DIGESTIVE ENZYME IDENTIFICATION AND EFFECTS OF FEEDING SERINE PROTEINASE INHIBITORS TO *SOLENOPSIS INVICTA* (HYMENOPTERA:FORMICIDAE)

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ABSTRACT

During the life cycle of the red imported fire ant, *Solenopsis invicta*, the queen and larvae require protein and amino acids for development. Blocking this nutritional pathway could negatively affect the colony by reducing the number of eggs and prevent larvae from developing. A major protein digestive enzyme was identified from the fourth instar larvae as chymotrypsin, and has been isolated, sequenced, inhibited and crystallized (Botos et al. 2000, Whitworth 1998). In an attempt to disrupt protein digestion several serine proteinase inhibitors were evaluated: two small molecule inhibitors (PMSF and AEBSF), and two protein trypsin/chymotrypsin inhibitors (aprotinin 6.5 kDa, and lima bean trypsin inhibitor 10 kDa). These inhibitors were formulated in dried egg yolk or liquid sucrose, and fed to fire ant colonies in the laboratory. The general health of the queen and brood were monitored by measuring the queen's weight, number of eggs laid, and larval enzyme specific activity for chymotrypsin and elastase.

INTRODUCTION

The workers of the fire ant, *Solenopsis invicta* Buren Hymenoptera: Formicidae, have several developmental stages: egg, instars larvae, pre-pupae, pupae, and worker ant. Adult worker ants depend largely on fats and carbohydrates for their nutritional supplements while developing larvae require more protein, and the queen utilizes all three food types (Abbot 1978, Lange 1967, Sorensen et al. 1981, Sorensen 1981). To facilitate this feeding process, the larvae secrete digestive enzymes externally in the antero-ventral region (Petralia and Vinson 1978). In this external "food-basket" digestion site, nurse ants place larger particles of food for digestion (Holldobler and Wilson 1990). Later, the nurse ants collect the liquid digestion products, amino acids and peptide fragments, for feeding the queen and other larvae. Of the worker development stages, only the fourth instar express serine proteinase chymotrypsin and elastase (Whitworth et al. 1999). By hindering this digestive process, the queen and the larvae would not be able to digest scavenged protein-rich foods and therefore would lack important developmental components. The first approach has been to characterize a common proteinase in the larval digestive fluids to determine its class and inhibitor possibilities. Following the initial work of Whitworth (1998), the most abundant serine proteinase was isolated from the larvae and characterized with various inhibitors: phenylmethylsulfonyl fluoride, aprotinin; 4-(2-aminoethyl) benzene-sulfonyl fluoride hydrochloride, and lima bean trypsin inhibitor. These inhibitors were fed to colonies while monitoring the queen's weight, egg laying rate, larval enzyme activity, and colony health.

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METHODS AND MATERIALS

Purification of C1. Purification of the prominent serine proteinase, chymotrypsin isozyme C1, was based on the method described by Whitworth (1998) with necessary modifications. Fourth instar larvae (10 g) were vigorously stirred for 8 hours in 100 ml in buffer A (50 mM Tris, pH 7.4) at 4°C. The ruptured larval solution was centrifuged at 12,100 g for 20 min; floating fats were removed with a spatula and the supernatant solution (115ml) decanted. Over 15 min, 230 ml of acetone (-20°C) was added and centrifuged at 3,820 g for 20 min. The pellet was dissolved in 200 ml buffer A and stirred for 45 min. The protein solution was adjusted to pH 7.4 and centrifuged for 20 min at 12,100 g, before loading onto a Q-Sepharose column (2.5 x 10 cm, Sigma Q-1126) equilibrated with buffer A. The column was washed with ten column volumes of buffer A and the chymotrypsin activity eluted with two 100 ml step gradients containing 300 mM and 400 mM NaCl in buffer A. The protein was dialyzed twice in 2 L of 10 mM Tris, pH 7.4, for three hours. The conductivity was measured to determine dialysis completion. The protein was loaded onto a DEAE Sepharose Cl-6B column (1.5 x 30 cm, Sigma DCL-6B-100) and eluted with a 250 x 250 ml linear gradient from 0 to 250 mM NaCl in buffer A. Fractions (5 ml) were collected and a column profile was generated with 280 nm absorbance and chymotrypsin activity. The first activity peak corresponds to C1. Appropriate fractions were concentrated to 40 ml in a stirred Amicon cell (200 ml, YM-10 membrane) and exchanged with buffer B (20 mM Tris-HCl, pH 7.4) three times. Finally, the protein was dialyzed overnight in 2 L buffer B. The protein solution was loaded onto a High performance Q-Sepharose (10 ml, Pharmacia 17-1014-01) and eluted with a 200 x 200 ml linear gradient from 0 to 150 mM NaCl. The column absorbance profile at 280 nm resulted in a baseline separation of C1 from C2 and C3 (fractions 98-104, 1.8 ml).

Enzyme Assay. Final concentrations in 400 µl included: 100 mM Tris-HCl (pH 7.5), 200 mM NaCl, 125 uM substrate (N-Succinyl-Ala-Ala-Pro-Phe-pNA, Sigma S-7388), at room temperature. The release of pNA was monitored with a Beckman DU-50 at 410 nm ($\varepsilon = 8800$ $M^{-1}cm^{-1}$). The protein concentration was determined using the Bradford assay (Pierce Inc., Rockford, Illinois), with bovine serum albumin as standard (Bradford 1976).

Inhibition. Purified C1 was incubated with inhibitors for 10 min and 1 hr followed by addition of substrate and activity determination. The predominance of chymotrypsin encouraged the testing of potential serine proteinase inhibitors: 4-(2-aminoethyl) benzene-sulfonyl fluoride hydrochloride (AEBSF), aprotinin, and lima bean trypsin inhibitor (LBTI, *Phaseolus limensis*,). The small covalent inhibitor, AEBSF, has greater solubility and stability in aqueous solutions, than the more potent phenylmethylsulfonyl fluoride (PMSF). Aprotinin is an effective but selective inhibitor of mammalian serine proteinases (Ki 0.06 pM for trypsin, Ki 9 nM chymotrypsin and Ki 3.5 mM elastase (Fritz, H. and G. Wunderer. 1983)). With three disulfides and molecular weight of 6.5 kDa, aprotinin is a small and stable protein that could be transported by worker ants to the larvae. Alternately, a larger serine inhibitor protein LBTI (10 kDa), was selected in that it might be transported directly to the larval antero-ventral region or "feeding –basket".

Preliminary laboratory assay of digestive enzyme inhibition formulations: Eight colonies (a control and three inhibitors for each formulation) were cultivated in a plastic container 16.5 x 9 x 31 cm. Fluon® AD1 (Asahi Glass Company Charlotte, N.C.) lined the upper interior to prevent ants escaping. Water was available continuously in test-tubes plugged with cotton. The nest was a covered 60 mm Petri dish containing castone (Castone Dental Stone Type III, Dentsply International, York, PA), and was moistened three times a week with water. Each single queen colony was collected in the field (Brazos County, Texas) and subsequently reduced to 0.5 g larvae, approximately 2000 workers and single queen.

During ten weeks, a weekly feeding schedule was followed. On day 1, the remaining food was removed. This was to deplete any reserves and ensure consumption of the inhibitor

formulations that were given the next day. The next day, each colony was analyzed: 1) queen's mass (mg), 2) number of eggs laid in two hours, and 3) enzyme activity. Following the measurements, four colonies were fed dried egg yolk (three contained separate inhibitor), and four colonies were fed liquid sucrose (three contained separate inhibitor). On day 3 and 4, no additional food was provided to encourage complete consumption of the inhibition formulation. This was to ensure that all the formulation was consumed. Days 5, 6, and 7, the colonies resumed normal feeding days (i.e., no inhibitors present) which included mealworms, honey, starch and sesame oil (Petralia and Vinson 1978). Two control colonies received the same treatments but the egg yolk or sucrose did not contain inhibitors.

Inhibitor Feeding. Colonies were fed inhibitor present in sucrose or egg yolk. The inhibitor quantity used was to ensure inhibition of the larval chymotrypsin concentrations present in the colony (see below). Three inhibitors were: aprotinin (25 μg, 3.8 nmol, 15.4 μM, Sigma A6279), lima bean trypsin inhibitor (250 μg, 27.7 nmol, 111 uM Sigma T9378), and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 0.125 mg, 511 nmol, 2.04 mM, Sigma A8456). These inhibitors were mixed separately with either liquid sucrose (250 μl, 116 mM, 40 g/l) or dried egg yolk (100 mg, Sigma E0625). An homogeneous suspension of egg yolk, water, and inhibitor was stirred and then air-dried prior to feeding. While sucrose is a simple food source containing only carbohydrates; dried egg yolk is a more complex food, containing fats and proteins. As a control, blue food coloring was added to a single inhibitor feeding to confirm that the dye/food (egg yolk or sucrose) reached the larvae. Nourishment delivery and consumption was confirmed when larvae turned blue. Each week, the same inhibitor was fed to the same colony (Fig. 2, 3). For an upper limit estimation on the amount necessary for covalent inhibition, 20 worker larvae yielded 90 μg protein. If all proteins were C1, 4.0 nmoles inhibitor would be required. Based on the purification table (Table 1), 0.6 % of the total protein was C1; thus ensuring enough inhibitor for the whole colony.

Larval enzyme activity was assayed twice a week following the third week of feeding (Fig. 2, 3). This delay allowed the inhibitor to take effect on the colony. Each colony was anesthetized with carbon dioxide, and 10 to 20 fourth instar larvae were randomly collected. The larvae were crushed and suspended in 700 μl of 20 mM Tris (pH 7.4), vortex-stirred for 10 sec, and centrifuged for 10 min. The supernatant solution (500 μl) was removed and precipitated with 1.0 ml acetone (4°C), centrifuged for 10 min, and re-suspended in 200 μl of 20 mM Tris (pH 7.4). Aliquots were assayed as described previously. When larvae were not present, values were recorded as zero.

RESULTS

Using fourth instar larvae, a fire ant chymotrypsin was purified (Table 1, Fig. 1). The enzyme activity was reduced with several chymotrypsin/serine protienase inhibitors (Table 2). Aprotinin, lima bean trypsin inhibitor, and PMSF were all effective against the purified fire ant chymotrypsin *in vitro*., while AEBSF was less effective. In addition, these compounds can inhibit other serine proteinases, like elastase and trypsin, which suggested broader inhibition during the feeding trials.

Colonies Studies. Feeding serine proteinase inhibitors to fire ant laboratory colonies resulted in some observable effects in the variables measured (queen's weight, egg production, and chymotrypsin and elastase activity). The queens weight normalized: 13.2 ± 2.6 mg for sucrose fed colonies (Fig. 2a), 13.7 ± 2.8 mg for egg yolk fed colonies (Fig. 2b). In both groups (sucrose and egg yolk fed colonies), this initial decrease was probably due to reduction in colony size for experimental comparison and a new feeding scheme. Likewise the queens'

ability to lay eggs was affected in this initial adjustment period (Fig. 2c, 2d). For the egg yolk fed colonies (Fig. 2d), the rate normalized at 23 ± 6 eggs in two hours, while the more nutritionally deprived sucrose fed colonies averaged 6 ± 6 eggs in two hours (Fig. 2c).
With respect to chymotrypsin activity, the egg yolks fed colonies (Fig. 3b) gradually increased their specific activity (0.3 to 0.5 μmol/min/mg), while the colonies fed sucrose (Fig. 3a) had more data variation. The higher specific activity with sucrose may be diet specific, by causing an increase in chymotrypsin pools (under utilized, not distributed, decreased trophallaxis or not down regulated,) due to the protein-lean diet. Conversely, the specific activity of larval elastase from both egg yolks and sucrose remained around 0.1 μmol/min/mg (Fig. 3c, 3d), with the sucrose fed colonies having more data variation. The colonies with too few larvae for assay analysis were recorded as zero.

TABLE 1. Purification of Chymotrypsin

Purification Step	mgs	S.A.
Crude	142	0.8
Acetone Precipitation	78	1.6
Q-Sepharose Column	28.8	3.3
Cl-6B Sepharose Column	1.15	21.5
Mono-Q Column	0.85	38

Values for the purification procedure using 10 g larvae are listed as the total protein (mgs) and specific activity (S.A., μmol/min/mg total protein) using the previously described assay.

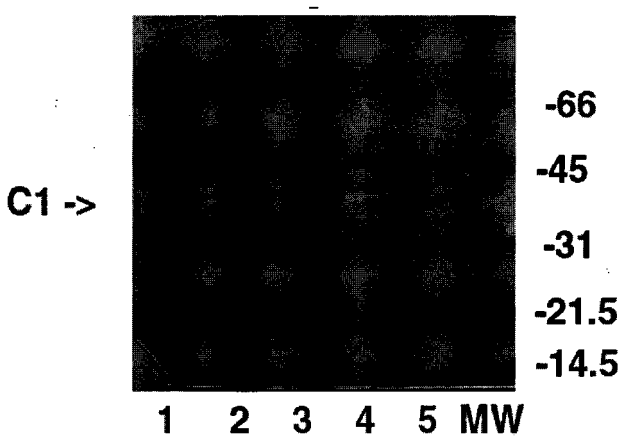

FIG.1 10 % PAGE gel for the purification of chymotrpysin C1 with the following lanes 1) crude lysate, 2) acetone precipitation, 3) Q-sepharose, 4) Cl-6B, 5) High performance Q-sepharose, 6) molecular weight markers: Serum albumin 66.2kDa, Ovalbumin 45kDa, Carbonic anhydrase 31 kDa, Trypsin inhibitor 21.5 kDa, Lysozyme 14.4 kDa, SDS-PAGE Molecular weights Standards, broad Range BioRad 161-0317.

TABLE 2. Inhibition of Chymotrypsin

Inhibitor	Concentration	10 min	1 hr
AEBSF	3.8 mM	90 %	75 %
Aprotinin	11 uM	0	0
Lima Bean	1 uM	0	0
PMSF	1 uM	0	0

Remaining activity of C1 (0.42 mUnits) after incubation with the inhibitor in assay buffer. Reaction was initiated with addition of substrate.

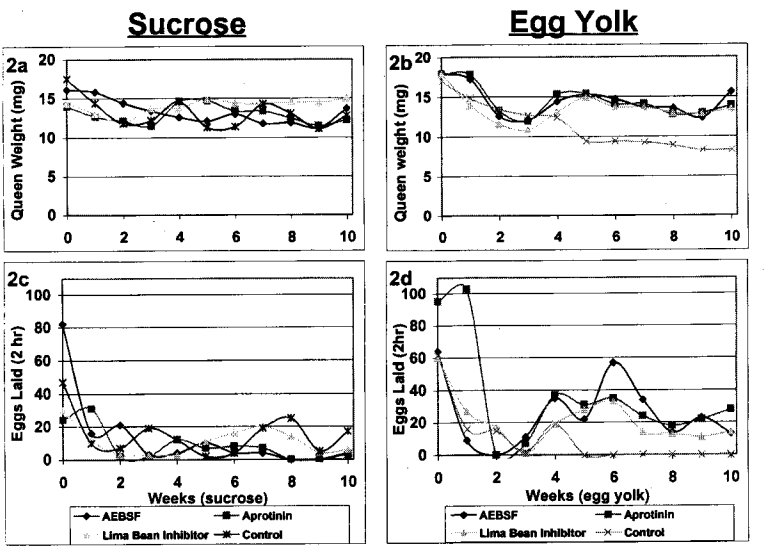

FIG. 2 Measurement of Queens Mass and Eggs Laid: the mass of the queen measured once a week in milligrams (a, b), the number of eggs laid by the queen during a two hours (c, d). The colonies fed sucrose (a, c) and egg yolk (b, d) appear in the same column. Each inhibitor is marked with the same symbol in all the graphs.

DISCUSSION

The compounds for this feeding study were inhibitors of purified fire ant chymotrypsin (C1); however, their effect in reducing the ant colony (queen weight and egg production) were not detected. Formulations containing food coloring provided visual evidence of nutrient transfer. During the inhibitor feeding by forager ants, reserve ants, and nurse ants the trophallaxis process might have decreased potency prior to feeding nutrients to larvae (Sorensen et al. 1983). Other proteinases may be present in the worker ants (aspartic, metalo and cysteine) to inactivate the inhibitor. Using only three days for regular feeding had a greater impact on the colony's health by decreasing the queen's weight and number of eggs laid, than any of the inhibitor.

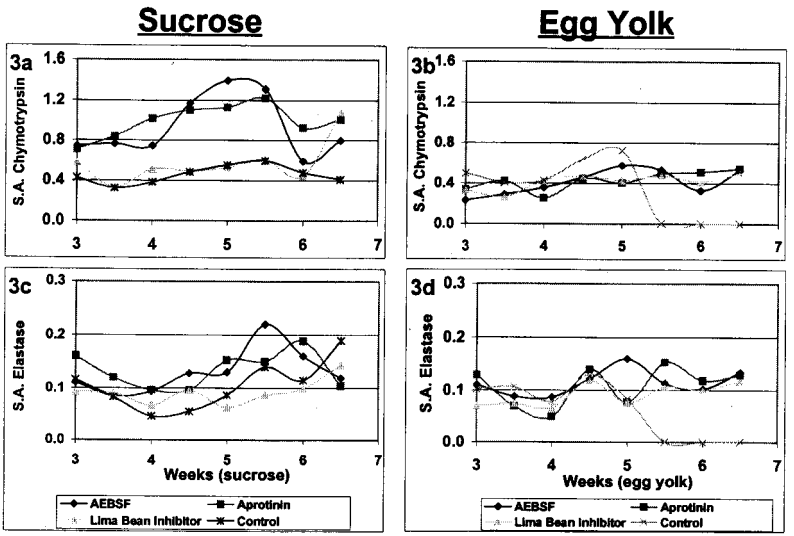

FIG. 3 Measurement of Specific Activity: The fourth instar larvae were assayed for enzymatic activity using a chymotrypsin substrate (a, b) and elastase substrate (c, d) and calculated as micro moles pre minute per milligram protein. The colonies fed sucrose (a and c) and egg yolk (b and d) appear in the same column.

The selection of sucrose and egg yolk in the forulation were chosen by nutritional type. Sucrose is more likely utilized by the worker ants, while egg yolk proteins utilized by the larvae. Not knowing the effect of trophallaxis on the inhibitor transport, the chance of protein being delivered to the antero-ventral region seemed greater than in the liquid sucrose. This would allow for more direct inhibitor delivery, thus affecting the desired digestion. Unfortunately, detecting inhibitor delivery was not possible with the current methods. While the food coloring provided a qualitative confirmation, a more quantitative method might include some type of tag (radio label or fluorescence). Since sucrose and egg yolk were not nutritionally balanced, the regular feed was also provided to the colony. Cannibalism was not observed. The sucrose fed colonies had fewer eggs and more varied queen's weight, while the egg yolk fed colonies had more eggs and uniform queen's weight. The number of eggs laid during each counting was varied and provided a general indication of the queen's health. Additional colonies and multiple egg countings per week could provide a better statistical analysis of queen's health in relation to fed inhibitor, eggs and larval enzyme activity.

The enzymatic assay of chymotrypsin and elastase in larvae revealed that activity was present even after the inhibitors became part of the diet. Both enzymes in all colonies studied suggested that the larvae were able to either express more enzyme to compensate the inhibition, or that inhibitor modification occurred during trophallaxis to counter its intended effect. While the protein inhibitors (aprotinin, and lima bean trypsin inhibitor) were chosen to avoid trophallaxis, their inhibitory properties were not detected. These results show that the general enzymatic activity was similar within each feeding group. While these are preliminary data, some general observations can be extracted from the graphs. With the sucrose fed colony, two colonies had a 2-3 fold greater chymotrypsin specific activity. This may be the

result of the feeding scheme, or a response to inhibitor in the diet. Further studies are required, including base level activity prior to inhibitor, and a more accurate correlation to queen weight to detect chymotrypsin expression variation between the species.

One control colony appeared to have lost larvae due to reduction in egg production by the queen. This was a risk in using single queen colonies versus a multi-queen colony. The loss of queen weight did correlate to loss in egg production in this control. In general, the egg yolk diet, rather than the sucrose diet, provided additional nutritional value in maintaining the queen's egg laying capabilities.

A rapid inhibition of the protein digestion process was expected based on the *in vitro* studies. While a continuous feeding method (food with and without inhibitor present) provides continual nourishment and a more realistic environment for inhibitor effectiveness, we wanted to encourage inhibitor consumption. By providing the colony with inhibitor laced food as the only source for nourishment for a few days, a direct impact on egg production by the queen, larval development, and brood replacement would be expected to be more dramatic.

As a result of these inhibitor feeds, alternate approaches are being devised. Structural analysis of chymotrypsin C1 (Botos et al. 2000) and C3 (in preparation) now provides a unique opportunity to design a specific inhibitor for these enzymes which would significantly differ from mammalian digestive enzymes. In addition, alternative formulation procedures for inhibitor delivery need to be implemented.

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