Altered proteolytic and amydolytic activity in insecticide-susceptible and -resistant strains of the maize weevil, *Sitophilus zeamais*

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Abstract

Fitness cost is usually associated with insecticide resistance and may be mitigated by increased energy accumulation and mobilization. Preliminarily evidence from tests with *Sitophilus zeamais* Motschulsky, the maize weevil (Coleoptera: Curculionidae) suggested possible involvement of proteinases and amylases in such a phenomenon. Therefore, trypsin-like serine-proteinases, cysteine-proteinases and α-amylases were purified and characterized from an insecticide-susceptible and two insecticide-resistant strains (one with associated fitness cost [resistant cost strain], and the other without it [resistant no-cost strain]). Trypsin-like serine-proteinases were purified by aprotinin-agarose affinity chromatography, while cysteine-proteinases were purified using thiol-sepharose affinity chromatography, and the main α-amylase of each strain was purified by glycogen precipitation and ion-exchange chromatography. The activity and inhibition profile differed among strains for each group of purified enzyme. The higher levels of activity observed for trypsin-like proteinases and amylase in the resistant no-cost strain, as well as their susceptibility to inhibition provide support for the hypothesis that enhanced trypsin-like protease and α-amylase activity may be playing a major role in mitigating fitness costs associated with insecticide resistance. In contrast, enhanced cysteine-proteinase activity is likely to play only a secondary role, if any, in mitigating the costs usually associated with insecticide resistance.

Keywords: Fitness cost mitigation, Insecticide resistance, Digestive enzymes, Amylases, Proteinases.

1. Introduction

Insecticide resistance is a frequent consequence of overreliance on insecticide use, which is particularly acute among stored product insects in warmer climates (Champ and Dyte, 1976; Badmin, 1999). Resistance can make a positive contribution to an individual's fitness under insecticide exposure, but also may place resistant individuals at a fitness disadvantage in the absence of the insecticide (Coustau et al., 2000; Oliveira et al., 2007). Although not universal, such fitness costs associated with insecticide resistance are commonly reported among insect pests and they are a frequent assumption in models of insecticide resistance evolution (Coustau et al., 2000; Guedes et al., 2006).

The expression of insecticide resistance mechanisms usually cause higher energy demands, consequently leading to higher metabolic rate. Therefore, increases in metabolic rate may be necessary for the maintenance of insecticide resistance mechanisms. Unless the energy metabolism in resistant insects is enhanced, energy relocation may deprive other basic physiological processes leading to the expression of fitness costs associated with insecticide resistance (Harak et al. 1999; Guedes et al., 2006; Araújo et al., 2008ab). Therefore, larger stores of energy reserve molecules may constitute an additional energy supply for the maintenance of insecticide resistance mechanisms, without compromising the energy demanded for the other physiological processes, thereby mitigating the costs associated with insecticide resistance (Guedes et al., 2006; Araújo et al., 2008ab).

Earlier studies with the maize weevil (*Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)) recognized pyrethroid-resistant strains with and without associated fitness costs (Guedes et al., 2006; Oliveira et al., 2007). The pyrethroid-resistant strain lacking fitness cost led to higher grain loss, and exhibited higher respiration rate, higher body mass, and larger energy reserve cells (i.e., trophocytes)

than the pyrethroid-susceptible and –resistant (with associated fitness cost) strains of *S. zeamais* (Guedes et al., 2006; Oliveira et al., 2007). Subsequent investigation surveying hydrolytic enzymes in these same strains associated high proteinase and particularly high amylase activity with the pyrethroid-resistant strain lacking associated fitness costs (Araújo et al., 2008ab). Such findings led to the hypothesis that high proteinase and high amylase activity may be underlying physiological mechanisms mitigating fitness costs associated with insecticide resistance in the maize weevil.

We reported here the purification and characterization of trypsin-like serine-proteinases, cysteine-proteinases and α-amylases from a susceptible and two insecticide-resistant strains *S. zeamais* (with and without associated fitness cost), which have been used as models for the study of mechanisms underlying the mitigation of fitness costs associated with insecticide resistance. Higher activity levels and distinct inhibition profile of the purified enzymes were expected in the pyrethroid-resistant strain without associated fitness costs, particularly in contrast with the pyrethroid-resistant strain with associated fitness costs, if these hydrolytic enzymes are indeed involved in the mitigation of fitness costs associated with insecticide resistance mechanisms, as previously suggested (Araújo et al., 2008ab).

2. Material and Methods

2.1. Insects

Three strains of *S. zeamais* were used. The susceptible strain, here referred as "susceptible", has been maintained for nearly 20 years without insecticide exposure; its insecticide susceptibility is periodically checked (Araújo et al., 2008a). Both the insecticide-resistant strains exhibit over 100-fold resistance to pyrethroids, which is also periodically checked (Araújo et al., 2008a). The strain here referred as "resistant cost" has reduced fitness in the absence of pyrethroid insecticides, while the strain here referred as "resistant no-cost" does not exhibit reduced fitness in the absence of pyrethroids (Guedes et al., 2006; Oliveira et al., 2007; Araújo et al., 2008a). All three insect strains were maintained in whole maize grains free of insecticides under controlled temperature (25 ± 2ºC), relative humidity (70 ± 5%) and photoperiod (12:12 L:D).

2.2. Chemicals

Enzymatic kits were used for determination of amylase activity (K003) and purchased from BIOCLIN (QUIBASA – Química Básica Ltda, Belo Horizonte, MG, Brazil). Glucose, potassium sodium tartarate, and potassium chloride were purchased from Merk S. A. Ind. Quím. (Porto Alegre, RS, Brazil), while the DEAE-Sephacel chromatographic resin was purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All of the remaining reagents used were purchased from Sigma-Aldrich Química Brazil (São Paulo, Brazil).

2.3. Preparation of crude insect extract

For purification of proteinases, frozen adult maize weevils (0.2 g mL^{-1}) were homogenized in 0.1 M Tris-HCl (pH 8.0) and used as enzyme source after cell lyses by a series of nitrogen freezing and thawing at 37 ºC water bath. Aliquots of 1 mL of crude extract were centrifuged at 100,000 *g* for 60 min at 4 ºC. The resulting supernatant was dialyzed against 100 volumes of 0.01 M Tris-HCl buffer (pH 7.5). The supernatant was subsequently recentrifuged at 100,000 *g* for 45 min at 4ºC and loaded onto a aprotinin-agarose affinity column (Sigma-Aldrich Química, São Paulo, Brazil) equilibrated with 0.01 M Tris-HCl and 5 mM $CaCl_2$ at a flow of 1 mL min^{-1} to retain serine-proteinases. The remaining sample (with serine-proteinases removed) was subsequently loaded onto a thiol sepharose 4 B column (Sigma-Aldrich Química, São Paulo, Brazil) equilibrated with 0.1 M Tris-HCl pH 7.5 containing 0.5 M $NaCl_2$ and 1 mM ethylenediaminetetraacetic acid (EDTA). After loading the sample, the medium was rinsed with binding buffer until the baseline was stable. Bound molecules were eluted with 0.1 M Tris-HCl pH 7.5 containing 25 mM dithiothreitol (DTT) and 1 mM EDTA. A flow rate of 0.5 mL/min was used, and 1 mL fractions were collected.

Non-sexed adult weevils (10.5 g for each strain) were used for α-amylase purification. The adult insects were nitrogen-frozen and homogenized in 20 mM sodium acetate buffer (pH 5.0) containing 20 mM NaCl and 0.1 mM $CaCl_2$ at a ratio of 3 mL buffer for 1 g of insect, following Baker and Woo (1985). The crude homogenates were filtered through glass-wool and centrifuged at 5,300 *g* for 30 min. Lipids were removed by collecting the supernatant and centrifuging it once more under the same conditions. The

resulting lipid-free supernatant was dialyzed in 20 mM sodium acetate buffer (pH 5.0) containing 20 mM NaCl and 0.1 mM $CaCl_2$ for 20 h at 4°C. The supernatant was subsequently recentrifuged at 5,300 *g* for 30 min. The soluble extract obtained was subjected to glycogen precipitation following Loyter and Schramm (1962), and the sample obtained was subjected to ion-exchange chromatography using a DEAE-Sephacel column (10 x 2 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM $CaCl_2$. The unbound proteins were eluted from the column with 30 mL of buffer, followed by a subsequent increase in the saline gradient to 0.4 M NaCl to elute bound α-amylases. A flow rate of 0.5 $mL.min^{-1}$ at 4°C was used and 1.5 mL fractions were collected. The fractions corresponding to the same activity peak were pooled and only the fractions corresponding to the main (i.e., more active) α-amylase isoform of each strain were used for subsequent characterization. Polyacrylamide gel electrophoresis (PAGE) was carried out following Laemmli (1970) using 12% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulphate (SDS) to check the enzyme purification and to estimate de molecular mass of the purified enzymes.

2.4. Protein determination and enzyme activity

Protein concentration was measured following the method of Bradford (1976). Bovine serum albumin (BSA) solutions of 0-0.02 mg mL^{-1} were used as the standard. Amidolytic activity of serine-proteinases was determined using *N*-α-benzoil-L-Arg-*p*-nitroanilide (L-BApNA) as substrate at a final concentration of 0.5 mM in 0.1 M Tris-HCl buffer (pH 8.2). Esterolytic activity of serine-proteinases was determined using *N*- α-*p*-tosyl-L-Arg methyl ester (L-TAME; 0.5 mM) as a substrate in 0.1M Tris-HCl buffer (pH 8.2). Amidolytic activity was also determined for the purified cysteine-proteinases as described by Erlanger et al. (1961), using L-BapNA as a substrate at 0.5 mM in 0.1 M Tris-HCl buffer (pH 8.0), with 20 mM $CaCl_2$ and 5 mM DTT, and 100 µL of the serine-proteinase inhibitor benzamidine at 1 mM in order to measure only the cysteine-proteinase activity. Enzyme activity was determined by formation of *p*-nitroanilide, through the measurement of absorbance at 405 nm, and using the molar absorption coefficient 8800 $M^{-1}.cm^{-1}$. α-Amylase activity was determined with the K003 BIOCLIN enzymatic kit containing a substrate solution (starch) and color reagent (iodine), following methods adapted from Caraway (1959). Activity values of amylase were expressed as amylase units ($AU.dL^{-1}$), which refers to the amount of amylase that hydrolyzes 10 mg starch in 30 min at 37°C.

2.5. Effect of enzyme inhibitors and enzyme kinetics

Selected proteinase and amylase inhibitors were tested for their effect on the purified enzyme activity using concentration ranges covering their estimated K_i from published papers. Partially purified enzyme samples were incubated for either 7.5 or 15 min (amylase and proteinase inhibitors respectively) with the different inhibitors and enzyme activity was subsequently determined as previously described. Determination of the kinetic parameters K_M and V_{max} was carried out with increasing substrate concentrations using the methods previously described subjected to non-linear regression using the curve-fitting procedure for enzyme kinetics of SigmaPlot (SPSS, 2000).

3. Results

3.1. Trypsin-like serine-proteinases

Serine-proteinases from the susceptible strain and the two resistant strains were partially purified using an aprotinin-agarose affinity column providing purification factors ranging from 36.5 to 51.2%, with yields between 10 and 15% and activity between 529 and 875 µM min^{-1} mg^{-1} protein with the substrate L-BApNA. SDS-PAGE of the purified fraction revealed a 56,000 Da molecular mass band in all strains and a 70,000 Da band more visible in the resistant no-cost strain. The purified proteinases from all strains were inhibited by *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), aprotinin, benzamidine and soybean trypsin inhibitor (SBTI) characterizing them as trypsin-like serine-proteinases (Table 1). Trypsin-like proteinases from the resistant strains exhibited higher affinity for L-BApNA. The resistant no-cost strain exhibited V_{max}-values 1.5- and 1.7-fold higher than the susceptible and resistance cost strains, respectively (Table 2). A similar trend was also observed when using L-TAME as substrate (Table 2).

3.2. Cysteine-proteinases

Purification of the cysteine-proteinases revealed a single 74,000 Da molecular mass band in the susceptible strain, two bands of 72,000 and 83,000 Da in the resistant cost strain, and two bands of

68,000 and 74,000 Da in the resistant no-cost strain. Purified cysteine-proteinases of the three strains were different regarding substrate degradation and inhibition; the proteinases least sensitive to inhibition by the specific cysteine-proteinase inhibitor E-64 were those from the resistant no-cost strain (Table 1). The pH and temperature profile of cysteine-proteinase activity differed among strains and although affinity (i.e. K_M) of the cysteine-proteinases were similar, the V_{max} value for cysteine-proteinases from the resistant cost strain was 3x and 5x higher than V_{max} values for the resistant no-cost and susceptible strains respectively (Table 2). Cysteine-proteinase activity was highest for the resistant cost strain rather than the resistant no-cost (Table 2).

Table 1 Concentration required to inhibit 50% of enzyme activity (I_{50}) (± SEM) for selected inhibitors towards the activity of proteinases and α-amylases purified from a susceptible and two pyrethroid-resistant strains (resistant cost and resistant no-cost) of *S. zeamais.* Results are reported as the mean ± standard error (n = 3). Means followed by the same letter in a row are not significantly different by Fisher's LSD test ($p < 0.05$).

		I_{50}		
Purified enzyme	**Inhibitor**	**Susceptible**	**Resistant cost**	**Resistant no-cost**
Trypsin-like serine-proteinases	Benzamidine (mM)	0.54 ± 0.02 a	0.41 ± 0.01 a	0.13 ± 0.01 b
	TLCK (mM)	0.56 ± 0.05 a	0.14 ± 0.03 b	0.11 ± 0.05 b
	Aprotinin (µM)	0.37 ± 0.10 a	0.60 ± 0.20 a	0.90 ± 0.31 a
	SBTI (µg.mL^{-1})	140.17 ± 0.50 a	75.20 ± 6.60 b	36.90 ± 3.10 c
Cysteine-proteinases	E-64 (mM)	0.0031 ± 0.0008 b	0.0057 ± 0.0021 b	0.0150 ± 0.0050 a
α-Amylases	Acarbose (mM)	41.01 ± 6.42 b	24.49 ± 2.37 c	63.80 ± 7.23 a
	Wheat amylase inhibitors (µg.mL^{-1})	4.89 ± 0.55 b	4.22 ± 0.58 b	6.38 ± 0.67 a

Table 2 Kinetic parameters (± SEM) of proteinases and α-amylases purified from a susceptible and two pyrethroid-resistant strains (resistant cost and resistant no-cost) of *S. zeamais.* Results are reported as the mean ± standard error (n = 3). Means for each enzyme followed by the same letter in a column are not significantly different by Fisher's LSD test ($p < 0.05$).

		K_M		V_{max}	
Enzyme	**Strain**	**L-BapNA (mM)**	**L-TAME (mM)**	**L-BapNA (µM/s/mg)**	**L-TAME (µM/s/mg)**
Trypsin-like serine-proteinases	Susceptible	0.34 ± 0.05 a	0.27 ± 0.03 a	0.044 ± 0.002 c	0.85 ± 0.03 b
	Resistant cost	0.26 ± 0.03 b	0.28 ± 0.04 a	0.050 ± 0.002 b	0.72 ± 0.03 b
	Resistant no-cost	0.22 ± 0.02 b	0.21 ± 0.03 a	0.076 ± 0.002 a	1.29 ± 0.05 a
Cysteine-proteinases	Susceptible	0.38 ± 0.04 a	-	10.40 ± 0.32 c	-
	Resistant cost	0.39 ± 0.04 a	-	53.10 ± 1.90 a	-
	Resistant no-cost	0.42 ± 0.05 a	-	32.04 ± 1.20 b	-
		K_M (starch; g.L^{-1})		**V_{max} (starch; AU.dL^{-1})**	
α-Amylases	Susceptible	0.24 ± 0.04 a		640.09 ± 26.67 a	
	Resistant cost	0.14 ± 0.02 b		500.12 ± 15.07 b	
	Resistant no-cost	0.23 ± 0.01 a		674.34 ± 7.18 a	

3.3. α-Amylases

The main α-amylase of each maize weevil strain was purified by glycogen precipitation and ion-exchange chromatography (≥ 70-fold purification, ≤ 19% yield). Single α-amylase bands with the same molecular mass (53,700 Da) were revealed for each insect strain. Higher activity was obtained at 35-40°C and at pH 5.0-7.0 for all of the strains. The α-amylase from the resistant no-cost strain exhibited higher activity towards starch and lower inhibition by acarbose and wheat amylase inhibitors (Table 1). Opposite results were observed for the α-amylase from the resistant cost strain (Table 1). Although the α-amylase from the resistant cost strain exhibited higher affinity to starch (i.e., lower K_m), its V_{max} value was the lowest among the strains, particularly the resistant no-cost strain (Table 2).

4. Discussion

The enzymes partially purified with aprotinin-agarose affinity column from the susceptible, resistant cost and resistant no-cost strains of *S. zeamais* were trypsin-like serine-proteinases because they were able to hydrolyze L-BApNA and L-TAME (Oliveira et al., 2005). Furthermore, they were inhibited by typical trypsin-like inhibitors, namely aprotinin, benzamidine, SBTI and TLCK (Oliveira et al., 2005). The resistant strains showed greater sensitivity to inhibition and the trypsin-like proteinases from the resistant no-cost strain were particularly sensitive to inhibition by benzamidine, TLCK and SBTI compared to trypsin-like proteinases from the susceptible strain. Trypsin-like proteinases purified from the resistant no-cost strain showed a nearly two-fold higher activity towards the two substrates investigated, which is in agreement with our previous finding in crude preparations (Araújo et al. 2008b).

The recognition of the purified proteinases as cysteine-proteinases was achieved through their subsequent characterization via kinetic studies and inhibitor analysis carried out with fractions eluted from thiol-sepharose affinity column. E-64 (a specific cysteine-proteinase inhibitor) efficiently inhibited such activity, unlike inhibitors of other proteinase classes (i.e., EDTA for metalloproteinases, pepstatin for aspartato-proteinases, and TLCK for serine-proteinases), as expected for cysteine-proteinases (D'Avila-Levy et al., 2003; Mohamed et al., 2005). The proteinases least sensitive to inhibition by the specific cysteine-proteinase inhibitor E-64 were those from the resistant cost strain. Although affinity (i.e. K_M) of the cysteine-proteinases was similar among them, the V_{max} value for cysteine-proteinases from the resistant cost strain was 3x and 5x higher than V_{max} values for the resistant no-cost and susceptible strains, respectively. These combined results indicate the existence of different isoforms of cysteine proteinases and consequently qualitative differences associated with the insect strains studied here.

The biochemical characterization of the purified α-amylases provided results consistent with insect α-amylases (Baker, 1983; Mendiola-Olaya et al., 2000; Cinco-Moroyoqui et al., 2008). They were similar among the maize weevil strains, although the activity levels of α-amylases obtained for the resistant no-cost strain were always higher, particularly when compared with the α-amylase from the resistant cost strain. The inhibition profile of the purified α-amylases from each strain were however significantly different not only for acarbose, but also for wheat amylase inhibitors. Both are recognized inhibitors of α-amylase, acarbose is an anti-diabetic drug (Brzozowski and Davies, 1997), while wheat amylase inhibitors are natural compounds obtained from wheat grains (Cinco-Moroyoqui et al., 2006).

We earlier hypothesized that higher proteinase and amylase activity in insecticide-resistant strains of maize weevil might contribute in mitigating the physiological costs (and consequently the fitness costs) usually associated with insecticide resistance, as initially suggested by studies with these insect strains (Guedes et al., 2006; Araújo et al., 2008ab). If so, higher activity levels and distinct inhibition profile of hydrolytic enzymes would occur in the resistant no-cost strain, particularly in contrast with the resistant cost strain. Indeed, higher levels of trypsin-like serine-proteinase and α-amylase activity were observed in the resistant no-cost strain, whose main isoforms also exhibited different inhibition profiles. The higher levels of α-amylase activity in the resistant no-cost strain were likely due to a higher expression of this enzyme rather than to a more efficient isoform of amylase, since it exhibits lower affinity to starch. In contrast, different isoforms of trypsin-like proteinases are probably the main determinants for the higher activity observed in the resistant no-cost strain. Such results provide support for the hypothesis that enhanced trypsin-like proteinase and α-amylase activity may be playing a major role in mitigating fitness costs associated with insecticide resistance (Araújo et al., 2008ab), unlike cysteine-proteinases, which may be playing only a secondary role, if any, in mitigating the costs usually associated with insecticide resistance.

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