TÜBİTAK

Research Article

Turk J Agric For
33 (2009) 285-294
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doi:10.3906/tar-0802-5

Identification and characterization of hydrolytic enzymes from the midgut of the cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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Received: 13.02.2008

Abstract: Midgut hydrolytic enzymes of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) were identified and partially characterized. K_m, V_{max}, optimum pH, and specific activity were determined for proteolytic enzymes and α-amylases. All hydrolytic enzyme activity had an optimum pH value in the alkaline pH range. We observed major serine protease activity, together with minor cysteine-like activity, the former being significantly inhibited by soybean trypsin inhibitor (SBTI) and aprotinin. Moreover, different degrees of inhibition were observed with synthetic protease inhibitors. Electrophoretic methods revealed 3 isozymes of α-amylases, of which 2 had higher molecular weight and were more active than the other. Inhibition of amylolytic activity was observed with wheat α-amylase inhibitor (WAAI), whereas partially purified maize, chickpea, and bean seed crude extracts did not exhibit inhibitory activity toward α-amylases. To the best of our knowledge this is the first report on the properties of α-amylases from *Helicoverpa armigera* and the effects of several plant-originated a-amylase inhibitors on them.

Key words: *Helicoverpa armigera*, protease, protease inhibitors, α-amylase, α-amylase inhibitors

Pamukta yeşil kurt (*Helicoverpa armigera*, Hübner) (Lepidoptera: Noctuidae) zararlısının hidrolitik enzimlerinin tanısı ve karakterizasyonu

Özet: Bu çalışmada pamukta yeşil kurt (*Helicoverpa armigera*) zararlısının sindirim sisteminde bulunan hidrolitik enzimler tanımlanmış ve karakterize edilmiştir. K_m, V_{max}, optimum pH değerleri ve spesifik aktiviteler tüm proteolitik enzimler ve α-amilaz enzimi için belirlenmiştir. Hidrolitik enzimlerinin tamamı bazik pH aralığında optimum aktivite değerleri vermiştir. Pamukta yeşil kurt zararlısının sindirim sisteminde çoğunluğu serin ve metaloproteaz benzeri proteolitik aktiviteye rastlanmıştır. Serin proteaz benzeri aktivite doğal proteaz inhibitörleri olan soya fasulyesi tripsin inhibitörü ve aprotinin ile önemli ölçüde inhibe edilmiştir. Ayrıca sentetik proteaz inhibitörleri (PMSF, E-64, TPCK, $CdCl_2$, $CuCl_2$ ve chymostatin) ile de inhibisyon gözlenmiştir. Jel elektroforez yöntemi ile üç farklı α-amilaz izo-enzimine rastlanmıştır. Bunlardan yüksek moleküler ağırlıktaki ikisi diğerine göre daha yüksek enzim aktivitesi göstermiştir. Alfa-amilaz aktivitesi mısır, nohut ve fasülye α-amilaz inhibitörlerine karşı direnç gösterirken, buğday α-amilaz inhibitörü ile % 60 oranında inhibe olmuştur. Bu çalışma, *Helicoverpa armigera*'nın α-amilaz aktivitesinin belirlenmesi ve bitki kökenli inhibitörlerin denenmesi açısından literatürde ilk kez yayınlanmaktadır.

Anahtar sözcükler: *Helicoverpa armigera*, proteaz, proteaz inhibitörleri, α-amilaz, α-amilaz inhibitörleri

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Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae), a major pest of many economically important crop species, including cotton, tomato, maize, and potato, as well as many ornamental plants, is distributed worldwide. It is responsible for significant economic losses throughout its range (EPPO/CABI, 1996). The pest is primarily controlled by biological and chemical means, yet the insect's wide-ranging host preference, high population density throughout the breeding season, and tendency to develop resistance to insecticides hamper attempts to overcome the damage it causes. Similar to other lepidopteran insects, protein digestion in the cotton bollworm is facilitated mainly by the trypsin- and chymotrypsin-like activity of serine proteases, which are extracellular and active at an alkaline pH (Bown et al., 1997, 1998). Proteolytic enzymes of insect pests are currently being investigated due to their potential involvement in plant transgenic studies of the genes encoding proteins that adversely affect insect proteolytic activity (e.g. plant protease inhibitors). There are several reports on the efficiency of plant protease inhibitors in preventing insect development (reviewed in Hilder and Boulter, 1999; Carlini and Grossi-de-Sa, 2002); however, recent studies have shown that some insects, including *H. armigera*, have a tendency to develop resistance to protease inhibitors via the induction of novel proteolytic proteins that are unaffected by the inhibitors (Bown et al., 1997; Gatehouse et al., 1998; Nandi et al., 1999; Chougule et al., 2005).

α-Amylases, a group of enzymes with a central role in carbohydrate digestion, are very important for insects that feed on seeds. Hence, they could be considered alternative targets for the development of insect resistant transgenic plants through the use of α-amylase inhibitors. Legume seeds are known as rich sources of α-amylase inhibitors and were reported to be effective in combating *Bruchus* spp. in several studies (Ishimoto and Kitamura, 1989; Shade et al., 1994; Ishimoto et al., 1996; Morton et al., 2000).

To find an effective inhibitor against a pest it is important to know the entire hydrolytic enzyme profile of the pest. Herein we report the extensive proteolytic activity and amylolytic activity present in the gut of *H. armigera*, together with kinetic property and inhibition data. To the best of our knowledge this is the first report on the properties of α-amylases from the midgut of *H. armigera* to evaluate the inhibitory potential of several plant-derived α-amylase inhibitors on *H. armigera* α-amylase activity.

Materials and methods

Preparation of *H. armigera* gut extract

H. armigera larvae were reared on an artificial diet, as reported (Nagarkatti and Satyaprakash, 1974). Mid-to-late instar midguts were isolated via dissection, suspended in 0.15 M NaCl solution, and stored at −20 °C until use. Extraction was carried out in 0.15 M NaCl solution (5 midguts mL^{-1}) using a glass-Teflon homogenizer; the suspension was centrifuged at 10,000 ×g for 10 min at 4 °C. The resulting supernatant was used for the enzyme assays. Protein concentration of extracts was determined by the Bradford (1976) method, using bovine serum albumin as a standard protein.

Enzyme assays

All proteolytic enzymes were assayed spectrophotometrically (Shimadzu UV-160A double beam spectrophotometer) using synthetic substrates specific for each protease. Trypsin-, chymotrypsin-, elastase-, and leucine aminopeptidase-like activity were measured according to Ortego et al. (1996) using BApNa ((Nα-benzoyl-DL-arginine *p*-nitroanilide), SA_2PPpNa (N-succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide), SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide), and LpNa (L-leucine p-nitroanilide) as substrates, respectively. Papain-like activity was measured according to Delledone et al. (2001) using azo-albumin as a substrate. Total protease activity was measured according to Wilhite et al. (2000) using azocasein as a substrate. Carboxypeptidase-A activity was measured according to the method of Folk and Schirmer (1963), and carboxypeptidase-B activity was measured according to the method of Folk et al. (1960) using HPA (hippuryl-phenylalanine) and HA (hippuryl-L-arginine) as substrates, respectively. α-Amylase activity was measured according to the method of Bernfeld (1955) using soluble starch as a substrate.

The optimum pH value of each enzyme was determined at a pH range of 7.0-12.0 using 0.1 M K-PO_4 buffer for pH 7-7.5, 0.1 M Tris-HCl buffer for pH 8.0-9.0, 0.1 M gly-NaOH buffer for pH 9.5-11.0, and 0.1 M Na-PO_4 buffer for pH 12.0.

Inhibition assays

The inhibitory activity of several synthetic and natural inhibitors was tested against the proteolytic and amylolytic activity of *H. armigera* midgut extract. The effects of the inhibitors were determined by incubating midgut extract containing 20 or 50 µg of protein and by varying the concentration of inhibitors for 10 min at 30 °C before measuring the enzyme activity. Trypsin inhibition was determined in the presence of 0.1-1 mM PMSF (phenylmethyl-sulfonyl fluoride), 2.5-50 nM soybean trypsin inhibitor (SBTI; Sigma), and 7.5-75 nM aprotinin (Sigma), separately. Chymotrypsin inhibition was determined in the presence of 7.5-150 nM SBTI, 0.5-10 µM aprotinin, 3-60 µM TPCK (tosyl phenylalanyl chloromethyl ketone, Sigma), 20-150 µM PMSF, and 3-33 nM chymostatin (Sigma), separately. Elastase inhibition was analyzed in the presence of 2.5-50 nM SBTI, 0.5-1.5 µM aprotinin, 0.1-3 mM PMSF, and 5-100 µM TPCK. Leucine aminopeptidase inhibition was measured using 15-150 µM PMSF, 0.3-1.5 mM $CuCl_2$, and 3-60 µM $CdCl_2$. Papain inhibition was analyzed using 0.5-4 mM PMSF and 8-40 µM E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane). Inhibition of general protease activity was determined using all the inhibitors listed.

α-Amylase activity inhibition was analyzed in the presence of 0.5-10 µM wheat α-amylase inhibitor (WAAI) (Sigma), and partially purified seed extracts of bean, chickpea, and maize, each containing 50 -500 µg of protein.

Partial purification of bean, chickpea, and maize α-amylase inhibitors

For the partial purification of bean and chickpea α-amylase inhibitors, 2 g of dry seeds were ground in a cold mortar and pestle with liquid nitrogen until a fine powder was obtained. The powder was suspended in 40 mL of 0.02 M sodium phosphate buffer containing 20 mM NaCl and 0.1 mM $CaCl_2$ at pH 6.7. The suspension was stirred on a magnetic stirrer at 4 °C for 1 h and centrifuged at 11,900 ×g for 10 min. The supernatant was submitted to fractionation by ammonium sulfate precipitation (20%-60% saturation). The precipitate was recovered by centrifugation at 15,500 ×g for 60 min at 4 °C and dissolving the pellet in 0.02 M sodium phosphate buffer at pH 6.7. The suspension was dialyzed against 1.0 L of the same buffer overnight at 4 °C. The extract was stored at −20 °C until use.

Partial purification of maize seed α-amylase inhibitor was carried out according to a slightly modified method of Figueira et al. (2003). Corn seeds were finely ground using a mortar and pestle with liquid nitrogen. The flour was defatted by 15 min of shaking with acetone (1:1 v/v) 4 times and air-dried at room temperature. The corn flour (20 g) was mixed with 100 mL of 0.1 M acetate buffer at pH 6.0 and continuously stirred for 90 min at 4 °C. The soluble protein was obtained by centrifugation at 30,000 ×g for 20 min at 4 °C. The supernatant was submitted to fractionation by ammonium sulfate precipitation (30%-60% saturation). The precipitate was recovered by centrifugation at 30,000 × g for 20 min at 4 °C and dissolving the pellet in 0.01 M Tris-HCl buffer (pH 8.0). The suspension was dialyzed against the same buffer overnight at 4 °C and the partially purified maize seed extracts were stored at −20 °C until use.

The protein concentration of the extracts was determined according to the Bradford method (1976). The inhibitory activity of each extract was assayed against commercial α-amylase enzyme (Sigma) by incubating extracts containing 50-500 µg of protein with 1 unit of commercial α-amylase enzyme for 10 min at 37 °C before measuring the enzyme activity.

Starch zymography for α-amylase activity

Zymography for α-amylase activity was carried out according to the method of Titarenko and Chrispeels (2000), with minor modification. Samples were prepared in sample dilution buffer (0.4 M Tris [pH 6.8], 5% SDS, 20% glycerol, and 0.03% bromophenol blue) (Laemmli, 1970) without β-mercaptoethanol, and were incubated at RT for at least 10 min before loading. SDS-PAGE (6%) containing 0.5% soluble starch was used. Gels were run at a constant 12-mA current in a MiniGel electrophoresis system (EC 120 mini vertical gel system, E/C Apparatus Cooperation) until the blue tracking dye reached the bottom of the

gel. The gels were washed with 50 mL of 2.5% triton X-100 solution for 1 h to remove SDS and then were incubated in 50 mL of 0.1 M glycine-NaOH buffer (pH 10.0) containing 1% soluble starch for 1 h at 37 °C. Gels were then stained with 50 mL of 3% KI/I_2 solution for 5 min, washed with distilled water, and photographed. For in-gel inhibition assays, wheat α-amylase inhibitor (Sigma) and partially purified seed extracts of chickpea, maize, and bean were incubated with midgut extracts for 10 min. Samples were then treated with sample dilution buffer and loaded.

Results

For all enzymatic activity, K_m and V_{max} values, optimum pH, and specific activities were determined using enzyme-specific substrates. Kinetic analysis of enzyme activity at optimum pH values gave linear reciprocal Michealis-Menten (Lineweaver-Burk) plots, enabling estimation of K_m and V_{max} values (graphs not shown). Comparison of *p*-nitroanilide substrates shows that SA_2PPpNa, SA_3pNa, and LpNa bound more strongly than BApNa to their enzymes (Table 1). The affinity for the trypsin substrate was lower compared to the other *p*-nitroanilide substrates, as BApNa contains only a single amino acid residue for the binding of the enzyme. The rate of hydrolysis of the chymotrypsin and leucine aminopeptidase substrates was approximately 3 times higher than that of the trypsin substrate, while a very low hydrolysis rate was observed for the elastase substrate (Table 1). When specific activities were considered, serine-type proteolytic activity, together with a metalloprotease (Leucine aminopeptidase-like) activity predominated in the *H. armigera* midgut. Minor proteolytic activity was detected with elastase and papain. No carboxypeptidase-A or carboxypeptidase-B-like activity was detected using enzyme-specific synthetic substrates HPA and HA, respectively. The effect of pH on the hydrolytic activity of the *H. armigera* larval midgut extracts was determined using a discontinuous buffer system between pH 7.0 and 12.0. The synthetic *p*-nitroanilide substrates were all hydrolyzed in an extremely alkaline pH range (10.5-11.0), except LpNa hydrolysis, which occurred at pH 8.0. Optimal hydrolysis of the α-amylase substrate and starch occurred at pH 9.5, and the specific activity was detected as 620 μmol of maltose produced min^{-1} mg^{-1} of protein from the *H. armigera* midgut (Table 1).

The effect of pH on general protease activity was analyzed at the pH range of 8-11 and the greatest activity was detected at pH 11.0, which was 400 units mg^{-1} of protein (Table 2). General protease activity was also assayed in the presence of different concentrations of protease inhibitors at different pH values and the results, as maximum percent inhibition, are shown in Table 2. Buffer pH had varying effects on inhibitor capacity. The inhibition

Table 1. Activity of hydrolytic enzymes from the midgut of *H. armigera* against enzyme-specific substrates. K_m and V_{max} values were calculated using Lineweaver-Burk plots. Specific activity was calculated through enzyme titration at excess substrate concentrations. Each datum is the average of at least 3 independent experiments (± SEM).

Substrate (enzyme specificity)	K_m mM (± SEM)	V_{max} μmoles min^{-1} mg^{-1} protein (± SEM)	pH optimum	Specific Activity μmoles min^{-1} mg^{-1} (± SEM)
Starch (α-amylase)	0.30 (± 0.001)	824.8 (± 80)	9.5	620 (± 23)
BApNa (trypsin)	2.3 (± 0.3)	0.43 (± 0.04)	10.5	0.22 (± 0.02)
SA_2PPpNa(chymotrypsin)	0.12 (± 0.02)	1.33 (± 0.24)	10.5	0.64 (± 0.02)
SA_3pNa (elastase)	0.22 (± 0.05)	0.024 (±0.002)	11.0	0.01 (± 0.01)
LpNa (LAP)	0.52 (± 0.04)	1.22 (± 0.11)	8.0	0.44 (± 0.03)
Azo-albumin (papain)	0.25 (± 0.06)[1]	0.41 (±0.08)[2]	11.0	0.071 (± 0.005)[3]

[1]Units of K_m for starch and azo-albumin hydrolysis are defined as mg mL^{-1}.
[2]Units of V_{max} and SA for azo-albumin hydrolysis are defined as mU ΔOD min^{-1} mg^{-1}.

Table 2. General protease activity of midgut extracts from *H. armigera* at 4 different pH values and the effects of protease inhibitors on general protease activity.

Control (units mg^{-1} protein)	pH 8.0	pH 9.0	pH 10.0	pH 11.0
	298 ± 23	320 ± 32	341 ± 22	400 ± 32
			% Inhibition[a]	
SBTI (5 nM)	23.0 ± 5.5	33.1 ± 6.1	34.4 ± 3.7	48.0 ± 6.02
Aprotinin (0.15 µM)	25.4 ± 9.5	39.8 ± 4.6	46.6 ± 10.2	55.0 ± 8.4
Chymostatin (1 µM)	47.8 ± 0.5	40.6 ± 0.2	38.4 ± 5.9	36.2 ± 6.6
TPCK (0.1 mM)	n.i.[b]	n.i.	n.i.	n.i.
PMSF (1 mM)	61.6 ± 2.5	55.6 ± 1.2	54.5 ± 4.7	60.7 ± 5.7
EDTA (0.1 mM)	n.i.	n.i.	n.i.	n.i.
$CdCl_2$ (1 mM)	35.2 ± 0.6	20.1 ± 7.5	21.6 ± 4	13.4 ± 4.5
$CuCl_2$ (1 mM)	46.7 ± 4.2	27.5 ± 3.9	22.7 ± 5.9	15.4 ± 7.9
E-64 (0.1 mM)	n.i.	n.i.	n.i.	n.i.

[a] Values are given as % inhibition $((1 - V_i/V_o) \times 100) \pm$ SEM with respect to their corresponding controls without inhibitor, except the control values, which are given as units mg^{-1} (1 unit of azocaseinolytic activity was defined as 0.001 units ΔOD_{335} min^{-1} mg^{-1} of protein).

[b] Inhibition lower than 10% was considered no inhibition (n.i). Each item of data is the average of at least 3 independent experiments.

rate of the serine protease inhibitors SBTI and aprotinin, which are protein in structure, increased as pH increased. This was correlated with the optimum pH values for the serine-type proteases detected in the midgut (Table 1). On the other hand, inhibition by chymostatin, an inhibitor of chymotrypsin-like activity, was greatest at pH 8. Among the inhibitors that are protein in structure, the highest potency was observed for SBTI, causing 48% inhibition at the 5-nM concentration at pH 11. A general protease inhibitor, PMSF, caused 55%-60% inhibition at all pH values tested. This may imply that PMSF was not affected by variation in pH and it inhibited a broad range of proteolytic activity present at different pH values. The metalloprotease inhibitors $CdCl_2$ and $CuCl_2$ caused 35.2% and 46.7% general protease activity inhibition at pH 8.0, respectively (Table 2). This value was consistent with the optimum pH value of leucine aminopeptidase-like (a metalloprotease) activity (Table 1). TPCK and E-64 did not cause any significant difference in the general protease activity when included in the assay medium at given concentrations, most probably due to the low specific activity of elastase- and papain-like proteases in the insect midgut (Table 2).

The effect of inhibitors on the hydrolysis of enzyme-specific substrates was determined by incubating varying concentrations of inhibitors with midgut extract before measuring proteolytic activity. The inhibitors that are protein in structure, namely SBTI, aprotinin, and chymostatin, were very effective, causing 70%-90% inhibition of proteolytic activity at nM concentrations (Table 3). The metalloprotease inhibitors $CdCl_2$ and $CuCl_2$ almost completely inhibited LpNa hydrolysis, verifying the presence of leucine aminopeptidase-like activity. Similarly, inhibition by E-64, which specifically inhibits cysteine proteases, proved the presence of papain-like protease in the *H. armigera* midgut. A general protease inhibitor, PMSF, caused varying degrees of inhibition of proteolytic activity. A comparison of the effectiveness of inhibitors is shown in Table 4 in the form of IC_{50} values.

Spectrophotometric assay for α-amylase activity was carried out in the presence of plant-derived α-amylase inhibitors. A 60% inhibition rate was observed when 2 µM of WAAI was incubated with *H. armigera* α-amylase for 10 min before enzymatic assaying (Figure 1). Inhibition assays were also carried out with partially purified seed extracts of chickpea,

Table 3. Effect of inhibitors on *H. armigera* midgut proteolytic activity.

Inhibitor	Substrate (enzyme specificity)	Effective concentration[a]	% Inhibition[b]
SBTI	BApNa (trypsin)	20 nM	70.8 ± 0.8
	SA_2PPNa (chymotrypsin)	75 nM	79.2 ± 0.6
Aprotinin	BApNa (trypsin)	150 nM	82.3 ± 0.7
	SA_2PPNa (chymotrypsin)	10 µM	84.6 ± 1.25
Chymostatin	SA_2PPNa (chymotrypsin)	30 nM	91.2 ± 0.87
TPCK	SA_2PPNa (chymotrypsin)	130 µM	29.8 ± 2.3
	SA_3PNa (elastase)	50 µM	59.1 ± 4.3
PMSF	BApNa (trypsin)	1 mM	33.0 ± 3.9
	SA_2PPNa (chymotrypsin)	50 µM	88.14 ± 1.24
	SA_3PNa (elastase)	1 mM	73.2 ± 1.7
	LpNa (LAP)	30 µM	91.6 ± 1.9
	Azo-albumin (papain)	4 mM	76.9 ± 7.8
$CuCl_2$	LpNa (LAP)	1.3 mM	98.4 ± 0.4
$CdCl_2$	LpNa (LAP)	66 µM	90.2 ± 0.4
E-64	Azo-albumin (papain)	40 µM	93.3 ± 0.8

[a] Values are the inhibitor concentrations that caused maximal inhibition.

[b] Values are given as maximum % inhibitions $((1 - V_i/V_o) \times 100$; where V_i is the initial rate with inhibitor, V_o is the initial rate without inhibitor). Each item of data is the average of at least 3 replicates ± SEM. LAP: Leucine aminopeptidase.

Table 4. The potency of inhibitors against the hydrolytic enzymes of the *H. armigera* midgut, given as IC_{50} values. The values were calculated from [S]/v vs. [S] graphs, and are mean ± SEM values of 3 independent experiments.

Inhibitor	Substrates				
	(Trypsin) BApNa	(Chymotrypsin) SA_2PPNa	(Elastase) SA_3PNa	(LAP) LpNa	(Papain) Azocasein
SBTI (nM)	7.8 ± 0.5	12.5 ± 1	n.i.	-	-
Aprotinin (nM)	15.8 ± 1	1010 ± 140	n.i.	-	-
Chymostatin (nM)	-	10 ± 1	-	-	-
TPCK (µM)		167 ± 19	44 ± 7		
PMSF (µM)	1025 ± 80	17.9 ± 0.4	67 ± 5	18 ± 0	821 ± 114
$CuCl_2$ (µM)				360 ± 1	
$CdCl_2$ (µM)	-	-	-	21.6 ± 0.4	-
E-64 (µM)					21.0 ± 0.4

maize, and bean; however, no inhibitory activity was detected, although commercial α-amylase (from porcine pancreas, Sigma) activity was inhibited by these extracts (Table 5).

Native starch-polyacrylamide gel electrophoresis revealed 3 isozymes of α-amylase—Amy-1, Amy-2, and Amy-3—the first 2 were much more active than the other. Zymographic data also indicates the

Table 5. Inhibition of commercial α-amylase activity with commercial wheat α-amylase inhibitor, and extracts of bean, chickpea, and maize, at given concentrations.

	% inhibition of commercial α-amylase (± SEM) WAAI
(30 μM)[1]	100.0 ± 0.0 BAAI
(100 μg)[1]	100.0 ± 0.0 CpAAI
(100 μg)[1]	86 ± 3.2 MAAI
(50 μg)[1]	80 ± 5.4

[1] Indicates the amount of inhibitor per assay medium at which maximal inhibition was obtained. BAAI, CpAAI, and MAAI quantities per assay medium are given as protein concentration in partially purified extract

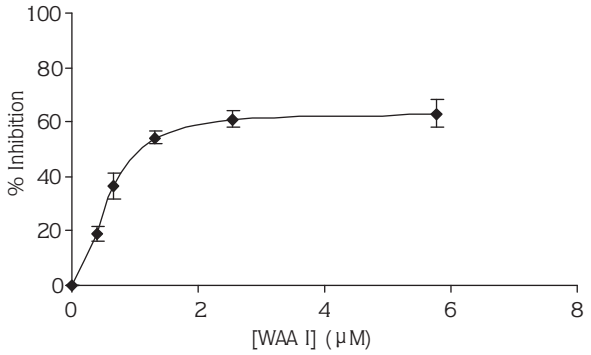

Figure 1. Inhibition of *H. armigera* α-amylase activity by wheat α-amylase inhibitor (WAAI). Data are the means of 3 measurements and bars indicate the SEM at the 95% confidence interval.

inhibition of α-amylase activity by WAAI, while the seed extracts used in this study had no effect on *H. armigera* midgut α-amylase activity. WAAI almost completely inhibited Amy-1 and Amy-2 (Figure 2). On the other hand, a slight increase in activity was observed in the Amy-3 isozyme in the presence of WAAI.

Discussion

While there are sufficient data on the properties of proteases from *H. armigera* and some other lepidopteran insects in the literature, to the best of our knowledge this is the first report on the kinetic properties of proteases and α-amylase in the *H. armigera* midgut.

All of the reports concerning the proteolytic properties of the midgut of *H. armigera* and other Lepidopteran insects indicate an alkaline pH as optimal and that serine protease activity is dominant (Johnston et al., 1991; Christeller et al., 1992). Our results also correlate well with these findings. Major serine proteases detected in the *H. armigera* midgut were trypsin-like, chymotrypsin-like, and leucine aminopeptidase-like serine proteases, in addition to minor elastase-like protease activity. We also detected a low level of cysteine-like activity. This finding was confirmed by the use of the cysteine protease-specific inhibitor E-64, which had an IC_{50} value of 21.02 μM; however, when it was used in the general protease assay no significant inhibition was detected, indicating the presence of minor papain-like activity in the *H. armigera* midgut.

We were unable to detect either carboxypeptidase A- or carboxypeptidase B-like activity against the substrates used; however, there are reports indicating the presence of carboxypeptidase activity in the *H. armigera* midgut (Bown et al., 1998; Estebanez-Perpina et al., 2001). There are also some reports that *H. armigera* proteolytic enzymes, as a part of its resistance mechanism, are induced when they are fed a diet containing protease inhibitors (Gatehouse et al., 1998; Chougule et al., 2005). Bown et al. (1998) also reported that chronic ingestion of soybean Kunitz trypsin inhibitor by *H. armigera* larvae results in both increased accumulation of carboxypeptidase mRNA in the midgut cells and an increase in carboxypeptidase activity in gut extracts. Therefore, due to the diversity of proteolytic activity present in the insect midgut, it can adapt its proteolytic activity rapidly in response to changes in diet (Srinivasan et al., 2006). It was reported that when the insect is fed a protein-rich diet, trypsin- and chymotrypsin-like proteolytic activity predominate (Patankar et al., 2001).

The kinetic parameters determined for the proteolytic enzymes in *H. armigera* are in the line with earlier estimates for enzymes from *H. virescens* (Johnston et al., 1995) and *L. oleracea* (Gatehouse et al., 1999). Peptide substrates showed greater affinity than BApNa, which only contains residue of a single amino acid. When V_{max} values were compared, the

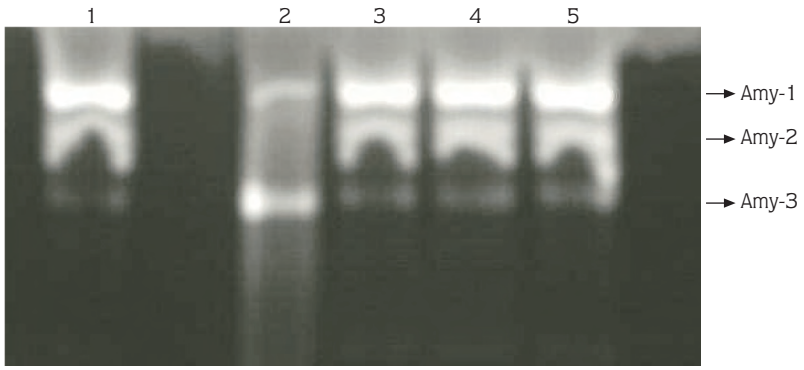

Figure 2. Zymograms of α-amylase activity from the midgut of *H. armigera* subjected to 6% SDS-PAGE containing 0.5% soluble starch. Samples were prepared by incubating in standard sample dilution buffer (without β-mercaptoethanol) for 10 min at room temperature (no heat treatment). Midgut extracts containing 100 µg of protein were loaded into each well. For inhibition, 100 µg of midgut extract was incubated with each inhibitor separately at room temperature and then treated with sample dilution buffer. Gels were run at a 12-mA constant current and stained with KI/I_2 solution
Lane 1: 100 µg of midgut extract without inhibitor
Lane 2: 100 µg of extract + 5 µg of WAAI
Lane 3: 100 µg of midgut extract + maize extract containing 10 µg of protein
Lane 4: 100 µg of extract + chickpea extract containing 40 µg of protein
Lane 5: 100 µg of midgut extract + bean extract containing 40 µg of protein

highest level of hydrolysis was observed against the chymotrypsin substrate, SA_2PPpNa. This is in agreement with earlier studies in which higher hydrolysis rates were observed for phenylalanine-based substrates (Johnston et al., 1995; Gatehouse et al., 1999)

Inhibition experiments against proteases showed that SBTI was a very potent inhibitor for trypsin- and chymotrypsin-like activity, while elastase activity was unaffected by SBTI. The same held true for another natural protease inhibitor, aprotinin, except that when compared to SBTI it had a lower potency against chymotrypsin-like activity, as shown by the IC_{50} values. A general serine protease inhibitor, PMSF, caused inhibition of all proteolytic activity, with varying degrees of potency, and caused around 60% inhibition of general protease activity at all pH values tested. The irreversible metalloprotease inhibitors $CdCl_2$ and $CuCl_2$ caused complete inhibition of leucine aminopeptidase activity, the former being more potent, as shown by the IC_{50} values. General protease activity was inhibited by these inhibitors more strongly at lower pH values, which correlates well with the pH optimum of leucine aminopeptidase activity. Chymostatin caused complete inhibition of chymotrypsin activity at nM concentrations, which was also apparent from its IC_{50} value. It also significantly reduced general protease activity. A chymotrypsin inhibitor, TPCK, caused only 29.8% inhibition of chymotrypsin activity and caused no inhibition in general protease activity, meaning that chymotrypsin-like activity in the *H. armigera* midgut is slightly sensitive to inhibition by TPCK, or that TPCK is unstable at an alkaline pH; good results were not obtained with this inhibitor.

Our results show that α-amylase activity in *H. armigera* is optimum at an alkaline pH, which is consistent with the optimum pH reported for other lepidopteran species (Abraham et al., 1992; Markwick et al., 1996, Alfonso et al., 1997; Valencia-Jimenez et al., 2008). α-Amylase zymography showed that the midgut of *H. armigera* has 3 α-amylase isozymes—Amy-1, Amy-2, and Amy-3. The first 2 isozymes have high molecular weights and major amylolytic activity, as shown by the electrophoretic results. The occurrence of several isozymes of α-amylase from lepidopteran insect midguts was previously reported. Valecia-Jimenez et al. (2008) reported the presence of

4 isozymes of α-amylase activity from the midgut of *Tecia solanivora*. Similar studies with *Spodoptera frugiperda* revealed 1 major and 3 minor α-amylase activities. Spectrophotometric and electrophoretic studies indicated that α-amylase activity is sensitive to inhibition by wheat α-amylase inhibitor, while all were insensitive to inhibition by partially purified seed extracts of maize, chickpea, and bean. Their inhibitory activity, however, was confirmed by the spectrophotometric assays against commercial α-amylase from the porcine pancreas (Sigma) (Table 5). Wheat α-amylase inhibitor caused 60% inhibition at the 2-μM concentration, and had an IC_{50} value of 1.04 μM. This result is in agreement with previous data indicating the sensitivity of lepidopteran α-amylases towards wheat α-amylase inhibitor (Baker, 1989; Markwick et al., 1996; Alfonso et al., 1997).

Although the possible use of PIs for the development of *H. armigera* resistant host plants is widely studied, reports concerning the properties of *H. armigera* α-amylases and the possible use of plant-derived α-amylase inhibitors for transgenic studies are not available. Herein we reported that legume seed α-amylase inhibitors (chickpea, maize, and bean) were ineffective against *H. armigera* midgut α-amylase activity, whereas WAAI may be a good candidate for transgenic studies.

Acknowledgements

This work was supported by grant AFP-2002-07-02-00-54. The authors thank Dr Sakine Uğurlu of the Central Research Institute of Crop Protection, Ankara, for supplying the insect culture.

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