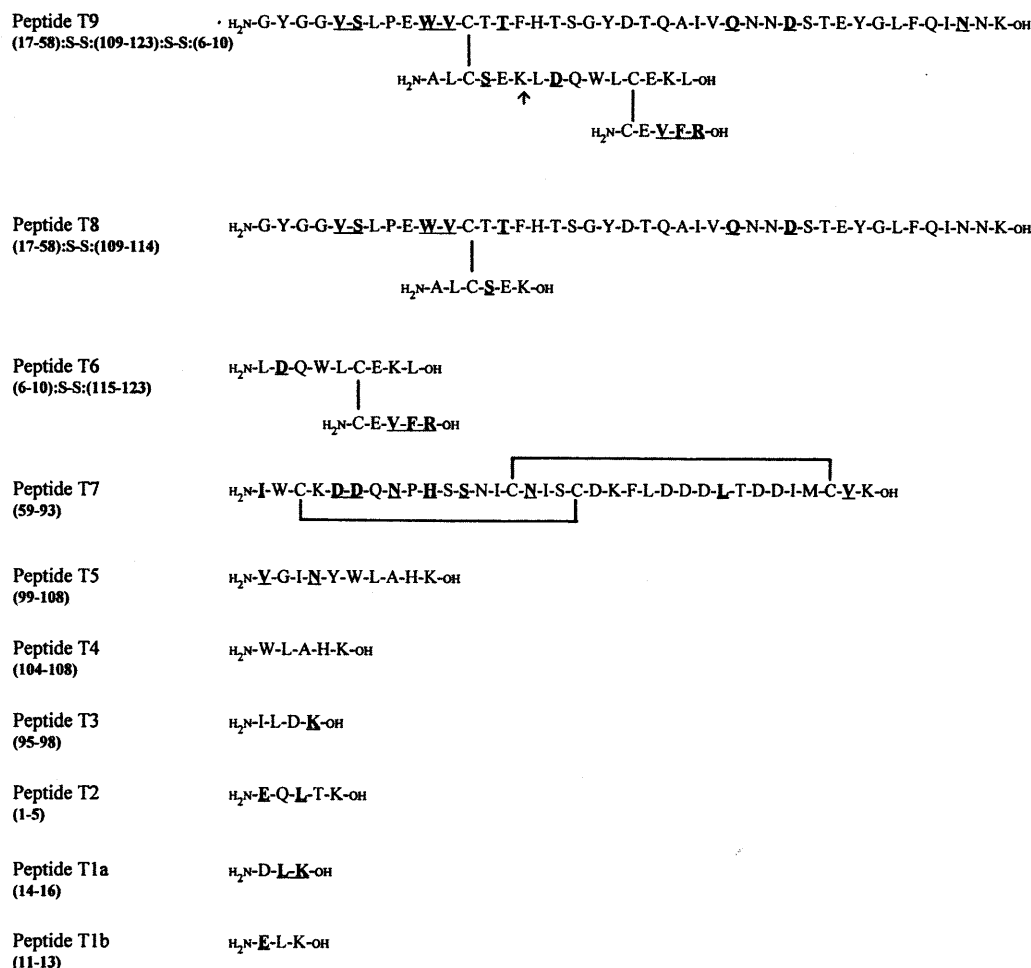
Due to the small quantities disposable of some peptides and the low volumes of human sera available, a second series of trials was performed using immobilized peptides, in order to increase the sensitivity of competitive ELISA inhibition. Only human sera that gave significant specific IgE responses on coated peptides in direct ELISA were used. Binding of specific IgE to immobilized peptides was inhibited by the same peptide (native or reduced) in solution as

well as the corresponding protein (α -La N or α -La R). With all sera tested, the inhibition was significant (25–91%) although not always complete. This could be related either to a too low concentration of inhibitor and/or a decreased IgE affinity towards soluble peptides versus immobilized ones.

Moreover, we tested the inhibitory activity of small peptides which had been shown unable to bind IgE when immobilized in direct ELISA. At a concentration of 1 μ mol/ml, peptides R5 (109A-L123) and T6 (6C-R10):S-S:(115L-L123) in solution inhibited the IgE binding (up to 47 and 77%, respectively, with serum S4) to immobilized peptides T9 and T8, whereas no IgE binding to the coated peptides R5 and T6 was previously found in direct ELISA. At the same concentration, no significant inhibition was observed with the synthetic control peptide.

On the other hand, peptides T7 and R3 (0.5 μ mol/ml) were tested for their ability to inhibit the IgE binding to immobilized α -La N. Specific inhibitions (>25%) were ob-

Peptides from α -La N



Peptides from α -La R

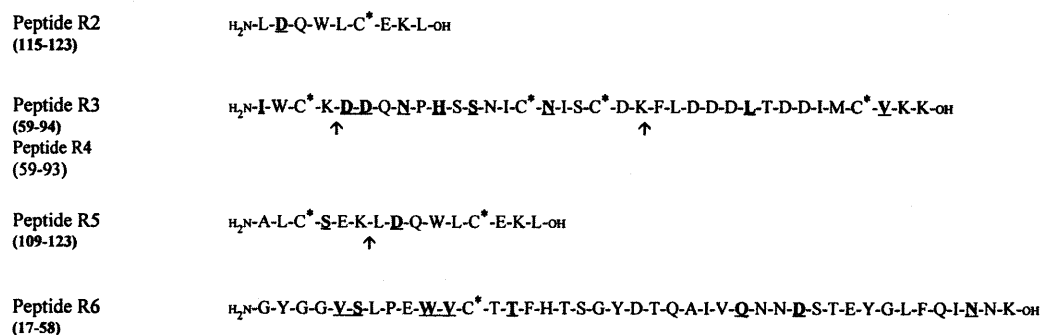


Fig. 2. Primary sequences of tryptic peptides from α -La N and α -La R. Vertical bars represent disulfide bonds and C* carboxymethylamidocysteine. Underlined residues correspond to nonhomologous amino acids with human α -La. Arrows indicate uncleaved specific tryptic sites.

tained that confirmed the IgE binding capacities observed with these two peptides in direct ELISA. With serum S1, the reduced sequence (59I-K94; peptide R3) was more inhibitory (50%) than its unreduced equivalent T7 (59I-K93; 40%).

The competitive ELISA on T7-coated microplates also showed that the reduced sequence (59I-K94; peptide R3) had a better IgE binding capacity than peptide T7 corresponding to the same native sequence (70 vs. 44% inhibition with serum S7). Indeed, at the same concentration, a higher inhibition of IgE binding to immobilized peptide T7 was obtained with reduced peptide R3 than with T7 itself using S1 and S7 sera. This means that sera S1 and S7 contained a particular IgE population reactive to the reduced sequence (59I-K94) and that the reduction of disulfide bridges allowed a better exposure of the sequential epitope (s) located in this part of bovine α -La.

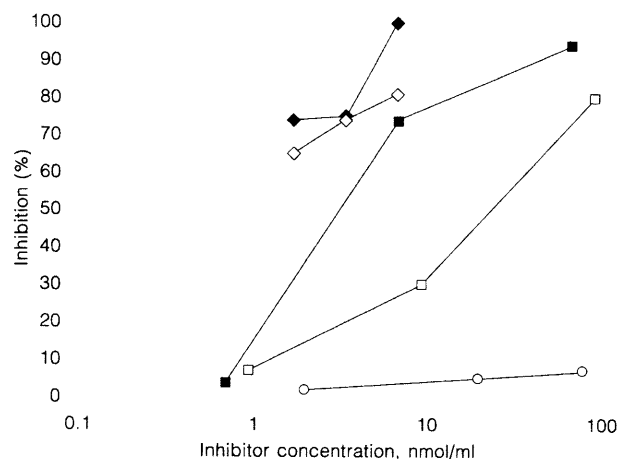


Fig. 3. Competitive ELISA inhibition. Increasing concentrations of α -La N ($\blacklozenge$), α -La R ($\diamond$), peptides T9 ($\blacksquare$) and T8 ($\square$) were preincubated with serum S4 in order to inhibit the IgE binding to immobilized native bovine α -La. $\circ$ = Synthetic peptide.

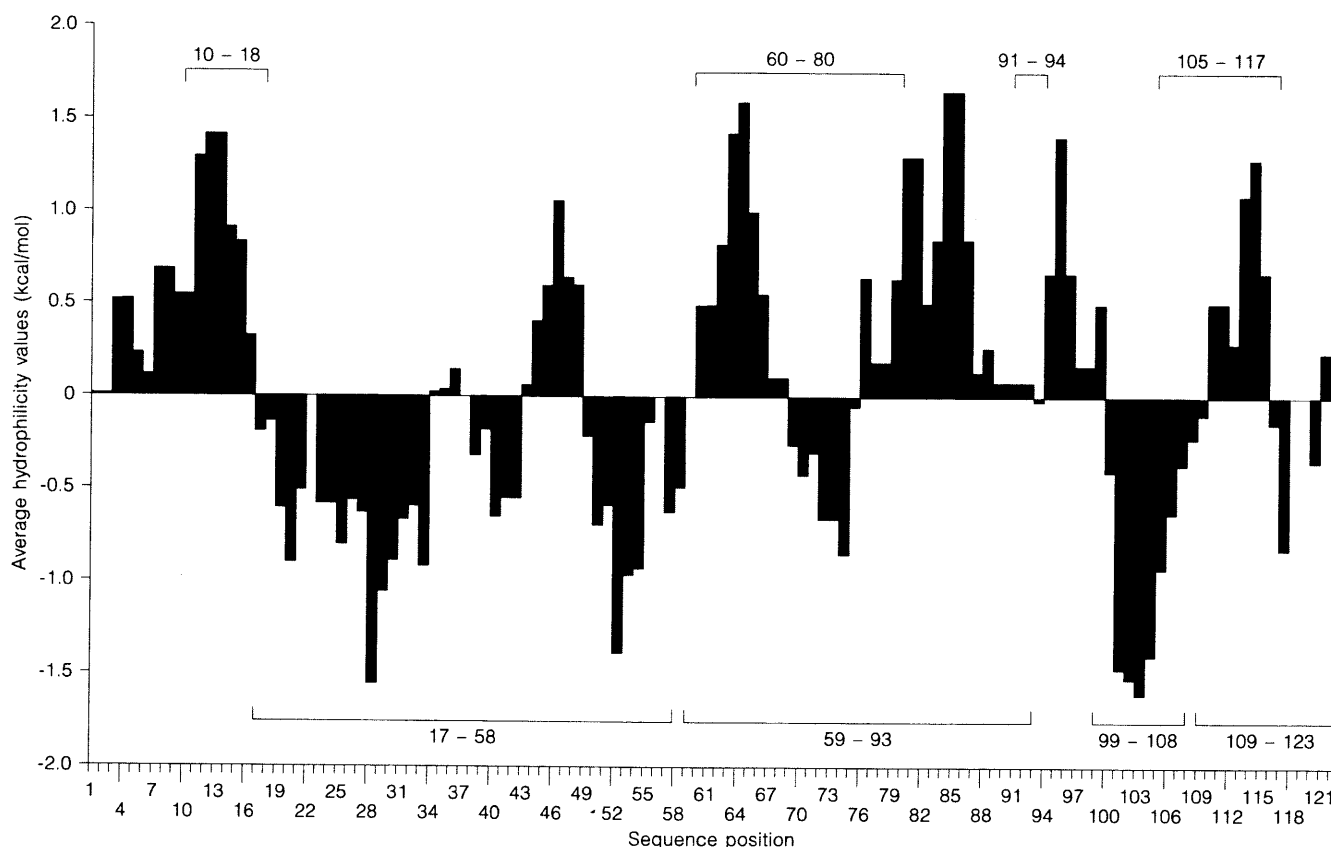


Fig. 4. Hydrophilicity profile of bovine α -La according to Hopp and Woods [14, 15]. Predicted antigenic determinants are reported at the top and tryptic peptides identified as allergenic epitopes at the bottom.

Discussion

The tryptic peptides from native and disulfide-bond-reduced bovine α -La were separated by RP-FPLC followed by repurification to reach a high degree of purity. Each purified peptide appeared as a single homogeneous peak in RP-HPLC. Moreover, the amino acid composition of pure peptides was in excellent correspondence with assigned sequences meaning that each of them was in a single pure form.

According to the narrow cleavage specificity of the TPCK-treated trypsin, most of the peptides resulted from cleavage of Lys-X and Arg-X bonds. However, peptide T4 assigned to sequence (104W-K108) corresponded to a part of peptide T5 (99V-K108). It was due to a nonspecific splitting of the Tyr103-Trp104 bond by the TPCK-treated trypsin, probably related to the presence of pseudotrypsin (autodigestion product of trypsin), the latter exhibiting a chymotrypsin-like activity [23]. On the other hand, peptide T9 [T6 (6C-R10):S-S:(115L-L123) plus T8 (17G-K58):S-S:(109A-K114)] and peptide R5 (109A-L123) both resulted from an incomplete cleavage of the Lys 114-Leu115 bond. Ionic interactions between Asp113 and Lys114 side chains probably contributed to restrain the accessibility of this tryptic bond. Likewise, the Lys62-Asp63 and Lys79-Phe80 bonds were not split by trypsin since sequence R3(59I-K94) was identified in the α -La R hydrolysate. The proximity of Asp63 and Asp78 also suggested the implication of negative charges. Although amino acid and mass spectrometry analyses did not allow to strictly assign a sequence for peptide R1, it likely corresponds to sequence (6-10) that was the only fragment not recovered in the tryptic digest of α -La R. The previous reduction of bovine α -La disulfide bonds led to the formation of shorter fragments than the native tryptic peptides, allowing to study more precisely the location of allergenic determinants of this protein. Indeed, peptide R6 (17G-K58) represented the longest continuous sequence of peptides T9 and T8, whereas peptide R5 (109A-L123) corresponded to the central segment of peptide T9. Moreover, the influence of structure on IgE binding capacity could also be tested as both, the native sequence T7 (59I-K93) and the reduced corresponding one (peptide R3) were available.

The mapping of IgE binding sites (allergenic determinants) of bovine α -La was investigated using both direct and competitive ELISA inhibition. The direct ELISA technique was suitable for protein (α -La N) and large-peptide specific IgE determinations. The sensitivity of this technique allowed to work with small volumes of diluted sera and required rather low peptide quantities for coating. The IgE response was expressed in international units per milliliter for

proteins while the absence of calibrated validation for peptides lead us to express the results in percents of IgE response to proteins. Such expression could induce a distortion since in case of very low response (sera S5 and S6), the reactivity with peptide was at the detection limit.

A great variability in anti-bovine α -La-specific IgE titers was observed in the sera of patients allergic to cow's milk proteins. Some of them exhibited particularly high specific IgE levels showing that bovine α -La could be a very strong allergen. 60% of the sera contained IgE exclusively directed towards α -La N unable to recognize the different peptides. This means that specific IgE of these sera were more likely directed to conformational epitope(s) of bovine α -La. However, 40% of the sera also reacted with tryptic peptides. The polyclonal IgE population repertoire appears to be variable and this variability is apparently not related to the specific anti- α -La IgE concentration. In some sera, specific IgE response to tryptic peptides was much lower than to native bovine α -La while, in others, it was in the same order of magnitude to that observed with α -La.

Most tryptic peptides immobilized by passive adsorption were recognized by IgE in the human sera. Some of these peptides reacted with the majority of sera while others were only occasionally recognized. The largest peptides either native or reduced exhibited a high IgE binding capacity and were the most frequently recognized. Three of them share the common (17-58) sequence. Small peptides, i.e. peptides T6 and R5, were not able to bind IgE in direct ELISA. This could be related either to the absence of epitope(s) located in their sequences or structural modification or steric hindrance for IgE binding due to solid phase-peptide interactions.

Competitive ELISA studies with immobilized proteins demonstrated the competitive inhibition potential of large peptides that confirmed both their IgE binding capacities and the specificity of the direct ELISA response. These competitive ELISA experiments required high peptide concentrations (0.1-1 μ mol/ml) to obtain significant inhibitions. The high peptide concentrations used could potentially induce nonspecific inhibitions. Competitive ELISA also showed the ability of peptides T6 and R5, not recognized in direct ELISA, to inhibit IgE binding to immobilized peptides T9 and T8 from which they were derived.

However, 100% inhibition was not always reached in all competitive ELISA experiments. Since sera of patients allergic to cow's milk are characterized by polyclonal anti- α -La IgE populations, the inhibition of IgE binding to immobilized α -La can hardly be complete when using a single purified peptide as competitor. On peptide-coated plates, incomplete inhibition could be due either to insufficient in-

hibitor concentration or more likely to higher IgE affinity for the immobilized than the soluble antigen. Partial structural modification or better presentation of immobilized antigen could explain this higher affinity.

Several reports have dealt with the theoretical prediction of protein antigenic determinants from their amino acid sequences [14–16, 24], assuming that antigenic sites are surface features of the proteins and should be associated with hydrophilic regions along the polypeptide chain. The hydrophilicity profile of bovine α -La, according to Hopp and Woods [14, 15], is represented in figure 4. Antigenic determinants of this protein have been associated with residues 10–18, 60–80, 91–94 and 105–117. Our results on IgE binding for tryptic peptides (59I-K93; peptide R3) and (109A-L123; peptide R5) seem in good agreement with this theory. However, this is not the case for peptides R6 (17G-K58) and T5 (99V-K108) which both correspond to strongly hydrophobic regions. This suggests that allergenic sites of bovine α -La do not systematically correspond to antigenic determinants. This suggestion is reinforced by the fact that sequence (60–80):2S-2S:(91–96) of bovine α -La, also qualified as 'peptide loop', was found to have the highest antigenic activity with rabbit antisera [12]. Moreover, the same corresponding structure of hen's egg white lysozyme had also been identified as major antigenic determinant (site 2) [25]. In our study, we showed that native peptide T7 (59I-K93), which also contained two intact disulfide bonds and corresponded to a large part of the 'peptide loop', was not the most frequently recognized among the tryptic peptides tested, and did not show highest IgE binding capacity. This means that allergenic epitopes are not necessarily identical with antigenic determinants, and such prediction methods may not be relevant in allergy studies [26].

A majority of patients (60% of sera tested) responded to native α -La only, indicating that in these patients, anti- α -La IgE were specific to conformational epitopes. However, a large number of patients was also responding to fragments of either native or denatured (i.e. reduced and S-carboxymethylated) α -La. In this fraction of milk-allergic patients, IgE binding to sequential epitopes was thus observed.

Sometimes, the human sera tested were more reactive to the corresponding reduced sequence (59I-K94; peptide R3) than to the native one (T7). IgE that recognized this bovine α -La region were more specific to the sequential epitope contained in sequence (59–93) than to the conformational S-S bond-stabilized epitope (peptide T7). This high specificity towards a denatured part of the protein evidenced by ELISA probably reflects a specific mechanism of food allergy. Stimulation of the immune system and IgE response induction take place after gastric and intestinal digestion of

native α -La. The immuno-stimulating fragments could therefore be different to those present in the native structure.

Evolutionary amino acid substitutions may influence antigenicity/allergenicity of homologous proteins. It was suggested that parts of bovine α -La which present a high degree of variability with human α -La would be potential locations of antigenic and/or allergenic determinants [27]. Using two human sera, Adams et al. [13] showed that synthetic peptide (5–18) exhibited an IgE binding capacity although the RAST value measured was very low. This part of bovine α -La is characterized by a low degree of sequence homology compared to the homologous sequence of human α -La. Due to tryptic digestion performed to prepare our peptides, this sequence was not obtained in a tryptic digest and therefore the results of Adams et al. [13] could not be verified.

Immunological cross-reactions with IgE binding are often observed between more or less related proteins. They may involve low-affinity specific IgE and cannot always be related to clinical symptoms. The clinical or pathological significance of such in vitro immunological cross-reactions should be confirmed by in vivo studies such as skin tests and double-blind placebo-controlled provocation tests.

Our study shows unambiguously that a high IgE binding capacity was associated with sequence (17G-K58; peptide R6) and to the C-terminal end (109A-L123; peptide R5) of bovine α -La. Indeed, the competitive inhibition demonstrated that 15–80% inhibition can be reached with these two peptides, and these sequences were recognized with a high frequency. The peptides (17G-K58) and (109A-L123) have 81 and 87%, respectively, homology with the corresponding human sequences, showing that a high degree of homology does not exclude allergenic reactivity.

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