

Rice Cystatin: Bacterial Expression, Purification, Cysteine Proteinase Inhibitory Activity, and Insect Growth Suppressing Activity of a Truncated Form of the Protein

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A cDNA clone that encodes oryzacystatin, a cysteine protease inhibitor from rice, was isolated and expressed in *Escherichia coli* BL-21 (DE3) using an expression plasmid under the control of a T7 RNA polymerase promoter. The construct pT7OC 9b encoded a fusion protein containing 11 amino acid residues of the NH₂ terminus of the bacterial protein ϕ 10 and 79 residues of oryzacystatin lacking 23 NH₂-terminal residues of the wild-type protein. Recombinant oryzacystatin (ROC) constituted approximately 10% of the total bacterial protein mass and was purified in a single step by anion-exchange chromatography. The inhibitory activity of ROC toward papain ($K_i = 3 \times 10^{-6}$ M) was comparable with that of the naturally occurring protein isolated from rice. Caseinolytic activity in midgut homogenates from seven species of stored product insects was inhibited from 18 to 85% by ROC, whereas the same activity was inhibited from 14 to 69% by the serine proteinase inhibitor phenylmethylsulfonyl fluoride. Midguts of stored product insects apparently contain both cysteine proteinases and serine proteinases, but the relative amounts vary with the species. When fed to the red flour beetle, *Tribolium castaneum*, 10 wt% ROC in the diet suppressed growth approximately 35% relative to that of the control group of insects. © 1992 Academic Press, Inc.

Cystatins are proteins that specifically inhibit cysteine proteinases. Animal cystatins are classified into

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three families on the basis of their molecular structure and gene organization (1-5). These include family I, or the stefin family, for cystatins lacking disulfide bonds; family II, or the cystatin family, for members with two disulfide bonds; and family III for inhibitors with a multidomain structure such as kininogens. A fourth family, the phytocystatins, was recently identified for plant homologs. The phytocystatin family includes the so-called oryzacystatins I and II isolated from rice endosperm (6). The phytocystatin and animal cystatin genes differ with respect to intron boundaries and length (6).

The two oryzacystatins, OC-I and OC-II, have considerable amino acid sequence similarity, 55% identity (7,8), but their genes exhibit no sequence similarity in the 5'-upstream regulatory regions (6). They also show different inhibitory specificities for cysteine proteases (9); OC-I inhibits papain more effectively than cathepsin H, whereas the converse holds for OC-II.

We are interested in cystatins because of their potential role or use in protecting plants against insect attack. Midgut proteinases from stored product Coleoptera are strongly inhibited by OC-I (10). Because the content of oryzacystatin in rice seeds is rather low (5,9), it is extremely difficult to accumulate sufficient amounts of protein for extensive biochemical and toxicological studies. Abe *et al.* (11) expressed OC-I in *Escherichia coli* as a fusion protein, but the level of expression was rather low and the purification procedure not simple. Therefore, we have prepared a cDNA that encodes a new recombinant truncated fusion protein containing 79 of the 103 residues of OC-I. Here we report the efficient expression of a very basic recombinant form of oryzacystatin (ROC) in *E. coli* using the T7RNA polymerase promoter (12) and a single-step purification of the pro-

tein. We also report the inhibitory activity of this protein toward papain and gut proteinases from several species of stored product insects *in vitro* and suppression of the growth of the red flour beetle, *Tribolium castaneum* by ROC.

MATERIALS AND METHODS

Materials

All chemicals were reagent or HPLC grade and were obtained from Fisher Scientific or Sigma Chemical Co. unless otherwise noted. Nylon membranes were from Micron Separations, Inc. The λ gt 10 cDNA library made from endosperm of developing rice seeds (Nato, CI 8998) was a gift from Dr. Susan Wessler, University of Georgia (Athens, GA). pBluescript KS plasmid was from Stratagene. pKK233-3 was from Pharmacia. pT7-7 was provided by Dr. F. William Studier, Biology Department, Brookhaven National Laboratory (Upton, NY). The Sequenase kit (version 2) was from United States Biochemical Corp. Oligodeoxynucleotides were synthesized in the Department of Chemistry, University of Kansas (Lawrence, KS). DNases, RNases, restriction enzymes, Taq polymerase, and T4 polynucleotide kinase were purchased from Stratagene or Promega. LB and NZCYM media were from GIBCO. *trans*-Epoxy succinyl-L-leucylamide (4-guanidino) butane (E-64), chicken cystatin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma.

Isolation of cDNA Clones of Oryzacystatin

A 30-mer (5' CGTCACCGAGCACAACAAGAAGGCCAATTC 3') oligodeoxynucleotide was synthesized based on positions 157–186 of the 5'-coding region of the cDNA clone λ OC 26 (7), labeled with [γ - 32 P]dATP by T4 polynucleotide kinase (13), and used as a probe. The rice cDNA library was plated at a density of about 1200 plaques per 150-mm plate, and duplicate plaque lifts were made of each plate using nylon membranes following standard procedures (13). After being air-dried for 30 min, the membranes were autoclaved for 2 min. Washing, prehybridizing, and hybridizing were performed following the procedures described in Section 6.4 of "Current Protocols in Molecular Biology" (14).

cDNA inserts were excised from positive clones by *Eco*RI digestion, subcloned into pBluescript KS plasmid (pBS) following standard procedures (14), and sequenced using Sequenase (Stratagene). Sequences were confirmed by additional subcloning and by using synthetic primers.

Expression Constructs

1. Expression construct pKKOC 9b. A 25-mer primer, which contains an *Eco*RI linker at the 5' end followed by 17 nucleotides of the oryzacystatin cDNA sequence

starting with the translation start ATG codon (position 68), was synthesized. The 25-mer primer and a universal sequencing primer (T3) were annealed to the pBS plasmid containing the oryzacystatin cDNA insert pBSOC 9b and used for amplification by polymerase chain reaction (PCR). PCR amplification and cloning of the PCR product were carried out following the method of Hemsley *et al.* (15). The pBSOC 9b clone carrying an *Eco*RI site adjacent to the ATG starting codon of the oryzacystatin cDNA was cut with *Eco*RI and ligated into the expression vector pKK233-3, resulting in the expression construct pKKOC 9b (Fig. 2A). The insert of oryzacystatin cDNA was under the control of the strong *tac* promoter and a ribosome-binding site on the vector.

2. Expression construct pT7OC 9b. pBSOC 9b plasmid DNA was subjected to double-digestion with *Sal*I and *Cla*I. The released insert DNA was isolated and ligated into the vector pT7-7, which was also double-digested with the same two enzymes. The resulting expression construct, which was called pT7OC 9b, encoded a hybrid protein under control of a T7 promoter and a ribosome-binding site on the vector (Fig. 2B). The hybrid protein was predicted to be a fusion protein in which 11 amino acid residues at the N terminus were derived from the vector polylinker and the remaining 79 amino acid residues were derived from the C terminus of oryzacystatin (Fig. 2C). Therefore, in the recombinant oryzacystatin coded by this construct, the NH₂-terminal 23 amino acid residues of the wild-type inhibitor were replaced by 11 amino acid residues derived from the vector polylinker sequence.

Expression and Isolation of Recombinant Proteins

The orientations of the two expression constructs in pKK233-3 were determined by DNA sequencing. The plasmid that contained the wrong orientation of the oryzacystatin cDNA was used as a control. pKKOC 9b was expressed in host JM 109 following the procedures of Yang and Wells (16).

pT7OC 9b was expressed in *E. coli* BL21 (DE3) by a modified procedure of Studier *et al.* (12). A single colony containing the construct pT7OC 9b was inoculated on an LB plate containing 200 μ g ml⁻¹ ampicillin and incubated overnight at 37°C. Bacteria from the LB plate were transferred to 300 ml LB containing 200 μ g ml⁻¹ ampicillin and incubated for 3 h with shaking at 37°C. Then ampicillin was added again to a final concentration of 400 μ g ml⁻¹, and the cells were treated with isopropyl β -D-thiogalactopyranoside to a final concentration of 0.04 mM to induce the production of T7 RNA polymerase. The culture was incubated for 3 h, after which the cells were harvested by centrifugation. The pellet was resuspended in 10 mM Tris, pH 8 containing 2 mM EDTA. The suspension was sonicated for 5 min using a microprobe and sonic dismembrater Model 300

(Fisher), and the supernatant was collected by centrifugation. After addition of ammonium sulfate to 60% saturation, the precipitate was collected, resuspended in 20 mM Tris, at pH 7.5, and dialyzed extensively against the same buffer. The dialysate was applied to a Q-Sepharose or a Mono-Q (Pharmacia) column equilibrated with the same buffer. The flowthrough material was collected and analyzed for protein using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nearly homogeneous recombinant oryzacystatin (>95% pure as determined by HPLC) was present in the flowthrough fraction and was used for gel electrophoresis, NH₂-terminal sequencing, and inhibition studies.

Determination of NH₂-Terminal Amino Acid Sequence of Recombinant Oryzacystatin

For NH₂-terminal amino acid sequence analysis of ROC, samples were prepared following the method of Matsudaira (17) and sequences were determined by Dr. Mark A. Hermodson, Department of Biochemistry, Purdue University (West Lafayette, IN).

Protease Assays and Determination of K_i

Protease activity and inhibition constants (K_i) were measured following the procedure of Abe *et al.* (5). Briefly, papain was incubated with or without various amounts of inhibitor in 0.1 ml assay buffer (0.1 M sodium phosphate, 2 mM EDTA, and 5 mM dithiothreitol, pH 6) at 37°C for 10 min. Then 0.05 ml *N*- α -benzoyl-DL-arginine β -naphthylamide (BANA) was added, and the mixture was further incubated for 20 min before addition of 2 ml of coupling reagent (10 mM 4-chloromercuribenzoic acid, 50 mM EDTA, and 4% Brij 35). Color was developed and measured as described by Barrett (18). One unit of cysteine proteinase inhibitor was defined as the amount that decreased the papain activity by one enzyme unit (19).

The inhibitory activity assay of ROC and other inhibitors toward insect gut proteases has been described previously (10). Briefly, casein was labeled with tritium by reductive methylation in the presence of formaldehyde and high specific activity [³H]NaBH₄ (sp act 10–20 Ci mmol⁻¹) and purified by gel permeation on a Sephadex G-25 column. The protein concentration was adjusted to 2 mg ml⁻¹ with unlabeled casein and stored at -70°C. Ten microliters insect gut protease preparation (10), 1–6 μ l inhibitor solution, 74 μ l assay buffer (0.1 M PO₄³⁻, 2 mM EDTA, 5 mM dithiothreitol, pH 6), and 0–5 μ l deionized water were incubated at 37°C for 10 min. Then 10 μ l of the [³H]casein solution was added to each of the reaction mixtures, which were further incubated for 20 min. The reaction was stopped by adding 0.1 ml of 10% TCA. After they stood in ice for 20 min, the samples were centrifuged for 15 min in a microcentrifuge

and 0.15 ml of the supernatant was collected for scintillation counting. Background levels of TCA-soluble radioactivity were determined by the same procedure but without adding enzyme.

Bioassay of Recombinant Protein Using the Red Flour Beetle

The ROC and a commercially available protease inhibitor, E-64, were bioassayed with a laboratory colony of the red flour beetle, *T. castaneum* (Herbst). The diet was similar to that used by Applebaum and Konijn (20) and consisted of a mixture of corn starch, wheat gluten, cellulose, Torula yeast, and the salt mixture of Medici and Taylor (21). Ten percent ROC or 1% E-64 (wt/wt) was added to the diet and ground in a mortar and pestle with 20% (vol/wt) of water. Twelve neonate larvae were placed in a disposable beaker (ca. 12 mm diameter and 21 mm deep) with 300 mg of diet at 27°C and 75% R.H. A similar group of newly hatched larvae was weighed on a Cahn C-31 Microbalance to provide the average initial weight of the larvae. After 9 days on the diet, 10 larvae were selected randomly (any additional larvae were discarded). Individual weights were recorded for the 10 larvae, and they were placed in individual 1.5-ml, disposable centrifuge tubes with ca. 10% of the original diet from the beaker. Individual weights were recorded at several later time intervals. Statistical tests of significant differences between diet treatments were performed using the Tukey HSD multiple comparison analysis (22).

RESULTS

Isolation of cDNA Clones of Oryzacystatin

Screening of 10⁵ primary cDNA clones yielded 21 positive cystatin clones, eight of which were subjected to sequence analysis. All eight clones had the same nucleotide sequence, and seven of them contained the entire coding region as well as more than 70 residues flanking both the 5'- and 3'-noncoding regions.

Figure 1 shows the nucleotide sequence, with putative polyadenylation sites noted, and the inferred amino acid sequence of clone λ OC 9b. The 5'-noncoding and the coding regions of clone λ OC 9b are identical with those of clone λ OC 26 (6,7), although nine residues (including four single base deletions) are different within the 3'-noncoding region. In addition λ OC 9b extends 27 nucleotides further upstream than λ OC 26.

Expression of Recombinant Oryzacystatin in *E. coli*

Expression of the recombinant construct pKKOC 9b (Fig. 2A), which encodes the entire amino acid sequence of oryzacystatin and no residues from the vector, was

GCATTCG 7

CTAGCCACGCCGCTCCGCTCAGGCCGAGGGCCATCGCGCAGGGGAGAAGGGGAGGAGAAG 67

ATGTCGAGCGACGGAGGGCCGGTGTGGCGCGCTCGAGCCGGTGGGAACGAGAACGAC 127
MetSerSerAspGlyGlyProValLeuGlyGlyValGluProValGlyAsnGluAsnAsp 20

CTCCACCTCGTACGACCTCGCCCGCTTCGCGGTCCAGCAGCACAACAAGAAGGCCAATTC 187
LeuHisLeuValAspLeuAlaArgPheAlaValThrGluHisAsnLysLysAlaAsnSer 40

CTGCTGGAGTTCGAGAAGCTTGTGAGTGTGAAGCAGCAAGTTGTGCTGGCACTTTGTAC 247
LeuLeuGluPheGluLysLeuValSerValLysGlnGlnValValAlaGlyThrLeuTyr 60

TATTTCAAAATTGAGGTGAAGGAAGGGGATGCCAAGAAGCTCTATGAAGCTAAGGTCTGG 307
TyrPheThrIleGluValLysGluGlyAspAlaLysLysLeuTyrGluAlaLysValTrp 80

GAGAAACCATGGATGGACTTCAAGGAGCTCCAGGAGTTCAAGCTGTGATGCCAGTGCA 367
GluLysProTrpMetAspPheLysGluLeuGlnGluPheLysProValAspAlaSerAla 100

AATGCCTAAGGCCCATCTCGTATCCCTATGTGTATCAAGTTATCAAGAAGATGGGAATAA 427
AsnAlaTer 102

TATGGTGTGGATATAGCTATTGGACATGTAATTATCCACATGATAATATGGCTGGATA 487

TAAGGATCTCACACGATAATATGGCTGGATATATAGCTATTAAAGATTTTACCTATGGC 547

ATATTTCAATGTGTATTAGTACTAAGTAAGAAATGATTGCAAGGTGATTAACTACAATA 607

TTGCAATAAAGTCCCTGTTACTACAAGTACAAGG 643

FIG. 1. Nucleotide sequence of λ OC 9b and the inferred amino acid sequence of its encoded protein. Differences in nucleotide sequences of λ OC 9b compared with λ OCg 1 are shown in parentheses. Those between λ OC 9b and λ OC 26 are indicated above the main sequence of λ OC 9b. * represents a deletion in λ OC 26 or λ OCg 1. Δ represents a polyadenylation site.

attempted in *E. coli* JM 109. Upon electrophoresis of an extract of the total proteins of the bacteria, no new protein bands were seen when compared to the control, and furthermore, there was no evidence of an increase in intensity of any protein band in the region of the gel expected to contain oryzacystatin when compared to the pattern of proteins from bacteria that contained a plasmid with the oryzacystatin gene in the wrong orientation (data not shown). However, some papain inhibitory activity was detected in the induced sample, but not in the control, indicating that oryzacystatin was expressed in this system at a low level.

The construct pT7OC 9b (Fig. 2B), which encodes a fusion protein containing 11 extra amino acids at the NH₂ terminus and 79 amino acids of the COOH terminus of oryzacystatin (Fig. 2C), was expressed in the host BL-21 (DE3). Upon electrophoresis followed by Coomassie blue staining of an extract of the total proteins of *E. coli*, an intense protein band with a mobility corresponding to a 10-kDa protein, presumably ROC, was observed in the sample from the bacterial cells that contained the construct pT7OC 9b (Fig. 3, lane 4). An extract from a clone with the opposite orientation of cDNA did not contain the 10-kDa protein (lane 5).

Purification of the Recombinant Oryzacystatin

ROC was extracted after sonication of bacterial cells in 10 mM Tris-HCl, pH 8. After centrifugation of the extract, about 70% of the recombinant protein was present in the supernatant (Fig. 3, lane 3). After ammonium sulfate precipitation and dialysis against 20 mM Tris-HCl, pH 7.5, the supernatant was applied to a Q-Sepharose or a Mono-Q anion-exchange column. At this pH, essentially all of the bacterial proteins bound to the ma-

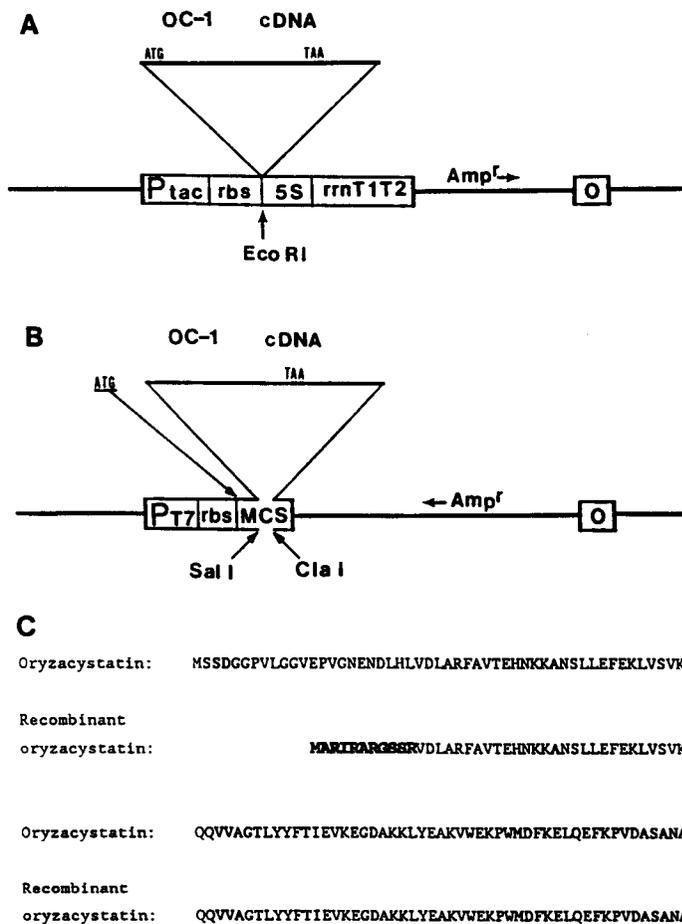


FIG. 2. Schematic diagram of expression constructs and amino acid sequences of natural oryzacystatin and recombinant oryzacystatin fusion protein. (A) The expression construct pKKOC 9b. The oryzacystatin gene is under the control of the tac promoter (P_{tac}) and a ribosome binding site (rbs) on the vector. 5S represents the 5 S ribosomal RNA gene and rrnT1T2, the ribosomal transcription terminator. O represents a replication origin. The insert encodes the entire length of oryzacystatin. (B) The expression construct pT7OC 9b. The oryzacystatin cDNA was cloned into the EcoRI site of the polylinker and is under the control of the T7 bacteriophage promoter (P_{T7}) and a ribosome-binding site on the vector. The recombinant product is a fusion protein, which lacks the N-terminal 23 amino acid residues of oryzacystatin and has, instead, 11 amino acid residues from the bacterial protein ϕ 10 and polylinker residues. MCS represents multiple cloning sites. (C) Amino acid sequences of natural oryzacystatin and the recombinant oryzacystatin encoded by pT7OC 9b. Amino acid residues noted in bold type were derived from the vector.

trix, but ROC did not. The inhibitor was thus purified to apparent homogeneity by this step, as indicated by SDS-PAGE (Fig. 3) and HPLC analyses. From 100 ml of sonicated bacterial culture extract, approximately 10 mg ROC was obtained.

Because the recombinant inhibitor did not bind to Q-Sepharose at pH 7.5, the purification method was further simplified by using a Q-Sepharose batch method. To the sonicated supernatant (pH 7.5), an appropriate amount of Q-Sepharose was added; the mixture was incubated for 30 min with stirring and centrifuged to collect the supernatant. At this step, most of the *E. coli* proteins bound to the anion-exchange resin and sedimented with Q-Sepharose, whereas ROC remained in the supernatant. The supernatant was adjusted to pH 8.5 and subjected to another Q-Sepharose adsorption as described above. The recombinant inhibitor bound to Q-Sepharose at pH 8.5. Homogeneous inhibitor was then desorbed from Q-Sepharose by addition of 0.2 M NaCl.

Determination of Amino Acid Sequence of Recombinant *Oryzacystatin*

Fifty amino acid residues of the NH₂ terminus of purified ROC were determined and found to be the same as

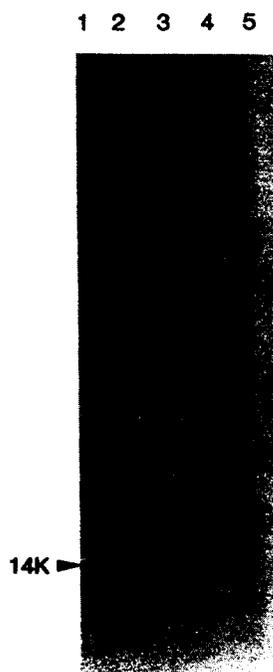


FIG. 3. SDS-PAGE of bacterial proteins containing recombinant oryzacystatin. Lane 1, molecular weight marker proteins; lane 2, purified recombinant oryzacystatin; lane 3, supernatant of bacterial cells containing recombinant oryzacystatin after sonication; lane 4, total bacterial proteins containing the expressed recombinant oryzacystatin; lane 5, total bacterial proteins obtained from cells with a construct containing the opposite orientation of cDNA. Samples were prepared as described under Materials and Methods.

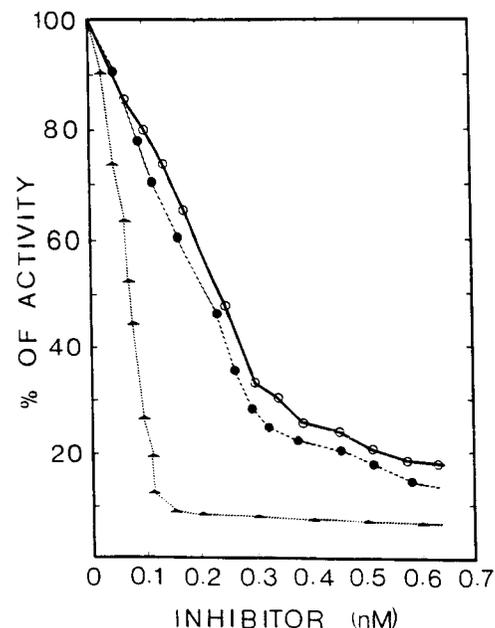


FIG. 4. Effect of inhibitor concentration on papain activity. Activities were determined by the [³H]casein digestion method (10) at pH 6 using 1 μg papain in the presence of E-64 (▲), chicken cystatin (●), and recombinant oryzacystatin (○).

those predicted for the expression construct pT7OC 9b (Fig. 2C).

Inhibitory Activity

The inhibition constant for ROC toward papain was determined to be comparable ($K_i = 3.2 \times 10^{-8} \text{M}$) with that for wild-type oryzacystatin isolated from rice seeds ($2.4 \times 10^{-8} \text{M}$; Ref. (5)) and also that for chicken cystatin (4) (Fig. 4). E-64, the microbial cysteine proteinase inhibitor, is approximately a threefold stronger inhibitor than ROC or chicken cystatin.

Insect gut proteinases were also inhibited by ROC. Figure 5 shows the effect of various amounts of inhibitor on the caseinolytic activity of gut homogenates of the rice weevil, *Sitophilus oryzae*, and the yellow meal worm, *Tenebrio molitor*. There was a linear relationship between the amount of inhibitor and the percentage inhibition of the gut proteinases for the rice weevil and yellow meal worm, until the extent of inhibition reached about 80% and 30% inhibition, respectively. Increasing the amount of ROC thereafter did not cause more inhibition, indicating that proteinases other than cysteine may be present in the gut homogenates.

Inhibition curves for other insects, including the flour beetles (*Tribolium confusum* and *T. castaneum*), the dark meal worm (*T. obscurus*), the cadelle beetle (*Tenebriodes mauritanicus*), and the Indianmeal moth (*Plodia interpunctella*), were similar (data not shown). The per-

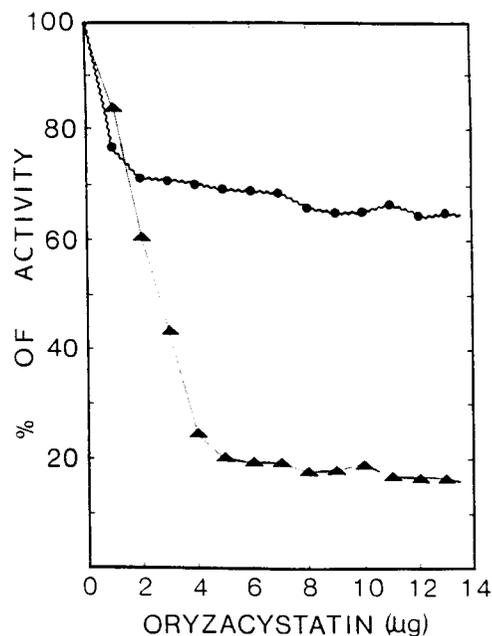


FIG. 5. Effect of recombinant oryzacystatin concentration on the activity of gut proteinases of rice weevil (▲) and yellow mealworm (●).

centages of maximum inhibition are summarized in Table 1. About 80% of the caseinolytic activities of gut homogenates from the rice weevil, the flour beetles, and

TABLE 1

Inhibitory Activities of Recombinant Oryzacystatin on the Gut Proteases of Different Stored Product Insects^a

Insect	Maximum % of inhibition		
	ROC	ROC + PMSF	PMSF
Confused flour beetle	84.8 ± 8.4	100	15.2 ± 3.6
Rice weevil	81.2 ± 6.2	100	22.8 ± 4.3
Red flour beetle	80.1 ± 7.3	100	13.5 ± 5.5
Cadelle beetle	79.8 ± 5.2	92.4 ± 4.1	18.4 ± 2.7
Dark mealworm	39.1 ± 2.5	65.7 ± 3.7	38.5 ± 3.9
Yellow mealworm	31.4 ± 5.6	66.3 ± 5.2	35.6 ± 4.1
Indianmeal moth	17.8 ± 5.1	95.6 ± 4.6	68.9 ± 3.3

^a Ten microliters of midgut extracts (3–9 μg protein) was incubated with inhibitors at pH 6 (80 mM Na₂HPO₄, 5 mM dithiothreitol, and 5 mM ethylenediaminetetraacetic acid) and 37°C for 10 min; then 10 μl of [³H]casein (4000 cpm) was added, and the mixture was further incubated for 20 min (see Materials and Methods). The percentage of inhibition was calculated by comparison to results of control incubations containing no inhibitor. ROC and PMSF represent recombinant oryzacystatin and phenylmethylsulfonyl fluoride. Mean values ± SE (*n* = 3) are reported for ROC (~0.6 nM), and mean values ± range (*n* = 2) for treatments with PMSF (0.1 mM) and ROC + PMSF.

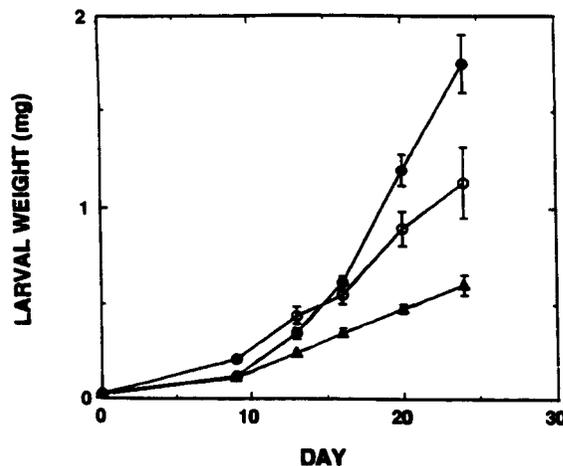


FIG. 6. Growth curves of *Tribolium castaneum* fed diets containing 1% E-64 (▲), 10% ROC (○), or a control diet (●). Mean values ± SE (*n* = 10).

the cadelle beetle were inhibited by ROC at pH 6. ROC also inhibited 30–40% of the caseinolytic activities of gut homogenates from the dark and yellow meal worms. Less inhibition (about 17%) was observed toward the gut proteinases from the Indianmeal moth.

The caseinolytic activities of gut homogenates of the red flour beetle, the confused flour beetle, and the rice weevil were completely inhibited by a mixture of oryzacystatin and PMSF, an inhibitor of serine proteases (Table 1). More than 90% of the activities of the gut proteases of the cadelle beetle and the Indianmeal moth were inhibited by the same combination. However, only about 65% of the caseinolytic activities of the gut homogenates of the yellow meal worm and the dark meal worm were inhibited by the same mixture. PMSF alone inhibited gut proteinase activity from 14 to 69%, depending upon the species tested.

Inhibition of Red Flour Beetle Growth

The effect of two cysteine proteinase inhibitors, E-64 and ROC, on the growth of the red flour beetle, *T. castaneum*, was determined by incorporation of the inhibitor into the diet (Fig. 6). Like oryzacystatin, E-64 inhibits casein digestion by midgut homogenates of red flour beetle larvae (10). Mean larval weights of the three treatments on Days 20 and 24 were significantly different from each other ($\alpha < 0.05$). One percent E-64 suppressed growth by approximately 65% after 24 days, the time when control larvae were close to pupation, whereas 10% ROC suppressed growth by only 35%. The ROC-supplemented, E-64-supplemented, and control diets yielded adult insects after approximately 48, 60, and 37 days, respectively. The mortalities resulting from these treatments were 50, 60, and 10%, respec-

tively. On a molar basis, the amount of E-64 in the diet was more than 10-fold greater than the amount of ROC. Thus, the two inhibitors appear to be comparable in ability to suppress flour beetle larval growth *in vivo*.

DISCUSSION

Abe *et al.* (7) isolated and sequenced a cDNA clone (λ OC 26) of oryzacystatin that lacked a poly(A) tail. Our clone is identical to Abe's in both the 5'-noncoding and coding regions, but nine residues are different within the 3'-noncoding region. Although Southern blot analysis indicated that oryzacystatin was encoded by a single gene (Ref. (8) and our unpublished data), a second gene, OC-II, was subsequently identified in rice (6). However, OC-II differs from OC-I in 45% of the amino acid sequence and, therefore, is not detected by the OC-I or OC-9b probe. Our clone is apparently identical to the gene from OC-I (7,8).

The enzyme inhibitory activity of ROC is comparable with that of oryzacystatin I isolated from rice seeds, even though the first 24 amino acid residues of oryzacystatin were absent and 11 additional amino acid residues from a bacterial protein were present instead. This result is consistent with previous reports demonstrating that the NH₂-terminal 21 amino acid residues are not required for the inhibitory activity of OC-I (11,23).

Cysteine proteinase inhibitors are distributed rather widely in nature, but their levels are rather low. For example, large amounts of fresh blood were required to obtain enough inhibitor protein for the characterization of human stefin A (24), and, likewise, kilogram amounts of rice seeds yielded only microgram amounts of oryzacystatin (5). Thus, it is rather difficult and time consuming to isolate cysteine proteinase inhibitors from natural sources directly. A better method to obtain larger amounts of purified inhibitors is to produce these proteins in *E. coli* or other microbial expression systems.

Bacterial expression of several cysteine proteinase inhibitors, including human stefin A, human stefin B, and rat stefin A, has been reported (11,25-29). Oryzacystatin I has been expressed in *E. coli* by Abe *et al.* (11). A common problem in these studies was a low level of expression of recombinant cystatins, in spite of the use of widely different vectors (30) and chemically synthesized genes, in which the codons were optimized for bacteria (30,31). In our study, we wished to achieve a high level of expression by using the tRNA polymerase system to produce a recombinant fusion protein (12). As expected, the level of expression that we achieved using construct pT7OC 9b was quite high. The fusion protein was visible as a major band in SDS-PAGE analysis. On the other hand, we observed a low level of expression with pKKOC 9b. The exact reason for the large difference in levels of expression between these two constructs is un-

known. It may have been due to a greater stability of the hybrid mRNA or to a higher efficiency of translation of the hybrid mRNA relative to that of non-fusion oryzacystatin mRNA in *E. coli*.

To facilitate purification of the recombinant protein, a cDNA encoding a relatively high pI form of oryzacystatin was prepared. First, the DNA encoding the NH₂-terminal 23 residues of OC-I, which contains four acidic amino acids, was deleted since that portion of the polypeptide is not required for inhibitory activity (11,27). Second, DNA which encodes 11 residues from the polylinker portion of the vector and contains four basic amino acids, was added. The substitution of a sequence containing basic instead of acidic residues at the NH₂ terminus rendered the recombinant fusion protein substantially more basic than other proteins synthesized by *E. coli*. The recombinant oryzacystatin was purified to homogeneity in high yield by a single anion-exchange chromatographic step. Approximately 10 mg of the recombinant inhibitor was purified from 100 ml of bacterial culture. The simplified Q-Sepharose batch purification method allowed us to isolate milligram amounts of protein in a few hours. This method also may be applicable to other high pI proteins, including other enzyme inhibitors in plant seeds (32).

Because important insect pests use cysteine proteinases as their predominant digestive enzymes (33-36), it may be possible to use cysteine proteinase inhibitors to protect plants against insect attack. We previously demonstrated that OC-I strongly inhibited caseinolytic activity in gut homogenates of the rice weevil and the red flour beetle (10). In the present study, we used the recombinant inhibitor to investigate the inhibition by OC-I of the gut proteinases of seven economically important species of stored product insects. ROC inhibited approximately 80% of the activity of gut proteinases of the confused flour beetle, the rice weevil, the red flour beetle, and the cadelle beetle, and 18 to 39% of that of two species of mealworms and the Indianmeal moth (Fig. 5 and Table 1).

The caseinolytic activities of the gut homogenates of the confused flour beetle, the rice weevil, and the red flour beetle were completely inhibited by a mixture of ROC and PMSF. The activities of the cadelle beetle and the Indianmeal moth were nearly completely inhibited (92 to 95%) by this combination. Assuming that the inhibitors are diagnostic for specific classes of proteinases, these data suggest that about 80% of the caseinolytic activities of the gut homogenates of the two flour beetles, the rice weevil, and the cadelle beetle were attributable to cysteine proteinases and 20% to serine proteinases.

ROC also inhibited about 35% of the caseinolytic activities of the gut homogenates of the dark meal worm and the yellow meal worm. The yellow meal worm was reported previously to use serine proteinases as major

digestive enzymes (35,37). However, our results suggest that meal worms use both serine proteinases and cysteine proteinases as digestive enzymes. About 30% of the proteinase activities of the two meal worms was not inhibited by either oryzacystatin or PMSF. The uninhibited activity may be attributed to proteinases other than serine or cysteine. Thie and Houseman (38) found cysteine proteinase activity in the yellow meal worm to be higher in the anterior relative to the posterior portion of the midgut, whereas serine proteinase activity predominated in the posterior portion.

Both ROC and E-64 inhibited red flour beetle growth and development by 35–65%. Apparently, the two inhibitors block the digestion of dietary protein by inhibiting midgut cysteine proteinases (10). The utilization of phytocystatins to protect seeds against stored product insect pests whose major digestive enzymes are cysteine proteinases appears to be a reasonable approach to insect control.

Nonproteinaceous cysteine proteinase inhibitors previously have been shown to suppress the growth and development of several beetle species (37–42). The expression of oryzacystatin in *E. coli* at high levels and the simple but efficient purification method allowed us to isolate a rather large quantity of ROC and to demonstrate the ability of this proteinaceous inhibitor to retard the growth of beetle larvae. Research is currently under way to evaluate in more detail the effects of the recombinant inhibitor on stored grain insect growth and development by rearing several species of insects on diets containing the recombinant protein.

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