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What is the role of the hevein-like domain of fruit class I chitinases in their allergenic capacity?

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Summary

Background Class I chitinases are the major panallergens in fruits associated with the latex–fruit syndrome. These enzymes contain an N-terminal hevein-like domain homologous to latex hevein, and a larger catalytic domain. The role of these domains in their allergenic capacity is still controversial.

Objective We sought to evaluate the role of both domains of class I chitinases in their IgE-binding properties, using Cas s 5, the major allergen from chestnut, as a model.

Methods Recombinant Cas s 5 and its deleted form, lacking the hevein-like domain, designated rCat, were expressed in *Pichia pastoris* using the pPIC 9 vector. Both recombinant products were purified from the supernatants of transformed yeast cultures by gel-filtration and cation-exchange chromatography. The isolated proteins were characterized by N-terminal sequencing, enzymatic activity and N-glycosylation tests, anti-chitinase and specific IgE immunodetection. Immunoblot, RAST and CAP inhibition assays were also performed.

Results Both purified rCas s 5 and rCat showed the expected N-terminal amino acid sequences and an enzymatic activity similar to that of their natural counterparts isolated from chestnut seeds, and were strongly recognized by anti-chitinase antibodies. In contrast, only rCas s 5, but not rCat, bound specific IgE from sera of patients suffering from the latex–fruit syndrome, and fully inhibited IgE-binding to natural Cas s 5 in immunoblot inhibition assays. Latex hevein also exerted a strong immunoblot inhibition of IgE-binding to chestnut Cas s 5. RAST and CAP inhibition using whole chestnut extract on the solid phase, rendered inhibition levels around 70–90% for rCas s 5 and 60% for rCat, in contrast to the immunoblotting results.

Conclusions Recombinant Cas s 5 behaves like natural Cas s 5 in IgE-binding assays *in vitro*. The hevein-like domain of allergenic class I chitinases seems to include all their main IgE-binding epitopes when tested by immunodetection and immunoblot inhibition experiments. RAST and CAP inhibition assays, on the contrary, suggest that relevant epitopes are also harboured in the catalytic domain of these allergens.

Keywords chestnut, class I chitinases, hevein-like domain, latex hevein, latex–fruit syndrome, plant panallergens

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Introduction

IgE-mediated reactions to latex products has become a prominent occupational disease, mainly in glove-wearing groups, such as health care workers [1]. An unexpectedly high rate (30–50%) of these allergic patients shows an associated hypersensitivity to some plant-derived foods, especially freshly consumed fruits [2–5]. Therefore, a latex–fruit syndrome has been postulated. Avocado, banana, chestnut and kiwi are the foods most frequently associated with latex allergy and most prone to induce systemic anaphylaxis [6].

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The main allergens that cross-react with latex have been isolated and characterized in chestnut [7,8], avocado [7–10] and banana [11,12]. Class I chitinases with an N-terminal hevein-like domain have been found in all three fruits to be the panallergens responsible for the latex–fruit syndrome (recent reviews in 13,14).

Latex hevein (Hev b 6.02), a defence-related polypeptide of 43 amino acids, has been described as a major allergen recognized by up to 80% of sera from latex allergic patients [15,16]. The close relationship between the N-terminal hevein-like domains of allergenic class I chitinases and latex hevein (65–70% sequence identity) suggests that this protein domain plays a key role in latex–fruit cosensitization [13]. The strong inhibition displayed by hevein on the IgE-binding capacity of fruit class I chitinases [10,11,17], and the lack of allergenic activity of the homologous class II enzymes without hevein-like domain [7,8],

support this hypothesis. In contrast to these results, unexpectedly low levels of inhibition of IgE-binding to latex hevein have been found using avocado and banana extracts, or even banana class I chitinase as inhibitors [10,11,17]. Moreover, class II chitinases that are not reactive when tested by immunodetection and immunoblot inhibition assays and skin prick test (SPT), exert around 45% IgE-binding inhibition to their corresponding whole extracts in CAP inhibition assays [8]. Joint consideration of the above-mentioned data leads to the conclusion that further studies are needed to establish the actual role of the hevein-like domain in the allergenicity of plant class I chitinases. A direct approach to this problem is here reported, using Cas s 5, the major allergen and class I chitinase from chestnut seed, as a model. Recombinant Cas s 5, as well as its recombinant deleted form, lacking the hevein-like domain, were expressed and purified, and their IgE-binding properties were compared.

Materials and methods

Patient sera

A pool of sera from eight patients was used in this study. Patients were selected from a population with latex allergy and all of them fulfil the following criteria: (1) an IgE-mediated hypersensitivity to chestnut, banana and/or avocado according to the clinical history; (2) a specific IgE level higher than 3.50 kU/L to at least one of the reactive fruits, as determined by the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden); and (3) a positive prick by prick test response to the corresponding fruits, as defined by a mean weal diameter greater than 3 mm, 15 min after puncture, compared with the saline control. Clinical data and total and specific IgE to chestnut and latex of the eight selected patients are summarized in Table 1. A serum pool from eight atopic subjects sensitized to mites but not to fruits, paired for age, sex and total IgE levels, was used as negative control.

Isolation of cDNAs encoding Cas s 5 and its catalytic domain (Cat)

A cDNA clone for Cas s 5, previosly isolated by Allona et al. [18] (accesion number U48687; kindly provided by Drs C. Aragoncillo and L. Gomez, ETS Ingenieros de Montes, UPM, Madrid, Spain) was used as starting preparation. The coding sequence of this clone (without signal peptide) is presented in Fig. 1.

The coding regions for mature Cas s 5 and its catalytic domain (Cat) were amplified by PCR using the following oligonucleotide 5′-primers: 5′-CCGCTCGAGAAAAGAG AACAATGTGGCA-3′ for Cas s 5, and 5′-CCGCTCGA GAAAAGAGAAGCTAGTAGTGGTGG-3′ for Cat (*Xho* I restriction site underlined). For the reverse direction, the 3′-primer: 5′-CGGGAAACGGATTTATCGAATTCCGGT-3′ (*Eco* RI restriction site underlined) was used in both cases. The primers were designed from the nucleotide sequence of the cDNA clone for Cas s 5, incorporating the *Xho* I/*Eco* RI restriction sites to insert the DNA fragment into the pPIC 9 vector. The sense primers allowed also fusion of the encoding regions in frame with the sequence coding for the prepro-α-factor of *Saccharomyces cerevisiae* present in plasmid pPIC 9. The signal peptidase cleavage site lies between Arg and Glu. The N-terminal glutamic acid of the processed recombinant product was already present in the Cas s 5 sequence, but this residue had to be recreated by adding the codon GAA located before the first codon (GCT) for mature Cat.

The amplification was carried out with Taq-Gold DNA polymerase (Perkin-Elmer, Roche Molecular System, Branchburg, NJ, USA) as recommended by the manufacturer. The PCR programme was 4 min denaturation at 94 °C, followed by 25 cycles each of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C, and final extension for 12 min at 72 °C.

The isolated PCR products were cloned into pGEM TEasy vector (Promega, Madison, WI, USA), and the constructs used to transform DH5αF′ *Escherichia coli* cells. The plasmidic DNA from several clones with each construct, was sequenced using the ABI PRISM Dye Terminator Kit and a ABI 377 sequencer (Perkin-Elmer Biosystem, Warrington, UK).

Expression of recombinant Cas s 5 and Cat in Pichia pastoris

The PCR products cloned in pGEM TEasy were digested with *Xho* I/*Eco* RI restriction enzymes and inserted into the same sites of the pPIC 9 vector (pPIC/Cas s 5 and pPIC/Cat) to transform DH5αF′ *E. coli*, and the plasmidic DNA from several

Table 1. Clinical data of the selected latex-fruit allergic patients

			Latex			Chestnut		
Patient No.	Age/sex	Total IgE (kU/L)	Symptoms	SPT*	IgE‡	Symptoms	PPT†	IgE‡
1	39/F	268	LU/AE/BA	12	46.0	OAS	9	3.5
2	28/F	337	LU/RC/AE	8	> 100	A	8	18.0
3	30/F	78	LU/RC/AE	7	0.9	U	6	2.4
4	25/F	145	A	9	19.4	AE	7	1.1
5	39/F	141	A	7	> 100	OAS	5	23.6
6	25/F	213	LU/AE/BA	14	34.0	A	6	1.4
7	23/F	1900	LU/AE	8	> 100	OAS	7	6.5
8	61/F	236	U	5	43.2	OAS	7	4.4

F = female; LU = local urticaria; AE = angioedema; BA = bronchial asthma; RC = rhinoconjunctivitis; A = systemic anaphylaxis; U = urticaria; OAS = oral allergy syndrome. *Skin prick test using a commercial latex extract (ALK-Abello S.A., Madrid, Spain) and †prick by prick test with chestnut, expressed as the mean weal diameter in mm. ‡Specific IgE in kU/L, as determined by the Pharmacia CAP System.

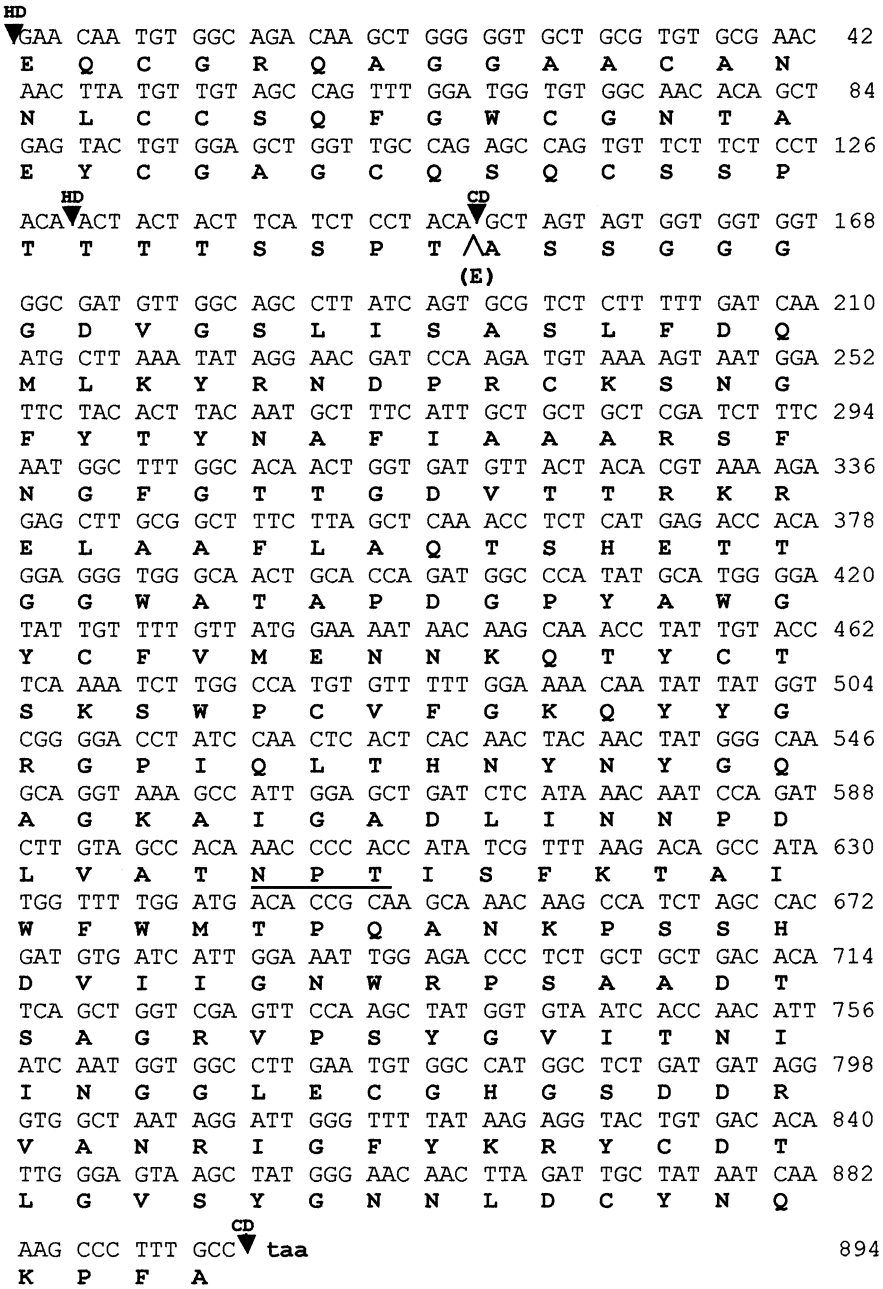

Fig. 1. Nucleotide and deduced amino acid sequences of the coding region for mature allergen Cas s 5 [18]. The hevein-like (HD) and catalytic (CD) domains, as well as the N-terminal glutamic acid added to rCat (E), are indicated. A potential N-glycosylation site is underlined.

positive clones was again sequenced to confirm the in-frame arrangements described above.

The pPIC/Cas s 5 and pPIC/Cat constructs were then digested with *Sac* I restriction enzyme, and the larger purified fragments were used to transform GS115 *P. pastoris* cells by electroporation, using an Electro Cell Manipulator 600 BTX (BTX Inc., San Diego, CA, USA) and following the Invitrogen manual recommendations (Invitrogen Corp., De Schelp, the Netherlands). Transformed cells were grown on minimal glucose plates at 30 °C during 3–4 days. Screening for positive clones was then carried out by patching the colonies in replica-plating on minimal glucose vs. minimal methanol plates.

The selected transformants were grown in buffered glycerol minimal medium at 30 °C for 4 days. Cells were then collected by centrifugation, and expression of the recombinant proteins was then induced by re-suspension in buffered methanol minimal medium. The production of rCas s 5 and rCat in the supernatant of the corresponding culture medium was checked by taking samples at different times and analysing by SDS-PAGE. Maximum expression was achieved after 4 days.

Isolation and characterization of rCas s 5 and rCat

The supernatant of the culture medium containing rCas s 5 or rCat was dialysed against 0.2 M ammonium acetate and freeze-dried. Each preparation (3 mg of protein) was fractionated by gel-filtration on a Superdex 75 HR 16/60 column (Amersham Pharmacia Biotech, Uppsala, Sweden) using 0.2 M ammonium acetate as elution buffer (1 mL/min). Fractions enriched in rCas s 5 or rCat, as judged by SDS-PAGE, were pooled, freeze-dried and then re-purified by cation-exchange chromatography on a

Mono S HR 5/5 column (Amersham Pharmacia Biotech; 1 mL total volume). The column was equilibrated with 10 mM sodium acetate, pH 5.3, and eluted with a salt gradient to 0.5 M NaCl in the same buffer (0% to 50% in 40 min and 50% to 100% in 20 min; 1 mL/min).

Defatted flour from mature nuts of chestnut (*Castanea sativa*) were extracted with PBS buffer (0.1 M sodium phosphate, pH 7.0, 0.15 M NaCl, 1×; 1 : 5 (w/v); 1 h; 4 °C), and after centrifugation, the supernatant was dialysed against H_2O and freeze dried (crude PBS extract). A protein preparation enriched in chitinases was obtained from the PBS extract (supernatant) by addition of 0.1 M HCl (final pH 3.5). nCas s 5 and CsII were isolated from this protein preparation by affinity-chromatography on a regenerated chitin column, followed by cation-exhange chromatography, as in Diaz-Perales et al. [7].

Purified proteins were quantified by the bicinchoninic acid test [19].

SDS-PAGE was performed according to the method of Laemmli [20] on Bio-Rad Miniprotein II system gels (15% polyacrylamide), except that reducing agents and heat treatment of samples were omitted.

N-terminal amino acid sequences were determined by standard methods using an Applied Biosystems 477 A gas-phase sequenator.

Chitinase activity was assayed by a colourimetric microtest using carboxymethyl–chitin–Remazol Brilliant Violet 5R as substrate. Procedure I of Wirth and Wolf [21] was followed (30 min at 37 °C per assay). Preliminary tests were carried out with different protein amounts to fit the Δ absorbance per assay between 0 and 300×10^{-3} units.

Glycoproteins were identified with a glycan detection Kit (Roche Molecular Biochemicals, Mannhein, Germany) following the manufacturer's instructions.

Immunodetection and immunoblot inhibition assays

Samples were separated by SDS-PAGE. The gels were then soaked for 15 min in transfer buffer (50 mM Tris, 50 mM boric acid, pH 8.3) and electrotransferred to PVDF membranes on a Bio-Rad Mini Trans-Blot cell for 60 min at 80 V. Membranes were washed, blocked and incubated with a serum pool from allergic patients or with control sera (1 : 3 dilutions), then with alkaline phosphate-conjugated monoclonal anti-human IgE (clone GE-1, Sigma; 1 : 500 dilution), and finally revealed by adding BCIP/NBT solution [22].

Alternatively, blocked membranes were inmunodetected with rabbit monospecific polyclonal antibodies to seed chestnut chitinase CsII [23] (1 : 250 dilution), which recognized both class I and II chinitases [7]. Membranes were then incubated with an alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma; 1: 500 dilution) and a BCIP/NBT solution.

Immunoblot inhibition assays were performed as described above, except that the serum pool from latex-fruit allergic patients was pre-incubated for 3 h at room temperature (25 °C) with each of the inhibitors (5 μg of purified nCas s 5, rCas s 5, CsII, rCat, latex hevein and bovine serum albumin as negative control).

RAST and CAP inhibition assays

RAST inhibition was performed using BrCN-activated cellulose disks (7 mm Ø; 00H Munktell, Grycksbo, Sweden) coupled to chestnut crude PBS extract (1 mg/mL). Disks were incubated overnight at room temperature with sera (100 μL) pre-treated with each inhibitor in PBS buffer with 0.5% (w/v) BSA, washed with the same buffer containing 0.05% (v/v) Tween 20 instead of BSA, and then incubated with anti-human IgE-Mab HE-2 ^{125}I (ALK-Abello, Madrid, Spain; 50000 cpm per test; 3 h at room temperature). After washing, bound radiactivity was measured. A chestnut crude PBS extract (50 μg/mL) and purified nCas s 5, rCas s 5, CsII, rCat and latex hevein (kindly provided by Dr Z. Chen, Bochum, Germany) (5 μg/mL) were used as inhibitors. Three replicas of the serum pool from latex–fruit patients (1 : 3 dilution) were incubated with five progressive 1 : 5 dilutions of each inhibitor in PBS buffer with 0.5% BSA (w/v). Serum samples pre-incubated with this buffer and samples without serum were used to determine the 0% and 100% inhibition values, respectively.

CAP inhibition was carried out as in Blanco et al. [8]. Triplicate samples of undiluted serum pool were premixed with five progressive 1 : 10 dilutions of each inhibitor solution, and then incubated with commercial chestnut immuno-CAPs (Pharmacia Diagnostics). Specific IgE levels were assessed as indicated in the CAP System manual. A chestnut crude extract (250 μg/mL), purified natural and recombinant chestnut proteins (40 μg/mL) and latex hevein (10 μg/mL) were used as inhibitors. A *Dermatophagoides pteronyssinus* commercial extract (ALK-Abello, Madrid, Spain) was tested as negative control. Inhibition–dilution relationships were calculated by plotting the mean inhibition values as a function of the log inhibitor dilutions, and the assays were validated if the linear correlation coefficients were higher than 0.95.

Results

cDNAs encoding mature Cas s 5 and its deleted form Cat, including only its catalytic domain, were obtained by PCR amplification of the corresponding regions of a previously isolated Cas s 5 cDNA clone [18] (Fig. 1). Five independent clones for Cas s 5 and four for Cat were isolated and sequenced, the clones of each group revealing identical sequences and fully confirming the expected nucleotide sequences (Fig. 1). The Cas s 5 cDNA encoded a 298 amino acid protein (calculated molecular weight 31978 D), and the Cat cDNA a 249 residue protein (including the N-terminal glutamic acid added for its proper extracellular expression; calculated molecular weight 27125 D). The deduced amino acid sequence for Cas s 5 showed 66–71% identity with those deduced for allergenic class I chitinases from avocado (Prs a 1), banana and green bean [14]. The Cas s 5 hevein-like domain was also 67% identical to latex hevein.

The isolated Cas s 5 and Cat cDNAs were inserted into the pPIC 9 expression vector and the constructs were used to transform *P. pastoris* GS115 cells. Maximum expression of extracellular rCas s 5 and rCat was obtained 4 days after induction with methanol, as judged by SDS-PAGE analysis of the proteins secreted by the corresponding cell cultures (not shown). The yield was around 30 mg of rCas s 5 or rCat per litre of culture supernatant.

In order to isolate rCas s 5 and rCat, the dialysed extracellular medium of the culture expressing each recombinant product was first separated by gel-filtration chromatography. Fractions containing rCas s 5 or rCat were freeze-dried and re-purified by cation-exchange chromatography. The peak including rCas s 5

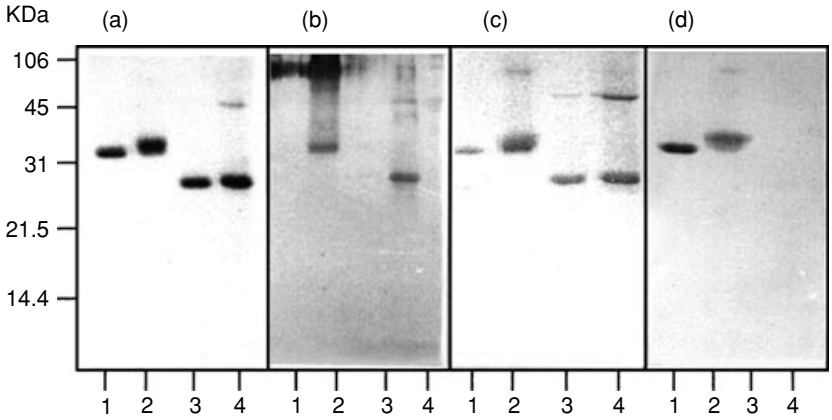

Fig. 2. SDS-PAGE separation of 2 μg of purified proteins nCas s 5 (1), rCas s 5 (2), Cs II (3) and rCat (4). Replica gels were stained with Coomassie Blue (a) or electrotransferred to PVDF membranes and immunoblotted with a glycan detection kit (b), monospecific polyclonal antibodies to CsII (c) or a serum pool from patients with latex–fruit allergy (d).

or rCat was checked by SDS-PAGE. Both purified recombinant proteins showed a single electrophoretic band of the expected molecular size (Fig. 2a). However, rCas s 5 exhibited a slightly higher apparent molecular weight than nCas s 5. It has been described that *P. pastoris* unspecifically glycosylates those recombinant proteins harbouring potential N-glycosylation sites. In fact, several natural non-glycosylated allergens, including the avocado class I chitinase Prs a 1, render glycosylated recombinant forms when expressed in this yeast [9,24]. The deduced amino acid sequence of Cas s 5 presented a potential N-glycosylation site (NPT, positions 201–203; Fig. 1) in its catalytic domain. The glycosylation of rCas s 5 and rCat, but not of nCas s 5 and the chestnut class II chitinase CsII, was verified by testing the four purified proteins by a glycan detection kit (Fig. 2b). This modification does not seem to affect the biochemical and immunological properties of the recombinant proteins when compared with their natural counterparts (see below).

The proper processing, and probably folding, of rCas s 5 and rCat was supported by three different experimental lines. First, the expected N-terminal amino acid sequences, EQCG for rCas s 5 and EASSG for rCat, were found when sequencing each isolated protein. Secondly, both recombinant proteins were strongly recognized by monospecific polyclonal antibodies to CsII (Fig. 2c). Thirdly, the enzymatic (chitinase) activity of rCas s 5 and rCat was similar to that of nCas s 5 and CsII, respectively (Table 2).

In spite of these data, only rCas s 5 and nCas s 5, but not rCat and CasII, were recognized by a pool of sera from latex–fruit allergic patients in IgE immunodetection assays (Fig. 2d). These results were strongly confirmed by the full IgE-binding inhibition showed by both Cas s 5 forms, and the lack of effect of rCat and Cas II, in immunoblot inhibition assays against purified rCas s 5 and nCas s 5 (Fig. 3). Interestingly, a strong, though not complete, inhibition was displayed by latex hevein in the same tests (Fig. 3, lane 6). Negative control sera did not react with any of the native or recombinant chestnut proteins analysed in Figs 2 and 3.

The high IgE-binding capacity of rCas s 5 and nCas s 5 was further confirmed by RAST and CAP inhibition tests (Fig. 4). Levels of specific IgE to chestnut were 6.41 kU/L in the pool of sera from the selected patients, as assesed by the commercial CAP system. Both allergen forms reached inhibition values over

Table 2. Chitinase activity of purified natural Cas s 5 and CsII and recombinant Cas s 5 and Cat proteins

Protein	ng/assay	Δ Absorbance $\times 10^{-3}$ (550 nm)
nCas s 5	100	205 ± 3
	500	283 ± 2
rCas s 5	100	173 ± 2
	500	246 ± 2
CsII	100	213 ± 3
	500	280 ± 2
rCAT	100	207 ± 3
	500	274 ± 2
BSA	500	1 ± 4

Bovine serum albumin (BSA) was included as negative control. Values are means ± SE ($n = 3$).

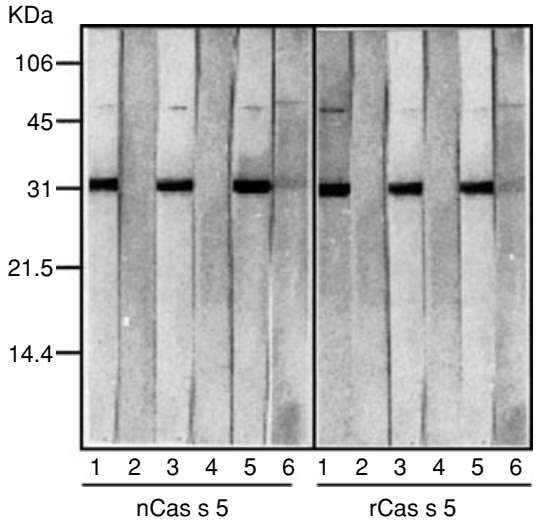

Fig. 3. Immunoblot inhibition assays against purified nCas s 5 and rCas s 5 (2 μg). Proteins were subjected to SDS-PAGE, electrotransferred to PDVF membranes and immunodetected with a pool of sera from latex–fruit allergic patients pre-incubated with bovine serum albumin (1, negative control), nCas s 5 (2), Cs II (3), rCas s 5 (4), rCat (5) and latex hevein (6). Five micrograms of each inhibitor were used.

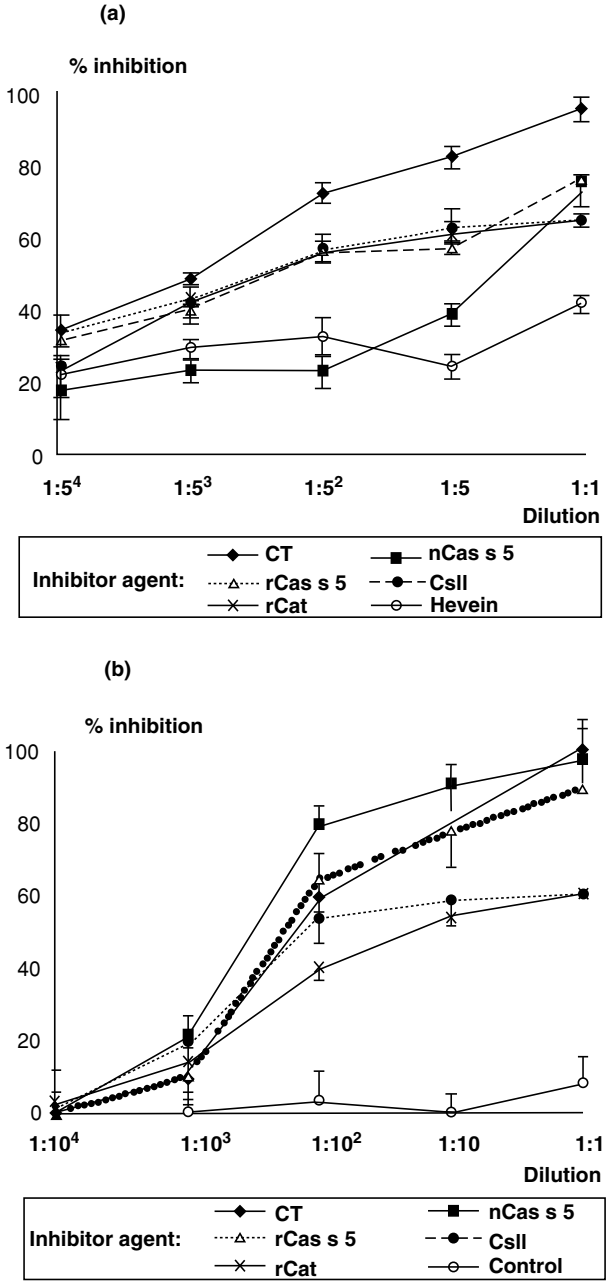

Fig. 4. (a) RAST inhibition by chestnut crude extract (CT) and purified natural and recombinant chestnut proteins, and latex hevein, using as solid phase chestnut crude PBS extract coupled to cellulose disks. A pool of sera from latex–fruit allergic patients was pre-incubated with progressive 1 : 5 dilutions of each inhibitor (initial concentrations: 50 μg/mL for chestnut crude extract and 5 μg/mL for each purified protein). (b) CAP inhibition by crude extract (CT) and purified natural and recombinant chestnut proteins, with a chestnut solid phase. A pool of sera from latex–fruit allergic patients was pre-incubated with progressive 1 : 10 dilutions of each inhibitor (initial concentrations: 250 μg/mL for CT and 40 μg/mL for each purified protein). A *D.pteronyssinus* commercial extract was used as negative control. Data represent means ± SE of triplicate determinations, in (a) and (b).

70% (RAST) and 90% (CAP) when tested against whole chestnut extract. However, and in contrast to the immunoblot results, significant inhibition levels, about 60% both in RAST and CAP, were also found for rCat and CsII (Fig. 4). Latex hevein showed an unexpectedly low inhibition capacity, with maximum inhibition levels of 42% in RAST (Fig. 4) and 65% ± 7% ($n = 3$) in CAP (10 μg/mL; neither higher amounts of inhibitor nor lower dilutions were assayed due to the lack of availability of purified protein).

Discussion

A wide body of evidence reported by different research groups points to plant food class I chitinases and latex hevein as the allergens mainly involved in the latex–fruit syndrome [7–14]. Moreover, the hevein-like domain located in the N-terminus of these enzymes seems to be essential for their allergenic reactivity, although some of the published data are not consistent with this hypothesis [14]. In contrast, class I chitinases from plant foods, including chestnut, do not react with sera from patients allergic to latex but not to fruit, thus suggesting that they do not play a specific role in allergy to latex non-associated with sensitization to fruits [22]. This background warranted the two objectives pursued in this study: to produce a recombinant class I chitinase showing an IgE-binding capacity similar to that of its natural form, and to further explore the role of the hevein-like and catalytic domains of these plant allergens in their allergenic properties. Both aims were approached by expressing the chestnut allergen rCas s 5 and its deleted form rCat, without hevein-like domain, in the yeast *Pichia pastoris*, as extracellular proteins. The appropriate processing and the integrity of purified rCas s 5 and rCat were supported by their determined N-terminal amino acid sequences, their recognition by anti-chitinase antibodies, and their enzymatic activities similar to those of their natural counterparts (Fig. 2c and Table 2).

IgE immunodetection and immunoblot inhibition assays using sera from latex–fruit allergic patients (Figs 2d and 3), as well as RAST and CAP inhibition tests, showed the close IgE-binding properties of rCas s 5 and nCas s 5. The recombinant allergen can thus be a useful tool to improve the diagnosis and, eventually, the future immunotherapy of patients suffering from the latex–fruit syndrome. Only another allergenic class I chitinase, the avocado major allergen Prs a 1, had been previously obtained as a recombinant nonfusion product [9].

The strong reactivity of rCas s 5 to IgE of sera from allergic patients, both in immunodetection and immunoblot inhibition assays, was in full contrast to the lack of reactivity of rCat in the same experiments (Figs 2d and 3). The behaviour of nCas s 5 when compared with that of the chestnut class II chitinase CsII, without hevein-like domain, fully agrees with these results. Joint consideration of these data strongly suggests that all relevant IgE-binding epitopes of Cas s 5 are located in its hevein-like domain. Thus, the deletion of this protein domain, as in rCat, leads to a complete loss of the IgE-binding capacity. The strong immunoblot inhibition of the IgE-binding to both rCas s 5 and nCas s 5 exerted by latex hevein, is in line with this hypothesis.

This clear-cut evidence was partially contradicted by the results obtained in RAST and CAP inhibition assays (Fig. 4). Although rCas s 5 and nCas s 5 showed increasing and expected inhibition values of up to 70–80%, both rCat and CsII (which exhibited very similar inhibition profiles) reached unexpected maximum inhibition levels of around 60%. Additionally, nCas s 5 seemed to exert lower inhibition capacity than the other purified

proteins tested when progressive dilutions were proved in the RAST inhibition assays. However, this result was not confirmed in the CAP tests, where nCas s 5 showed the highest inhibition levels among the proteins assayed, throughout the decreasing dilutions used. Furthermore, unexpected inhibition values, lower than those reported against banana and avocado extracts [10,11,17], were also obtained for latex hevein. These data suggest that the catalytic domain of class I (rCat) and II (CsII) plant chitinases also includes IgE-binding epitopes, non-reactive by immunoblotting but uncovered in RAST and CAP inhibition tests. It could be hypothesized that these epitopes are conformational, losing their tertiary structure and, thus, their IgE-binding capacity in the presence of SDS. However, the absence of *in vivo* reactivity (SPT) of purified chestnut and avocado class II chitinases [8], is not consistent with this hypothesis.

It seems clear that further studies are necessary to establish the actual role of both hevein-like and catalytic domains of plant class I chitinases in their allergenic capacity. This point is also relevant to understanding the molecular basis of latex–plant food cross-reactions, particularly the involvement of latex hevein. The availability of the recombinant Cas s 5 hevein-like domain could help to elucidate these questions. Unfortunately, we have not yet succeeded in expressing and isolating it from *P. pastoris* culture media in a proper and highly purified form. New efforts to reach this objective are currently being undertaken.

In conclusion, although we have demonstrated that the main IgE-binding epitopes of class I chitinases are located in their N-terminal hevein-like domain, our data suggest that at least another IgE-binding epitope, probably of conformational nature, is located in their catalytic domain.

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References

1 Poley GE, Slater JE. Latex allergy. J Allergy Clin Immunol 2000; 105:1054–62.
2 Blanco C, Carrillo T, Castillo R, Quiralte J, Cuevas M. Latex allergy: clinical features and cross-reactivity with fruits. Ann Allergy 1994; 73:309–14.
3 Beezhold DH, Sussman GL, Liss GM, Chang NS. Latex allergy can induce clinical reactions to specific foods. Clin Exp Allergy 1996; 26:416–22.
4 Brehler R, Theissen U, Mohr C, Luger T. 'Latex–fruit syndrome': frequency of cross-reacting IgE antibodies. Allergy 1997; 52:404–10.
5 Levy DA, Mounedji N, Noirot C, Leynadier F. Allergic sensitization and clinical reactions to latex, food and pollen in adult patients. Clin Exp Allergy 2000; 30:270–5.
6 Blanco C. The latex–fruit syndrome: a review on clinical features. Internet Symp Food Allergens 2000; 2:125–35.
7 Diaz-Perales A, Collada C, Blanco C et al. Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. J Allergy Clin Immunol 1998; 102:127–33.
8 Blanco C, Diaz-Perales A, Collada C et al. Class I chitinases as potential panallergens involved in the latex–fruit syndrome. J Allergy Clin Immunol 1999; 103:507–13.
9 Sowka S, Hsieh LS, Krebitz M et al. Identification and cloning of Prs a 1, a 32 kDa endochitinase and major allergen of avocado, and its expression in the yeast *Pichia pastoris*. J Biol Chem 1998; 273:28091–7.
10 Posch A, Wheeler CH, Chen Z et al. Class I endochitinase containing a hevein domain is the causative allergen in latex-associated avocado allergy. Clin Exp Allergy 1999; 29:667–72.
11 Mikkola JH, Alenius H, Kalkkinen N et al. Hevein-like protein domains as a possible cause for allergen cross-reactivity between latex and banana. J Allergy Clin Immunol 1998; 102:1005–12.
12 Sanchez-Monge R, Blanco C, Diaz-Perales A et al. Isolation and characterization of major banana allergens: identification as fruit class I chitinases. Clin Exp Allergy 1999; 29:673–80.
13 Sanchez-Monge R, Diaz-Perales A, Blanco C, Salcedo G. Class I chitinases and the latex–fruit syndrome. Internet Symp Food Allergens 2000; 2:137–44.
14 Salcedo G, Diaz-Perales A, Sanchez-Monge R. The role of plant panallergens in sensitization to natual rubber latex. Curr Opin Allergy Clin Immunol 2001; 1:177–83.
15 Alenius H, Kalkkinen N, Reunala T, Turjanmaa K, Palosuo T. The main IgE-binding epitope of a major latex allergen, prohevein, is present in its N-terminal 43-amino acid fragment, hevein. J Immunol 1996; 156:1618–25.
16 Chen Z, Posch A, Lohaus C, Raulf-Heimsoth M, Meyer HE, Baur X. Isolation and identification of hevein as a major IgE-binding polypeptide in *Hevea* latex. J Allergy Clin Immunol 1997; 99:402–9.
17 Chen Z, Posch A, Cremer R, Raulf-Heimsoth M, Baur X. Identifiction of hevein (Hev b 6.02) in *Hevea* latex as a major cross-reacting allergen with avocado fruit in patients with latex allergy. J Allergy Clin Immunol 1998; 102:476–81.
18 Allona I, Collada C, Casado R, Paz-Ares J, Aragoncillo C. Bacterial expression of an active class Ib chitinase from *Castanea sativa* cotyledons. Plant Mol Biol 1996; 32:1171–6.
19 Smith PK, Krohn RI, Hermanson GT et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150:76–85.
20 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–5.
21 Wirth SJ, Wolf GA. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J Microbiol Methods 1990; 12:197–205.
22 Diaz-Perales A, Collada C, Blanco C et al. Cross-reactions in the latex–fruit syndrome: a relevant role of chitinases but not of complex asparagine-linked glycans. J Allergy Clin Immunol 1999; 104:681–7.
23 Collada C, Casado R, Fraile A, Aragoncillo C. Basic endochitinases are major proteins in *Castanea sativa* cotyledons. Plant Physiol 1992; 100:778–83.
24 Sowka S, Wagner S, Krebitz M et al. cDNA cloning of the 43 kDa latex allergen Hev b 7 with sequence similarity to patatins and its expression in the yeast. *Pichia Pastoris* Eur J Biochem 1998; 255:213–9.