

Der p 1 and Der f 1, the highly related and major allergens from house dust mites, are differentially affected by a plant cystatin

M. PERNAS*, I. SANCHEZ-RAMOS†, R. SANCHEZ-MONGE*, M. LOMBARDERO‡, C. ARTEAGA‡, P. CASTAÑERA† and G. SALCEDO*

*Unidad de Bioquímica, Departamento de Biotecnología, ETS Ingenieros Agrónomos, Madrid, †Unidad de Entomología, Centro de Investigaciones Biológicas, CSIC, Madrid and ‡ALK-Abelló, Madrid, Spain

Summary

Background Der p 1 and Der f 1 are highly related allergens from the two main house dust mite species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively. A link between the cysteine proteinase activity of Der p 1 and its allergenicity has recently been demonstrated.

Objective To test the effects of several proteinase inhibitors, mainly a chestnut cystatin (CsC), on the enzymatic activity of Der p 1 and Der f 1, as well as to study the potential acaricide properties of the inhibitors.

Methods Inhibition tests were performed using mite extracts, as well as isolated Der p 1 and Der f 1 as targets. Immunodetection after SDS-PAGE and N-terminal amino acid sequencing were used to demonstrate the inactivation of chestnut cystatin by Der p 1. Bioassays including different inhibitors in a semisynthetic diet were performed to evaluate a potential effect on larval survival.

Results CsC inhibited the crude digestive proteinase activity of *D. farinae*, but it was not active towards the equivalent enzyme from *D. pteronyssinus*. This differential behaviour was fully explained by testing CsC against the two purified allergens, Der f 1 and Der p 1; the former was highly susceptible, whereas the latter was not affected. In contrast, other cysteine proteinase inhibitors, such as egg white cystatin and E-64, inhibited the proteinase activity of both mite allergens. Der p 1 inactivated CsC by a specific proteolytic cleavage between Gly 6 and Val 7, thus given rise to a noninhibitory processed protein. Besides the *in vitro* activity, CsC also showed an *in vivo* effect on *D. farinae*, drastically increasing the larval mortality when added to a semisynthetic diet.

Conclusion Der p 1 and Der f 1 behave very differently towards a plant cystatin, thus indicating substantial differences in their proteolytic activity. This fact could be significant with regards to the immunogenic properties of both allergens.

Keywords: cystatin, cysteine proteinase activity, *Dermatophagoides*, differential inhibition, major allergen.

Clinical and Experimental Allergy, Vol. 30, pp. 972–978. Submitted 24 May 1999; revised 25 August 1999; accepted 19 October 1999.

Introduction

Allergens from house dust mites, mainly *Dermatophagoides pteronyssinus* and *D. farinae*, play a prominent role in

immediate hypersensitivity reactions, including asthma and other allergic diseases (perennial rhinitis and atopic dermatitis) [1–3]. Up to 11 groups of different *Dermatophagoides* allergens have been defined [3]. Group 1 allergens, which comprises Der p 1 from *D. pteronyssinus* and Der f 1 from *D. farinae*, are among the strongest mite immunogens in man. In fact, over 80% of mite-allergic

Correspondence: G. Salcedo, Unidad de Bioquímica, Departamento de Biotecnología, E. T. S. Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain.

patients show positive skin prick test and serum IgE to these group 1 allergens [1].

Der p 1 and Der f 1 are 25 Kd proteins, excreted in mite faeces that have cysteine proteinase activity [4,5]. Both enzymes (allergens) are closely related (84% of amino acid sequence identity) and seem to present identical residues both in the active site and substrate-binding pocket deduced from the three dimensional modelling of Der p 1 [6–9]. Recently, a link between the proteolytic activity and the allergenicity of Der p 1 via the cleavage of CD23 and CD25 receptors has been suggested [10], thus emphasizing the significance of the enzymatic activity of these mite allergens with respect to their immunogenic properties [9]. Furthermore, cytokine release (IL-6 and IL-8) from human bronchial epithelial cells induced by Der p 1 is inhibited by E-64, the diagnostic inhibitor of cysteine proteinases [11].

Several plant proteinaceous inhibitors of cysteine proteinases, named phytocystatins, have been isolated and characterized [12,13]. Most are proteins of around 12 Kd with no disulphide bonds. Although homology with members of the cystatin superfamily of animal origin, such as egg white cystatin, has been reported, the plant inhibitors cluster in a specific subfamily [14]. We have recently purified and characterized a cystatin, named CsC, from chestnut (*Castanea sativa* Mill.) seeds [15]. Its cDNA clone was also isolated, and the recombinant inhibitor expressed in *Escherichia coli*. CsC inhibits cysteine proteinases of both plant (papain, ficin and chymopapain) and animal (cathepsin B) origin, and shows a weak effect towards trypsin. Besides affecting enzymes from insect pests, preliminary data suggests that the chestnut cystatin has a very different behaviour against the digestive proteases from the two main house dust *Dermatophagoides* species.

We report here a differential activity of the chestnut cystatin towards Der p 1 and Der f 1. CsC, but not other cystatins (such as that from egg white) nor specific inhibitors (E-64), inhibits Der f 1, whereas it does not affect Der p 1. Additional evidence suggests that Der p 1 inactivates CsC by a proteolytic cleavage. Besides the *in vitro* activity against Der f 1, CsC also greatly increases the larval mortality of *D. farinae* *in vivo* when added to a semisynthetic diet.

Materials and methods

Mite extracts

Mites isolated from growth media (purity above 99%) were extracted with 20 mM ammonium bicarbonate for 4 h at 4 °C. The extracts were centrifuged, dialysed against H₂O, filtered (0.2 µm pore size), and freeze-dried. The proportion of the total protein accounted by Der p 1 and Der f 1 was 7.1% and 5.3%, respectively, in the corresponding extracts,

as determined by ELISA using the appropriate monoclonal antibodies (ALK-Abelló, Madrid, Spain).

Cystatin-enriched protein preparations from seeds

Mature seeds of chestnut (*Castanea sativa*), pine (*Pinus pinea*), ginkgo (*Ginkgo biloba*), and bread wheat (*Triticum aestivum*) were ground and defatted with acetone (2×; 1 : 5 [w/v] for 1 h at 4 °C). The defatted flour was then extracted with PBS buffer (0.1 M sodium phosphate pH 7.0, 0.15 M NaCl; 1×; 1 : 5 [w/v] for 1 h at 4 °C), centrifuged, dialysed against 0.1 M ammonium acetate pH 6.8, and freeze-dried. Cystatin-enriched protein preparations were obtained from PBS extracts by gel filtration on Sephadex G-100 (80 cm × 2.5 cm column; 25 mL/h), using 0.1 M ammonium acetate pH 6.8 as elution buffer. Fractions showing a high antipapain activity were pooled and freeze-dried.

Protein purification

Bacterial expression and purification of recombinant CsC were performed as previously described [15]. Besides CsC, a recombinant protein containing the full CsC amino acid sequence fused to a 30 amino acid peptide with six histidines, hereafter named CsCHis, was also expressed in *E. coli* following the same method. CsCHis was isolated from a bacterial extract [15] by affinity chromatography on a Ni²⁺-NTA column (Novagen), using a standard protocol [16].

The mite cysteine proteinases (allergens), Der f 1 and Der p 1, were isolated from an aqueous extract of the corresponding mite species by monoclonal affinity chromatography as described previously [17].

Homogeneity of the purified recombinant cystatins, as well as of the isolated mite allergens, is illustrated in Fig. 1.

N-terminal amino acid sequencing

The N-terminal sequence of the modified form of CsC was determined analysing the corresponding stained band excised from PVDF membranes. Sequencing was performed by standard methods with an Applied Biosystem 477 A gas-phase sequenator.

Inhibition assays

The inhibitory activity of cystatin-enriched seed preparations and of purified CsC, as well as of the commercial proteinase inhibitors E-64 (an inhibitor of most cysteine proteinases) and soybean Bowman–Birk inhibitor (both from Sigma, St Louis, MO, USA), was tested against the crude proteinase extract of mites using azocasein as substrate [15]. The crude mite extract was pre-incubated with the appropriate inhibitor for 30 min in 0.1 M sodium

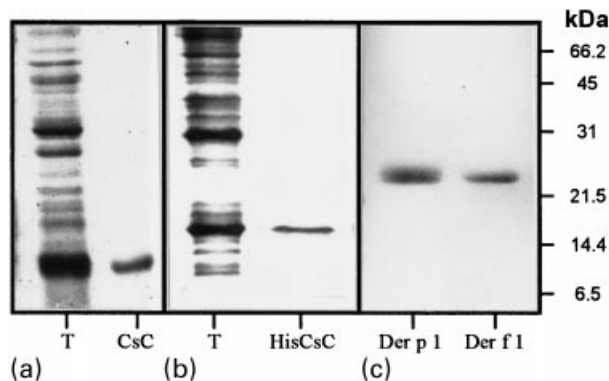


Fig. 1. SDS-PAGE separation of a crude extract from *E. coli* transformed cells (T), and of the corresponding purified recombinant cystatin: (a) CsC; (b) CsCHis; (c) SDS-PAGE separation of isolated mite allergens Der p 1 and Der f 1.

phosphate pH 6.5, 10 mM EDTA, 10 mM cysteine (preliminary tests were performed to determine the optimum pH range), in a final volume of 100 μ L. Azocasein solution in reaction buffer (500 μ L; 1 mg/mL) was then added, and the mixture was incubated for 3 h at 30 °C. After stopping the reaction with 250 μ L of ice-cold 10% trichloroacetic acid (30 min), samples were centrifuged and the A_{420} of supernatants was measured.

The inhibition of purified Der p 1 and Der f 1 was assayed following Twining [18] with minor modifications, using resorufin-labelled casein (Boehringer Mannheim) as substrate. Briefly, samples (0.625 μ g of Der p 1, or 1.5 μ g of Der f 1) were dissolved in 0.1 M sodium phosphate pH 6.5, 10 mM EDTA, 10 mM cysteine, 0.01% Brij-35 buffer, together with the test inhibitor (final volume of 75 μ L), and pre-incubated for 10 min at 37 °C. After adding 20 μ L of substrate solution in H₂O (0.4% w/v), the mixture was incubated for 1 h at 37 °C, and then the reaction was stopped with 150 μ L of 10% trichloroacetic acid (10 min at 37 °C). Samples were centrifuged, and the A_{574} of 200 μ L of the supernatant plus 300 μ L of 0.5 M Tris-HCl pH 8.8 was determined.

Determination of K_m and K_i values

Kinetic assays were carried out using resorufin-labelled casein as substrate to determine K_m (for both isolated Der p 1 and Der f 1) and K_i (of purified CsC for Der f 1) values, using conditions described above. Values were determined from a double reciprocal plot of the data [19], expressing the velocity of the reaction as $\Delta A_{574} \text{ min}^{-1} \text{ ml}^{-1}$.

Electrophoresis and immunodetection

Samples were separated by SDS-PAGE and then electrotransferred to PVDF membranes as previously reported [20].

After washing and blocking, membranes were immunodetected with previously raised rabbit monospecific polyclonal antibodies to CsC (1 : 1000 dilution), then incubated with an alkaline phosphatase-conjugated antirabbit IgG (Sigma; 1 : 500 dilution) and developed by adding BCIP/NBT (5-bromo-4 chloro-3 indolyl phosphate/nitro blue tetrazolium) solution.

Bioassays

To determine the effect of proteinase inhibitors (CsC, E-64 and soybean Bowman-Birk inhibitor) on *D. farinae* larval development, 10 eggs laid within 24 h were placed in a concavity slide (15–18 mm concavity diameter and 0.5–0.8 mm depth of well) and confined with a cover slip (22 mm $\times$ 22 mm). Larvae were fed on brewer's yeast flake (around 1.3 mg) and were treated, just prior to egg hatching, with 1% (w/w) of the appropriate inhibitor added in 2 μ L of distilled water. Instars and mortality were recorded every 24 h under a stereomicroscope until the larvae died or became protonymphs (when assay was finished). Each test was repeated 10 times. Assays were performed in an environmental chamber at 24 ± 0.5 °C, $80 \pm 5\%$ relative humidity under dark conditions.

Results

Chestnut cystatin inhibits Der f 1, but not Der p 1

The effect of CsC, as well as of other inhibitors, on crude proteinase preparations from *D. farinae* and *D. pteronyssinus* was tested (Fig. 2). E-64 strongly inhibited proteinases in both mite extracts, whereas a very weak inhibition was exerted by the soybean Bowman-Birk inhibitor (Fig. 2a). Contrary to what occurs with E-64, the *D. farinae* proteinase activity was affected by CsC (up to 55% inhibition at 1.5 μ M; Fig. 2b), whereas that of *D. pteronyssinus* was not when tested at the same cystatin levels.

To ascertain whether or not this differential behaviour against highly related mite proteinases is a common property of cystatins, a member of animal origin (egg white cystatin, designated Cew), as well as protein preparations from different plant seeds enriched in these inhibitors, were also assayed against the mite proteinases (Fig. 3). Unlike CsC, Cew inhibited the proteolytic activity in both mite extracts. Furthermore, when cystatin enriched preparations from plant seeds highly active towards papain were assayed, the wheat preparation had an effect in both mite extracts, unlike the chestnut, pine and ginkgo preparations, which only showed inhibition in the *D. farinae* extract.

To confirm that the differential activity of CsC shown in Figs 2 and 3 was due to a different susceptibility of the major cysteine proteinase present in each mite species, Der f 1

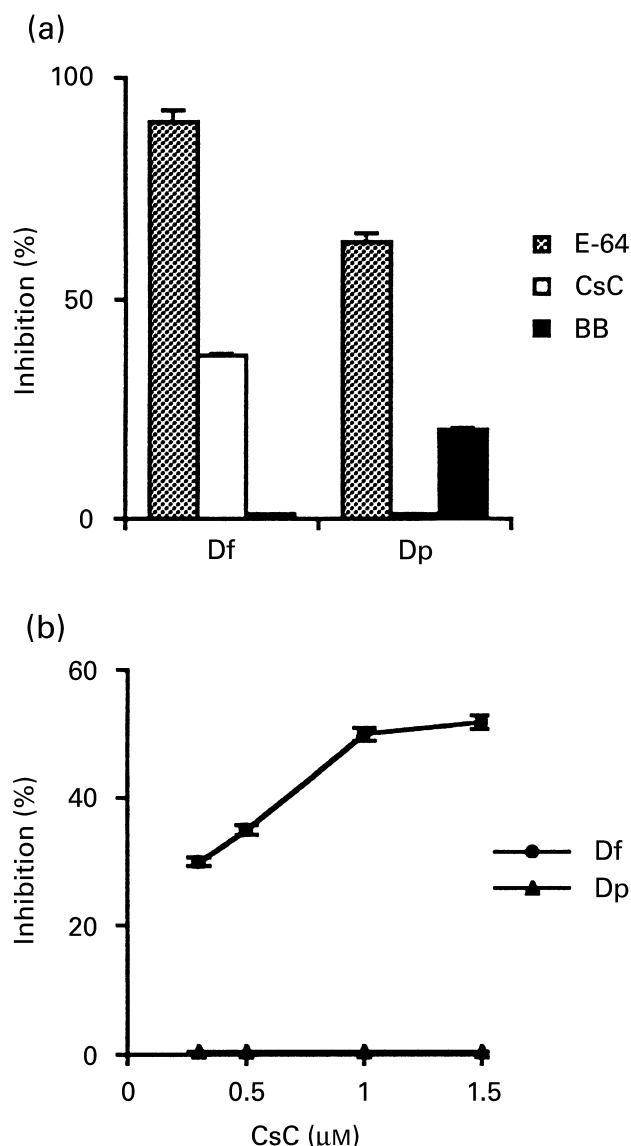


Fig. 2. (a) Inhibition of the proteolytic activity in *D. farinae* (Df) and *D. pteronyssinus* (Dp) extracts by 5 μ g of chestnut cystatin (CsC), E-64, and soybean Bowman-Birk inhibitor (BB). Values are means $\pm$ SE ($n=3$). Final concentration of E-64 was 140 μ M. (b) Inhibition of the same proteolytic activities by different amounts of CsC. Values are means $\pm$ SE ($n=3$). The enzymatic activities of both mite extracts were adjusted in preliminary test to obtain an $A_{420} \sim 0.4$ units, both in panels a and b.

from *D. farinae* and its homologue from *D. pteronyssinus*, Der p 1, were isolated (Fig. 1). The inhibition of both purified enzymes by E-64 and egg white cystatin, and the strong effect of CsC on Der f 1, but null towards Der p 1 (Table 1), fully confirmed the results discussed above. Additionally, kinetic assays were performed to determine K_m and K_i values, using resorufin-labelled casein as

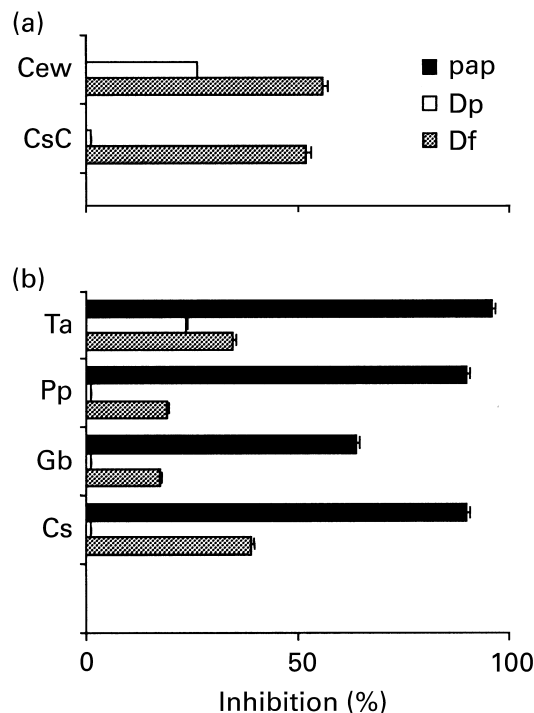


Fig. 3. Inhibition of the proteolytic activity in *D. farinae* (Df) and *D. pteronyssinus* (Dp) extracts by (a) 1.5 μ M of purified chestnut (CsC) and egg white (Cew) cystatins, or by (b) cystatin-enriched preparations (20 μ g of protein) from seeds of wheat (Ta), pine (Pp), ginkgo (Gb) and chestnut (Cs). Papain (pap) inhibition was used as positive control in b. Values are means $\pm$ SE ($n=5$).

substrate. K_m values of $33 \pm 3 \mu$ M for Der f 1 and $17 \pm 2 \mu$ M for Der p 1 were obtained. The K_i value of CsC for Der f 1 was 31 ± 4 nM, whereas no K_i could be determined for Der p 1 due to the lack of activity of the cystatin against this allergen in all conditions assayed. Interestingly, the double reciprocal plots (not shown) indicated an acompetitive inhibition kinetics in the case of Der f 1 vs CsC.

Der p 1 inactivates CsC by proteolytic cleavage

Partial digestion and inactivation of CsC by Der p 1 but not by Der f 1 could support the differential chestnut cystatin-sensitivity of both mite proteinases. This hypothesis was tested by SDS-PAGE and immunodetection analysis of purified CsC and the recombinant fusion protein CscHis (which comprised the full CsC amino acid sequence plus an N-terminal peptide with 30 residues including six histidines) after treatment with the crude proteinase extract from each mite (Fig. 4a). A shift to apparent low molecular sizes, although still showing well-defined bands, was obtained in both proteins when incubated with the *D. pteronyssinus* extract, thus suggesting that CsC, as well as CscHis, were specifically cleaved.

Table 1. Inhibition of purified mite cysteine proteinases Der p 1 and Der f 1 by soybean Bowman–Birk inhibitor, E-64, chestnut cystatin CsC and egg white cystatin

	Inhibition (%)				
	BSA*	BB	E-64	CsC	Cew
Der p 1†	0	13 ± 2	93 ± 1	0	24 ± 2
Der f 1	0	9 ± 1	95 ± 1	81 ± 1	75 ± 2

*Each inhibitor was tested at 5 µg/assay. Values are means ± SE ($n=3$). BB, soybean Bowman–Birk inhibitor; Cew, egg white cystatin; BSA, bovine serum albumin (negative control). †The amounts of purified Der p 1 (0.625 µg) and Der f 1 (1.5 µg) were adjusted in preliminary tests to obtain an $A_{574} \sim 0.4$ units.

On the contrary, no effect was shown in the samples treated with the corresponding *D. farinae* extract. These results were further supported when Der p 1 was used instead of the crude extract (Fig. 4b). The isolated proteinase also processed CsC in an apparently similar manner, whereas egg white cystatin (which is active against Der p 1) was not modified.

The electrophoretic band corresponding to the cleaved form of CsC (line CsC +, in Fig. 4b) was subjected directly to N-terminal sequencing (Fig. 4c). The amino acid sequence obtained (VSDV) clearly matched that from residue 7–10 of the full CsC sequence previously reported [15]. Therefore, Der p 1 seems to cleave the chestnut cystatin between Gly 6 and Val 7, leading to the loss of the N-terminal hexapeptide AALVGG (~350 D), which is in line with the slight change in electrophoretic mobility shown in Fig. 4. The inactivation of CsC when treated with *D. pteronyssinus* proteinases was proved by testing its effect

on Der f 1 (Fig. 5). The treatment fully abolished the inhibitory activity of the chestnut cystatin, whereas CsC incubated with the equivalent *D. farinae* extract in similar conditions was as active as the untreated inhibitor. None of the CsC samples inhibited Der p 1, as expected.

CsC affects the larval development of *D. farinae* in vivo

Finally, and taking into account the susceptibility of Der f 1 to CsC and its role in mite digestion, the *in vivo* effect of the cystatin on the larval development of *D. farinae* was also tested (Table 2). Chestnut cystatin, as well as E-64, drastically increased the larval mortality when compared with the control sample, probably through Der f 1 inhibition, and, thus, interfered with larval digestion processes. No effect was shown by addition of the soybean Bowman–Birk inhibitor, in agreement with its weak *in vitro* activity towards *D. farinae* proteinases (Fig. 2 and Table 1). None of the inhibitors tested caused a significant change in the larval period (not shown).

Discussion

Amino acid sequence comparison of Der p 1 and Der f 1 has demonstrated their high degree of sequence identity [6,7]. Moreover, no significant differences between both allergens have been reported as regards their enzymatic activity and susceptibility to inhibitors, or in the putative residues of the active site and substrate-binding pocket [8,9]. On the other hand, increased evidence is uncovering the significance of the proteolytic activity of Der p 1 in relation to its immunogenicity [9–11]. Thus, the study of this activity in group 1 allergens is warranted considering its connection with their allergenic properties.

Der p 1 and Der f 1 behave differentially towards the

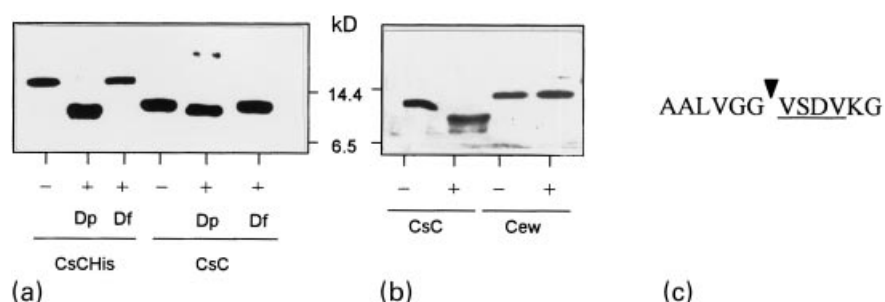


Fig. 4. (a) Immunodetection of chestnut cystatin (CsC) and the recombinant fusion protein CscHis (see material and methods) by monospecific polyclonal antibodies to CsC. Samples were separated by SDS-PAGE, electrotransferred to PVDF membranes, and immunodetected. Purified proteins (2 µg) were previously incubated (6 h; 37 °C) with buffer (–) or with crude proteinase extracts (10 µg) of *D. pteronyssinus* (+ Dp) or *D. farinae* (+ Df). (b) SDS-PAGE, electrotransfer to PVDF membrane and protein (Amido Black) staining of 2 µg of purified CsC and egg white cystatin (Cew), previously incubated (6 h; 37 °C) with buffer (–) or with 2 µg of isolated Der p 1 (+). (c) Putative proteolytic cleavage of CsC by Der p 1. The N-terminal amino acid sequence of the CsC fragment obtained after incubation with Der p 1 is underlined. The partial sequence of CsC was taken with permission from Pernas *et al.* [15].

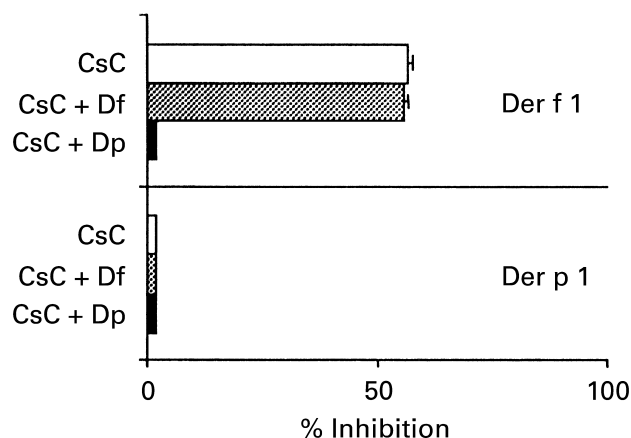


Fig. 5. Effect of crude proteinase extract from *D. farinae* (+Df) or *D. pteronyssinus* (+Dp) on the inhibitory activity of chestnut cystatin (CsC) against the purified enzymes Der f 1 and Der p 1. Two micrograms of CsC were incubated (6 h; 37°C) with the appropriate extract (1 µg) or with buffer (CsC), and then tested towards the purified enzymes in conditions similar to those of Table 1. Values are means $\pm$ SE ($n = 4$).

plant cystatin CsC in spite of their close similarity. The *D. pteronyssinus* allergen was not affected by CsC, whereas Der f 1 activity was strongly reduced. The K_i value (31 nM) of CsC for Der f 1 is in the range of those previously determined for papain (29 nM) and ficin (65 nM) [15], with the same inhibitor. However, a competitive inhibition kinetics has been found in the case of the mite allergen, whereas noncompetitive inhibition had been observed for cysteine proteinases of plant origin. In contrast to CsC, E-64, a nonprotein inhibitor of most cysteine proteinases, was highly active towards both Der p 1 and Der f 1. Other cystatins of animal (egg white), and even plant (wheat) origin also inhibited the two group 1 *Dermatophagoides* allergens, indicating that other members of the cystatin superfamily do not share the specificity of CsC. However,

Table 2. Effect of the proteinase inhibitors E-64, chestnut cystatin CsC and soybean Bowman–Birk in the larval mortality of *D. farinae*

	Larval mortality (%)
Control	23.7 $\pm$ 7
E-64*	100 $\pm$ 0
CsC	80.8 $\pm$ 8
BB	23.0 $\pm$ 4

*Each inhibitor was assayed at 1% (w/w) of the diet. Values are means $\pm$ SE ($n = 10$, with 10 larvae in each repetition). BB, soybean Bowman–Birk inhibitor.

the results obtained when testing antipapain-enriched preparations from chestnut, pine and ginkgo (Fig. 3b) suggest that cystatins from seeds of these forest species, discriminate, as CsC, between Der f 1 and Der p 1. The weak inhibition of the purified allergens exerted by the soybean Bowman–Birk inhibitor (specific of serine-type proteinases) could be explained considering the residual serine proteinase activity detected in purified Der p 1 by Hewitt *et al.* [21]. Interestingly, this soybean inhibitor also has a slight effect on the crude extracts from both *Dermatophagoides* species, whereas it strongly inhibits those from the storage mites *Lepidoglyphus destructor* and *Tyrophagus putrescentiae* [15]. The proteinases of both storage mites are either not affected or weakly affected by E-64 or CsC [15], in line with the lack of detection of group 1 allergens in these mite species.

Some cases of pest digestive proteinases insensitive to the corresponding plant inhibitors have been reported [22–24]. Proteolytic digestion of the inhibitor has been documented in at least one of them [24]. The data presented in Figs 4 and 5 indicate that Der p 1 inactivates CsC by such a mechanism. A proteolytic cleavage between residues Gly 6 and Val 7 of the chestnut inhibitor removes the six N-terminal amino acids, thus leading to an inactive form of CsC. The lost N-terminal hexapeptide includes a Gly (position 5) which is conserved throughout the cystatin family and seems to be essential for the inhibitory activity towards papain [12,14,25]. Interestingly, the cleavage site of CsC (Gly in P1 and Val in P1' positions, respectively) is uncommon when compared to that reported for Der p 1 on other protein substrates, where a preference for charged residues (Glu/Asp/Arg) in P1 and small hydrophylic/hydrophobic residues (Ser/Ala/Thr/Gly) in P1' positions was found [9,10,21]. No modification of CsC was detected when treated with Der f 1. Joint consideration of the data presented above strongly indicates a differential behaviour of Der p 1 and Der f 1 with respect to their proteinase activity. The potential relevance of this difference to their allergenicity is now under study.

Finally, the *in vitro* inhibition of Der f 1 can probably explain the *in vivo* effect of CsC on the larval development of *D. farinae* (Table 2). The acaricide activity of the cystatin inhibitor family has not yet been documented, as far as we know. Therefore, it would be worth exploring the potential use of phytocystatins in the control of in-house mite populations, thus reducing the production of allergens.

Acknowledgements

We thank D. Lamóneda and J. García-Guijarro for technical assistance, J. Varela (CSIC, Madrid) for protein sequencing, and Drs D. Barber and F. Juan (ALK-Abelló, Madrid) for facilities in the use of mite cultures. This work was supported by Dirección General de Enseñanza Superior, MEC (grant PB95–0035).

References

- 1 Chapman MD, Platts-Mills TAE. Dust mites: immunology, allergic disease and environmental control. *J Allergy Clin Immunol* 1987; 80:755–75.
- 2 Platts-Mills TAE, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: report of the third international workshop. *J Allergy Clin Immunol* 1997; 100:S2–24.
- 3 Thomas WR, Smith W. House-dust-mite allergens. *Allergy* 1998; 53:821–32.
- 4 Stewart GA, Lake FR, Thompson PJ. Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: physicochemical characterisation of potential allergens. *Int Arch Allergy Appl Immunol* 1991; 95:248–56.
- 5 Anto T, Ino Y, Haida M *et al.* Isolation of a cysteine protease in the crude mite extract, *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* 1991; 96:199–205.
- 6 Chua KY, Stewart GA, Thomas WR *et al.* Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* 1988; 167:175–82.
- 7 Dilworth RJ, Chua KY, Thomas WR. Sequence analysis of cDNA coding for a major house dust mite allergen, Der f 1. *Clin Exp Allergy* 1991; 21:25–32.
- 8 Topham CM, Srinivasan N, Thorpe CJ, Overington JP, Kalsheker NA. Comparative modelling of major house dust mite allergen Der p 1: structure validation using an extended environmental amino acid propensity table. *Protein Engng* 1994; 7:869–94.
- 9 Robinson C, Kalsheker NA, Srinivasan N *et al.* On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. *Clin Exp Allergy* 1997; 27:10–21.
- 10 Shakib F, Schulz O, Sewell H. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 1998; 19:313–6.
- 11 King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998; 161:3645–51.
- 12 Turk B, Turk V, Turk D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol Chem* 1997; 378:141–50.
- 13 Shewry PR, Lucas JA. Plant proteins that confer resistance to pest and pathogens. *Adv Bot Res* 1997; 26:135–92.
- 14 Margis R, Reis EM, Villeret V. Structural and phylogenetic relationships among plant and animal cystatins. *Arch Biochem Biophys* 1998; 359:24–30.
- 15 Pernas M, Sanchez-Monge R, Gomez L, Salcedo G. A chestnut cystatin differentially effective against cysteine proteinases from closely related pests. *Plant Mol Biol* 1998; 38:1235–42.
- 16 Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Biotechnology* 1988; 6:1321–5.
- 17 Lombardero M, Heymann PW, Platts-Mills TAE, Fox JW, Chapman MD. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens. *J Immunol* 1990; 144:1353–60.
- 18 Twining SS. Fluorescein isothiocyanate-labelled casein assay for proteolytic enzymes. *Anal Biochem* 1984; 143:30–4.
- 19 Dixon M, Webb EC, Throne CJR, Tipton KJ. Enzyme inhibitors. In: Dixon M, Webb EC. eds. *Enzymes* New York: Academic Press, 1979, 332–81.
- 20 Sanchez-Monge R, Gomez L, Lopez-Otín C, Armentia A, Salcedo G. Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the α -amylase inhibitor family have enhanced IgE-binding capacity. *Biochem J* 1992; 281:401–5.
- 21 Hewitt CRA, Horton H, Jones RM, Pritchard DI. Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen Der p 1. *Clin Exp Allergy* 1997; 27:201–7.
- 22 Jongsma M, Bakker PL, Jeroen P, Bosch D, Stiekema W. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proc Natl Acad Sci USA* 1996; 92:8041–5.
- 23 Broadway RM. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. *J Insect Physiol* 1997; 43:855–74.
- 24 Giri AP, Harsulkar AM, Deshpande VV, Sainini MN, Gupta VS, Ranjekar PK. Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. *Plant Physiol* 1998; 116:393–401.
- 25 Urwin PE, Atkinson HJ, McPherson MJ. Involvement of the NH₂-terminal region of oryzacystatin-I in cysteine proteinase inhibition. *Protein Engng* 1995; 12:1303–7.