

Original article

Further characterization of IgE-binding antigens from guinea pig hair as new members of the lipocalin family

Background: Guinea pigs are important sources of inhalant allergens in home and working environments. However, little is known about the molecular characteristics and the relevant epitopes of guinea pig allergens. Recently, several allergens have been identified in hair extract and urine, and the major allergen Cav p 1 (20 kDa) has been characterized.

Objective: The aim of the present study was to isolate and to characterize a further major allergen from guinea pig hair with 17 kDa.

Methods: Guinea pig hair extract was fractionated using anion exchange chromatography and reverse-phase high performance liquid chromatography. Analyses were carried out by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, 2D-PAGE, immunoblotting, immunoblot inhibition, glycoprotein detection, and N-terminal amino acid sequencing.

Results: The nonglycosylated 17 kDa allergen, which was named Cav p 2, was purified to homogeneity. On the basis its 15 N-terminal residues, there was 69% identity with a sequence of Bos d 2, an allergenic protein from cow dander belonging to the lipocalin family. The 2D-immunoblotting analyses of guinea pig hair extract demonstrated that Cav p 2 and Cav p 1, contained several isoforms with pI values ranging from 3.6 to 5.3. The 2D-immunoblot inhibition disclosed cross-reactive IgE epitopes on the allergens Cav p 2 and Cav p 1. Furthermore, Cav p 1 can form both monomers (20 kDa) and dimers (40–42 kDa).

Conclusion: These studies provide important information on the isoallergen character of two relevant guinea pig allergens Cav p 1 and Cav p 2 as well as on their cross-reactive properties.

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Allergic reactions to guinea pigs constitute an occupational hazard among laboratory workers and veterinarians. In addition, guinea pigs may contribute to household allergen levels because they have become increasingly popular household pets. Until now, limited information is available on the properties of guinea pig allergens (1–3). Recently, several allergens with molecular masses between 8 and 70 kDa have been identified in hair extract and urine of guinea pigs using immunoblot analyses with 40 individual patient sera (4). The prevalence of IgE binding to 8, 17 and 20 kDa allergens was 65, 55 and 70% respectively in subjects allergic to guinea pig. Thus, these allergens are strong candidates to be major allergens in guinea pig hair extract. N-terminal amino acid sequencing of the 20 kDa allergen identified it as member of the lipocalin family of proteins (4). Lipocalins are a group of functionally and structurally related proteins (5) and many major allergens from different animals (horse, cow, dog, mouse, rat and cockroach) belong to this superfamily (6). The 20 kDa

allergen existing in guinea pig hair and urine has been named Cav p 1 [<http://www.allergen.org>].

This paper describes the purification of an additional guinea pig major allergen (17 kDa) from hair extract by chromatographical techniques. Furthermore, the N-terminal amino acid sequence of the 17 kDa allergen has been determined and its cross-reactivity to the Cav p 1 allergen has been studied.

Materials and methods

Patient sera

Sera were obtained from patients selected on the basis of atopic symptoms [rhinoconjunctivitis, asthma, dermatitis (7)], positive skin prick test results to guinea pig, and positive specific IgE results (>0.7 kU/l) to guinea pig (allergencode e6) using the CAPTM system (Pharmacia, Upjohn, Sweden). To identify allergens, a serum pool from eight patients with CAP class >4 to e6 was used as an immunochemical reagent in immunoblot studies and inhibition

assays. A serum pool from 20 nonallergic individuals was used as negative control.

Source material and purification of the 17 kDa allergen

A crude extract was prepared by extraction of guinea pig hair with a 0.125 M NH_4HCO_3 buffer as described previously (4). An enrichment of the protein fraction containing the 17 kDa allergen was accomplished by anion exchange chromatography of the hair extract using a RESOURCE QTM column (AmershamPharmacia Biotech AB, Uppsala, Sweden) and a linear gradient elution with 0–0.5 M NaCl [as described before (4)]. The fraction eluted at 0.26 M NaCl was dialysed against distilled water and lyophilized. Subsequently, the lyophilizate was dissolved in water containing 0.05% trifluoroacetic acid (TFA) and loaded on a ProRPC[®] column (1 ml; Pharmacia, Uppsala, Sweden) equilibrated with 0.05% TFA. The proteins were eluted with a linear gradient of 0–80% acetonitrile in 0.05% TFA in 35 min. Protein profiles were obtained by monitoring the absorbance of the eluate at 220 and 280 nm, and the main fractions were collected. The solvent was evaporated by vacuum drying and the fractions analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. Protein concentrations were determined using the modified Bradford assay with bovine albumin standards (8).

Gel electrophoresis

SDS–PAGE. Hair extract or separated proteins (each 10 µg/lane) were analysed with ready-to-use Excel polyacrylamide gradient gels (8–18%; Amersham Pharmacia Biotech, Uppsala, Sweden) under reducing conditions. Proteins were visualized by silver staining (9).

2D-gel electrophoresis

A 2D-PAGE was performed according to the instructions of the manufacturer (Amersham Biosciences, Uppsala, Sweden). Briefly, in the first dimension isoelectric focusing (IEF) was performed with precast immobilized pH gradient (IPG) strips (ImmobilineTM DryStrips pH 3–10; Amersham Biosciences, Uppsala, Sweden). The rehydration solution contained 8 M urea, 2% (w/v) 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 0.2% (w/v) dithiothreitol (DTT), 0.5% IPG Buffer.

A total of 30 µg protein of guinea pig hair extract were applied per IPG strip and separated at 3500 V and 2 mA for 4 h. After incubation of the strips for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8, containing 2% SDS, 6 M urea, 30% glycerol and 1% DTT) and a second equilibration (15 min) with iodoacetamide-containing SDS equilibration buffer (without DTT), SDS–PAGE was carried out in the second dimension as described above. One 2D gel was silver-stained and the other gel was used for blotting.

Immunoblot analyses

Proteins separated by gradient SDS–PAGE were transferred to nitrocellulose (NC) (BA 0.2 µm; Schleicher & Schuell, Dassel, Germany) according to Towbin (10). For immunological studies of 2D-PAGE, thermoblotting was performed. To this end the gel was blotted three times onto different sheets of NC-membrane by means of modified diffusion blotting (11) at 50°C to obtain identical replica.

After protein transfer, unreacted binding sites were blocked by incubation of the NC-membranes in phosphate buffer saline/Tween 20 (0.1%) and the membranes incubated overnight with a pool of patient or control sera (diluted 1 : 5). Bound IgE was detected by

sequential incubation with rabbit anti-human IgE (1 : 4000; 1 h; DAKO, Hamburg, Germany), biotinylated goat anti-rabbit IgG (1 : 6,000; 1 h; DAKO), streptavidin AP (1 : 5,000; 1 h; Roche Diagnostics GmbH, Mannheim, Germany) as well as 4-nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma, St. Louis, MO) as substrate.

Immunoblot inhibition assays were carried out accordingly, except that a serum pool from patients was preincubated for 1 h at RT with each inhibitor (purified allergen) before exposure to the membranes.

Glycoprotein detection of purified and blotted 17 kDa allergen was performed using the DIG Glycan Detection Kit (Boehringer Mannheim, Germany) and several digoxigenin-labelled lectins (Boehringer Mannheim, Germany) (12).

Amino acid sequencing

The N-terminal amino acid sequences of protein samples were determined by automated Edman degradation on the Applied Biosystems model 494 sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Purification and characterization of the 17 kDa allergen of guinea pig

The procedure to purify the protein was similar to that previously used for the other major allergen in guinea pig hair, i.e., Cav p 1 (4). The first step was an enrichment of the 17 kDa allergen from guinea pig hair extract by anion exchange chromatography using a RESOURCE QTM column and was described in more detail recently (4). The fraction 2 resulting from this chromatography still contained several proteins (Fig. 1A, lane 2). Further separation of this fraction by reverse phase chromatography yielded several UV-absorption peaks (Fig. 2). The fraction with a retention time of 10.7 min gave a single strong band at 17 kDa and good IgE binding in immunoblotting (Fig. 1A and B, lane 3). The final yield of the isolated guinea pig allergen was 0.5% of the total protein content in the hair extract. However, the isolated 17 kDa allergen has to be judged as major allergen on the basis of immunoblot analyses with individual sera (Fig. 3).

This purified allergen was used for the N-terminal sequence analysis too. Table 1 lists the 15 N-terminal amino acid residues of the 17 kDa guinea pig allergen. On the basis of these 15 residues, a computer search in the Swiss protein database was performed and the alignment showed 69% identity with a subsequence including the residues 7–19 of Bos d 2, an allergenic protein from cow dander belonging to the lipocalin family. An allergenic relationship between Cav p 2 and Bos d 2 could be excluded by supplementary immunoblot inhibition experiments. The N-terminal sequence of the 17 kDa allergen is different from that of another major guinea pig allergen reported recently (Cav p 1; 20 kDa) (4). Thus, the major guinea pig allergen with 17 kDa will appear in

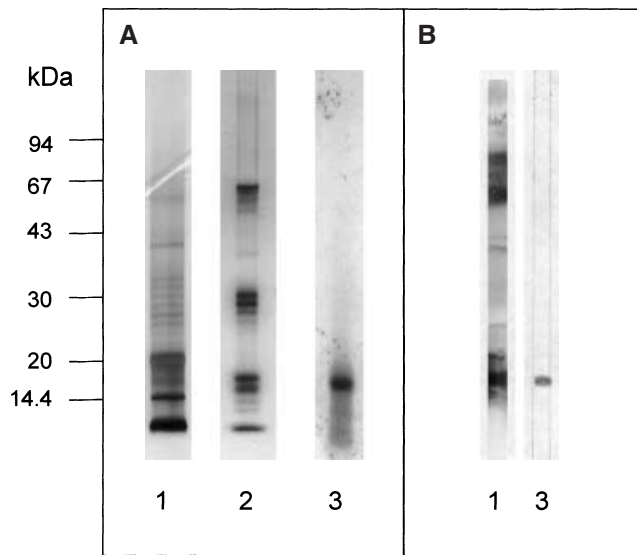


Figure 1. (A) sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of guinea pig hair extract (lane 1), fraction 2 obtained by anion exchange chromatography (4) (lane 2), protein isolated from ProRPC® column (see Fig. 2) (lane 3). Proteins were silver-stained. (B) Immunoglobulin E reactivity of pooled sera from guinea pig allergic patients to NC-blotted guinea pig hair extract (lane 1) and the protein isolated from ProRPC® column with a retention time of 10.7 min (lane 3).

the allergen nomenclature (<http://www.allergen.org>) as Cav p 2 according to (13).

The presence of carbohydrate moieties in Cav p 2 was evaluated by incubation of blotted samples with several digoxigenin-labelled lectins from *Galanthus nivalis* (GNA), *Sambucus nigra* (SNA), *Maackia amurensis* (MAA), *peanut* (PNA), *Datura stramonium* (DAS), *Aleuria aurantia* (AAA) and *Canavalia ensiformis* (Con A), which show different affinities against sugars. None of

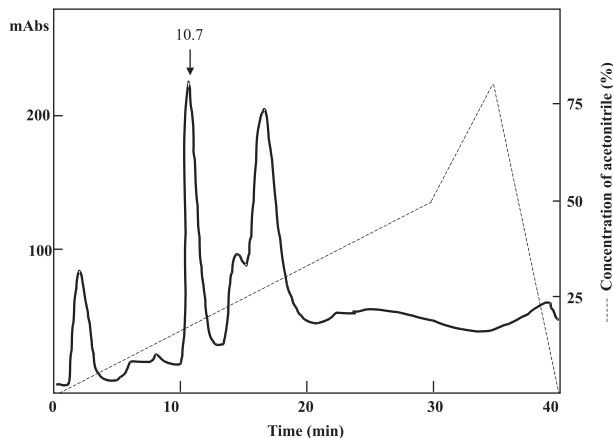


Figure 2. Elution profile of the last chromatographic step to isolate the guinea pig allergen on a ProRPC® column (1 ml) using an acetonitrile gradient. The arrow indicates the elution of Cav p 2 at retention time of 10.7 min.

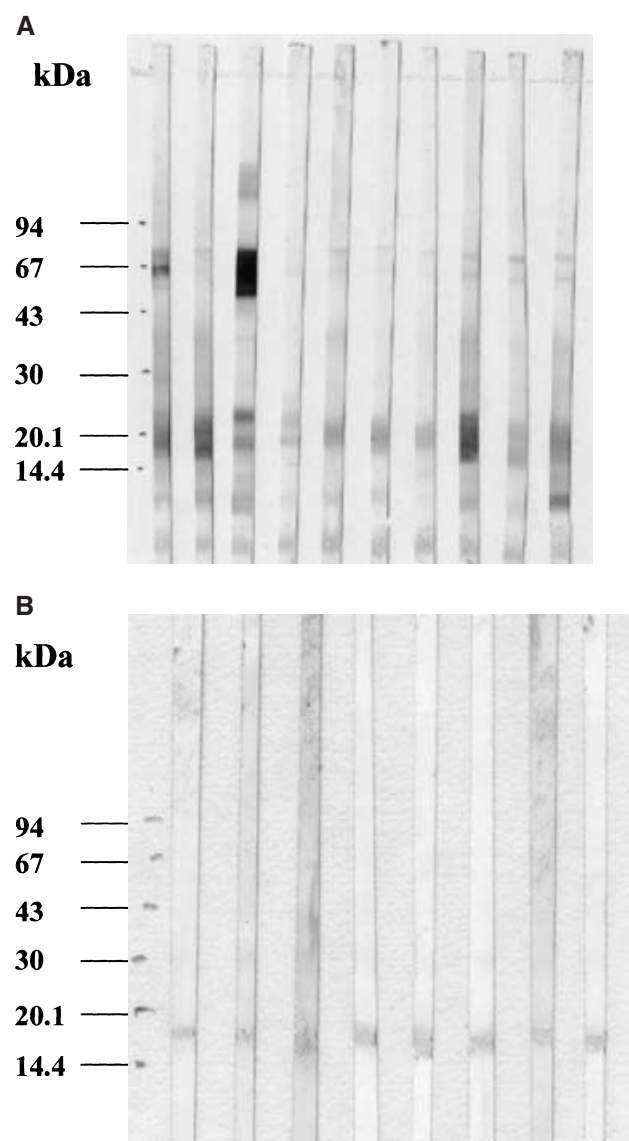


Figure 3. Immunoglobulin E reactivity of individual sera from guinea pig allergic patients to NC-blotted guinea pig hair extract (A) and isolated Cav p 2 (B).

Table 1. N-terminal amino acid sequences of the Cav p 2 (17 kDa) and Cav p 1 (20 kDa) allergens isolated from guinea pig hair extract including the homology to other proteins determined by reference to the Swiss-Prot data base

Cav p 2 (P83508)†	D	S	I	D	Y	S	K	V	P	G	N	W	R	T	I	15
*				I	D		S	K	+	P	G	W	R		I	
Bos d 2 (Q28133)†				I	D	P	S	K	I	P	G	E	W	R	I	19
Cav p 1 (P83507)† (4)	S	E	I	N	G	D	W	N	T	I	A	L	S	A	D	15

* Identities are echoed and substitutions to which the BLOSUM-62 matrix (18) gives a positive score are indicated by a '+' symbol.
† Accession number for Swiss-Prot data base.
The characteristic lipocalin sequence is indicated in bold letters.

the tested lectins bound to Cav p 2. Further glycoprotein analysis of blotted Cav p 2 using a commercial kit based on mild periodate treatment also gave negative results. These results suggested that Cav p 2 is not a glycoprotein. The same findings were obtained for Cav p 1.

2D-analysis of guinea pig hair proteins and allergens

To identify and characterize the components of crude guinea pig hair extract, we performed 2D-electrophoresis.

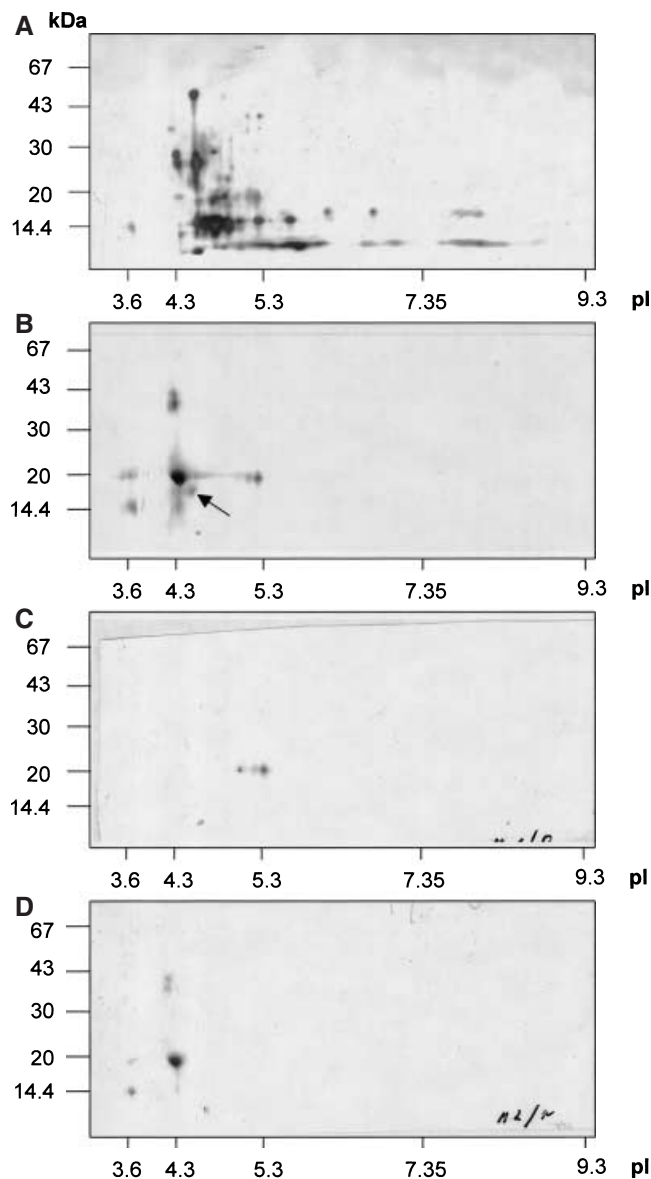


Figure 4. Inhibition of immunoglobulin E (IgE) binding to guinea pig hair extract. Gels were either silver-stained (A) or transferred onto NC for immunoblot analysis (B–C). The NC blots were probed for IgE binding with a serum pool of patients only (B) or the same serum pool preincubated either with Cav p 1 (C) or Cav p 2 (D). The arrow indicates the position of Cav p 2 (17 kDa and pI 4.5).

As shown in Fig. 4A, there are more than 50 components in the crude extract detectable by silver staining. In order to find out which of these proteins were allergens, we performed immunoblotting using serum pool of patients (Fig. 4B). Allergens were located mainly around five different molecular masses (40–42, 20, 17, 14 and 8 kDa) and showed acidic pI values in the range between 3.6–5.3. A marked heterogeneity of the pI for allergens with 20, 17 and 14 kDa pointed to the existence of isoforms.

The 20 kDa allergen is equivalent to Cav p 1 (4) and showed isoforms with pI ranging from 3.6 to 5.3. The isoform with 20 kDa and a pI of 4.3 could be considered as immunodominant. Furthermore, allergens were detected at 40–42 kDa with the same pI of 4.3 also found for the dominant isoform of Cav p 1. The allergen located at 17 kDa appeared as a doublet with similar pI values (pI 4.3–4.5). Further allergens were detected at 14 kDa (two distinct spots with pIs of 3.8 and 4.3) and of 8 kDa (pI of 4.6).

To study possible cross-reactive components between the different isoallergens, immunoblot inhibition experiments were performed with 2D-blots.

Identification of cross-reactive isoforms of Cav p 1 and Cav p 2

The 2D-blots were incubated either with pooled patient serum alone (Fig. 4B) or with serum preincubated with preparations of Cav p 1 (Fig. 4C) and Cav p 2 (Fig. 4D), respectively.

The preincubation with Cav p 1 (20 kDa) led to almost complete inhibition of IgE binding except for binding to 8 kDa allergen (pI 4.6) and to the isoforms of 20 kDa with a pI of 5.0 and 5.3.

On the contrary, preincubation with Cav p 2 inhibited specific IgE-binding to the 17 kDa allergens with pI of 4.3 and 4.5, but also to the 20 kDa allergens with pI of 5.0 and 5.3. Furthermore, preincubation with the 17 kDa allergen had no apparent effect on the IgE-binding to the 8 kDa (pI 4.6), 14 kDa (pI 3.8), 20 kDa (pI 3.8 and 4.3), and 40–42 kDa (pI 4.3) allergens.

Discussion

Guinea-pigs are important sources of inhalant allergens in home and working environments. Recently, we have published the characterization and isolation of the predominant guinea pig allergen with 20 kDa, which was designated Cav p 1 (4). In this report we describe a more detailed analysis of another major allergen isolated from guinea pig hair. The high degree of purification of the 17 kDa allergen allowed the sequencing of its 15 N-terminal residues. The sequence of Cav p 2 showed 69% identity with a sequence of Bos d 2, a mammalian lipocalin allergen in cow dander (14). In recent years, it has become evident that a number of important mammalian allergens are members of the lipocalin family. The

current list includes mouse Mus m 1, rat Rat n 1, dog Can f 1 and Can f 2, horse Equ c 1 and Equ c 2 as well as cow Bos d 2 and Bos d 5 (15). We have previously shown that Cav p 1, a major allergen in guinea pig hair and urine, is also a lipocalin (4). Although the sequence homology among the members of the lipocalin family is generally low (10–20%), short stretches of conserved sequences can be identified. The most conserved motif is the GXW triplet, which is present in all lipocalins including Cav p 1 and Cav p 2 (Table 1).

In the present study, the biochemical properties (e.g. glycosylation, mw, pI) of Cav p 1 and Cav p 2 were studied for the first time and compared with those of known lipocalin allergens. Notably, several lipocalin allergens are nonglycosylated (Bos d 2, Bos d 5 and Mus m 1), while others have putative N-glycosylation sites (Can f 1, Can f 2, Equ c 1). Our own findings suggest that the allergens Cav p 1 and Cav p 2 are also nonglycosylated lipocalins.

Some lipocalins, like Bos d 5 (an important food allergen) and Equ c 1 (16), are present in a dimeric state. We suppose that Cav p 1 can also form dimers. By means of 2D-immunoblotting analysis of guinea pig hair extract we detected allergens at 20 kDa [corresponding to Cav p 1 (4)] and at 40–42 kDa with the same pI of 4.3 (Fig. 4b). As Cav p 1 (20 kDa) prevented IgE binding to allergens with 20 and 40–42 kDa (Fig. 4C), we conclude that identical IgE binding epitopes may be recognized on both allergens because of Cav p 1 dimerization and/or aggregation. Final clarification of the question whether the 20 and 40 kDa allergens from guinea pig hair extract are indeed monomer/dimer components of the same protein Cav p 1, however requires further studies (e.g. amino acid analysis).

Furthermore, the results of 2D-immunoblotting inhibition suggest that Cav p 1 belongs to a group of related

allergens (so-called isoallergens) with different mw's and pI's, as has been shown also for other allergens (17). In fact, Cav p 1 inhibited the IgE binding to allergens with 20 kDa (pI 3.6–3.8 and 4.3) and 14 and 17 kDa (equivalent to Cav p 2). The result suggests that these allergens may share cross-reactive IgE epitopes. The same applies to the allergen Cav p 2, which inhibited the IgE binding to allergens with 17 kDa and those with 20 kDa and a pI of 5.0 and 5.3. Whether the present allergens in guinea pig hair extract are isoallergens of Cav p 1 or Cav p 2 or are independent molecules is unclear at present. Because Cav p 1 (20 kDa) and Cav p 2 (17 kDa) show different N-terminal amino acid sequences, it is more likely that both allergens are independent proteins with cross-reacting structures. We suppose that the most allergenic determinants of the guinea pig allergens are present on Cav p 1 but that only some determinants are present on Cav p 2. Thus, identical as well as nonrelated IgE epitopes may be present on the two different allergens. Despite the diversity in their physical characteristics (mw, pI) and N-terminal amino acid sequence, some cross-reactive IgE epitopes on Cav p 1 and Cav p 2 appear to be highly conserved.

In conclusion, the present study has identified a new major guinea pig allergen Cav p 2 as a member of the lipocalin family of proteins. Furthermore, the results provide important information on the isoallergen character of two relevant guinea pig allergens Cav p 1 and Cav p 2, as well as on their cross-reactive properties. Although much remains to be discovered on the guinea pig major allergens, our results represent first important steps towards their structural characterization. Accumulation of such information is essential for detailed studies of the interactions in allergenic reactions, as well as for clinical diagnosis and therapy.

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