Phage display selection can differentiate insecticidal activity of soybean cystatins

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Summary

Plant cysteine proteinase inhibitors (phytocystatins) have been implicated as defensive molecules against Coleopteran and Hemipteran insect pests. Two soybean cystatins, soyacystatin N (scN) and soyacystatin L (scL), have 70% sequence identity but scN is a much more potent inhibitor of papain, vicilin peptidohydrolase and insect gut proteinases. When these cystatins were displayed on phage particles, papain-binding affinity and CPI activity of scN were substantially greater than those of scL, in direct correlation with their relative CPI activity as soluble recombinant proteins. Furthermore, scN substantially delayed cowpea weevil (Callosobruchus maculatus (F.)) growth and development in insect feeding bioassays, whereas scL was essentially inactive as an insecticide. Papain biopanning selection of phage-displayed soya-cystatins resulted in a 200–1000-fold greater enrichment for scN relative to scL. These results establish that binding affinity of cystatins can be used in phage display biopanning procedures to select variants with greater insecticidal activity, illustrating the potential of phage display and biopanning selection for directed molecular evolution of biological activity of these plant defensive proteins.

Introduction

The plant defense response to herbivorous insect attack includes accumulation of proteinaceous proteinase inhibitors (PI) (Koiwa et al., 1997; Ryan, 1990). PI are presumably a tactical plant protection strategy, because these attenuate nutrient assimilation in insect guts by inhibiting the activity of digestive proteinases. The defensive function of PI is derived from data which show that PI effectively inhibit insect digestive proteinases in vitro, and transgenic plants overproducing PI acquire enhanced resistance against feeding insects (Duan et al., 1996; Hilder et al., 1987; Johnson et al., 1989), although it is feasible that PI perturb other critical metabolic processes of the pest. The preponderance of available information about PI details insecticidal properties of serine proteinase inhibitors (SPI), typically trypsin inhibitors. However, the discovery that many Coleopteran and Hemipteran insects utilize cysteine proteinase inhibitors (CPI) as plant defensive proteins (Chenet al., 1992; Hines et al., 1991; Kuroda et al., 1996; Liang et al., 1991; Michaud et al., 1993, 1996; Murdock et al., 1988; Zhao et al., 1996). Among the major pests in these insect Orders are seed-feeding beetles and western corn rootworm (Liang et al., 1991; Orr et al., 1994). Recently, a plant CPI has been shown to reduce infection by cyst nematodes (Urwin et al., 1995).

Plant defensive CPI are, predominantly, cystatin superfamily inhibitors of the papain family CP. Cystatins form reversible, tight-binding complexes that mask the reactive site of the CP, making it inaccessible to protein substrates that are normally hydrolyzed by the proteinase. The proteins of the cystatin superfAMILY occur ubiquitously among animals, plants and micro-organisms, and are categorized generally, based on primary sequence similarities, into four families: family I, stefin; family II, cystatin; family III, kininogen; and family IV, phytocystatin (Turk et al., 1997), with the latter including almost all plant CPI. In animals, cystatins are involved in the regulation of protein turnover and have been implicated in the regulation of pathological processes resulting in disease mitigation (Henskens et al., 1996). Amongst the pathological processes presumed to be attenuated by cystatins are viral replication, bacterial infection, protozoan parasite host invasion, chronic inflammation and tumor metastasis.

Efficacy of phytocystatin-mediated pest control will be dependent on the degree of insect vulnerability to a cystatin(s), and this is based on the importance of the target CP to the life cycle of the pest, the effectiveness of the cystatin as an inhibitor of the target CP, and the
adaptive capacity of the pest or pathogen in response to an inhibitor challenge. Insect adaptation to PI includes production of inhibitor-insensitive proteinases that not only facilitate nutrient assimilation but also degrade the inhibitor protein (Jongsma et al., 1995a; Michaud, 1997). Furthermore, deployment of an insecticidal PI will impose selection pressure on the insect that will lead to evolution of proteinases with lower PI affinity, as exemplified when Plodia interpunctella developed resistance to Bacillus thuringiensis (BT) toxin within a few generations (Sparber, 1985). Consequently, an extensive molecular resource of proteinases must be available from which variants can be selected and used to develop an effective and genetically durable insect control strategy. To date, plant biotechnology strategies have been limited by the genetic variation that exists in natural gene pools. However, recent developments in genetic engineering offer the potential for utilization of in vitro-directed molecular evolution to obtain phytocystatin variants with effective target specificity for insect pest CP.

Currently available in vitro mutagenesis techniques can provide a high degree of molecular variation; however, in vitro protein evolution has been limited by availability of effective screening or selection techniques that result in isolation of functional peptide variants. The filamentous M13 phage display system is readily applicable for effective selection of peptides from a large population of variants, and subsequent isolation of the encoding DNA (Scott and Smith, 1990), provided that binding affinity to the substrate is functionally involved in activity. With the phage display system, successive rounds of amplification and biopanning selection can be imposed on populations of variants under increasingly stringent conditions. The potato SPI, PI–II, has been functionally displayed on the surface of phage particles and the phage display system has been used to confirm the involvement of domains I and II in binding interactions with SP. Furthermore, biopanning selection enriched a phage population for functional PI–II from a population mixed with mutated non-functional PI–II variants (Jongsma et al., 1995b). Chicken cystatin has also been successfully displayed on the phage (Tanaka et al., 1995) but, to date, neither phage display of a phytocystatin nor biopanning selection of any cystatin has been reported.

In this research, we establish, for the first time, that scN substantially inhibited growth and development of cowpea weevil (Callosobruchus maculatus) when added to an artificial diet, whereas scL had virtually no effect at the same concentrations. Phage-displayed scN had substantially higher papain-binding affinity and greater CPI activity than scL. Furthermore, repeated biopanning selection differentially enriched phage populations for soyacystatin isoforms according to their papain affinity and insecticidal activity.

### Results

**Preparation of soyacystatin proteins**

Soyacystatins scL and scN were produced as phage-displayed fusion proteins as well as soluble inhibitors (Figure 1). The coding sequences of the soyacystatins were cloned either into pET28a (pETNM<sup>8–103</sup> and pETLM<sup>3–93</sup>) or pSSHisA (pSSNM<sup>8–103</sup> and pSSLML<sup>3–93</sup>) to produce soluble and phage-displayed soyacystatins, respectively (Figure 2a,b). Expression of fusion proteins on phage particles was confirmed by immunoblot analysis using antibodies raised against purified scNM<sup>8–103</sup> (Figure 2a). Anti-scNM<sup>8–103</sup> detected proteins of about 50 kDa in phage particles produced from cells expressing scLM<sup>3–93</sup>–pIII or scNM<sup>8–103</sup>–pIII fusion proteins (Figure 2a). This migration of the fusion proteins was consistent with the calculated size of the fusion proteins based on the molecular weights of the soyacystatins and pIII.

**Specific interaction between immobilized papain and phage-displayed inhibitors**

The interaction between phage particles displaying soyacystatin–pIII fusion protein and immobilized papain was determined using micropanning assays in 96-well micro-
Phage display selection of soyacystatin

Figure 2. Soluble and phage-displayed recombinant soyacystatin proteins separated by SDS–PAGE.
(a) Immunoblot analysis of phage-displayed soyacystatin proteins, scL and scN. Phage particles were purified from culture supernatant of helper phage-infected bacteria harboring pBluescript (lane 1), pSSLM–3–93 (lane 2) or pSSNM8–103 (lane 3). Proteins were isolated from purified phage particles, fractionated by SDS–PAGE on a 10% gel, blotted onto nitrocellulose filter and probed with antibody raised against purified scNM8–103.
(b) Tris-Tricine SDS–PAGE separated (16% gel) soluble recombinant soyacystatins. Proteins are from crude extracts prepared from IPTG-induced bacteria, equivalent to 100 µl of culture harboring pET28a (lane 1), pETLM–3–93 (lane 2) or pETNM8–103 (lane 3), or 1 µg of purified scLM–3–93 (lane 4) or scNM8–103 (lane 5).

Figure 3. Data from one representative experiment of biopanning selection of phage-displayed soyacystatins. Each well of the microtiter plate was coated with solution of PBS without (–) or with (+) 30 μg ml–1 of papain. Phage particles were purified from bacteria harboring pBluescript, pSSLM–3–93 (scLM–3–93) or pSSNM8–103 (scNM8–103) and the binding reaction was performed in the absence (–) or presence (+) of chicken cystatin (competitor). Number of bound phage particles was determined as XL-1 blue CFU and normalized to CFU of the papain/competitor treatment for each phage preparation. Bars indicate standard deviations.

Figure 4. Differential papain inhibitory activity of soyacystatins is analogous for phage-displayed and soluble recombinant proteins. Preactivated papain was incubated with N-benzoyl-DL-arginine-β-naphthylamide to establish 100% activity and each point is the average of five replicate samples.
(a) Phage particles and (b) soluble recombinant proteins.

Inhibition assays (Figure 4a). The scNM8–103 phage substantially inhibited papain activity and, differentially, to a much greater extent than scLM–3–93, confirming the relationship between binding affinity and inhibitor activity of the displayed proteins. The ~ 10-fold difference in relative binding to papain (Figure 3) was very analogous to the 10-fold difference in phage particles required for 50% inhibition of papain activity (Figure 4a). Prevailing information has established that between 10% and 25% of phage particles are infectious (Basset al., 1990; Wang et al., 1995) and 10–30% of phage particles express one copy of the fusion protein (Röttgen and Collins, 1995; Wang et al., 1995) in monovalent phagemid systems such as pSurfscript. Consequently, in these experiments, it can be estimated that ~ 2 × 10¹² scNM8–103 phage particles inhibited 20 pmol (2 × 10¹² molecules) of papain. This estimation agrees well with the established 1:1 stoichiometry for the cystatin–papain interaction (Bode and Huber, 1992).

To establish that the binding affinity difference between the inhibitors was sufficient to differentiate, by selection, the two isoforms, experiments were conducted to demonstrate relative enrichment for scNM8–103 phage particles over scLM–3–93 particles (Table 1 and Figure 5). Two rounds of biopanning selection were sufficient for substantial enrichment (200- to 1000-fold) of scNM8–103 phage particles (Table 1 and Figure 5). After three rounds of papain biopan-
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Figure 5. Papain biopanning selection differentially enriches for scNM8–103 phage particles to a substantially greater extent than for scLM3–93 phage particles. The initial mixture (0) contained the following ratio of phage particles – pBluescript:pSSLM3–93:pSSNM8–103 = 10^3:10^1:10^1 CFU. After the first (1) and second (2) rounds of biopanning selection, bacteria (XL-1 blue) were infected with the eluted phage particles to amplify the selected population. Then the JM105 cells were infected with an aliquot of the population to produce about 400–450 colonies plate^{-1} (w/IPTG, X-gal), i.e. pBluescript colonies were blue (X-gal) and the cystatin (scN or scL)-containing colonies were white. Colony hybridization with an oligonucleotide probe (GGTGCTAGAAACATTGACTG) specific for a unique region of scN cDNA facilitated detection of pSSNM8–103 from pSSLM3–93 amongst the white colonies (hybridization with scN). The bottom row illustrates hybridization of the scN-specific oligonucleotide probe to an equivalent number of pBluescript, pSSLM3–93 or pSSNM8–103 colonies. Note: After three rounds of biopanning, only pSSNM8–103 colonies were detected.

Table 1. Biopanning enrichment of soyacystatin phage based on binding affinity to papain-coated wells

<table>
<thead>
<tr>
<th>Phage</th>
<th>Total CFU</th>
<th>Cystatin phage/ pBluescript phage</th>
<th>Fold enrichment†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>scN*</td>
<td>scL†</td>
<td>scN/scL</td>
</tr>
<tr>
<td>Original mixture</td>
<td>Cystatin</td>
<td>1.0 × 10^6 1.0 × 10^6</td>
<td>1.0 × 10^{-3} 1.0 × 10^{-3}</td>
</tr>
<tr>
<td>pBluescript</td>
<td>1.0 × 10^5 1.0 × 10^5</td>
<td>8.5 × 10^{-1} 2.8 × 10^{-2}</td>
<td>8.5 × 10^2 2.8 × 10^1</td>
</tr>
<tr>
<td>Cystatin</td>
<td>1.1 × 10^3 3.0 × 10^3</td>
<td>3.5 × 10^2 2.5 × 10^{-1}</td>
<td>3.5 × 10^5 2.5 × 10^2</td>
</tr>
<tr>
<td>pBluescript</td>
<td>3.3 × 10^2 1.2 × 10^2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Original mixture contains scNM8–103 phage and pBluescript phage.
†Original mixture contains scLM3–93 phage and pBluescript phage.
‡Fold enrichment was calculated by dividing cystatin phage/pBluescript phage ratio of each step by that of the original mixture.

Enzyme inhibition and insecticidal activity of soluble inhibitors

To establish the relationship between functionality of soyacystatins as phage-displayed proteins and biological activity, soyacystatins were overexpressed in Escherichia coli as soluble recombinant proteins (pETNM8–103 and pETLM3–93 vectors) independent of peptide fusions (Figures 1a and 2b). The scNM8–103 protein effectively inhibited papain while scLM3–93 was essentially inactive at the same concentrations (Figure 4b). However, in large excess, scLM3–93 protein weakly inhibited papain (data not shown), consistent with our previous results with soluble (Botella et al., 1996; Zhao et al., 1996) and phage-
Figure 6. The scNM\textsuperscript{8–103} protein (■) substantially inhibits cowpea weevil growth and development and affects high insect mortality, whereas the scLM\textsuperscript{3–93} protein (●) is relatively inactive.

An individual insect egg was placed onto an artificial seed (Shade et al., 1990) containing no or increasing concentration of soyacystatins, 10 seeds per treatment. Within-seed developmental time (upper panel) was determined as a period between when the eggs hatched and when adult insects emerged. Bars indicate standard deviation. Insect mortality (lower panel) was calculated, as a percentage of total, from the number of the insects that failed to emerge from artificial seeds.

displayed (Figure 4a) soyacystatins. The papain-binding affinity of soyacystatins displayed on the surface of phage particles and the inhibitory activity of soluble or phage-displayed proteins were tightly correlated. Furthermore, the modifications made to soyacystatins did not alter their differential level of activity. Purified scNM\textsuperscript{8–103} substantially retarded growth and increased mortality of cowpea weevil, while scLM\textsuperscript{3–93} affected neither insect growth nor mortality (Figure 6). Thus it can be concluded that the protein structure, which governs proteinase binding affinity of phage-displayed soyacystatins, is essential for insecticidal activity.

Interestingly, precise monitoring of insect-feeding events, by ultrasonic detection during the course of bioassay, indicated that growth of first and second instar larvae was remarkably retarded by 0.2% of scNM\textsuperscript{8–103} inhibitor in the diet. The molting event between the second and third instar was delayed 5 days by scNM\textsuperscript{8–103} and this was the basis for the overall retardation of development time that occurred during the experimental period (data not shown). Also, insect mortality recorded at the end of the experiment (Figure 6b) was attributable to effects of the soyacystatins that occurred during the first or second instar. These results indicate that the insecticidal properties of ingested soyacystatin are manifested mainly during the first and second instar of larval development, implying that a qualitative alteration in the digestive system is occurring that renders the insect insensitive to the inhibitor. This may be due to a developmental program or adaptation in response to the dietary phytocystatin.

Discussion

Presented here is a demonstration that the biological activity of a plant defensive protein can be predicted based on binding affinity to a target substrate. In this instance, the insecticidal activity of a soyacystatin can be extrapolated from the binding affinity of a phage-displayed protein to an immobilized proteinase, papain. Our results establish that inhibition of cowpea weevil proteinases is the mechanism of insecticidal activity of soyacystatin, since differential papain inhibitory activity of recombinant proteins was directly correlated with their capacity to inhibit growth and development of the insects. The substantial insecticidal activity of scNM\textsuperscript{8–103} supports the feasibility that expressing this protein in transgenic crops will increase host-plant resistance to insects that utilize CP for digestion or other critical metabolic processes.

The tactic of using proteinase inhibitors to attenuate assimilation of nutrients derived from proteolysis, and thereby inhibit insect growth and development, must be a component of a strategy that considers several aspects of insect biology. Insects utilize various proteolytic enzymes for digestion. The reduced impact of scNM\textsuperscript{8–103} in the later stages of cowpea weevil growth and development is indicative of the pests’ capacity to modify its response to soyacystatin challenge during the course of the bioassay and implicates the occurrence of adaptation that renders the inhibitor less active. Most probably this results from the capacity of the insect to produce an inhibitor-insensitive proteinase (Broadway, 1996; Jongsma et al., 1995a). Colorado potato beetle, an insect that is susceptible to CPI, altered its profile of digestive enzymes, presumably by producing inhibitor-insensitive cathepsin H-like CP, in response to ingested CPI that were induced in planta by jasmonate treatment (Bolter and Jongsma, 1995). At present, the full spectrum of insect proteinase diversity remains to be elucidated. However, there seem to be genetic constraints to the extent that an insect can overcome an inhibitor challenge either during a single life cycle or over many generations through evolution of resistant biotypes. Analyses of insect gut extracts indicate that the number of PI-insensitive target proteinases is limited, and the levels of inhibitor-insensitive proteinases produced by insects, in response to PI challenge, are insufficient to restore fully the normal level of proteolytic activity (Jongsma et al., 1995a, 1996a, b). Even when inhibitor-
resistant biotypes have evolved, it is most plausible that the phenotype will be mediated by minimal mutation resulting in the evolution or constitutive expression, perhaps, of a few inhibitor-insensitive proteinases. It is unlikely that mutation will result in production of a different class of proteinase since the activity of proteolytic enzymes is constrained by the environment of the gut, e.g. pH. Biotechnological engineering can be used to introduce into plants additional PI that are active against the relatively few PI-insensitive insect proteinases, and these, combined with native inhibitors (Burgess et al., 1994), will represent a defensive tactic that mediates effective and genetically durable host-plant resistance (Jongsma et al., 1996b). Regardless, a resource of molecular variation will be required in order to select new effective variants. To date, genetic variation has been restricted to germplasm pools; however, now it is likely that the new iterations of proteinase inhibitors will be obtained by a selection-based strategy using techniques of directed molecular evolution.

The data reported here, which establishes the functional relationship between the binding-affinity of a phage-displayed soyacystatin and its insecticidal activity, are the foundation for the experimental rationale for directed in vitro molecular evolution of this host-plant defensive protein. Candidate sequences for targeted incorporation of molecular diversity, which would enhance phyto cystatin efficacy, can be predicted based on current data defining the cystatin--CP interaction. Available information about insect gut proteinases and peptides deduced from cloned cDNA sequences suggests that cathepsin L- and H-like proteinases are the most plausible target CP against which to engineer a plant defense reaction that would inhibit proteolysis and nutrient assimilation (Bolter and Jongsma, 1995; Matsumoto et al., 1995, 1997; Ribolla and Bianchi, 1995). Although the three-dimensional structure of cathepsin L or H complexed with cystatin has yet to be determined, their amino acid sequences and structural similarities to papain can be the basis for a design strategy (Berti and Storer, 1995; Fujishima et al., 1997). Analysis of the X-ray structure of the papain-stef B complex indicates that cystatins inhibit their cognate enzymes via a simple blocking mechanism (Stubbs et al., 1990). The cystatin forms a tripartite wedge that slots into the active site cleft of the CP. In the center of the wedge is the first hairpin loop, which includes the conserved QxVxG motif, and this loop is flanked by an N-terminal trunk and a C-terminal second hairpin loop. These regions are primary targets for incorporation of molecular diversity since numerous intermolecular atomic interactions, principally hydrophobic interactions on the interface, occur between residues in these regions and residues in the catalytic center of proteinase. It can be presumed that these interactions govern binding tightness and specificity of the cystatin--CP interaction (Stubbs et al., 1990). Steric hindrances or electrostatic repulsion that attenuate interactions between the inhibitor-insensitive CP and the inhibitor presumably could be minimized in variant cystatins with appropriate amino acid substitutions in these regions. Another approach for generating molecular diversity is DNA shuffling, a technique that utilizes homologous recombination to generate combinatorial libraries (Cramer et al., 1996; Cramer et al., 1997; Stemmer, 1994a, b, 1995). DNA shuffling can efficiently pyramid multiple mutations throughout the peptide sequence that might be necessary if changes in the overall scaffold structure of the cystatin are required to facilitate a tighter binding interaction.

The relevance of the above approaches is clearly dependent on selection efficiency. Our results established that biopanning selection, using papain as the immobilized target, differentially enriched for the active scN isoform to a 3.5 \times 10^5- and 1.4 \times 10^5-fold greater extent than background vector or the weakly active scL isoform, respectively (Table 1). In a direct competition experiment, scN-phage particles were differentially enriched by 200-fold after two rounds of biopanning, and after the third round no scL-phage particles could be detected (Figure 5). The conditions of this experiment were quite similar to those that are likely to occur when performing directed molecular evolution, as variant forms of the protein with altered binding should be represented in the population at low, but equal, frequency relative to the vast majority of null variants. Our results are a demonstration that phage display selection can be used to select biologically active cystatin isoforms. In the future, highly insecticidal soyacystatin variants will be obtained by selection from combinatorial phage libraries based on binding affinity to immobilized recombinant insect proteinases or even crude gut enzymes. The selected soyacystatins variants can be used also to characterize the insect digestive system and to establish the role of phycystatins in the plant anti-insect defensive response.

Experimental procedures

Construction of the soluble and phage display recombinant protein vectors

The phage display vector pSSHisA was derived from pSurfscript (Stratagene, CA) by inserting into the Not- SpeI site a linker sequence (CCGGCCGAGGTCTCTCCTTACGAGCAACCAAGCACATTACGGCTCCGGAGCTCAACCATCATCATCATCATACTAGT) containing multiple cloning sites, a 6× His-tag and an amber stop codon. These modifications facilitate cloning of cDNA inserts into the vector, and production of protein, independent of the pIII peptide, when the vector is transformed into a non-suppresser bacterial strain that can recognize the amber codon. cDNA inserts encoding the soyacystatins scN and scL were subcloned into pSSHisA and pET28a (Novagen, WI). Since the soyacystatin cDNA clones did not encode a methionine for initiation of translation, or peptides of equivalent length (Botella et al., 1996), the inserts were modified...
to produce peptides as indicated in Figure 1. The scL protein, scLM3–93, included an additional three-residue N-terminus synthesized based on the scN sequence, and scN protein, scNM8–103, included a seven-residue truncation on the N-terminus and a one-residue deletion on the C-terminus (Figure 1). The cDNA was ligated into the Ncol–SpeI site of pSSHisA or Ncol–XhoI site of pET28a to produce pSSNM8–103 and pETNM8–103 that expressed scL, or pSSLM3–93 and pETLM3–93 that expressed scL.

Preparation of proteinase

Papain (2X crystallized; Sigma, MO) was further purified by chromatography with Mono-S (Pharmacia) to remove contaminating papaya proteinase IV, as described previously (Buttle et al., 1989). Purified papain was quantified by titration with chicken cystatin (Sigma).

Phage particles expressing soyacystatins and binding affinity assay pSSNM8–103 or pSSLM3–93 were introduced into host XL-1 blue cells for soyacystatin expression on the surface of phage particles. Phage particles were produced and purified from the bacterial culture supernatant according to the Stratagene protocol. As a negative control, pBluescript was introduced into XL-1 blue cells for phage production.

For binding assays, papain was immobilized to the bottom surface of 96-well microtiter plates (Falcon) by coating each well with 100 µl of 30 µg ml–1 papain in PBS (10 mM sodium phosphate, 1 mM potassium phosphate, 130 mM sodium chloride, 1 mM potassium chloride, pH 6.0) and overnight incubation. Titer wells incubated with PBS only were used as negative controls. The solution was removed and the wells were blocked by incubation with 5% skim milk in PBS for 2 h at 4°C. After washing six times with PBST (0.05% Tween-20 in PBS), 16.6 µl of 3X binding buffer (3% skim milk, 0.9% NaCl, 0.15% Tween-20, pH 7.5), or with or without 42 µg ml–1 of chicken cystatin, was added into each well and then followed by 33 µl of phage preparations [108 CFU (ampicillin-resistant colony-forming units)]. The solutions were mixed and allowed to react for 1 h at 4°C. Unbound phage particles were removed by washing the titer wells nine times with PBST and bound phage particles were eluted by incubating with 100 µl of 0.1 M triethylamine for 10 min at room temperature. The phage-particle eluate was neutralized immediately with 50 µl of 1 M Tris, pH 7.4, and titered with XL-1 blue cells. Phage particles produced by bacteria with pBluescript were used to monitor non-specific interactions with papain.

In vitro selection

Phage particles displaying scNM8–103 and/or scLM3–93 were mixed with an excess number (102 times) of particles obtained from cells that contained pBluescript. Biopanning selection from the mixture (105 CFU) was performed as described above, with modifications to the binding and wash buffers to increase stringency. The high stringency (HS) binding buffer contained 0.8 mM NaCl, 1% skim milk, 0.5% Triton X-100, and 0.5% Tween-20 in PBS. HS wash buffer was composed of 0.8 mM NaCl, 10 mM Tris, 1% SDS, 0.5% Triton X-100, and 0.5% Tween-20 at pH 8.8. Amplification of recovered phage particles was performed as described elsewhere (Wang et al., 1995). The enrichment of scLM3–93 or scNM8–103 phage particles relative to pBluescript phage particles was monitored as described elsewhere (Wang et al., 1995).

Overexpression and purification of soluble inhibitors

pETNM8–103 or pETLM3–93 were introduced into BL21 (DE3) cells for recombinant protein expression. Production of recombinant soyacystatins was induced by 0.4 mM IPTG (final concentration) and recombinant proteins were allowed to accumulate in cells grown for 16 h at room temperature. Cells were harvested by centrifugation and disrupted by sonication. scNM8–103 was purified to homogeneity by successive steps of ammonium sulfate precipitation and chromatography on phenyl sepharose CL-4B, and on DEAE sepharose fast flow. scLM3–93 protein obtained from DEAE fractionation was further purified using hydroxylapatite to achieve homogenity. Purified proteins were desalted and concentrated with an ultrafiltration unit (Amicon). These proteins were assayed for insecticidal activity. The purity of the proteins at different points in the fractionation process was assessed by Tricine SDS-PAGE according to the methods of Schägger and von Jagow (1987).

Inhibition of cysteine proteinases

Papain inhibitory activity of soyacystatins was determined using a modification of the method described previously (Zhao et al., 1996). Papain was preactivated by incubating in 25 mM sodium phosphate and 20 mM β-mercaptoethanol for 10 min at 40°C. Activated papain (0.5 µg in assays with soyacystatin-phage and 2 µg with soluble unconjugated inhibitor) was incubated with various quantities of inhibitor for 5 min at 40°C. The reaction was initiated by addition of 200 µl of N-benzoyl-DL-arginine-β-naphthylamide (final concentration of 1 mM), continued for 10 min at 40°C, and then terminated by addition of 1 ml of 2% HCl in ethanol. The color was developed by addition of 1 ml of 0.06% p-dimethylaminocinnamaldehyde and measured at A540.

Bioassay against cowpea weevil

Artificial seed bioassays with cowpea weevil (Callosobruchus maculatus) were performed exactly as described by Zhu et al. (1996). The feeding events were monitored with the ultrasonic detection system as described elsewhere (Shade et al., 1986, 1990).

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