# A recombinant form of chagasin from *Trypanosoma cruzi*: inhibitory activity on insect cysteine proteinases

Ana Carolina dos Santos Monteiro,[1,2] Osmundo Brilhante de Oliveira Neto,[1] Rafael Perseghini Del Sarto,[1,3] Mariana Torquato Q de Magalhães,[1,4] Janaina Nascimento Lima,[1,3] Ariane Ferreira Lacerda,[1,5] Raquel Sampaio Oliveira,[1,5] Julio Scharfstein,[2] Maria Cristina Mattar da Silva,[1] Jorge W Arboleda Valencia,[1,3,6] Arnubio Valencia Jiménez[1,3,7] and Maria Fatima Grossi-de-Sa[1,5]*

[1]*Embrapa Recursos Genéticos e Biotecnologia, PqEB, Final W5 Norte, 70770-900, Brasília, DF, Brazil*
[2]*Instituto de Biofísica Carlos Chagas Filho, UFRJ, 21990-400, Rio de Janeiro, RJ, Brazil*
[3]*Departamento de Biologia Celular, Universidade de Brasília, 70.910-900, Brasília, DF, Brazil*
[4]*Universidade Federal Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[5]*Pos Graduação em Ciências Genômicas – UCB, Brasília, DF, Brazil*
[6]*Facultad Ciencias para la Salud, Programa de Bacteriología, Universidad Católica de Manizales, Crra 23 # 60-63, Manizales, Colombia*
[7]*Facultad de Ciencias Agropecuárias, Departamento de Fitotecnia, Universidad de Caldas, Calle 65 # 26-10, Manizales, Colombia*

## Abstract

**BACKGROUND: The activity of the major digestive cysteine proteinase detected in the intestinal tract of larvae of the bean weevil, *Acanthoscelides obtectus* (Say), was efficiently inhibited by the well-characterized cysteine proteinase synthetic inhibitor E-64 and also by a recombinant form of chagasin (r-chagasin), a tight-binding cysteine proteinase inhibitor protein from *Trypanosoma cruzi*.**

**RESULTS: Incorporation of r-chagasin into an artificial diet system at 0.1 g kg$^{-1}$ retarded growth rate, decreased larval survival and led to complete mortality of *A. obtectus* at the end of the trial. The observed differences in growth rates occurred particularly in the first and second development stages. Artificial seeds containing high levels of r-chagasin (0.5–30 g kg$^{-1}$) completely inhibited larval penetration.**

**CONCLUSION: Together, the results reported in this paper support the hypothesis that the inhibitory activity of r-chagasin towards the major insect gut cysteine proteinase *in vitro* and *in vivo* is an accurate prediction of its insecticidal effects. The selectivity of this inhibitor against insect digestive proteinases supports the key role in parasite virulence by affecting the endogenous proteinase activity in its natural host.**


**Keywords:** *Acanthoscelides obtectus*; r-chagasin; cysteine proteinase; inhibitor; bean weevil

## 1 INTRODUCTION

*Acanthoscelides obtectus* (Say), a coleopteran insect that belongs to the Bruchidae family, is a serious insect pest of the common bean, *Phaseolus vulgaris* L., which is an important food source in Latin America and Africa and is highly susceptible to this bean weevil. Infestation of stored common bean seeds by this bruchid beetle causes economic and nutritional losses, mainly in developing countries where the food is stored inadequately. Damaged seeds are usually unsuitable for consumption or planting.[1] A number of wild strains of bean (*P. vulgaris*) originating from Mexico are resistant to *A. obtectus* and the Mexican bean weevil, *Zabrotes subfasciatus* (Boh.).[2–4] Apparently, this characteristic is related to factors that protect them from pest attack such as: (i) lectin-like defence proteins, including phytohemaglutinin, arcelin and $\alpha$-amylase inhibitors, (ii) inhibitors of digestive proteolytic enzymes and (iii) secondary metabolites such as alkaloids, saponins and cyanogenic glycosides.[5,6] However, in cultivated beans, these factors have been reduced or eliminated from the seeds during the natural process of selection owing to their toxicity to mammals.[7]

It is well known that proteolytic enzymes and their inhibitors are involved in several biological systems, including the degradation of dietary proteins, regulation of cellular protein catabolism and inhibition of pathogen extracellular proteases during infection.

* Correspondence to: Maria Fatima Grossi-de-Sa, Embrapa Recursos Genéticos e Biotecnologia, PqEB, Final W5 Norte, 70770-900, Brasília, DF, Brazil
E-mail: fatimasa@cenargen.embrapa.br
(*Received 27 July 2007; revised version received 29 October 2007; accepted 27 November 2007*)
Published online 5 March 2008; DOI: 10.1002/ps.1553

 *Pest Manag Sci* 1526–498X/2008/$30.00

Furthermore, it has been assumed that protein inhibitors of proteolytic enzymes, which are present in a variety of species, from mammals, plants and insects to primitive organisms, play an important regulatory role by inhibiting specific proteinase-related events.[8–10]

Most herbivorous insects have proteolytic enzymes to mediate the digestion of plant proteins.[11] The four major classes of these enzymes are found in insect midgut regions in variable amounts.[11–13] Lepidoptera and Diptera orders, with their typical alkaline midgut pH, generally have serine proteinase enzymes with high pH optima.[14,15] In contrast, most coleopteran insects have slightly acidic midguts where cysteine proteinases (CPs) comprise the major proteolytic activity.[12] There is strong evidence that insect species coevolved naturally with the food sources that they eat.[16] In the course of evolution they started to use alternative digestive systems, such as CPs in the case of coleopterans, probably to survive in an environment rich in serine proteinase inhibitors which are abundantly found in plant storage tissues.[17–19]

Therefore, the use of CP inhibitors against the bean weevil *A. obtectus* could represent an attractive way to protect the economically important *P. vulgaris* plant from this insect predation. In order to find and test new suitable control strategies of *A. obtectus*, the authors investigated the chagasin inhibitory activity against the digestive CP activities of this insect pest. Chagasin is a recently described CP inhibitor of 12 kDa, isolated from the pathogenic protozoan *Trypanosoma cruzi*,[20,21] and it has been suggested that chagasin regulates the endogenous activity of CP, thus indirectly modulating proteolytic functions that are essential for parasite differentiation and invasion of mammalian cells.

The lack of significant identity with proteins of the cystatin or other known classes of CP inhibitors suggested that chagasin is the prototype of a new family of proteinase inhibitors recently classified as clan IX, family I42, in the MEROPS database (http://merops.sanger.ac.uk). It is a tight-binding and reversible inhibitor of CP from the papain superfamily (clan CA, family C1), displaying broad-target specificities, being active against the endogenous major *T. cruzi* CP cruzipain, papain and other related CPs.[20] More recently, proteins similar to chagasin were found in certain prokaryotes and lower eukaryotes.[22] Chagasin-like genes from *Pseudomonas aeruginosa* (Schroet.) Mig.,[23] *Trypanosoma brucei* Steph., *Leishmania mexicana* and *Entamoeba histolytica*[24] were cloned and expressed, and also exhibited potent inhibitory activity against papain-like CPs.

The authors' results showed that bacterially expressed r-chagasin from *T. cruzi* (r-chagasin) could inhibit the major CP activity from the digestive tract of *A. obtectus*. Furthermore, feeding trials using artificial seeds made with flour from susceptible beans containing r-chagasin demonstrated a toxic effect of this protein on the development and survival of this bruchid. This study presents evidence for the potential of CP inhibitors as tools to obtain pest-resistant plants.

## 2 EXPERIMENTAL

### 2.1 Chemicals

Casein was purchased from Merck (Darmstadt, Germany), and glucose, Cbz-phe-arg-AMC (carbobenzoxy-phenylalanyl-arginyl-7-amido-4-methylcoumarin), E-64 [L-*trans*-epoxysuccinylleucylamido-(4-guanidino)butane] and DTT (dithiothreitol) were purchased from Sigma Chemicals Co. (St Louis, MO, USA).

### 2.2 Gut enzyme preparation

Initially, larvae of the last instar of *A. obtectus* were immobilized and their whole instestinal tracts removed. After this, midgut sections were excised and put into 250 mM sodium chloride solution. The midgut sections (100 midguts $mL^{-1}$) were homogenized with a 10 mM Tris solution, pH 6.0. Midgut tissue homogenates were centrifuged at $12\,000 \times g$ for 15 min at 4 °C, and the clear supernatants were stored at −20 °C and used as a source of digestive proteolytic enzymes.

### 2.3 Enzyme assays of midgut CPs

The molar concentration of r-chagasin was determined by titration with papain, which had been previously titrated with E-64.[20] Sequential dilutions of r-chagasin were incubated with papain in 100 mM sodium phosphate buffer, pH 6.5, containing 2 mM EDTA and 1 mM DTT for 30 min at room temperature. The substrate Bz-DL-Arg-pNA was added to give 2.5 mM final concentration, and the residual catalytic activity of papain was detected by measuring product generation as a function of absorbance at 410 nm in a Hitachi U2000 spectrophotometer.

Assays with midgut extracts were performed by incubating them at 37 °C with the substrate CBZ-Phe-Arg-AMC (carbobenzoxyphenylalanylarginyl-7-amido-4-methylcoumarin; 10 μM) in 100 mM sodium phosphate buffer, pH 6.5, containing 2 mM EDTA and 1 mM DTT. Stock solutions of the synthetic peptide substrates (1 mM) were made in 50% aqueous dimethyl sulfoxide (DMSO). Substrate hydrolysis was monitored in a Hitachi F4500 fluorimeter at 380 nm excitation and 440 nm emission wavelengths. Steady-state velocities before ($v_o$) and after ($v_i$) addition of E-64 (10 μM) or r-chagasin (10 nM) were obtained by linear regression of the substrate hydrolysis curves. All determinations of $v_o$ and $v_i$ were based on assays with less than 2% substrate hydrolysis and a linear regression coefficient at steady state greater than 0.990.

### 2.4 Purification of r-chagasin

r-Chagasin was expressed in the periplasmic space of *Eschericia coli* MC1061 with the plasmid pHD313/Tc18 as the expression vector (kindly

donated by Dr Magnus Abrahamson, University of Lund, Sweden), and purified as described elsewhere.[20] Briefly, the insert of one of several clones identified in an epimastigote λgt11 cDNA expression library after screening by ligand binding to carboxymethylated papain and cruzipain (clone Tc18; GenBank/EMBL accession no. AJ299433) was subcloned in the pHD313 plasmid for high-level expression in *E. coli*.[20] The construct was composed of: (1) the Omp A signal sequence, (2) a seven-residue linker (ASVSAEF) and (3) the Tc18 clone sequence (starting at nt 61 of the chagasin gene/AJ299433). The Omp A peptide and the linker peptides were removed from the purified recombinant protein (126 residues, $M_r$ 13 854) during the isolation procedure, since N-terminal sequencing (FKGTR) revealed that it started at residue 2 of the open reading frame predicted in the Tc18 cDNA.[20] The chromatography was performed at room temperature on Sephadex G 50–150 (Pharmacia) packed in a 1.6 × 100 cm column and equilibrated with 0.05 M sodium acetate and 0.05 M EDTA solution, pH 8.0. Fractions of 0.7 mL were collected at a flowrate of 20 mL $h^{-1}$ of the equilibrium solution. Samples of 3 mL from each batch of expressed proteins were applied onto the column. The column was calibrated using bovine serum albumin (BSA, 66 kDa), pepsin (34 kDa) and α-lactalbumin (14 kDa) (Sigma). Fractions containing homogeneous r-chagasin, as judged by SDS-PAGE, were pooled, dialysed and lyophilized. Electrophoretic reagents were from Bio-Rad (Richmond, CA).

### 2.5 Rearing of insects

The colony of *A. obtectus* was supplied originally by Dr Massaru Yokoyama of the EMBRAPA/CNPAF, Goiania, GO, Brazil. A stock culture of this species was established in Brasília, DF, Embrapa Recursos Genéticos e Biotecnologia. The insects were reared on *P. vulgaris* (cv. Jalo) in the dark and maintained at 28 ± 1 °C with a relative humidity of 65 ± 5%.

### 2.6 Artificial feeding experiments

*In vivo* feeding assays were carried out to investigate the biological effect of r-chagasin on *A. obtectus* development. It is well known that several insecticidal proteins, such as inhibitors of digestive enzymes, do not actually cause mortality, but instead retard insect growth and development.[25] Assays were performed by feeding insects (larvae) with a mixture of dry (14% moisture) common bean powder and different concentrations of r-chagasin (30, 15, 10, 7.5, 5.0, 2.5, 1.0, 0.75, 0.50 and 0.10 g $kg^{-1}$). These mixtures were then used to prepare artificial seeds with a columnar shape by pressing with a hand compressor. The artificial seeds were placed individually into plastic dishes, and each treatment was carried out in 12 separate replicates. For each experiment, four neonate larvae were introduced per seed. The seeds were analysed every 24 h during the assay period. After 20 days, one half of each separated artificial seed was opened and the dead larvae were counted. On day 40 (at the end of the experiment), the percentage of adult emergence was calculated from the number of neonate larvae introduced and the total adults emerged from each replicate. In the control treatment, the insecticidal protein was not added to the artificial diet.

### 2.7 Statistical analysis

A complete random design was used and the comparisons between means were made by Tukey's test at a 5% level of probability by using the general linear model procedure of the SAS statistical program.[26]

## 3 RESULTS

### 3.1 Inhibitory effect of r-chagasin against digestive CP activity from *Acanthoscelides obtectus*

By using the synthetic substrate CBZ-Phe-Arg-AMC, it was possible to detect CP activity in crude protein extracts from the larval midgut of *A. obtectus*. This activity, which was detected at optimal pH 6.0, was strongly inhibited by the inhibitors E-64 (10 μM) and r-chagasin (10 nM) (Table 1). Both inhibitors were previously titrated with papain using CBZ-Phe-Arg-AMC as substrate.

### 3.2 Effects of r-chagasin ingestion on larval development and survival

The insecticidal effects of r-chagasin were tested against *A. obtectus* by incorporating this protein at concentrations varying from 30 to 0.10 g $kg^{-1}$ into artificial seeds made of common bean flour. Control artificial seeds containing only the bean flour were used to observe normal larval insect growth and development. At the middle of the trial period (day 20), six seeds of each experiment were opened and the number of living larvae and prepupae was recorded (Fig. 1). The presence of r-chagasin had a significant effect on larval penetration into seeds containing 30–0.50 g $kg^{-1}$ of this protein, and no larvae were found in these seeds (data not shown). Insect development was substantially inhibited when

**Table 1.** Inhibition of a major cysteine proteinase activity from the digestive tract of *A. obtectus* larvae by r-chagasin

| | Enzyme activity[ab] ($V^{\#} \times 10^{-10}$ M $s^{-1}$) | Inhibition (%) |
|---|---|---|
| Midgut extract | 12.6 (±1.3) | 0 |
| Midgut extract + E-64 | 2.5 (±0.28) | 80.0 (±2) |
| Midgut extract + r-chagasin | 2.85 (±0.6) | 77.5 (±3.4) |

[a] The enzymatic activity was measured in 100 nM sodium phosphate solution containing 2 mM EDTA and 1 mM DTT at 37 °C. CBZ-Phe-Arg-AMC was used as substrate at pH 6.0. The assays were done in triplicate.

[b] $V^{\#}$ represents the initial velocity for the hydrolysis of the substrates before and after the addition of the inhibitors (E-64, 10 μM, and r-chagasin, 10 nM).

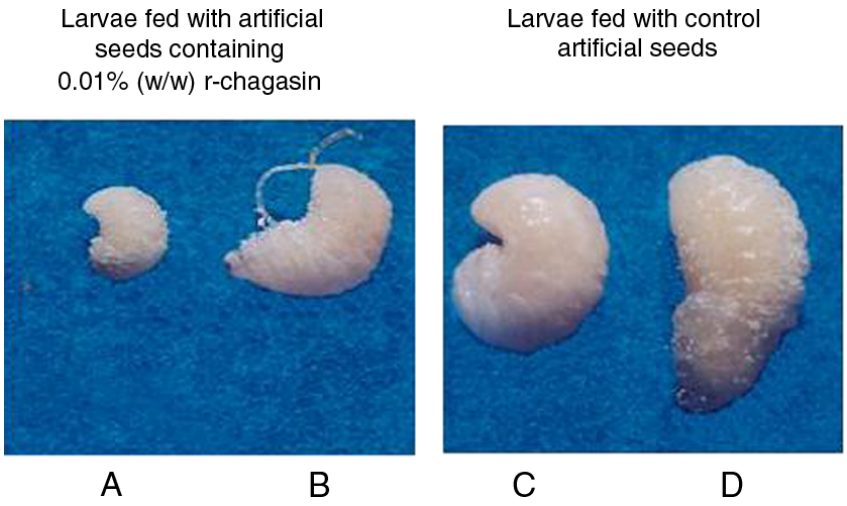


**Figure 1.** Effects of r-chagasin ingestion on insect larval growth and development. At day 20 of the feeding trials, half of the experimental seeds were opened and surviving larvae were recorded and analysed. A and B, first- and second-instar larvae found in the artificial seeds containing 0.10 g $kg^{-1}$ of r-chagasin; C and D, fourth-instar and pupae-stage forms found in control artificial seeds.

artificial seeds containing 0.10 g $kg^{-1}$ of r-chagasin were used (Table 2). When larvae in the control assay had reached the final instars, with the majority in the prepupae stage (3.7 mm), the larvae reared on insect diets containing r-chagasin only reached the first (0.8 mm) and second instars (1.3 mm). After 40 days, the percentage of adult emergence was recorded from all experimental seeds (Table 3). It was observed that seeds containing 0.10 g $kg^{-1}$ of r-chagasin remained intact, without any apparent damage, and contained dead larvae from first to second instars inside them. Larvae from control assays completed their entire life cycle, with 92% emergence (Table 3). It was thus possible to observe a striking retardation in insect development in those larvae that were reared on the diets containing r-chagasin.

### 3.3 Effects of r-chagasin ingestion on insect larval digestive proteinases

At day 20 of the feeding trials, the midguts from first- and second-instar surviving larvae and prepupae were dissected and the major CP activity was determined by enzymatic assays. There was a significant decrease in the enzyme activity (85%) in those larvae fed with seeds containing 0.10 g $kg^{-1}$ of r-chagasin in their diet when compared with those fed with artificial diet only (data not shown).

**Table 2.** Effects of r-chagasin, delivered in artificial seeds, on development of *Acanthoscelides obtectus* larvae

| r-Chagasin concentration (g $kg^{-1}$) | Larvae[a] first and second instars | Larvae[a] third and fourth instars | Pupae | Dead insects |
|---|---|---|---|---|
| 30–0.50 | 0 | 0 | 0 | 0 |
| 0.10 | 20 | 0 | 0 | 4 |
| Control | 0 | 5 | 17 | 2 |

[a] Number of surviving larvae in different instars of development at day 20 of the feeding trial.

**Table 3.** Effects of r-chagasin, delivered in artificial seeds, on *Acanthoscelides obtectus* adult emergence

| r-Chagasin concentration (g $kg^{-1}$) | Number of dead larvae | Adult emergence[a] (%) |
|---|---|---|
| 0.10 | 20 | 0 |
| Control | 2 | 92 |

[a] Recorded at the end of the feeding trial at day 40.

## 4 DISCUSSION

It has been previously described that the common bean weevil *A. obtectus* (Coleoptera: Bruchidae) is able to feed on leguminous crop seeds, especially on common bean (*P. vulgaris*) seeds, causing severe crop losses.[3] This insect pest is detected in dried seeds of *P. vulgaris*, and its developmental cycle appears very well adapted for reproduction in a storage environment. In general, bruchids are able to feed on bean seeds owing to a complex proteolytic system with different specificities, which is abundantly found in their midgut region.[11] In the case of *A. obtectus*, the authors report here the detection of a major papain-like CP activity in the intestinal tract content. These data are consistent with the fact that many coleopteran insects, which usually have midguts with a pH in the slightly acid range, use digestive CPs to catalyse the release of peptides and amino acids from dietary protein.[27]

Diverse plant defence factors have been evaluated for their toxicity towards the common bean weevil, as in the cases of $\alpha$-amylase inhibitors from wheat,[28] rye[29] and the Kunitz proteinase inhibitor from algarroba, which possesses inhibitory activity against CPs.[30] In addition, it has been shown by several research groups that both serine and CP inhibitors, when ingested as constituents of either artificial diets or in plant material, can retard insect growth and development.[18,19,31,32] Moreover, when expressed in transgenic plants, proteinase inhibitors have also been shown to be able to confer some protection

to plants and trees against attack from pests and pathogens.[18,19,33,34]

The present results have demonstrated that r-chagasin is a potent inhibitor *in vitro* and *in vivo* of CP activity from the digestive system of *A. obtectus* larvae, acting at very low concentration levels. Based on the experiments that are presented here, it appears that r-chagasin could be used to develop insect-resistant plants. When incorporated into artificial seeds at levels of 30–0.50 g $kg^{-1}$, r-chagasin had a significant effect upon survival and larval penetration into the seeds. When tested at 0.10 g $kg^{-1}$, r-chagasin completely blocked the development through instars of feeding larvae over the trial period. These early effects observed on larval development resulted in a significant mortality, making it possible to point out that r-chagasin appeared to be a particularly potent defence factor when compared with other proteins. For example, 15 g $kg^{-1}$ of $\alpha$-amylase inhibitors 1 and 2 was required to cause severe mortality to *Z. subfasciatus* and *Bruchus pisorum* L. respectively.[25]

Examination of the digestive proteinases extracted from the midguts of first and second larvae at the middle of the feeding trials (day 20) showed that r-chagasin is a potent inhibitor of the major insect CP activity, which was completely inhibited *in vivo*. It has been observed that *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) larvae presented a physiological adaptation to transgenic oilseed rape expressing oryzacystatin I (OCI).[35] The adaptation consisted of an increase in serine proteinase activity by more than twofold, which is consistent with the fact that OCI-I completely inhibited the insect CP activities *in vitro*. This adaptation was not observed in the present experiments (data not shown), predicting the success of r-chagasin as a protein defence factor against those insect pests expressing mainly CP proteinase activity in their intestinal tracts.

Together, the results reported in this paper support the hypothesis that the inhibitory activity of r-chagasin towards the major insect gut CP *in vitro* and *in vivo* is an accurate prediction of its insecticidal effects. Although important questions remain regarding CP inhibitor stability in plants, the results showed that r-chagasin could be very useful in controlling the bruchid pest *A. obtectus*. However, the interaction between proteinase inhibitors and the digestive physiology and biochemistry of the insect pests is clearly more complex than the original concept of simple inhibition of digestive proteinases. To be effective, a digestive enzyme inhibitor should not only inhibit the insect enzyme substantially at a low enough concentration but also be resistant to attack by insect intestinal proteinases. Indeed, insect pests have found diverse ways to avoid the negative effects of proteinase inhibitors on their host plants during evolution.

## ACKNOWLEDGMENTS

This work was supported by grants from the Brazilian government EMBRAPA, CNPq and CAPES. The authors would like to thank Dr Linda Adams Fothergill-Gilmore for critical reading and correction of the English.

## REFERENCES

1 Cardona C, Posso CE, Kornegay J, Valor J and Serrano M, Antibiosis effects of wild dry bean accessions on the mexican bean weevil and the bean weevil (Coleoptera: Bruchidae). *J Econ Entomol* **82**:310–315 (1989).
2 Schoonhoven A, Cardona C and Valor J, Resistance to the bean weevil and the mexican bean weevil (Coleoptera: Bruchidae) in noncultivated commom bean accessions. *J Econ Entomol* **76**:1255–1259 (1983).
3 Cardona C, Kornegay J, Posso CE, Morales F and Ramirez H, Comparative value of four arcelin variants in the development of dry bean lines resistant to the mexican bean weevil. *Entomol Experimentalis et Applicata* **56**:197–206 (1990).
4 Kornegay J and Cardona C, Inheritance of resistance to *Acanthoscelides obtectus* in a wild commom bean accession crossed to commercial bean cultivars. *Euphytica* **52**:103–111 (1991).
5 Chrispeels MJ and Raikhel NV, Lectins, lectin genes and their role in plant defense. *Plant Cell* **3**:1–19 (1991).
6 Carlini CR and Grossi-de-Sa MF, Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon* **40**:1515–1539 (2002).
7 Osborn TC, Alexander DC, Sun SSM, Cardona C and Bliss FA, Insecticidal activity and lectin homology of arcelin seed protein. *Science (Washington)* **240**:207–210 (1988).
8 Rawlings ND and Barrett AJ, Evolutionary families of peptidases. *Biochem J* **290**:205–218 (1993).
9 Rawlings ND, Tolle DP and Barrett AJ. Evolutionary families of peptidase inhibitors. *Biochem J* **378**:705–716 (2004).
10 Otlewski J, Jelen F, Zakrzewska M and Oleksy A, The many faces of protease–protein inhibitor interaction. *EMBO Journal* **24**:1303–1310 (2005).
11 Terra WR and Ferreira C, Insect digestive enzymes: properties, compartmentalization and function. *Comp Biochem Physiol* **109B**:1–62 (1994).
12 Wolfson JL and Murdock LL, Diversity in digestive proteinase activity among insects. *J Chem Ecol* **16**:1089–1101 (1990).
13 Purcell JP, Greenplate JT and Sammons RD, Examination of midgut luminal proteinase activities in six economically important insects. *Insect Biochem Mol Biol* **22**:41–47 (1992).
14 Christeller JT, Laing WA, Markwick NP and Burgess EP, Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. *Insect Biochem Mol Biol* **24**:103–109 (1992).
15 Applebaum SW, Biochemistry of digestion, in *Comparative Physiology and Pharmacology of Insects*, ed. by Kerkut GA and Gilbert LI. Pergamon, Toronto, Canada, Vol. IV, pp. 279–311 (1985).
16 Erlich PR and Raven PH, Butterflies and plants: a study in coevolution. *Evolution* **18**:586–608 (1964).
17 Ryan CA, Protease inhibitor in plants: genes for improving defenses against insects and pathogens. *Annu Rev Phytopathol* **28**:425–449 (1990).
18 Jongsma MA and Bolter C, The adaptation of insects to plant protease inhibitors. *J Ins Physiol* **43**:885–895 (1997).
19 Valueva TA and Mosolov VV, Role of inhibitors of proteolytic enzymes in plant defense against phytopathogenic microorganisms. *Biochemistry (Moscow)* **69**:1305–1309 (2004).
20 Monteiro ACS, Abrahamson M, Lima APCA, Vannier-Santos MA and Scharfstein J, Identification, characterization and localization of chagasin, a tight-binding cysteine protease inhibitor in *Trypanosoma cruzi*. *J Cell Sci* **114**:3933–3942 (2001).
21 Rigden DJ, Monteiro AC and Grossi-de-Sa MF, The protease inhibitor chagasin of *Trypanosoma cruzi* adopts an

immunoglobulin-type fold and may have arisen by horizontal gene transfer. *FEBS Lett* **504**:41–44 (2001).
22 Rigden DJ, Mosolov VV and Galperin MY, Sequence conservation in the chagasin family suggests a common trend in cysteine proteinase binding by unrelated protein inhibitors. *Protein Sci* **11**:1971–1977 (2002).
23 Sanderson SJ, Westrop GD, Scharfstein J, Mottram GH and Coombs GH, Functional conservation of a natural cysteine peptidase inhibitor in protozoan and bacterial pathogens. *FEBS Lett* **542**:12–16 (2003).
24 Riekenberg S, Witjes B, Saric M, Bruchhaus I and Scholze H, Identification of EhICP1, a chagasin-like cysteine protease inhibitor of *Entamoeba histolytica*. *FEBS Lett* **579**:1573–1578 (2005).
25 Morton RL, Schroeder HE, Bateman KS, Chrispeels MJ, Armstrong E and Higgins TJV, Bean $\alpha$-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proc Natl Acad Sci* **97**:3820–3825 (2000).
26 *SAS/STAT (Users Guide)*. SAS Institute Inc., Cary, NC (2000).
27 Murdock LL, Brookhart G, Dunn PE, Foard DE, Kelley S, Kitch L, *et al.*, Cysteine digestive proteinases in Coleoptera. *Comp Biochem Physiol Part B Biochem Mol Biol* **87**:783–787 (1987).
28 Franco OL, Rigden DJ, Melo FR, Bloch C, Jr, Silva CP and Grossi-de-Sa MF, Activity of wheat $\alpha$-amylase inhibitors towards bruchid $\alpha$-amylases and structural explanation of observed specificities. *Eur J Biochem* **267**:1466–1473 (2000).
29 Iulek J, Franco OL, Silva M, Slivinski CT, Bloch C, Jr, Rigden DJ, *et al.*, Purification, biochemical characterization and partial primary structure of a new $\alpha$-amylase inhibitor from *Secale cereale* (Rye). *Internat J Biochem Cell Biol* **32**:1195–1204 (2000).
30 Oliveira AS, Pereira RA, Lima LM, Morais AAH, Melo FR, Franco OL, *et al.*, Activity toward bruchid pest of a Kunitz-type inhibitor from seeds of the algaroba tree (*Prosopis juliflora* D.C.). *Pestic Biochem Physiol* **72**:122–132 (2002).
31 Gomes APG, Dias SC, Bloch C, Jr, Melo FR, Furtado JR, Monnerat RG, *et al.*, Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds. *Biochem Mol Biol* **140**:313–319 (2005).
32 Calderon LA, Teles RC, Leite JR, Franco OL, Grossi-de-Sa MF, Medrano FJ, *et al.*, Purification of a 6.5 kDa protease inhibitor from Amazon *Inga umbratica* seeds effective against serine proteases of the boll weevil *Anthonomus grandis*. *Prot Pept Lett* **12**:583–587 (2005).
33 Irie K, Hosoyama H, Takeuchi T, Iwabuchi K, Watanabe H, Abe M, *et al.*, Transgenic rice established express corn cystatin exhibits strong inhibitory activity against insect gut proteinases. *Plant Mol Biol* **30**:149–157 (1996).
34 Jouanin L, Bonadé-Bottino M, Girard C, Morrot G and Giband M, Transgenic plants for insect resistance. *Plant Sci* **131**:1–11 (1998).
35 Jouanin L, Pham-Delegue M, Bonade-Bottino M, Williams I, Bartlet E, Zaccomer B, *et al.*, Growth stimulation of beetle larvae reared on a transgenic oilseed rape expressing a cysteine proteinase inhibitor. *J Insect Physiol* **44**:263–270 (1998).