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Isolation and purification of cat albumin from cat serum by copper ion affinity chromatography: further analysis of its primary structure

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ABSTRACT

Proteins, regardless of their origin, have to be highly purified, particularly from the immunochemical point of view, if they are to be used to study their allergenicity. It is shown that cat albumin, a highly potent allergen for cat-sensitive humans, can be isolated and purified from cat serum using immobilized metal ion affinity chromatography (copper ions) instead of a salting-out process or precipitation with alcohol, techniques generally used for the preparation of serum proteins. During the process described, immunoglobulins are concomitantly isolated in a relatively pure form. Cat albumin amino acid composition and sequence were analysed after an ultimate purification by ion-exchange chromatography. The highest homology (>80%) was found with the rat serum albumin.

INTRODUCTION

Previous studies have shown that several allergens [1–3] may be extracted from cat pelts. One of them is the major feline allergen Fel dI [4] and the others, to which cat-sensitive patients are less frequently sensitive, are albumin [2,3,5] and immunoglobulins [5,6]. Nevertheless, cat albumin is a very potent allergen, hence a highly purified form is obviously useful. Pure allergens are needed either for diagnosis or for therapy of hypersensitivity.

A better knowledge of the allergen structures could lead to a better understanding of their allergenic potency. The purpose of this work was to prepare a highly purified cat albumin, to analyse its primary structure and to compare it with other mammalian albumins for which cross-reactions have been reported either *versus* rabbit IgG antibodies or *versus* human IgE antibodies [5,7].

The cat serum albumin (CSA) purification process described here is essentially

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based on immobilized metal ion affinity chromatography (IMAC). We chose IMAC taking in account the previous observations by other workers during human serum protein fractionation [8,9], who stated that the adsorption capacity of the metal chelates for serum proteins, particularly for albumin, decreased in the order Cu > Zn > Ni > Mn [10]. We examined a Cu^{2+} charged chelating Sepharose fast flow gel packed in a glass column, the ligand-exchange principle being involved in the elution process.

EXPERIMENTAL

Materials

Cat sera were a kind gift from Dr. Prel, a veterinary clinician.

Rabbit IgG antibodies against cat serum were purchased from Sigma (St. Louis, MO, U.S.A.).

As standard references for cat serum proteins, cat albumin (Cohn fraction V) and cat immunoglobulins were purchased from Sigma.

Immobilized metal ion affinity chromatography

IMAC was performed on a cross-linked agarose gel to which imminodiacetate was coupled, *i.e.*, chelating Sepharose fast flow from Pharmacia (Uppsala, Sweden). After being washed according to the manufacturer's suggestions, the gel was packed in an HR 10/10 column and charged until saturation with copper ions from 0.5% (w/v) copper(II) chloride solution. After thorough rinsing with water to eliminate unbound copper ions, the column was washed again with a solution containing 1 m*M* imidazole and 1 *M* sodium chloride buffered with 0.02 *M* sodium phosphate buffer (pH 7.0). Saturation with imidazole was performed by injecting 30 ml of 10 m*M* imidazole dissolved in the same salt-buffered solution.

A pre- and a post-column packed with the chelating gel without metal ions were incorporated in the system to remove any free metal ions that might interfere with the chelating process; the columns used were of the HR 5/5 type.

After loading the cat serum sample onto the column, elution was performed in three steps: first an isocratic run, then either a linear concentration gradient of imidazole followed by a plateau at 100% solution B, or stepwise elution with different solutions of increasing imidazole concentration. The compositions of the solutions were as follows: solution A, 1 m*M* imidazole in 1 *M* NaCl buffered with 0.02 *M* sodium phosphate (pH 7.0) (see above); solution B, 20, 40 or 50 m*M* imidazole in 1 *M* NaCl buffered with 0.02 *M* sodium phosphate (pH 7.0).

The whole process was controlled by a fast protein liquid chromatography system (FPLC) (Pharmacia).

Chromatofocusing

A Mono P column of the HR 5/20 type (Pharmacia) was used after being equilibrated with 0.025 *M* sodium acetate buffer (pH 5.58). The protein of interest was dissolved in the same buffer and loaded onto the column. Elution was effected with a Servalytes 2–4 solution at 40% (Serva, Heidelberg, Germany), diluted with water to 0.2% and adjusted to pH 2.05 with 1 *M* hydrochloric acid. The pH gradient was recorded using a pH monitor (Pharmacia).

Anion-exchange chromatography

A Mono Q column of the HR 10/10 type (Pharmacia) was previously equilibrated with 0.03 *M* NaCl solution, buffered with a 0.02 *M* Tris–HCl (pH 8.00) (solution).

Protein sample dissolved in solution A was loaded onto the column. Elution began with an isocratic run with solution A, followed by a linear concentration gradient of NaCl from 0.03 to 1 *M* (50% solution B). Solution B consisted of 2 *M* NaCl solution buffered with 0.02 *M* Tris–HCl (pH 8.00). The third run was a plateau at 100% B to desorb all the strongly bound material. Thereafter, the Mono Q column can be washed and equilibrated with solution A to make it ready for the next separation process.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Analyses were performed with a PHAST apparatus using PHAST gels 8–25 (Pharmacia) according to the manufacturer's instructions.

Amino acid analysis

The amino acid composition was determined with a Beckman 7300 amino acid analyser after acid hydrolysis. Hydrolysis was performed in the vapour phase with 6 *M* hydrochloric acid containing 0.1% phenol for 24 h at 110°C *in vacuo* [11].

Micro-sequencing

Proteins were sequenced on a Model 470 A gas-phase protein sequencer (Applied Biosystems, Roissy, France). Phenylthiohydantoin (PTH) derivatives of amino acids were separated and identified by on-line reversed-phase high-performance liquid chromatography with a Model 120 A-PTH analyser (Applied Biosystems) added to prevent wash-out and to improve the initial yields [12].

An exhaustive search of homology cases was performed on the PSEQIP database.

Crossed immunoelectrophoresis methods

To follow the purification steps of the cat serum albumin (CSA), crossed immunoelectrophoresis (CIE) methods were used as previously described [13], using rabbit IgG raised against the whole cat serum (Sigma). Each fraction obtained by chromatographic techniques was tested by crossed line rocket immunoelectrophoresis (CLRIE), *i.e.*, the intermediate gel of a crossed line immunoelectrophoresis (CLIE) was lengthened on the right-hand part, allowing a rocket-line experiment (RLIE) to be performed, leading to an easy identification of each antigen [14].

RESULTS

Immobilized metal ion affinity chromatography

A 1-ml volume of cat serum was loaded onto a copper column, prepared as described under Experimental. For identification purposes this method was named copper chelate chromatography (CCC).

In a first series of experiments we applied, after an isocratic run of 40 min with solution A (1 m*M* imidazole), a linear concentration gradient of imidazole from 1 to 20

m*M*. Two peaks were eluted at 6 and 8 m*M*, the strongly bound material being eluted during the last run at 100% B, *i.e.*, 20 m*M* imidazole. The last peak was broad with pronounced tailing. We therefore increased the imidazole concentration up to 50 m*M* and a sharper peak without tailing was obtained. Finally, the complete process was defined as a stepwise elution at 0, 12, 16 and 100% B (Fig. 1).

Fractions of 1 ml were collected and pooled according to the recorded elution scheme, dialysed against neutral distilled water and freeze-dried.

Using cat albumin and immunoglobulins from Sigma chromatographed under the same conditions, we were able to identify peak Cu3 as probably being cat albumin and Cu4 as essentially being cat immunoglobulins.

Chromatofocusing

In order to compare the degree of purity of cat albumin from a Cohn fraction V (Sigma) and that of cat albumin prepared by IMAC (fraction Cu3), we performed a chromatofocusing as described under Experimental on the two protein batches at the same concentration, *i.e.*, 20 mg/ml, of 200 µl were loaded onto the column.

Fig. 2 shows that cat albumin obtained by copper chelate chromatography (CSA-CCC) is as pure as that obtained by the Cohn method. It should be noted that the last peaks on the chromatogram correspond to ampholytes in the pH 2 zone, which absorb in the UV region at 280 nm.

SDS-PAGE

Homogeneity of the cat albumin Cu3 was compared with that of Cohn fraction V (Sigma) by SDS-PAGE in an 8–25% gradient gel (PHAST gel). Fig. 3 shows that Cu3 is at least as pure as the Cohn fraction.

Anion-exchange chromatography

Considering the results obtained either by chromatofocusing or by SDS-PAGE, and also those obtained by immunochemical analysis, we concluded that some other unidentified proteins contaminate cat albumin preparations whatever the purification

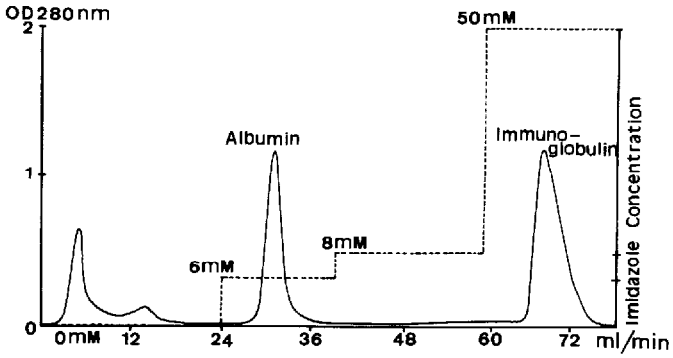

Fig. 1. Copper chelate chromatography. Column, HR 10/10 packed with chelating Sepharose fast flow (Pharmacia), charged with Cu^{2+}, washed with 1 m*M* imidazole in 1 *M* NaCl solution buffered with 0.02 *M* sodium phosphate buffer (pH 7.0), saturated with 10 m*M* imidazole in the same buffered salt solution. A 1-ml volume of cat serum, previously dialysed against 1 m*M* imidazole, was loaded onto the column. Stepwise elution was performed at a flow-rate of 1.0 ml/min.

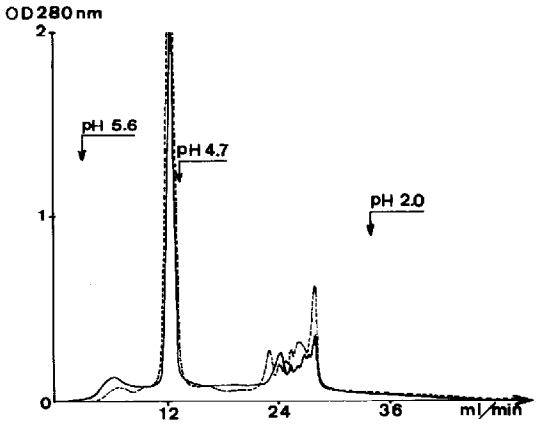

Fig. 2. Chromatofocusing with a Mono P HR 5/20 column equilibrated with 0.025 *M* sodium acetate buffer (pH 5.58). A 200-μl aliquot (4 mg of CSA) was loaded onto the column. Elution was performed at a flow-rate of 1.0 ml/min with a Servalytes 2–4 solution (Serva) at 0.2% adjusted to pH 2.05. The pH gradient was recorded using a pH monitor (Pharmacia). Solid curve, CSA-CCC; dashed curve, CSA Cohn fraction V.

process used. To eliminate these contaminants from the CSA-CCC, anion-exchange chromatography was carried out on Cu3. A 20-mg amount of lyophilized Cu3, solubilized in 1 ml of solution A (0.03 *M* NaCl), was loaded on the Mono Q column previously equilibrated with the same buffered salt solution. After a short isocratic elution during which no material was eluted, a linear concentration gradient of NaCl was applied, which gave five elution peaks (Fig. 4). Fractions from four of them, the quantitatively most important, *i.e.*, P2, P3, P4 and P5 were pooled, then dialysed against neutral water and freeze-dried.

Analyses were performed by SDS-PAGE and CLRIE, the results of which are shown in Figs. 3 and 5, respectively. P4 appears as a highly pure cat albumin preparation, while P5, found to be pure in CLRIE, contains two components, as shown by SDS-PAGE, with M_r 67 and 90 kilodalton, respectively.

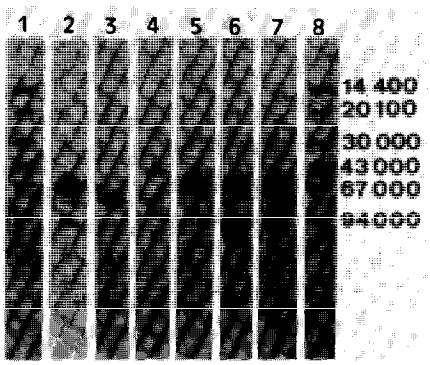

Fig. 3. SDS-PAGE using PHAST gels 8–25 on a PHAST apparatus (Pharmacia). Lanes 1 and 8, standard protein markers; lane 2, cat albumin CCC, 1.20 μg; lane 3, fraction P2, 0.40 μg; lane 4, fraction P3, 0.35 μg; lane 5, fraction P4, 0.64 μg; lane 6, fraction P5, 1.65 μg; lane 7, cat albumin (Sigma), 1.20 μg.

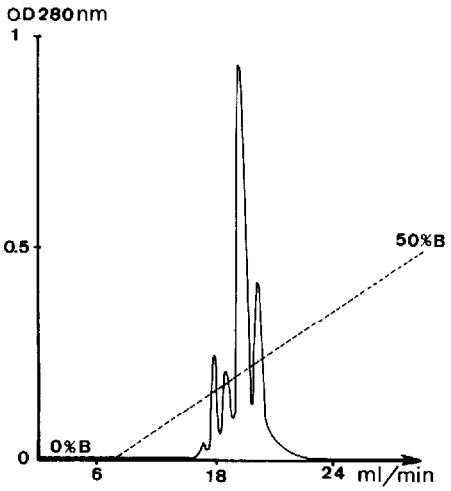

Fig. 4. Anion-exchange chromatography on Mono Q HR 10/10 column equilibrated with 0.03 *M* NaCl solution buffered with 0.02 *M* Tris–HCl (pH 8.00) (solution A). Elution was performed at a flow-rate of 1.0 ml/min with a linear concentration gradient of NaCl from 0.03 to 1 *M* (50% solution B). Solution B consisted of 2 *M* NaCl solution buffered with 0.02 *M* Tris–HCl (pH 8.00). A 1-ml volume of CSA-CCC (20 mg) was loaded onto the column.

Immunochemical analyses

The above results on the immunochemical purity of the different fractions obtained in the purification of cat albumin from cat serum were obtained using the method described under Experimental (Fig. 5). Rabbit IgG raised against whole cat serum was used in a CLIE, the LIE part was lengthened on the righ-hand part and rockets were included which allowed the proteins of interest to be identified commercially available preparations were used to identify albumin and immunoglobulins.

From an immunological point of view, albumin from fraction Cu3 seems to be less contaminated than the Cohn fraction V and P4, the most homogeneous cat albumin that we prepared, appears to be totally free from contaminants, at least under the conditions of this experiment, *i.e.*, antigen concentrations as indicated in Fig. 5 and using rabbit IgG antibodies.

Amino acid analysis

As Cu3P4 could be considered as a highly pure cat albumin, its amino acid composition was determined (Table I) and it showed differences in comparison with three other serum albumin amino acid compositions cited in the literature [15], *e.g.*, glycine is twice as concentrated and tryptophan seems to be absent.

Sequencing of the N-terminal peptide

The amino acid sequence of a 30-residue N-terminal peptide was determined and homologies were investigated (Table II).

The highest homologies were observed with RSA (83%), then with BSA (80%)

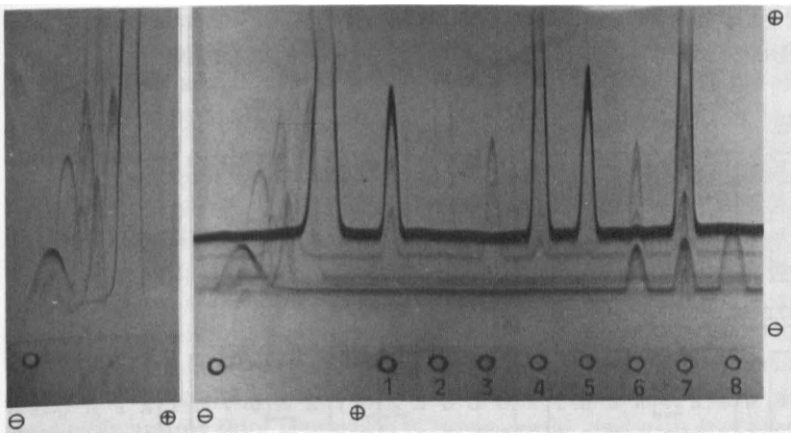

Fig. 5. CIE and CLRIE performed as described. The antibody containing agarose gel was poured in two parts; the upper part contained 1.5 μl/cm^2 of antibodies and the lower past 0.5 μl/cm^2. The amount of each "antigen" deposited was as follows. In the first dimension either in the CIE or in the CLRIE experiments, 10 μl of 1:50 diluted cat serum were submitted to zone electrophoresis. The intermediate gel, in the CLRIE, contained a dilution of the same cat serum enriched with cat IgG (Sigma). For the RLIE, the antigens were as follows: 1, Cu3 (partially purified CSA), 54 μg in 10 μl; 2, Cu3P2, 13 μg in 10 μl; 3, Cu3P3, 10 μg in 10 μl; 4, Cu3P4, 100 μg in 10 μl; 5, Cu3P5, 50 μg in 10 μl; 6, Cu4, 60 μg in 10 μl; 7, whole cat serum (protein equivalent), 58 μg in 5 μl; 8, cat immunoglobulins (Cohn fraction V, Sigma), 5 μg in 10 μl.

TABLE I

AMINO ACID COMPOSITION OF FOUR SERUM ALBUMINS

Amino acid	CSA[a]	BSA [15][b]	HSA [15][b]	RSA [15][b]
Asx	7.7	7.0	6.1	5.5
Asn	nd	2.2	2.9	3.4
Thr	4.8	5.8	4.8	5.6
Ser	6.0	4.8	4.1	4.1
Glx	15.3	10.1	10.6	9.7
Gln	nd	3.4	3.4	4.3
Pro	5.3	4.8	4.1	5.1
Gly	5.3	2.7	2.0	2.9
Ala	10.7	7.9	10.6	10.4
Cys	nd	6.0	6.0	6.0
Val	7.5	6.2	7.0	6.0
Met	1.2	0.7	1.0	1.0
Ile	1.4	2.4	1.3	2.2
Leu	10.5	10.5	10.4	9.6
Tyr	3.2	3.2	3.0	3.6
Phe	5.8	4.6	5.3	4.4
His	3.0	2.9	2.7	2.5
Lys	8.1	10.1	10.0	9.0
Arg	4.2	4.4	4.1	4.1
Try	–	0.3	0.1	0.1
Σ(mol-%)	100.0			

[a] nd = Not detected.
[b] Calculated from the amino acid sequences.

TABLE II

CAT SERUM ALBUMIN HOMOLOGIES (N-TERMINUS): N-TERMINUS COMPARISONS

COMMON RESIDUES [a]

BSA[1]	D	T	H	K	S	E	I	A	H	R	F	K	D	L	G	E	E	H	F	K	G	L	V	L	I	A	F	S	Q	Y
HSA[2,3]	D	A	H	K	S	E	V	A	H	R	F	K	D	L	G	E	E	N	F	K	A	L	V	L	I	A	F	A	Q	Y
RSA[4]	E	A	H	K	S	E	I	A	H	R	F	K	D	L	G	E	Q	H	F	K	G	L	V	L	I	A	F	S	Q	Y
CSA	E	A	H	Q	S	E	I	A	H	R	F	N	D	L	G	E	E	H	F	R	G	L	V	L	V	A	F	S	Q	Y

HOMOLOGOUS RESIDUES TO THOSE OF CSA [a]

BSA[1]	D	T	H	K	S	E	I	A	H	R	F	K	D	L	G	E	E	H	F	K	G	L	V	L	I	A	F	S	Q	Y
HSA[2,3]	D	A	H	K	S	E	V	A	H	R	F	K	D	L	G	E	E	N	F	K	A	L	V	L	I	A	F	A	Q	Y
RSA[4]	E	A	H	K	S	E	I	A	H	R	F	K	D	L	G	E	Q	H	F	K	G	L	V	L	I	A	F	S	Q	Y
CSA	E	A	H	Q	S	E	I	A	H	R	F	N	D	L	G	E	E	H	F	R	G	L	V	L	V	A	F	S	Q	Y

[a] 1 = Albusbovin, serum albumin precursor, bovine (bos taurus) [16]; 2 = Albushuman, serum albumin precursor, human (homo sapiens) [17]; 3 = Base8, preproalbumin human serum [18]; 4 = Albusrat, serum albumin precursor, rat (rattus norvegicus) [19].

and HSA (70%). For the RSA, three lysine residues are changed into glutamine, asparagine and arginine in positions 4, 12 and 20, respectively. The isoleucine in position 25 was replaced by a valine residue and glutamine in position 17 by a glutamic acid residue. The residues at position 28 in HSA and CSA are different.

DISCUSSION

Although cat albumin was stated to be a less important allergen than the major feline allergen Fel dI [2], it remains a protein of interest for the human patients sensitive to this albumin [7].

Identifying structures of allergenic molecules responsible for IgE antibody synthesis induction, apart their specific epitopes, is a difficult task and requires the availability of allergenic molecules of the highest degree of purity.

Sometimes, cloning of an allergenic protein as Der pI, a major allergen from *Dermatophagoides pteronyssinus* mite, is possible [20]. Otherwise, a major allergen such as Fel dI, the major feline allergen, can be purified either by using monoclonal antibody immunosorbent [21] or metal chelate chromatography [14].

Some proteins, such as albumins and immunoglobulins, have been obtained in a relatively pure form by a salting-out procedure, precipitation with alcohol or gel permeation. In order to improve their purification, we tried to use IMAC, a suitable chromatographic process for the large-scale fractionation and purification of proteins. IMAC has certainly already been used for serum protein fractionation [7,22] but, to our knowledge, copper chelate chromatography with a ligand-exchange process has never been used to isolate and purify cat serum albumin. Only human albumin was purified from a Cohn fraction on immobilized nickel by Andersson *et al.* [23].

IMAC, a method for protein fractionation, was developed about 15 years ago

[10]. It was essentially based on metal and amino acid interactions first described by Gurd and Wilcox [24], *i.e.*, histidine and cysteine, which have an electron donor atom in their side-chains, form fairly stable complexes with copper and zinc ions in nearly neutral aqueous solutions. That imidazole side-chains of histidine residues play the role of a ligand for divalent metal ions such as Cu^{2+} was further studied for HSA by Peters [15], since it was previously stated by Hearon [25] that exposed imidazole and thiol groups of the proteins, more particularly albumins, were involved in the protein–metal ions interactions. Subsequently a hydrophilic gel such as Sepharose, a cross-linked agarose, activated by a chelating agent (immunodiacetate) charged with metal ions, was shown to be a selective adsorbent for cysteine- and histidine-containing proteins [10]; although a relationship between the number of transition metal ions bound and the number of imidazole side-chains has never been clearly demonstrated [26], their chromatographic behaviour could be governed by the surface number of imidazole and/or thiol groups [9]. Consequently, and as it has been shown that the adsorption capacity of the metal chelate for serum proteins decreased in the order Cu > Zn > Ni > Mn [10], we chose copper chelate chromatography for the isolation and purification of cat serum albumin.

It is known that proteins can be eluted from IDA–M^{2+} columns (M = metal) at nearly neutral pH by a competing electron donor solute such as imidazole, which forms a stable metal complex with IDA–M^{2+}. However, the copper chelate gel has to be previously saturated with imidazole before an increasing concentration gradient of imidazole can be applied to the column [27].

The results reported show that cat serum loaded on a copper ion-charged chelating Sepharose fast flow column can be fractionated into four fractions, one of them relatively strongly bound to the IDA-Cu^{2+} containing a highly pure albumin, purer than a Cohn fraction V (Sigma), as judged by chromatofocusing, SDS-PAGE and immunochemical methods. Nevertheless, as we wished to analyse further structures of this potent allergen at chemical and immunological levels, we further purified this CSA preparation by anion-exchange chromatography. The resulting CSA was shown to be suitable for amino acid analysis and sequencing, and we therefore undertook an exhaustive search for homologies. It seemed that the CSA amino acid sequence, at least for the amino-terminal peptide, displayed only some small differences and showed the highest homology first with the rat serum albumin (83%), second with the bovine serum albumin (80%) and then the human serum albumin (70%).

If the primary structure of a protein is not totally responsible for its antigenic and/or allergenic potency, it is an important part of the constitution of its epitopes. It may be assumed that proteins having a high level of homologies in their amino acid sequence, even if the three-dimensional structures of homologous proteins are generally better conserved than primary ones, could display very important cross-reactions when tested against polyclonal and monoclonal antibodies, whatever their origin, *e.g.*, rabbit or human. It was recently shown that dog albumin and CSA cross-react with human IgE antibodies from cat-sensitive sera [7]; hence it would be interesting to analyse their homologies and also to test cross-reactions between RSA and CSA against the same IgE antibodies. This is currently under study.

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