



Luminal Proteinases From *Plodia interpunctella* and the Hydrolysis of *Bacillus thuringiensis* CryIA(c) Protoxin

BRENDA OPPERT,*†§ KARL J. KRAMER,*† DONOVAN JOHNSON,* STEVE J. UPTON,‡ WILLIAM H. MCGAUGHEY*

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The ability of proteinases in gut extracts of the Indianmeal moth, *Plodia interpunctella*, to hydrolyze *Bacillus thuringiensis* (Bt) protoxin, casein, and ρ -nitroanilide substrates was investigated. A polyclonal antiserum to protoxin CryIA(c) was used in Western blots to demonstrate slower protoxin processing by gut enzymes from Bt subspecies *entomocidus*-resistant larvae than enzymes from susceptible or *kurstaki*-resistant strains. Enzymes from all three strains hydrolyzed *N*- α -benzoyl-L-arginine ρ -nitroanilide, *N*-succinyl-ala-ala-pro-phenylalanine ρ -nitroanilide, and *N*-succinyl-ala-ala-pro-leucine ρ -nitroanilide. Zymograms and activity blots were used to estimate the apparent molecular masses, number of enzymes, and relative activities in each strain. Several serine proteinase inhibitors reduced gut enzyme activities, with two soybean trypsin inhibitors, two potato inhibitors, and chymostatin the most effective in preventing protoxin hydrolysis. Published by Elsevier Science Ltd

Insect digestive proteinase *Plodia interpunctella* *Bacillus thuringiensis* Proteinase inhibitor Protoxin activation Microbial insecticide

INTRODUCTION

The Indianmeal moth, *Plodia interpunctella*, is a major pest of stored products and is the first species reported to exhibit resistance to microbial insecticides from *Bacillus thuringiensis* (Bt) (McGaughey, 1985). Several laboratory-reared strains of this insect have been selected for resistance to Bt toxins (McGaughey, 1985; McGaughey

and Johnson, 1992, 1994). Although resistance to Bt in a *kurstaki*-resistant strain of this species has been explained by reduced toxin binding to the gut membrane (Van Rie *et al.*, 1990), other resistance mechanisms are possible. Since proteolytic enzymes are involved in the dissolution and activation of Bt protoxins, we wanted to determine whether a lack of gut proteinase activity might be a contributing factor to the insect's ability to avoid the toxicity of Bt.

Luminal proteinases are involved in the activation and/or solubilization of Bt crystal protoxins, thereby mediating Bt toxicity. Enzymes involved in protoxin activation/degradation have been described as trypsin- or chymotrypsin-like (Lecadet and Dedonder, 1967; Tojo and Aizawa, 1983; Milne and Kaplan, 1993). A chymotrypsin-like proteinase from larval intestinal extracts of the silkworm, *Bombyx mori*, is involved in the dissolution of Bt. subsp. *kurstaki* HD-I crystals (Tojo *et al.*, 1986). In the mosquito, *Culex quinquefasciatus*, a trypsin-like proteinase is apparently necessary for parasporal inclusion processing, while chymotrypsin- and thermolysin-like proteinases are apparently involved in processing of solubilized CryIVD protoxin (Dai and Gill, 1993).

Most Lepidoptera have highly alkaline midguts with a preponderance of serine proteinase activity (Ahmad *et*

Abbreviations: BSA, bovine serum albumin Bt, *Bacillus thuringiensis* pNa, ρ -nitroanilide; E-64, *N*-[*N*-(3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine EDTA, ethylenediamine tetraacetic acid HRP, horseradish peroxidase BApNa, *N*- α -benzoyl-L-arginine pNa PMSF, phenylmethylsulfonyl fluoride SAAPFpNa, *N*-succinyl-ala-ala-pro-phenylalanine pNa SAAPLpNa, *N*-succinyl-ala-ala-pro-leucine pNa SBBTI, soybean Bowman-Birk trypsin inhibitor SKTI, soybean Kunitz trypsin inhibitor PI-I, potato inhibitor I PI-II, potato inhibitor II TLCK, L-1-chloro-3-(4-tosylamide)-7-amino-2-heptanone TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone.

*U.S. Grain Marketing Research Laboratory, ARS-USDA, 1515 College Ave, Manhattan, KS 66502-2736, USA

†Department of Biochemistry, Kansas State University, Manhattan, KS 66506-3702, USA

‡Division of Biology, Kansas State University, Manhattan, KS 66506-3702, USA

§Author for correspondence: Tel. 913-776-2780, Fax 913-537-5584, Email bso@ksu.ksu.edu

al., 1976; Applebaum, 1985; Dow, 1986). Several studies have examined the properties of *Plodia* proteinases in gut extracts. Maximum casein digestion occurs at an alkaline pH > 9.0 (Johnson *et al.*, 1990; Christeller *et al.*, 1992). Major gelatin proteolytic activity is associated with multiple enzymes having apparent molecular masses of 20–50 kDa, as determined by zymography (Johnson *et al.*, 1990). Results of experiments using class-specific substrates and diagnostic inhibitors indicate that major *Plodia* enzymes are similar to trypsin and chymotrypsin (Johnson *et al.*, 1990; Christeller *et al.*, 1992).

We have previously described a substantially reduced soluble proteinase activity in gut extracts from a strain of the *P. interpunctella* that is resistant to toxins from Bt subsp. *entomocidus* (Oppert *et al.*, 1994). This observation was in contrast to the more efficient protoxin processing observed using extracts from susceptible and *kurstaki*-resistant strains of *P. interpunctella*. In that study, electrophoretic analyses of products obtained from the hydrolysis of protoxin by gut extracts were complicated by the presence of comigrating gut proteins. Therefore, we developed a polyclonal antiserum to CryIA(c) (HD-73) for use as a Bt protein-specific probe in Western blotting analyses of the products from the processing of Bt protoxin by *Plodia* gut proteinases. In this study, we demonstrate differential protoxin processing by gut extracts from three *Plodia* strains using a Bt toxin antiserum in Western blots. We have also evaluated the substrate specificity of enzymes from the three strains using the hydrolysis of several p -nitroanilide (pNA) substrates. Substrates hydrolyzed by gut enzymes were also used in activity blots and the results compared to zymogram analyses. In addition, class-specific diagnostic inhibitors were tested for effects on substrate or protoxin hydrolysis by enzymes from the Bt-susceptible strain of the Indian-meal moth.

MATERIALS AND METHODS

Materials

Substrates and inhibitors were obtained from Sigma, Boehringer Mannheim, or CalBiochem. Porcine pancreatic trypsin, bovine pancreatic α -chymotrypsin, and porcine pancreatic elastase were from Sigma. Precast gels, electrophoresis buffers, and molecular markers were from Novex. Bovine serum albumin (BSA) was from Research Products International. Anti-rat IgG conjugated to horseradish peroxidase (HRP) was from Sigma. The HRP developing kit was from Biorad. Supported nitrocellulose (BA-S) was from Schleicher and Schuell. Microwell plates were obtained from Continental Laboratory Products. Software for kinetic enzyme analysis, KinetiCalc, was obtained from Bio-Tek Instruments. Statistical analyses were performed using SYSTAT.

Insects

P. interpunctella were reared as previously described (McGaughey and Beeman, 1988). Strains used in this

study were 1) Bt susceptible-RC688^s; 2) Bt resistant-198^r, which are RC688^s insects selected for resistance to isolate HD-198 (Bt subsp. *entomocidus*); and 3) Bt resistant-Dpl^r, which are RC688^s insects selected for resistance to HD-1 (Dipel®; Bt subsp. *kurstaki*) (McGaughey and Johnson, 1992).

Protoxin

Bt subsp. *kurstaki* HD-73 was grown on glucose-yeast extract-salts medium at 30°C for 2–3 days with agitation, until sporulation was complete (Nickerson *et al.*, 1974). Crystals were purified by density gradient centrifugation (Ang and Nickerson, 1978). Crystals were washed with water and solubilized in 200 mM sodium carbonate, pH 10.0, 1.6 M NaCl, 20 mM ethylenediamine tetraacetic acid (EDTA), and 40 mM β -mercaptoethanol by grinding in a glass tissue homogenizer on ice. After spinning out insoluble material at 15 000 $\times g$ for 5 min, the supernatant was added to glycerol (1:1) and then stored at –20°C.

Antiserum

Approximately 50 mg of unpurified trypsin-activated CryIA(c) (HD-73) in phosphate buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 0.9% sodium chloride) mixed with Freund's complete adjuvant was injected subcutaneously into adult male outbred rats (Harlan Sprague-Dawley). Toxin in Freund's incomplete adjuvant was injected 2 weeks later, and toxin only injected 2 weeks after that. Four days following the last injection, animals were tail bled and the serum was collected. Following centrifugation, antiserum was aliquoted 1:1 in glycerol and stored at –20°C.

Insect gut extracts

Late fourth instars were chilled, and the posterior and anterior ends were removed. Guts were excised with forceps, immediately submerged in ice-cold 200 mM Tris, pH 8.0, 20 mM CaCl₂ (buffer A), aliquoted 5 per 50 μ l buffer, and frozen at –20°C until use. Samples were quick-thawed by spinning at 15 000 $\times g$ for 2 min, and the supernatant containing soluble gut proteinases was used in assays.

Enzyme assays

With pNA substrates, 50 μ l of gut extract diluted 1:100 in buffer A or selected mammalian enzymes was incubated with serially diluted substrates at 37°C in a microwell plate. Absorbance was monitored at 405 nm at 15 s intervals over a 5 min period. For gut enzymes, activities were compared using the change in absorbance per min per ml of gut extract. For mammalian enzymes, activities were given as the change in absorbance per min per mg protein (trypsin and chymotrypsin) or per unit (elastase). In gut enzyme inhibition assays, inhibitors were preincubated in microwell plates with extract for 30 min at room temperature prior to assay. IC₅₀ and IC₁₀₀ values were estimated using linear regression of the natu-

ral logarithm of the concentration of the inhibitor. Protoxin hydrolysis was evaluated using immunoblots (see below).

Immunoblots

Samples containing 2 μ l of gut extracts (approximately 6–8 μ g total protein) or mammalian trypsin (approximately 2 μ g) with or without inhibitors and 2 μ l of CryIA(c) protoxin (5 μ g) were incubated in 50 μ l total volume of buffer A at 37°C for the designated time. Reactions were stopped by incubating at 95°C for 5 min. After cooling to room temperature, reducing sample buffer was added and samples were again heated at 95°C for 5 min. Twenty microliter aliquots were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) on 8% gels and electroblotted onto nitrocellulose. After washing in 10 mM Tris, pH 7.5, 50 mM NaCl, 2 mM EDTA (buffer B), blots were incubated in buffer C (2% BSA in buffer B).

Blots were then incubated in buffer C containing Bt-anti-serum at a dilution of 1:1000. After washing with buffer B, blots were incubated with anti-rat IgG conjugated to HRP diluted 1:5000 in buffer C, washed as above, and finally incubated with HRP substrate to reveal blue immunoreactive bands. Regression analysis of the relative mobility of molecular markers was used to estimate apparent molecular masses.

Zymograms

Gut proteins (5 μ g per lane) were subjected to SDS-PAGE on 12% non-reducing gels and processed for zymogram analysis as described by García-Carreño *et al.* (1993). Gels were preincubated at 4°C with substrate in buffer A for 30 min, and then in the same solution at 25°C for 2 h. Gels were rinsed several times, stained with Coomassie brilliant blue, and destained for visualization of clear areas corresponding to active enzymes.

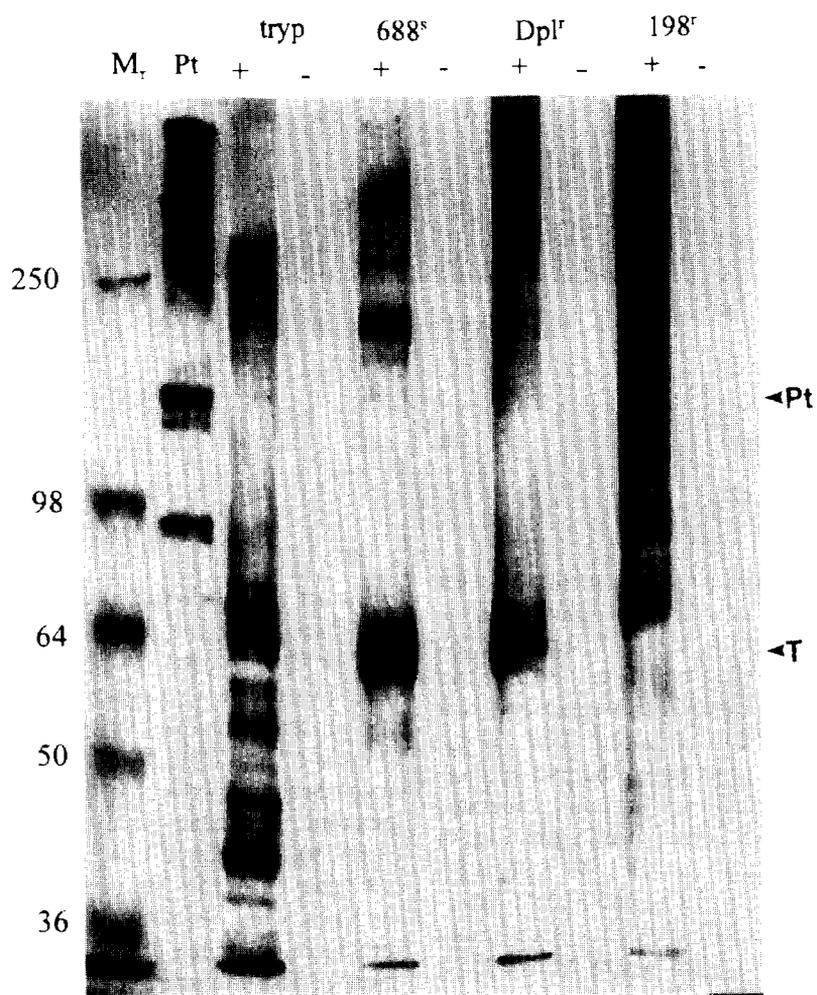
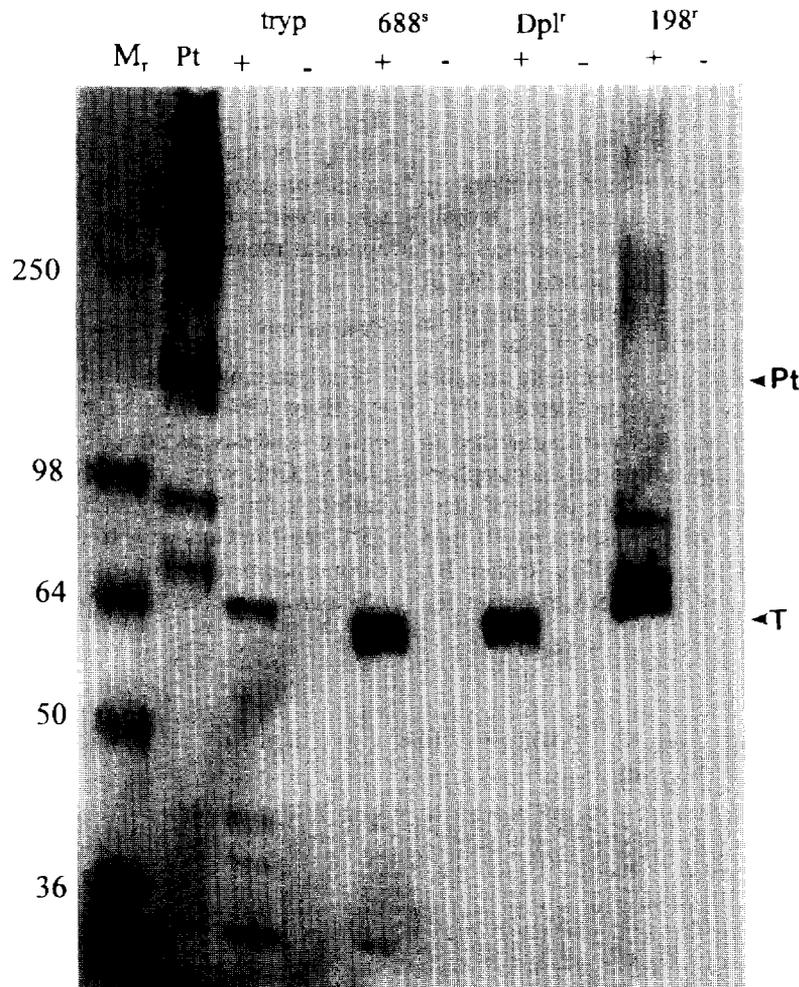


FIGURE 1. Immunoblot of CryIA(c) protoxin (Pt) or protoxin incubated with 2 μ g porcine trypsin or 2 μ l soluble gut enzymes (approximately 0.2 gut equivalents) from *Plodia interpunctella* strains 688^s, Dpl^r, or 198^r. Incubations of enzymes only (-) contained no protoxin. Incubations were stopped at a) 15 min or b) 90 min. Arrows denote the predicted migration of the protoxin (P+~135 kDa) and toxin (T~60 kDa) proteins.

FIGURE 1. *Continued.*

Activity blots

Enzymes capable of hydrolyzing pNA substrates were identified using a modified procedure of Ohlsson *et al.* (1986). Nitrocellulose blots of SDS-PAGE-separated gut proteins (10 μ g per lane) were incubated in 5 ml of buffer A containing 0.5 mg/ml pNA substrate for 1 h at room temperature with gentle agitation. Use of the Econoblot system (Lablogix) facilitated the use of small volumes of substrate containing buffers. Liberated nitroanilide was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% *N*-(1-naphthyl)-ethylenediamine in 47.5% ethanol. Membranes were placed in heat-sealed bags and stored at -20°C .

Protein assays

Protein concentration was determined by the dye-binding method of Bradford (1976) using BSA as the standard.

RESULTS

Use of a Bt toxin antiserum in Western blots revealed that *Plodia* 198^r enzymes were much less effective than 688^s or Dpl^r enzymes in hydrolyzing protoxin CryIA(c) (Fig. 1). After a 15 min incubation, enzymes from 688^s or Dpl^r strains almost completely processed the protoxin to a toxin form (Fig. 1a). After a 90 min incubation, the major protein products with 688^s or Dpl^r enzymes had apparent molecular masses of approximately 60 kDa (Fig. 1b). A different proteolytic pattern was produced with enzymes from strain 198^r. Many hydrolytic intermediates were observed after 15 min, and proteolysis remained incomplete even after a 4 h incubation (data not shown). The antiserum was specific for protoxin-derived proteins because none of the endogenous soluble gut proteins cross-reacted with the antiserum (- lanes). Mammalian trypsin was not as effective in hydrolyzing the protoxin as were enzymes from 688^s or Dpl^r in a 15 min incubation, but after 90 min the electrophoretic pattern

was similar to those obtained using 688^s and Dpl^r enzymes.

Zymograms using 2% casein or 1% Bt protoxin were used for both quantitative and qualitative analyses of soluble gut enzymes from strains 688^s and 198^r (Fig. 2). In the gut of 688^s insects, major caseinolytic enzymes had apparent molecular masses of approximately 25, 27, 29, 33, 38 and 43 kDa. All but the 43 kDa enzyme hydrolyzed CryIA(c) protoxin. The total enzyme activity was much less in extracts from the 198^r strain, with relatively low caseinolytic activity exhibited only by 35, 39, and 43 kDa enzymes. There was no detectable protoxin hydrolysis by 198^r enzymes.

The activity of soluble gut enzymes from the three *Plodia* strains was compared using several *p*-nitroanilide (pNA) substrates in a microwell plate assay. Enzymes from all three strains hydrolyzed *N*α-benzoyl-L-arginine pNA (BAPNA) (Fig. 3a), *N*-succinyl-ala-ala-pro-phe pNA (SAAPFPNA) (Fig. 3b), and *N*-succinyl-ala-ala-pro-leu pNA (SAAPLPNA) (Fig. 3c) to varying degrees.

These substrates are selectively hydrolyzed by trypsin-, chymotrypsin-, and elastase-like enzymes, respectively (Nagel *et al.*, 1965; Del Mar *et al.*, 1979, 1980). In the susceptible strain, chymotrypsin-like activity was predominant, followed by elastase- and trypsin-like activities. In strain Dpl^r, chymotrypsin- and elastase-like activities were similar and greater than the trypsin-like activity. Hydrolysis of all substrates was substantially less with enzymes from 198^r. In data not shown, soluble gut enzymes from all three stains were unable to hydrolyze *N*-succinyl-phenylalanine pNA (a substrate diagnostic for chymotrypsin-like enzymes), *N*-succinyl-ala-ala-pNA and *N*-succinyl-ala-ala-val-pNA (both diagnostic for elastase-like enzymes), and leucine pNA (diagnostic for leucine aminopeptidase-like enzymes).

Substrate-specific hydrolysis was compared in the three strains using activity blot analysis (Fig. 4). Two BAPNA-hydrolyzing enzymes had apparent molecular masses of 27 and 39 kDa in strain 688^s and 30 and 39 kDa in strain Dpl^r (Fig. 4a). Higher molecular mass enzymes (>100 kDa) were also observed in both strains. Only weak BAPNA activity was associated with a protein of approximately 34 kDa in the 198^r extract. SAAPFPNA-hydrolyzing enzymes had apparent molecular masses of approximately 30 kDa in strain 688^s and 33 kDa in strain Dpl^r, in addition to several higher molecular mass forms (Fig. 4b). While no major bands of SAAPFPNA hydrolyzing activity were observed with 198^r enzymes, a trace level of activity was apparent with enzymes of approximately 40 and 100 kDa. When SAAPLPNA was used as the substrate, a 688^s enzyme was detected at 30 kDa, whereas Dpl^r enzymes were detected at 34, 38, 46, and 49 kDa. Both extracts exhibited larger enzymes with molecular masses >100 kDa (Fig. 4c). No SAAPLPNA hydrolyzing activity was detected in the 198^r extract.

Class-specific inhibitors of proteolytic enzymes were used to further characterize gut enzyme activity from the susceptible strain of *Plodia* (Fig. 5). Of the low molecular mass inhibitors tested, leupeptin and antipain, which inhibit trypsin and papain (Aoyagi *et al.*, 1969; Suda *et al.*, 1972), were the most effective against BAPNA hydrolysis with IC₅₀ values of 2.7 pM and 0.2 μM, respectively (Fig. 5a). Chymostatin and TLCK, which are specific inhibitors of chymotrypsin- and trypsin-like proteinases, and E-64, a specific inhibitor of cysteine proteinases, also reduced 688^s hydrolysis of BAPNA. Benzamide was inhibitory at millimolar concentrations, while PMSF and APMSF were only slightly inhibitory at a concentration of 10 mM.

High molecular mass inhibitors that reduce both trypsin- and chymotrypsin-like activities, such as soybean Bowman-Birk trypsin inhibitor (IC₅₀≅0.1 μM), were the most effective in inhibiting 688^s hydrolysis of BAPNA (Fig. 5b). Aprotinin, soybean Kunitz trypsin inhibitor, and potato inhibitors I and II, which inhibit both trypsin- and/or chymotrypsin-like enzymes, also reduced BAPNA hydrolysis. Hen's egg white cystatin was similar to E-64

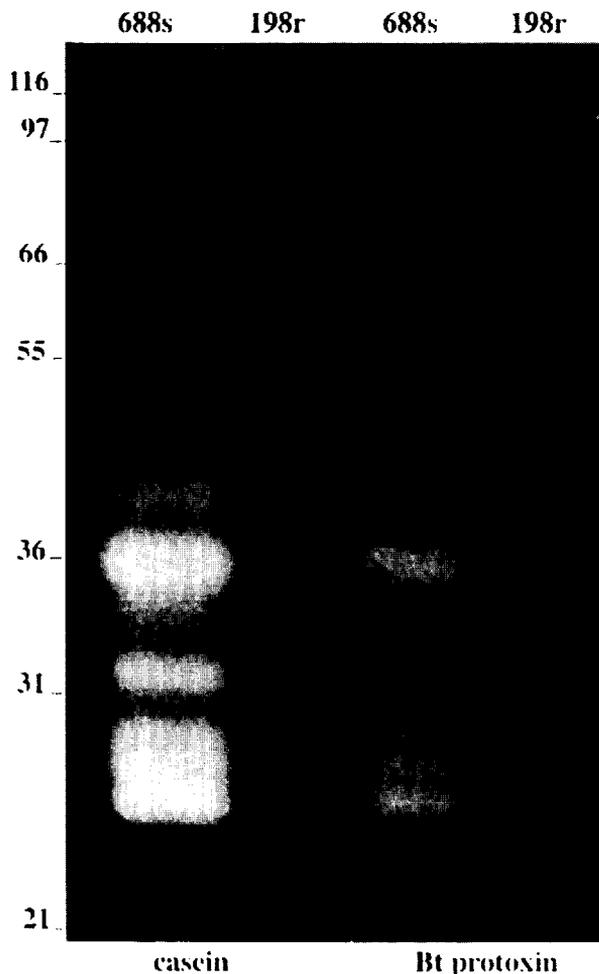


FIGURE 2. Zymogram of 5 μg soluble gut protein from *Plodia* strains 688^s or 198^r subjected to electrophoresis using 12% SDS polyacrylamide gels and incubated with 2% casein or 1% Bt protoxin CryIA(c).

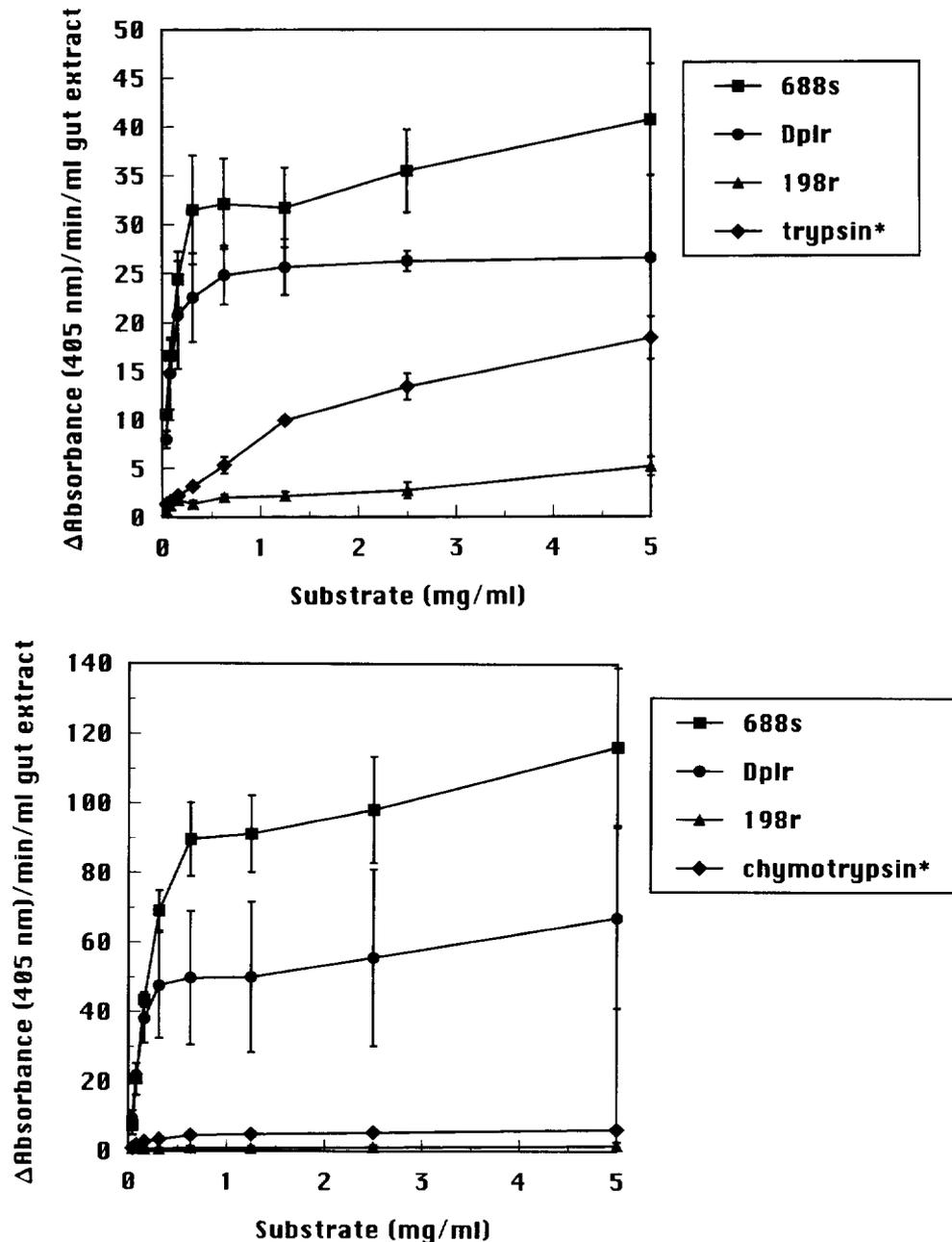
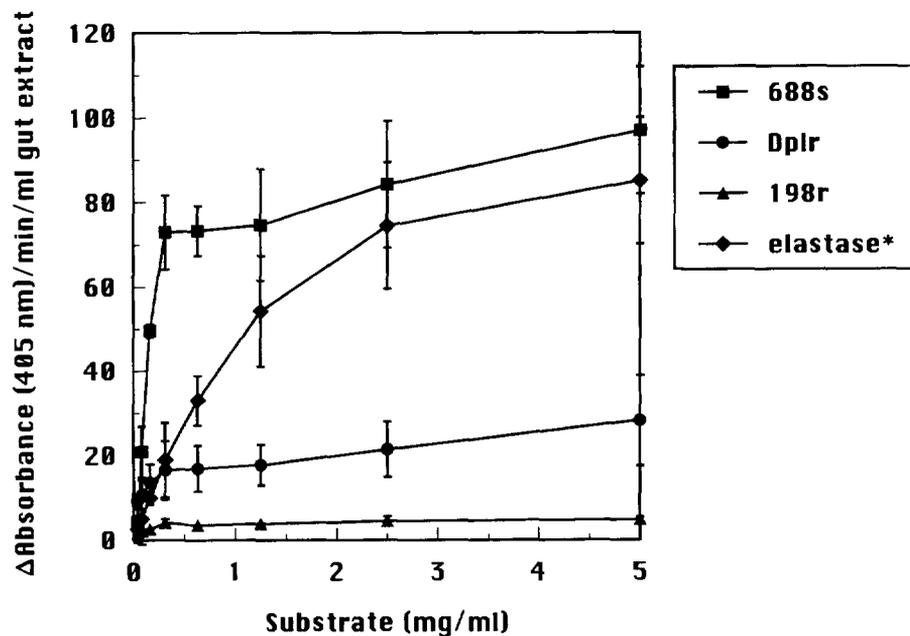


FIGURE 3. Enzyme assays using *Plodia* gut or mammalian enzymes incubated with serially diluted substrates: a) BApNA, b) SAAPFpNA, and c) SAAPLpNA. Mean values were obtained from 2–4 separate dissections. *Units of mammalian enzymes are in $\Delta A_{405}/\text{min}/\text{mg}$ (trypsin and chymotrypsin) or $\Delta A_{405}/\text{min}/\text{unit}$ (elastase).

in its inhibition of 688^s gut proteinase activity. Inhibitors that did not affect BApNA hydrolysis by gut enzymes included EDTA, bestatin, iodoacetamide, TPCK, elastatinal, calpain inhibitor I, and leech elastase inhibitor (data not shown). When an extract from 198^r guts was preincubated with 688^s gut enzymes, the BApNA hydrolytic activity was additive (data not shown), indicating that 198^r guts did not contain an endogenous inhibitor of 688^s gut enzymes and vice versa.

Inhibitors were also tested for their effect on

SAAPFpNA hydrolysis by 688^s gut enzymes (Fig. 5c). The most effective inhibitors were PI-I and PI-II, with IC_{50} values of 0.5 and 0.9 μM , respectively. Both of the soybean trypsin inhibitors and chymostatin were also inhibitory to SAAPFpNA hydrolysis at micromolar levels. Leech elastase inhibitor and PMSF were inhibitory at millimolar concentrations. Inhibitors that had no effect on SAAPFpNA hydrolysis were antipain, aprotinin, TPCK, TLCK, E-64, leupeptin, pepstatin, cystatin, EDTA, iodoacetamide, bestatin, elastatinal, benzamide, and extracts from 198^r guts (data not shown).

FIGURE 3. *Continued.*

Potato inhibitors I and II were also the most effective in reducing SAAPLpNA hydrolysis by 688^s gut enzymes, with IC₅₀ values of 0.7 and 0.5 μ M, respectively (Fig. 5d). The soybean trypsin inhibitors and chymostatin were also effective in reducing SAAPLpNA hydrolysis at micromolar concentrations, and leech elastase inhibitor and PMSF were inhibitory at millimolar concentrations. These results were similar to those obtained using SAAPFpNA as the substrate, and the same inhibitors that had no effect on SAAPFpNA hydrolysis were also ineffective in preventing SAAPLpNA hydrolysis.

Selected inhibitors were preincubated with gut enzymes from strain 688^s at a concentration that was estimated to give 100% inhibition of BApNA hydrolysis and evaluated by Western blot analysis for their ability to inhibit the hydrolysis of CryIA(c) protoxin (Fig. 6). Without inhibitors in the incubation mixture, 688^s enzymes degraded the protoxin to a protein of apparent molecular mass of 65 kDa. With the inhibitors antipain or leupeptin, the major products were proteins ranging in mass from 65–90 kDa. Inhibitors that were most effective in preventing toxin generation included chymostatin, PI-I, PI-II, SKTI, and SBBTI. Chymostatin was the best inhibitor based on the amount of protoxin (~135 kDa) that survived the incubations. All of the inhibitors tested slowed the rate of protoxin hydrolysis and resulted in the appearance of intermediate products ranging in apparent molecular masses from 70 to 90 kDa.

DISCUSSION

A polyclonal antiserum generated using activated CryIA(c) toxin reacted with protoxin, toxin, and inter-

mediate products produced during protoxin hydrolysis and was useful in monitoring the progression of protoxin activation and degradation. The antiserum was used in Western blot analyses of protoxin treated with various proteinases to illustrate that enzymes from *Plodia* strain 198^r were substantially less efficient in generating the toxin than enzymes from the other two strains. Isolation of the toxin products resulting from the incubations, followed by insect bioassay and *in vitro* binding studies, will be necessary to determine if and how these proteins differ in toxicity and receptor binding.

When the hydrolysis of several pNA substrates by *Plodia* gut enzymes were compared, the major proteolytic activities in extracts from the susceptible strain were similar to chymotrypsin- elastase-, and trypsin-like enzymes. The substrate specificities of chymotrypsin and elastase are similar because enzymes from the two strains comigrated to 30 kDa in the activity blots using SAAPFpNA and SAAPLpNA as the diagnostic substrates. Similar results were also obtained when surveying for inhibitor effects on the hydrolysis the substrates of SAAPFpNA and SAAPLpNA. This result was not unexpected, since mammalian α -chymotrypsin and elastase are less specific than trypsin for their respective substrates. Based on the fact that chymotrypsin-specific inhibitors were more effective in reducing SAAPFpNA and SAAPLpNA hydrolyses than were elastase inhibitors, this *Plodia* enzyme is probably more chymotrypsin-like in specificity. All pNA activities were highest in the susceptible strain, slightly reduced in the *kurstaki*-resistant strain, and substantially reduced in the *entomocidus*-resistant strain.

In general, inhibitors of serine proteinase activity were

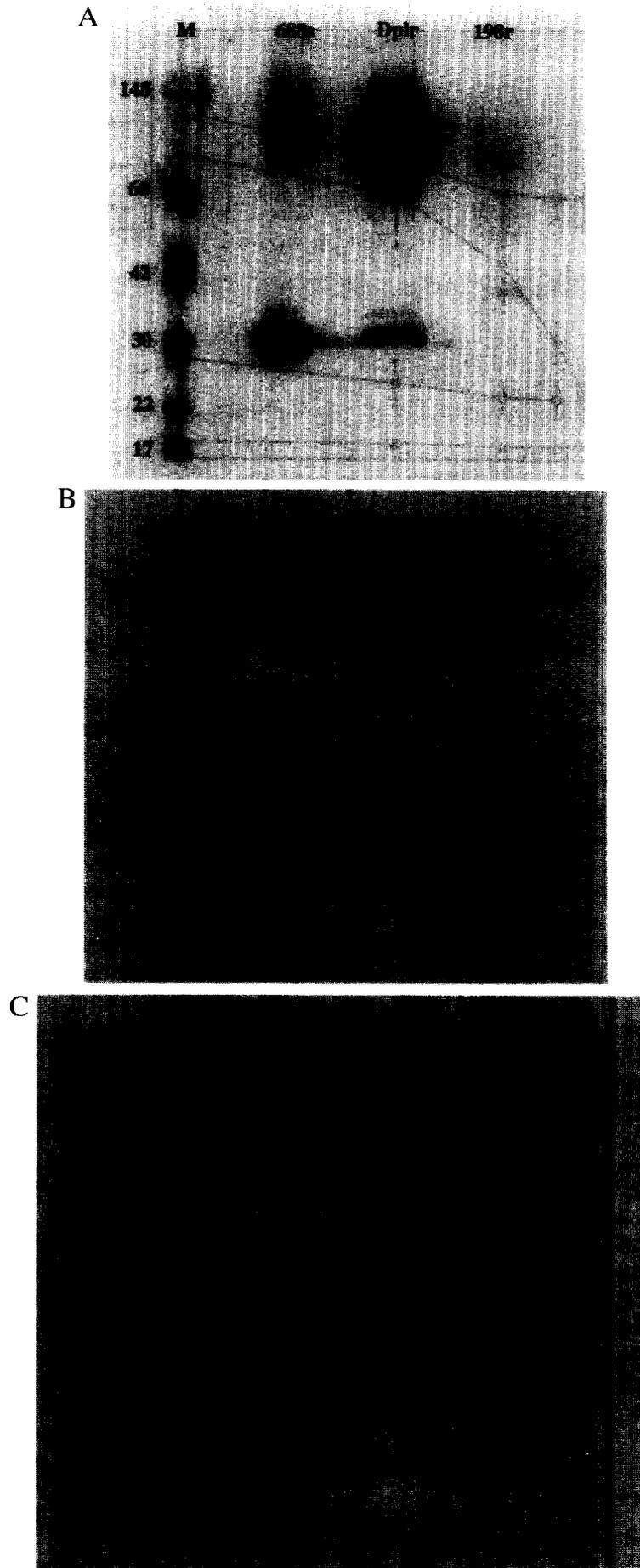


FIGURE 4. Activity blots of *Plodia* gut enzymes. Soluble gut enzymes from strains 688⁺, Dpl⁺, and 198⁺ were subjected to SDS-PAGE, transferred to nitrocellulose, incubated with pNA substrates, and diazotized to observe free nitroanilide. a) BApNA, b) SAAPFpNA, and c) SAAPLpNA.

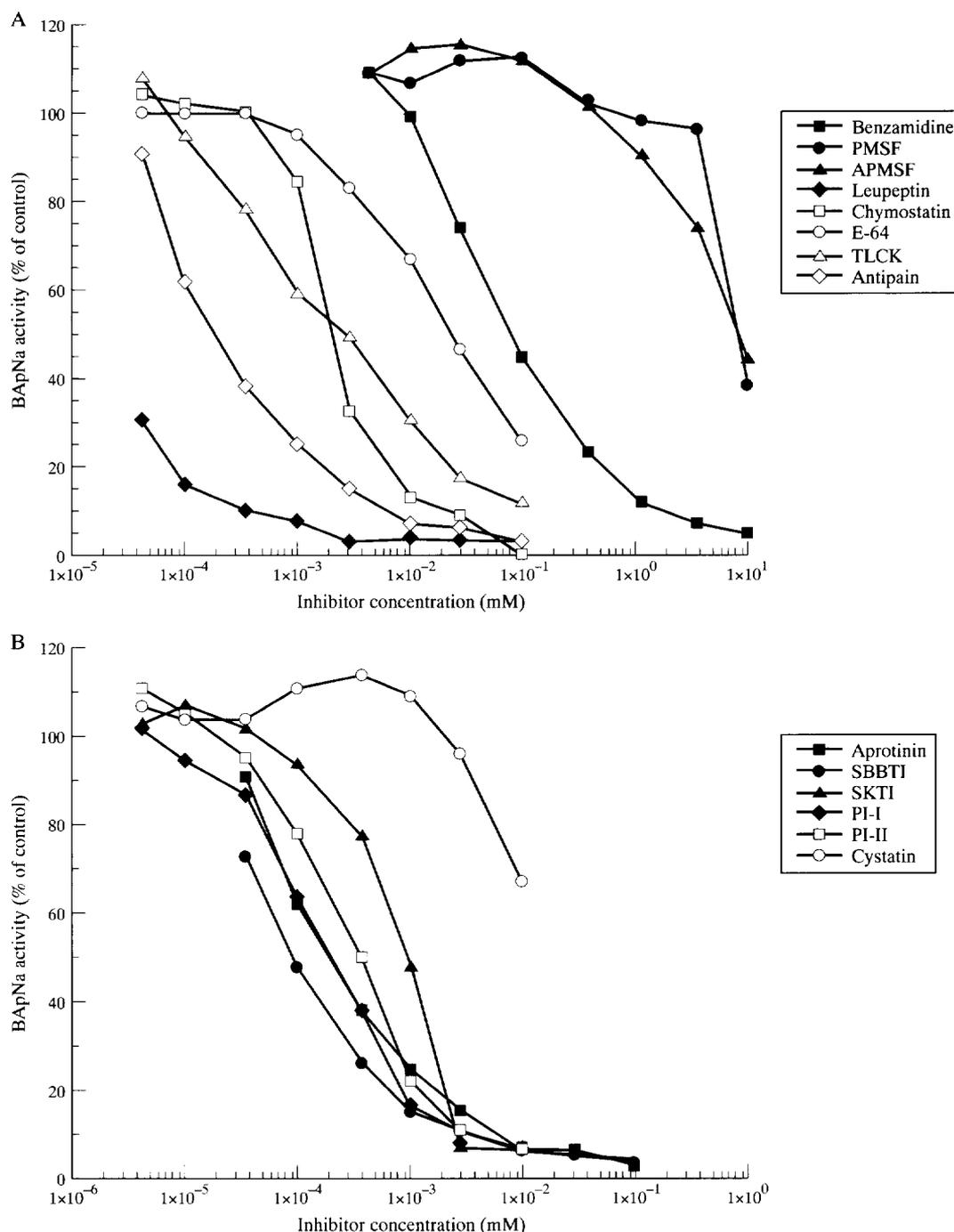
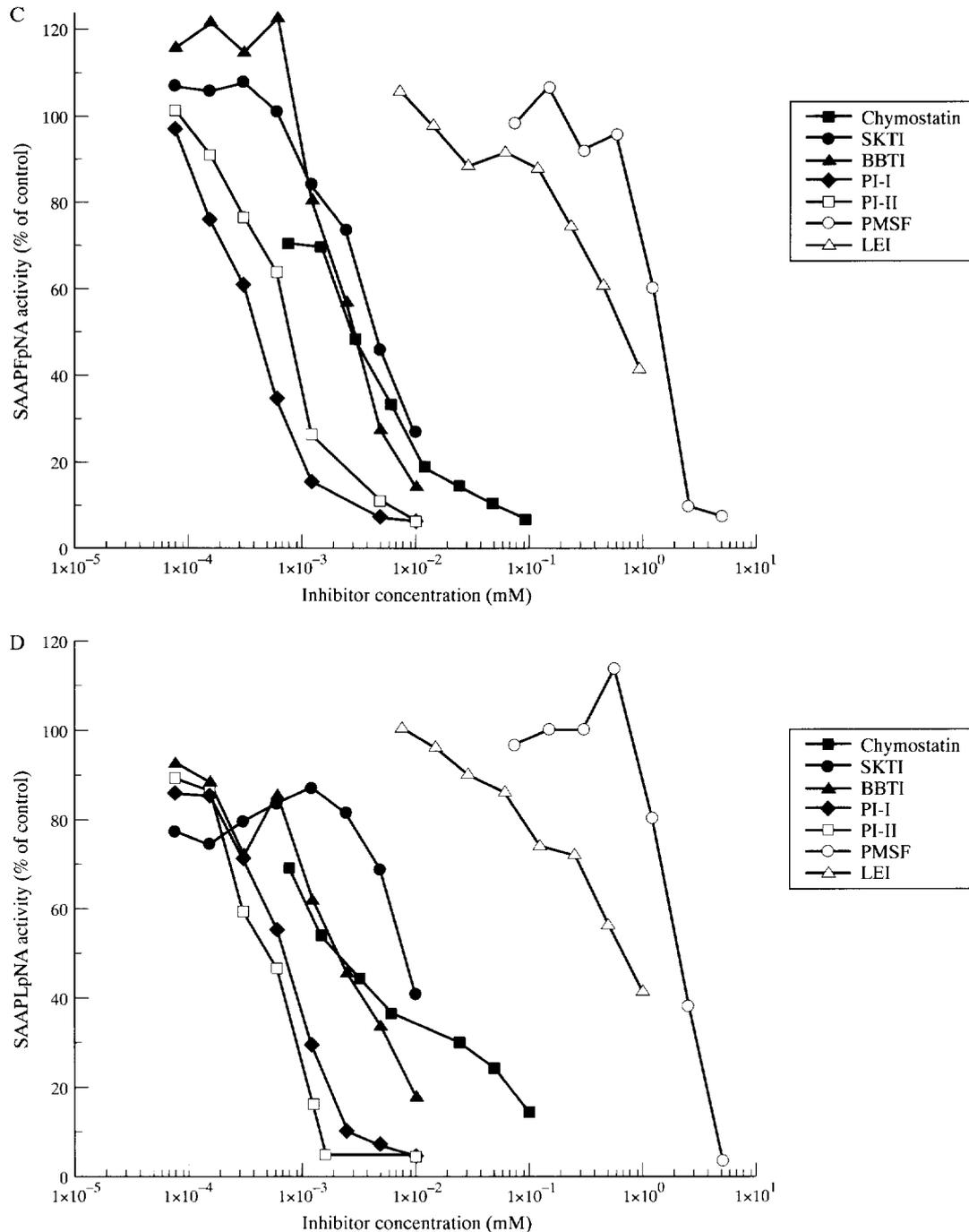


FIGURE 5. The effect of proteinase inhibitors on the hydrolysis of BApNA by 688s gut enzymes. Inhibitors were preincubated with gut extracts, and after addition of substrate absorbance at 405 nm was monitored for 5 min. Rates were calculated using the program KinetiCalc. Data points represent single determinations. a) Low molecular mass inhibitors, BApNA; b) High molecular mass inhibitors, BApNA; c) SAAPFpNA; d) SAAPLpNA.

good inhibitors of 688^s gut enzyme activity. Based on the results obtained using enzyme-specific substrates, the most effective inhibitors of BApNA hydrolysis were leupeptin, antipain, and SBBTI; those effective against SAAPFpNA and SAAPLpNA hydrolysis were the potato inhibitors I and II. A number of other trypsin- or chymotrypsin-specific inhibitors reduced 688^s proteinase

activity. Cysteine proteinase inhibitors were only slightly inhibitory and appeared to target trypsin-like enzyme activity. In general, inhibitors specific for trypsin- or chymotrypsin-like enzymes were the most effective in inhibiting soluble gut proteinases from *Plodia* larvae.

When CryIA(c) protoxin was used as the substrate, chymostatin, SBBTI, SKTI, and the potato inhibitors

FIGURE 5. *Continued.*

were most effective in reducing protoxin hydrolysis by 688^s gut enzymes, causing incomplete hydrolysis of the protoxin. Leupeptin, a potent inhibitor of trypsin-like enzymes, was not as effective in preventing protoxin activation as chymostatin, an inhibitor of chymotrypsin-like enzymes. These data indicate that, although both might play a role, chymotrypsin-like enzymes appear to be more important than trypsin-like enzymes in the activation of Bt protoxin.

Insect enzymes are likely to be unique in their interac-

tions with substrates and inhibitors. Although *Plodia* enzymes effectively hydrolyzed selected chymotrypsin and elastase substrates, they were unable to hydrolyze others. *Plodia* enzymes did not hydrolyze some elastase substrates, and elastatinal was not inhibitory to *Plodia* enzyme activity. *N*-Succinyl-phe-pNA is reported to be a specific substrate for chymotrypsin-like enzymes (Nagel *et al.*, 1965), but we were unable to hydrolyze this substrate, even with mammalian chymotrypsin (data not shown). Although E-64 and cystatin reduced gut



FIGURE 6. Immunoblot of CryIA(c) protoxin (Pt) and 688s gut enzyme-activated toxin without (T) or with inhibitors, as indicated. Incubations were stopped after 1 h. Inhibitors used were: PI-II, potato inhibitor II; PI-I, potato inhibitor I; SBBTI, soybean Bowman-Birk trypsin inhibitor; SKTI, soybean Kunitz trypsin inhibitor; CHY, chymostatin; AP, antipain; LP, leupeptin. Concentrations of inhibitors used were those resulting in 100% inhibition of BApNA hydrolysis and were estimated from regression analysis of data shown in Fig. 4.

enzyme activity toward BApNA, iodoacetamide had no effect. Previous work based on inhibitor effects suggested the presence of a minor cysteine proteinase activity in the *Plodia* gut (Chen *et al.*, 1992). However, our results demonstrated inhibition of *Plodia* trypsin-like activity by E-64. E-64 also reduced trypsin-like activity from the armyworm, *Spodoptera littoralis*, and it was suggested that a cysteine residue is present in the active site (Lee and Anstee, 1995).

Using casein or CryIA(c) protoxin as a substrate for *Plodia* enzymes, we identified at least five proteinases with apparent masses between 20 and 50 kDa in the gut of Bt susceptible insects. Soluble enzyme activity was substantially reduced in the 198^r strain, with caseinolytic activity observed in only three enzymes with apparent masses of 35, 39, and 43 kDa. Gut extracts from the 198^r strain produced no detectable activity bands in zymogram analysis when Bt was used as the substrate.

Based on activity blots with pNA substrates, 688^s and Dpl^r extracts appear to have BApNA hydrolyzing enzymes with apparent masses of 27–30 kDa and 39 kDa. Apparently, these enzymes are distinct from those that hydrolyzed the succinylated substrates. The smallest enzymes that hydrolyzed the succinylated substrates were slightly larger (approximately 31–34 kDa) than the smallest BApNA hydrolyzing enzymes (27–30 kDa). It is likely that the lower mass enzymes that hydrolyzed both chymotrypsin and elastase substrates in activity blots are the same. However, only the Dpl^r extract contained sev-

eral slower migrating enzymes, with apparent masses of 38, 46, and 49 kDa, all of which hydrolyzed the elastase substrate. With all of the substrates, activity >100 kDa was more prevalent in gut extracts from the Dpl^r strain. All of the enzyme activities were greatly reduced in the 198^r gut extract. Minor BApNA hydrolyzing activity in 198^r was associated with a 34 kDa protein, while minor SAAPFpNA hydrolyzing activity was observed as faint broad bands of about 40 and >100 kDa.

We evaluated a number of hypotheses to explain why strain 198^r has lower proteolytic activity than the other two strains of *Plodia*. We observed no differences between strains in gut pH, were unable to activate 198^r enzymes with other proteases, and were unable to inhibit 688^s gut enzymes with extracts from 198^r (data not shown). Although the mechanism responsible for suppressed activity in 198^r remains unknown, it might be due either to an inhibition of enzyme synthesis or secretion into the gut lumen or to a suppressed transcription and/or translation of proteinase genes in response to some unknown factor.

The midgut is the probable location of digestive proteinase secretion in Lepidoptera (Santos *et al.*, 1983). Neural, hormonal, and dietary stimuli have been suggested to trigger the release of digestive enzymes into the gut lumen (Christopher and Mathavan, 1985). For example, expression of a trypsin gene in the mosquito, *Aedes aegypti*, is induced by feeding (Barrilas-Mury *et al.*, 1991), and the quality and quantity of protein in the

bloodmeal affects the transcription of the gene (Noriega *et al.*, 1994). Apparently, a trypsin expressed early after feeding is involved in signal transduction of a later-expressed trypsin (Barrilas-Mury *et al.*, 1995). The relative abundance of proteinases in the insect midgut can also be affected by the kinds of proteins in the diet. For example, feeding proteinase inhibitors to *T. ni* causes a shift in the relative proportion of proteolytic enzymes in the midgut (Broadway, 1995). Wheat germ agglutinin fed to the honeybee, *Apis mellifera*, causes a dramatic decrease in trypsin activity (Belzunces *et al.*, 1994). Studies on the response of *Plodia* gut enzymes to dietary components, such as Bt protoxins, are needed. Purification of *Plodia* digestive enzymes and cloning of their genes are currently in progress in order to examine the regulation of proteinase genes at the transcriptional level. Results from these studies will be helpful in understanding the mechanism that causes a reduction in both proteolytic activity and Bt protoxin activation in the 198^r strain.

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