Use of phage display to select novel cystatins specific for *Acanthoscelides obtectus* cysteine proteinases

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Abstract

Cysteine proteinases from larvae of the common bean weevil, *Acanthoscelides obtectus* (Coleoptera: Bruchidae), were isolated by ion exchange affinity chromatography on a CM-Cellulose column and used to select mutant cystatins from a library made with the filamentous M13 phage display system. The library contained variant cystatins derived from the nematode *Onchocerca volvulus* cystatin through mutagenesis of loop 1, which contains the QVVAG motif that is involved in binding to proteinases. After three rounds of selection, the activity of variant cystatins against papain and cysteine proteinases from *A. obtectus* was assayed by ELISA. Two different variant cystatins (presenting amino acids DVVSA and NTSSA at positions 65–69) bound to *A. obtectus* cysteine proteinases more tightly than to papain. In contrast, the wild type had similar affinity for *A. obtectus* proteinases and for papain. These two selected variants cystatins have greater specificity towards *A. obtectus* cysteine proteinases than the original sequence and could represent good candidate genes for the production of transgenic plants resistant to this insect pest.

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1. Introduction

Although all plants are endowed with natural defence mechanisms for protection against insects and pathogens, some agriculturally important plants have been modified to meet human needs by breeding and selection, to the extent that they may have lost resistance to certain insects and pathogens [1]. The bean weevil *Acanthoscelides obtectus* (Coleoptera, Bruchid) is an important storage pest of the common bean (*Phaseolus vulgaris*) in Latin America and Africa [2]. The weevil causes serious damage to stored beans, affecting not only cooking quality, but also seed germination and seedling vigour [3].

Insects that feed on plants depend on the breakdown of ingested proteins for normal growth, development and reproduction [4]. Proteinase inhibitors, along with other digestive enzyme inhibitors such as α-amylase inhibitors, therefore have potential as pest resistance factors against a variety of insect classes [5,6]. The majority proteinases used by the common bean weevil larvae are cysteine proteinases [7,8]. This suggests the use of cysteine proteinase inhibitors (such as cystatins) as resistance factor against this insect pest [8]. The structure of chicken egg white cystatin has been solved by X-ray diffraction, revealing a wedge shape composed of a five-stranded anti-parallel β-pleated sheet wrapped around a central long α-helix folded into a wedge, with the partially flexible N-terminal segment and the first and a second β-hairpin loop forming a molecular edge [9]. The mode of interaction of cystatins with cysteine proteinases was later elucidated with the
determination of the structure of papain complexed with human stefin B (Ref. [10]; Fig. 1). This showed that the inhibitor binds mainly into the primed sub-region of papain through its two hairpin loops and that its N-terminal segment interacts with the non-primed sub-sites in a substrate-like manner. The first hairpin loop, loop 1, contains the QVVAG motif that is highly conserved (Fig. 1) throughout the cystatin superfamily and is involved in binding to proteinases [11].

The role of the N-terminal region has been studied in more detail. For the interaction of cystatin A with papain, it contributes to affinity predominantly by anchoring the inhibitor to enzyme after formation of the complex [12]. Similarly, when the binding affinity and kinetic interactions of different cystatin A truncated variants were determined with papain, cathepsin L and cathepsin B, it was found that the N-terminal region of cystatin A contributes appreciably to binding to all three cysteine proteinases [13]. A phage display library of cystatin A has been constructed in which variants with the four N-terminal amino acids randomly mutated were expressed on surface of filamentous phage. Screening of this library for binding to papain gave predominantly variants with a glycine residue in position 4. This finding was in agreement with previous conclusions that glycine in this position is essential for tight binding of cystatin A to cysteine proteinases. In contrast, the first three residues of the variants obtained by the screening were more variable [12]. The N-terminal region is also important in other members of the family; an N-terminally truncated cystatin B binds to cysteine proteinases with strongly reduced affinity [9,14].

Many others proteins, peptides and protein domains have been successfully displayed on M13 phage particles for a variety of purposes [15,16]. The utilization of phage display as a tool for redesigning proteinases was explored by fusing trypsin to either pIII or pVIII phage coat proteins allowing the study of stability, efficiency and catalytic properties of the resulting chimeric trypsin [17]. The potato serine proteinase inhibitor has been functionally displayed on the surface of phage particles and biopanning selection enriched a phage population for functional domains I and II from a population mixed with mutated non-functional variant domains [18]. Chicken egg white cystatin has also been functionally displayed and remained active in a recombinant phage display system [19]. Similarly, two different soybean cystatin isofoms have been displayed on phage particles. These cystatins, scN and scL, share 70% sequence identity yet the scN is a more potent inhibitor of papain, vicilin peptidohydrolase and insect gut proteinases than scL [20]. When these cystatins were displayed on phage particles, papain-binding affinity and inhibitory activity of scN was substantially greater than that of scL. Furthermore, scN substantially delayed cowpea weevil (Callosobruchus maculatus) growth and development in insect feeding bioassays, whereas scL was essentially inactive as an insecticide. Selection of these phage-displayed soya cystatins by panning against papain resulted in a 200–1000-fold greater enrichment for scN relative to scL [21]. Furthermore, combinatorial phage display libraries of scN variants that contain mutations in the motifs of the first (QVVAG) and second (EW) hairpin–loop regions were used to define essential residues for an optimal binding between scN-papain. The first loop was invariant in all functional scN proteins, and all selected variants had W79 in the second hairpin–loop motif. However, there was diversity for hydrophobic and basic amino acids in residue 78 [22].

To date, no cysteine proteinase inhibitor active against gut proteinases from A. obtectus has been obtained, although α-amylase inhibitors from wheat kernels (known as 0.19 and 0.53) and a Kunitz-type inhibitor from seeds of Prosopis juliflora (protease inhibitor PTPKI) have been shown to be effective against A. obtectus α-amylase in vitro [23–25]. In this study we report the use of a large phage display library of variant cystatins to select novel mutants active against proteinases from A. obtectus. The library used in this work contained variant cystatins derived from the nematode O. volvulus cystatin “Onchocystatin” through mutagenesis of loop 1, which contains the QVVAG motif that is highly conserved throughout the cystatin superfamily and is involved in binding to proteinases [11].

![Fig. 1. Ribbon diagrams of stefin B in complex with the cystein proteinase papain (PDB code 1STF [10]). Residues important for inhibitory activity (QVVAG) are shown in white. The figure was made using SPDBView 3.7 program [34].](image-url)
2. Materials and methods

2.1. Preparation of gut homogenates

Midguts from 17–20-day-old, 3rd to 4th instar larvae of *A. obtectus* were dissected in ice-cold 25 mM NaCl. Freshly dissected guts were homogenized and centrifuged at 4000 × g for 20 min at 4 °C to remove the gut walls and cellular debris. The supernatant was used for isolation of cysteine proteinases and activity assays.

2.2. Enzyme assays

Proteolytic activity was measured by a modification of the procedure described by Oliveira et al. [24] using 1.5% (w/v) azocaseine as substrate. The semi-purified gut preparations (0.5 ml) were added to 0.25 ml of pre-warmed (37 °C) azocaseine solution. The volume was brought to 0.8 ml with 0.1 M acetate buffer, 1 mM cysteine, 3 mM EDTA, pH 5.6. This mixture was incubated at 37 °C for 30 min and the reaction was stopped by the addition of 0.25 ml of 5% (w/v) trichloroacetic acid. After 15-min incubation the precipitate was removed by centrifugation at 2000 × g for 15 min at 4 °C and 0.5 ml of the supernatant was mixed with 0.5 ml of 2 M NaOH. The optical density was measured at 440 nm. *trans*-Epoxy succinyl-L-leucylamide-(4-guanidino) butane (E-64) was used to demonstrate that the proteolytic activity observed was due to the presence of cysteine proteinases.

2.3. Isolation of cysteine proteinases

The supernatant from 250 dissected guts from *A. obtectus* larvae (1.5 ml) was applied to an ion exchange column CM-Cellulose (1.0 × 15 cm) equilibrated with 0.05 M acetate buffer, pH 5.6 at 4 °C. The flow through was washed off with equilibration buffer while the adsorbed compounds were eluted from the column using a linear gradient of 0 to 0.5 M NaCl. Fractions (1.0 ml) were collected at a flow rate of 25 ml h⁻¹ and used to measure proteolytic activity. Fractions containing proteolytic activity were pooled, dialyzed overnight against distilled water and stored at −20 °C.

2.4. Electrophoresis

The semi-purified proteinases obtained from CM-Cellulose chromatography were analysed by SDS-PAGE (15% acrylamide) according to Laemmli [27]. The proteolytic activity of crude extract from midguts of *A. obtectus* larvae was measured on activity SDS-PAGE gels (10% acrylamide) containing 0.1% azocaseine. Following electrophoretic separation of proteins, the gel was incubated in a solution of 2.5% (v/v) Triton X-100 for 1 h at 37 °C to remove the SDS followed by an incubation in 0.1 M acetic acid, 1 mM cysteine, 3 mM EDTA, pH 5.0 overnight at 37 °C, with shaking, and finally the gel was stained with Coomassie brilliant blue (0.025%).

2.5. Construction of recombinant phagmids

A starting phagemid pCTB (4520 bp) was constructed from the phage display expression vector pCANTAB 5E 4522bp (Pharmacia). This plasmid was cleaved with *Sfi*I and *Not*I to remove a sequence of 51 bp and insert a 49-bp fragment containing the unique restriction sites *Kpn*I and *Pst*I. The phagemid pCTB was used to insert the cysteine proteinase inhibitor gene sequence from nematode *O. vulvulus* (OV7) as a fusion of the pIII protein to be integrated into M13 recombinant phages. The original cDNA sequence was silent mutated at positions Leu⁶⁶ (CTG → CTT), Arg⁸⁰ (CGA → AGA) and Ser⁸¹ (TCG → TCT) to insert the unique restriction sites *Afl*II and *Bgl*II, respectively. The restriction sites *Pst*I and *Kpn*I were inserted at the ends of the sequence to be cloned into the pCTB vector. The pCTB vector generated the recombinant pCTBOV7 by using the *Kpn*I restriction site to insert the OV7 gene. This construction was confirmed by sequencing.

2.6. Construction of OV7 mutant libraries

The phage-display library was constructed varying the amino acids at positions 65–69 in OV7, which correspond to wild-type amino acids QVVAG. The library containing 3 × 10⁶ different proteins contains all 20 amino acids at each mutated position. This number is obtained due to a technical limitation of random mutagenesis, corresponding only to a fraction of gene combinations possibilities (33,554,432). These possibilities are reduced by stop and degenerated codons. A degenerated oligonucleotide was synthesized which contains the nucleotide sequence NNK NNK NNK NNK NNK (K = G/T; N = A/C/G/T), which codes for any amino acids at the positions 65–69 of the inhibitor. A population of complementary DNA strands was constructed using the degenerated oligonucleotides and their respective backward primers with *Taq* polymerase (Invitrogen). The resulting double-stranded materials were cleaved with *Afl*II and *Bgl*II and ligated into dephosphorylated phagemid vector pCTBOV7 previously digested with *Afl*II and *Bgl*II restriction enzymes. Large-scale ligationes were carried out using T4 DNA ligase (Invitrogen) at 16 °C overnight. Ligated DNA was used to transform *E. coli* TG1 cells (K12(lac-pro), supE, thi, hsdD5/F’, traD36, proAB, lacIq, lacZΔM15) and to create the libraries. Libraries were plated on SOB containing 0.1 mg ml⁻¹ ampicillin and 2% glucose.

2.7. Selection of the phage display libraries

To rescue the library, 2.0 ml of 2 × YT culture media was added to plates containing the library in TG1 cells. The colonies were resuspended by scraping with a sterile glass
spread. The bacteria were pooled and diluted to OD$_{600}$ of 0.2–0.4 in 2 × YT with 100 μg ml$^{-1}$ carbenicillin and 2% glucose. This pool was incubated at 37 °C, with shaking for 2 h, until an OD$_{600}$ of 0.6–0.8 was reached. At which point 1 × 10$^8$ pfu/ml of the M13K07 helper phage was added. The cells were incubated for 30 min at 37 °C with shaking at 150–250 rpm and the cultures were then centrifuged at 800 × g for 10 min. The cells were resuspended in 2 × YT with 100 μg/ml carbenicillin and 50 μg ml$^{-1}$ kanamycin and incubated for 10 h with shaking at 37 °C for the production of recombinant phage particles. The cultures were then centrifuged at 8000 × g for 15 min and phage particles were precipitated by the addition of polyethylene glycol (MW 8000) and NaCl 1:5 (v/v) solution to the phage-containing supernatant. Tubes were mixed and incubated on ice for 60 min. The phage pellet was centrifuged at 10,000 × g for 20 min and the pellet was resuspended in 10-ml sterile PBS. Phages were filtered through a 0.45-μm filter and stored at 4 °C.

Five milligrams of semi-purified cysteine proteinases, purified by ion exchange chromatography, were dissolved in 5 ml of 0.2 M sodium acetate buffer, pH 5.6. This sample was used to coat tubes for 2 h at room temperature. The tubes were blocked for 1 h at room temperature with PBS containing 2% (w/v) non-fat dry milk powder. The phage, solved in PBS containing 2% milk powder (w/v), were incubated for 15 min at room temperature in milk-coated tubes to remove non-specific phage. Subsequently, non-bound phages were transferred to cysteine proteinase-coated tubes for panning. After incubation for 2 h at 37 °C, the tubes were extensively washed (20 × ) with PBS and PBS containing 0.1% Tween-20. Phages that remained bound to the immobilized cysteine proteinases were rescued by adding 5 ml of exponentially growing E. coli TG1 cells to the tubes and incubating for 60 min at 37 °C with shaking. Culture aliquots were retained for later titering and the remaining cells were spun down, plated on SOBAG culture media and incubated overnight at 30 °C. To determine the number of colonies obtained at each selection round, culture aliquots were diluted 100-fold and plated on SOBAG media. Isolated colonies were used for purification of high-copy plasmid DNA using the QIAprep Kit (Qiagen).

This procedure for panning, washing and amplification was repeated three times and clones obtained during the three rounds were sequenced.

2.8. ELISA binding assay

ELISA of phages was performed using the Detection Module-Recombinant Phage Antibody System (Pharmacia). Microtiter plates (Costar) were coated with papain (Sigma, positive control), bovine trypsin (Sigma, negative control) and a protein solution of 5 mg of semi-purified cysteine proteinases dissolved in 5 ml of 0.2 M sodium acetate buffer, pH 5.6. Wells were filled with 0.2 ml of these solutions and incubated overnight at 4 °C. After overnight incubation, wells were emptied and blocked for 2 h with 0.2-ml blocking buffer (2% (w/v) non-fat dry milk in PBS). Mixes of 0.1-ml blocking buffer and 0.1-ml 5 × 10$^8$ cfu of PBS purified phages were prepared 30 min in advance of addition to a microtiter well. Mixes with 10 μM E64 were also prepared. The mixes were added to the wells for 2 h. Non-specifically bound phages were removed by washing five times with PBS containing 0.5% Tween-20. To each well, horseradish peroxidase-labelled anti-M13 antibody (diluted 1:5.000 in blocking buffer) was then added. After 60-min incubation, the plates were washed six times with PBS containing 0.5% Tween-20. The plate-bound peroxidase was detected with ABTS (2’,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) substrate. Plates were read at 410 nm after 40-min incubation.

3. Results and discussion

3.1. Isolation of cysteine proteinases

The majority of Coleopteran insects, such as the bruchids cowpea weevil (C. maculatus) and the Mexican bean weevil (Z. subfasciatus), contain high levels of cysteine proteinase activity in their intestinal fluids [28–30]. According to Wieman and Nielsen [8], the crude gut extract and purified proteinases from A. obtectus, when incubated in the presence of potential enhancers of cysteine proteinase activity (cysteine, dithiothreitol ‘DTT’ and ethylenediamine-tetra-acetic acid ‘EDTA’), showed increased activity. Furthermore, when these proteinases were incubated with a synthetic inhibitor [1-trans-epoxysuccinylleucylamidine-(4-guanidino) butane, ‘E-64’], substantial inhibition was observed [8]. These studies indicated that A. obtectus also contains cysteine proteinases in its gut fluid. We monitored the proteolytic activity on peaks obtained from CM-cellulose chromatography by activity assay according to Kunitz [26] (Fig. 2). An increase on proteolytic activity in eluted peak ‘CM2’ was observed in the presence of cysteine and EDTA and this activity was substantially inhibited by the addition of E-64 (data not shown). The peak ‘CM2’, which showed high levels of cysteine proteinase activity (Fig. 2), was used to select mutant ‘onchocystatins’ on phage display library.

The molecular weight of a cysteine proteinase previously purified from A. obtectus was estimated to be 25 kDa based upon its mobility on SDS-PAGE [8]. The ‘CM2’ peak was analysed by SDS-PAGE revealing proteins with different molecular weights. A band with molecular weight around 25 kDa was found, but in addition, major bands of approximately 35 and 50 kDa were found (Fig. 3A). The crude extract was analysed using a polyacrylamide gel containing azocaseine 0.1% and showed two regions of proteinase activity at molecular weights of approximately 35 and 25 kDa, which correspond to proteinases isolated by CM-Cellulose chromatography (Fig. 3B). This experiment was
done under conditions favourable to cysteine proteinase activity, using a buffer containing cysteine and EDTA at pH 5.0. The pH optimum for activity of purified proteinases from \( A. \) obtectus and partially purified thiol proteinases from \( C. \) maculatus was 5.0 [8,31]. Presumably the activity found on the azocaseine-containing gel, corresponding to the band of 35 kDa, represents another cysteine protease from \( A. \) obtectus not previously described.

### 3.2. Selection of mutant cystatins by biopanning

The 25- and 35-kDa cysteine proteinases semi-purified from \( A. \) obtectus were used as bait to select novel variant cystatins displayed on phage particles. After each round of biopanning, the number of TGI cell colonies containing eluted phage was monitored by plating dilutions on SOBAG plates containing ampicillin. As observed by Kitch and Murdock [32] in studies of multi drug-resistance drug-binding peptides generated using a phage display library, the number of colonies rescued was highest in the latter rounds of panning (data not shown). To analyse the selected mutant cystatins, 50 individual clones belonging to the three different rounds were randomly chosen for sequencing. These cystatins contained several different sequences in place of the original QVVAG motif, but only two mutants, along with the wild type sequence, bound strongly to cysteine proteinases, as determined by ELISA assays. These two mutant clones were named A and B (Table 1).

Phages A and B selected on \( A. \) obtectus cysteine proteinases showed similar binding activities as phages carrying wild-type OV7 (WT) that were also selected. However, intriguingly, these phages (A and B) showed weaker binding to papain compared to the wild type OV7 (WT). All selected phages showed high specificity for cysteine proteinases since no signal could be detected against bovine trypsin (Table 1). In the presence of E64, a known potent cysteine proteinase inhibitor, ELISA signals were significantly lower for both proteinases. We observed ELISA signals of approximately 0.04 ± 0.01 for all mutants against papain and 0.02 ± 0.01 against AoCP, confirming the specificity of selected mutants (medium ± standard deviation). While both A and B differ at several positions from the wild-type sequence, it is notable that they have in common the substitution of AG in the wild-type sequence with SA. Since sequence conservation throughout the cystatin family, and particularly in the loop in question, ensures very similar overall structures for all these inhibitors, the sequences of A and B can be interpreted in the light of the structure of papain in complex with human stefin B (Fig. 1, Ref. [9]). Particularly important is the fact that the Gly in the wild-type sequence (numbered 57 in the papain–stefin B complex) occupies only a position in the Ramachandran plot that is readily accessible to glycine residues. Thus, its replacement with Ala, in both A and B, would necessarily lead to a local rearrangement of the loop backbone. Such changes in main change conformation are difficult to predict with certainty, but the likely scenario is that the \( A. \) obtectus cysteine proteinases are able, through differences in active site structure, to accommodate the reorganised inhibitor loop, and are therefore inhibited, while the new loop is not so readily accommodated into the papain active site and

### Table 1

| Phage clone | Residues | AoCP* | Papain | Bovine trypsin |
|-------------|----------|-------|--------|---------------|
| A           | NTSSA    | 1.940 ± 0.260 | 1.300 ± 0.150 | 0.130 ± 0.020 |
| B           | DVVSA    | 1.570 ± 0.220 | 0.530 ± 0.260 | 0.100 ± 0.050 |
| WT          | QVVAG    | 1.750 ± 0.090 | 2.190 ± 0.320 | 0.170 ± 0.050 |
| M-13b       | –        | 0.000 ± 0.005 | 0.010 ± 0.001 | 0.000 ± 0.000 |

Each values corresponds to medium of three replicates ± standard deviation.

* AoCP: \( A. \) obtectus cysteine proteinases.

b M-13 phage particles.
affinity is lost. The replacement of the preceding Ala with Ser in both mutants would, in a wild-type loop context, inevitably lead to steric clashes with the enzyme. However, in the reorganised Gly → Ala loops it is possible that the Ser forms hydrogen bonds with the enzyme.

Our results showed that the mutations on loop 1 of variants A and B, provided by phage display, probably were not capable of increasing the inhibitory activity against proteinases from *A. obtectus* when compared to wild-type cystatin (Table 1). Nevertheless, only after inhibitor expression will it be possible to measure with accuracy the inhibitory activity. Furthermore, it is important to observe that the mutant inhibitor’s strong binding is not the only important characteristic for a proteinase inhibitor to be considered as a candidate for use in genetic engineering of plants. The capacity to select between insect digestive enzymes and other non-target enzymes is also looked for in the plant defence field [23]. In this regard, while the mutations do not increase the inhibitory activity against *A. obtectus* cysteine proteinases, their ability to inhibit papain is significantly reduced (Table 1). Studies have shown that phage display could be used to select inhibitors with higher activity against pests [21,33] and also could change the class of an inhibitor, transforming a trypsin-like inhibitory activity into a new chymotrypsin-like inhibitory activity [33]. In the last case, the mustard inhibitor, MTI-2, a potent trypsin inhibitor with no activity towards chymotrypsin and highly toxic for lepidopteran insects, had its activity improved for aphids. This variant inhibitor, named Chy8, was highly toxic to nymphs of the aphid *Acyrthosiphon pisum*, and moderately toxic to nymphs of *Aphis gossypii* and *Myzus persicae* [33]. However, despite several examples cited above, this is the first time that the insect enzyme specificity of inhibitors, compared to non-target proteinases, has been improved. Thus, as well as screening for further inhibitors from natural sources, phage display technology may also be used in the search for inhibitors with properties desirable for plant defence studies.

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