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Inhibition of digestive proteinases of stored grain coleoptera by oryzacystatin, a cysteine proteinase inhibitor from rice seed

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Electrophoresis of midgut extracts from the rice weevil, *Sitophilus oryzae*, and the red flour beetle, *Tribolium castaneum*, in polyacrylamide gels containing sodium dodecyl sulfate and gelatin revealed there was one major proteinase (apparent molecular mass = 40000) in the rice weevil and two major proteinases (apparent molecular masses = 20000 and 17000) in the red flour beetle. The pH optima using [^{3}H]casein as substrate were about pH 6.8 for the rice weevil and pH 5.2 for the red flour beetle. Use of specific inhibitors, including L-*trans*-epoxysuccinyl-leucylamino-(4-guanidino) butane (E-64), *p*-chloromercuriphenylsulfonic acid (PCMS), and oryzacystatin, indicated that nearly all of the proteinase activity against casein was contributed by cysteine proteinases. The estimated IC_{50} values for oryzacystatin were 2×10^{-7} M and 4×10^{-7} M when tested against midgut extracts from *T. castaneum* and *S. oryzae*, respectively.

Oryzacystatin; *Sitophilus oryzae*; *Tribolium castaneum*; Midgut; Cysteine proteinase inhibitor; Rice weevil; Red flour beetle; Rice

1. INTRODUCTION

Cereal grains contain proteinaceous inhibitors of hydrolytic enzymes. These inhibitors are not well understood in terms of physiological importance during development or germination of the seed [1]. However, digestive enzymes of herbivorous insect species are putative targets of some of these inhibitors and, manifestly, the inhibitors may be important plant allelochemicals that impact insect growth and development. Diverse types of insect digestive enzymes release amino acids and sugars from dietary protein and carbohydrate [2]. One class of digestive enzymes is the cysteine proteinases, which are widely distributed in Coleopteran species [3]. Cysteine proteinase inhibitors have been purified from several cereal grains including rice [4], barley, rye, and corn [5]. However, no information is available about the effect of these inhibitors on the proteinases present in the midguts of stored grain insects. In the present study, we have partially purified and characterized the major proteinases extracted from midguts of the rice weevil, *Sitophilus oryzae*, and the red flour beetle, *Tribolium castaneum*. In addition, the effect of oryzacystatin, a cysteine proteinase inhibitor from rice seed, and other inhibitors on the midgut proteinases was examined. Our results show that the midgut cysteine proteinases of these two insects are inhibited by oryzacystatin, E-64, PCMS, and antipain.

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Abbreviations: E-64, L-*trans*-epoxysuccinyl-leucylamino-(4-guanidino)-butane; TCA, trichloroacetic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; PCMS, *p*-chloromercuriphenylsulfonic acid; DFP, diisopropyl phosphorofluoridate; SBTI, soybean trypsin inhibitor; CPTI, cowpea trypsin inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; antipain, [(*S*)-1-carboxyl-2-phenylethyl]carbonyl-L-arginyl-L-valyl-argininal; IC_{50}, the concentration of inhibitor required for 50% inhibition.

2. MATERIALS AND METHODS

2.1. Materials

Rice seed (*Oryzae sativa*, Newbonnet cultivar) was generously provided by Dr Robert Dilday of the USDA Rice Research Laboratory in Stuttgart, Arkansas. Papain (EC 3.4.22.2), E-64, PCMS, antipain, DFP, SBTI, CPTI, and casein (Hammersten) were purchased from Sigma (St. Louis, MO). [^{3}H]$NaBH_4$ (spec. act. 10–20 Ci nmol^{-1}) was purchased from New England Nuclear (Boston, MA).

2.2. Preparation of larval midgut proteinase extracts of rice weevil and red flour beetle

Midguts from mature larvae reared on a meridic diet (*Sitophilus* [6]) or wheat flour containing 5% brewer's yeast (*Tribolium*) were excised, placed immediately in cold deionized water (10–20 midguts ml^{-1}), homogenized with a glass tissue grinder, and centrifuged at 10 000 × g for 5 min. The supernatants were divided into aliquots and frozen at −80°C.

2.3. Assay of proteinase activity by electrophoresis

Proteinases present in midgut extracts of insects were analyzed electrophoretically using SDS-polyacrylamide gels containing porcine skin gelatin (Sigma) as substrate [7]. For this analysis, electrophoresis was conducted in 0.75 mm thick, 7.5% acrylamide minigels as described by Laemmli [8], except that 2-mercapthoethanol was omit-

ted. After electrophoresis, gels were washed briefly in 2.5% Triton X-100 to remove SDS and incubated overnight in 0.1 M Pipes buffer, pH 6 containing 5 mM DTT, at 37°C. Gels were then stained with Coomassie brilliant blue and destained briefly with 40% methanol 10% acetic acid and 50% water for several hours. This method visualized proteolytic activity as a clear zone against a dark blue background. Molecular masses were estimated by comparison of electrophoretic mobilities of unknown and standard proteins.

2.4. *Purification of oryzacystatin*

Oryzacystatin was purified by modification of the method of Abe [4]. Two kg of rice flour was suspended in 4 liters of 25 mM sodium phosphate (pH 7) containing 0.15 M NaCl and stirred at room temperature for 1 h. After centrifugation, the extract was heated to 75°C for 10 min and then fractionated by ammonium sulfate precipitation. Papain inhibitory activity was precipitated at 30–65% saturation with ammonium sulfate. To assay the inhibitory activity, we used recrystallized papain as enzyme and casein (Hammerstein) as substrate [9]. The 30–65% precipitate was chromatographed successively on Q-Sepharose (Pharmacia, 2.6 × 27 cm) with 20 mM Tris (pH 8) and a linear gradient of increasing NaCl concentration up to 0.5 M, on S-Sepharose (Pharmacia, 1.5 × 20 cm) with 25 mM formate (pH 4.1) and a linear gradient of increasing NaCl concentration up to 0.4 M. Final purification was achieved by chromatofocusing on a column of polybuffer exchanger 94 (BioRad, 1 × 30 cm) equilibrated with 25 mM imidazole-HCl (pH 7.4). The protein was eluted with diluted polybuffer 74 (BioRad) (pH 5). The polybuffer was removed by 80%-satd. $(NH_4)_2SO_4$ precipitation and dialysis against water.

2.5. *Proteinase assay using [^{3}H]casein digestion*

Details of preparing [^{3}H]casein and of the assay procedure have been described previously [3]. Briefly, casein was labeled with tritium by reductive methylation in the presence of high specific activity [^{3}H]$NaBH_4$ (spec. act. 10–20 Ci mmol^{-1}). Low molecular weight radioactive by-products of this reaction were removed by gel filtration on a Sephadex G-25 column (Pharmacia) eluted with 100 mM Tris-HCl, pH 7.8. Ci 25 fractions that contained [^{3}H]protein were combined and diluted to 4×10^6 cpm ml^{-1}. The protein concentration was adjusted to 2 mg ml^{-1} with unlabeled casein. The solution was divided into 0.1 ml portions and stored at −70°C. Twenty μl of [^{3}H]casein was mixed with buffer, insect midgut extract, and ten μl of 50 mM DTT to give a reaction volume of 0.1 ml contained in a 1.5 ml plastic centrifuge tube. The reaction was initiated by adding enzyme and quenched after 20 min at 37°C by adding 0.1 ml of 10% TCA. After standing on ice for 20 min, the samples were centrifuged at 14 000 rpm for 5 min and 0.175 ml aliquots of the supernatant were pipetted into counting vials containing 5 ml of scintillation fluid. Background levels of TCA-soluble radioactivity were determined by substituting an equal volume of buffer for the enzyme solution.

2.6. *Inhibitory activity of inhibitors*

Proteinase inhibitors were dissolved in either methanol or water at 10-times the desired concentration. Inhibitor solution (0.025 ml) was added to 0.225 ml of the enzyme in buffer. The solution was vortexed and incubated for 10 min at 37°C. Proteolytic activity was then assayed using 0.01 ml of the incubation mixture added to 0.05 ml of [^{3}H]casein and 0.04 ml 0.1 M Pipes buffer (pH 6.6).

2.7. *Protein concentration*

Protein concentrations were determined by the method of Smith et al. [10], using bovine serum albumin as a protein standard.

3. RESULTS

3.1. *Electrophoresis*

Electrophoresis in polyacrylamide gels containing gelatin as substrate revealed the presence of multiple

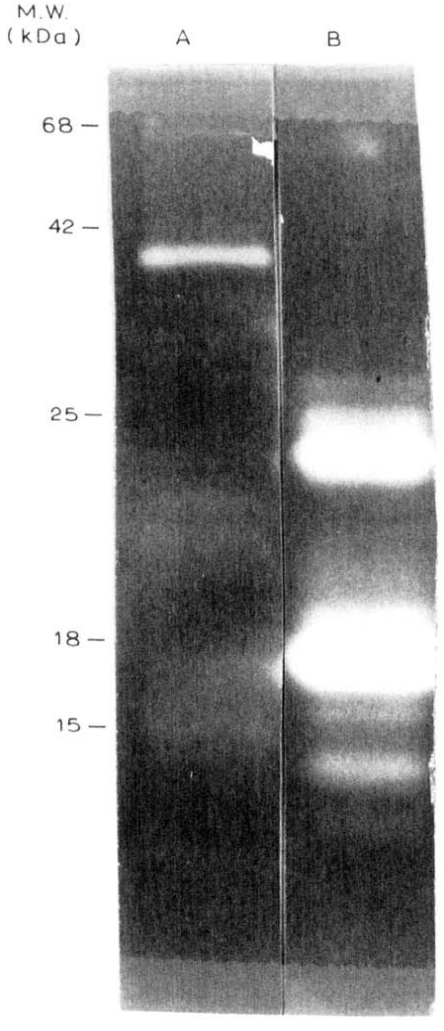

Fig. 1. Identification of proteinase activities of insect midgut extracts in polyacrylamide gel slabs containing gelatin. Gel slabs contain 11% polyacrylamide, 0.1% SDS, and 0.1% gelatin. (Lane A) rice weevil (approx. 1.84 μg protein), (lane B) red flour beetle (approx. 0.12 μg protein).

zones of proteinase activity in midgut extracts of rice weevil and red flour beetle (Fig. 1). Activity was substantially higher in the latter extract than in the former. The rice weevil midgut extract exhibited one major zone of proteolytic activity that corresponded to a protein with an apparent molecular mass of 40 kDa. Two major zones of proteolytic activity were observed in the red flour beetle midgut extract with apparent molecular masses of 20 kDa and 17 kDa. Minor red flour beetle proteinases had apparent molecular masses of 25 kDa, 19 kDa, and 14 kDa.

3.2. *pH optima*

The pH optima for caseinolytic activity were in the mildly acidic range: pH 6.8 for the rice weevil extract and pH 5.2 for the red flour beetle extract. Based on gut equivalents, the caseinolytic activity of the red flour

TABLE I

Effects of proteinase inhibitors on [^{3}H]casein digestion by midgut homogenates of rice weevil and red flour beetle

Inhibitor	Concentration (M)	Inhibition (%)	
		Rice weevil	Red flour beetle
E-64	1×10^{-5}	90	96
PCMS	1×10^{-3}	95	96
PCMS + DTT	$1 \times 10^{-3} + 5 \times 10^{-3}$	0	8
Antipain	1×10^{-5}	95	98
Oryzacystatin	2×10^{-7}	88	90
DFP	1×10^{-3}	0	9
SBTI	1×10^{-5}	0	4
CPTI	3×10^{-7}	0	10

*Ten μl of midgut extracts (approx. 9.2 μg protein for rice weevil and 3.2 μg protein for red flour beetle) were incubated first with inhibitors for 10 min and then with 20 μl of [^{3}H]casein (approx. 4000 cpm) in pH 6 buffer (0.1 M Na_2HPO_4, 2 mM EDTA, 5 mM DTT) for 30 min at 37°C. The percentage inhibition was calculated by comparing the results of control incubation with treatments containing various inhibitors. Data are the mean values of duplicate assays.

†The rice weevil gut preparation in the absence of DTT or inhibitors exhibited only 70% of the activity observed in the presence of DTT. Red flour beetle activity was stimulated 2-fold by DTT. All percent inhibition values were based on DTT-stimulated activity except for PCMS which was based on controls incubated in the absence of DTT.

beetle was approximately 25-times greater than that of the rice weevil.

3.3 *Inhibitor studies*

The inhibitor sensitivity profiles of the caseinolytic activities in the midgut extracts (Table I) suggested that the major proteolytic enzymes were cysteine proteinases. E-64 and PCMS are considered as diagnostic inhibitors for cysteine proteinases [11] and are highly effective against both midgut extracts. The addition of DTT rescued the caseinolytic activity from the effect of PCMS. The IC_{50} value for E-64 was 3×10^{-7} M for the rice weevil midgut extract. Serine proteinase inhibitors, such as DFP, SBTI, and CPTI, had no effect on the caseinolytic activity of the midgut extracts. Antipain, which inhibits both serine and cysteine proteinases [12], was inhibitory against both extracts.

Oryzacystatin was purified to homogeneity from Newbonnet rice (Fig. 2). It had an apparent molecular mass of 13 000 and properties similar to those reported previously [4]. Oryzacystatin fully inhibited the midgut proteolytic activities (Fig. 3). The IC_{50} values were estimated to be 4×10^{-7} M and 2×10^{-6} M for rice weevil and red flour beetle extracts, respectively.

5. DISCUSSION

Data obtained with low molecular weight inhibitors clearly indicated that proteinase activities in midgut extracts of rice weevil and red flour beetle are predominantly cysteine proteinases. These results agree with those of Murdock et al. [3], who first demonstrated that cysteine proteinases that are maximally active at mildly acidic pH are widespread among many species of Coleoptera including the red flour beetle. Serine proteinases may also be present, but these enzymes appear to be minor in the species examined here. However, in larvae and adults of the granary weevil, *Sitophilus granarius*, major proteinases were inhibited by SBTI and TLCK, indicating that a serine proteinase is functional in that species [13]. The novel aspect of our work is the finding that nearly all of the proteolytic activity against casein in midgut extracts of rice weevil

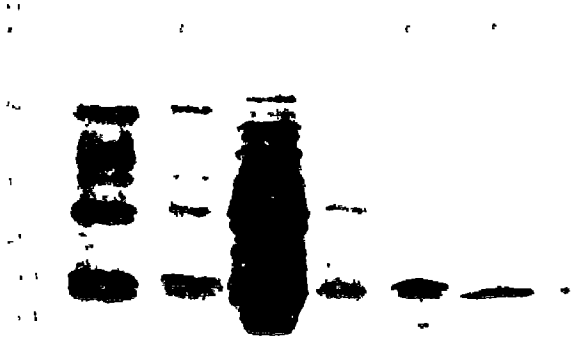

Fig. 2. SDS PAGE of oryzacystatin at different steps in the purification scheme. (Lane A) 0.15 M NaCl crude extract of rice seed flour. (Lane B) Soluble fraction after heating (75°C, 10 minutes) of 0.15 M NaCl extract. (Lane C) 30–65% saturated ammonium sulfate fraction of supernatant from heated extract. (Lane D) Fraction from Q-Sepharose column. (Lane E) Fraction from S-Sepharose column. (Lane F) Purified oryzacystatin from chromatofocusing column.

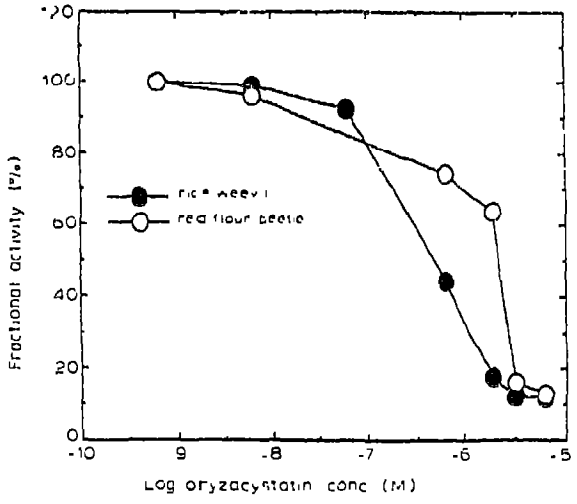

Fig. 3. Inhibition of midgut proteinase extracts from rice weevil and red flour beetle by oryzacystatin. Ten μl of midgut extracts (approx. 9.2 μg protein for rice weevil and 3.2 μg protein for red flour beetle) were incubated first with oryzacystatin for 10 min at 37°C and then with 20 μl of [^{3}H]casein (approx. 4000 cpm) in pH 6 buffer (0.1 M Na_2HPO_4, 2 mM EDTA, 5 mM DTT) for 30 min at 37°C. The percentage inhibition was calculated by comparing results of control incubation with treatments containing various amounts of oryzacystatin. Data are mean values of duplicate assays.

and red flour beetle larvae is inhibited by the plant proteinaceous inhibitor, oryzacystatin. To our knowledge this is the first demonstration of the effectiveness of a naturally occurring seed inhibitor towards insect midgut cysteine proteinases.

E-64 has been used previously in a similar manner to inhibit other insect midgut proteinases and also to demonstrate the vulnerability of two insect species to cysteine proteinase inhibitors. E-64 prolonged the developmental time and increased larval mortality of the cowpea weevil, *Callosobruchus maculatus*, in direct proportion to its concentration in artificial seeds at 0.01–0.25% by weight [14]. Consumption of E-64-treated potato leaves also delayed the growth and development of the Colorado potato beetle, *Leptinotarsa decemlineata* [15].

Cysteine proteinases are good target enzymes to inhibit for the purpose of insect control because higher animals do not utilize this class of enzyme for digestion. Oryzacystatin and other naturally occurring proteinaceous cysteine proteinase inhibitors could be manipulated by classical host plant resistance and genetic engineering approaches to create cereal varieties resistant to infestation by stored grain Coleoptera. A cDNA clone for oryzacystatin has already been isolated [16] and efforts to transform plants with cysteine proteinase inhibitor genes are underway.

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