The imported red fire ant (Solenopsis invicta) is a problematic pest in the Southern United States. The stages of development for these ants are as follows: egg, 1st, 2nd, 3rd, and 4th instar larvae, prepupa, pupae, and adult. The 4th instar larvae plays an important role in the survival of the colony in that it is totally responsible for the digestion of solid foods and the source of nutrients for the queen and adult workers. In our studies we have been successful in purifying and characterizing four proteinases from the 4th instar larvae. Based on substrate specificity, they appear to represent two chymotrypsin-like and two elastase-like proteinases. These are referred to as Soli C1, Soli C2, Soli E1, and Soli E2, with molecular masses of 25, 28, 23, and 24 kDa, respectively, based on SDS-PAGE. All enzymes were inhibited by diisopropyl fluorophosphate, a general serine class inhibitor. Various synthetic substrates with either Phe or Val in the P1 position, were readily cleaved by Soli C1/C2 or E1/E2, respectively. Each enzyme has been characterized as to pH optimum, pH stability, isoelectricfocusing and susceptibility to inhibition by a broad range of natural and synthetic proteinase inhibitors. Such compounds may prove useful for the development of insecticides to control fire ant infestation.

The imported red fire ant (Solenopsis invicta) is a major pest which, over the past 50 years, has spread across and infested the southern United States, while virtually eradicating the indigenous species (1). This organism is also a major nuisance to man and other animals since it possesses a powerful, painful sting from which its name was acquired and which, in some cases, can result in anaphylactic shock (2). Together with its medical impact and, therefore, require adequate control and/or total eradication. However, it seems clear that affecting their function through the addition of specific proteinase inhibitors could interfere with colony survival, primarily through starvation of the queen for essential amino acids. This report describes results obtained for the purification and characterization of four proteinases isolated from the 4th instar larvae of S. invicta, as well as inhibition profiles for each enzyme isolated (Table I).

**EXPERIMENTAL PROCEDURES**

**Materials**

N-Suc-Ala-Ala-Pro-Phenylalanine (NAP), N-tosyl-L-lysinechloromethyl ketone (TLCK), N-p-tosyl-L-lysinechloromethyl ketone (TPCK), lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), and Trizma were from Sigma. Diisopropyl fluorophosphate (DFP) was from Calbiochem. Trasylol and a-1-proteinase inhibitor (a-1-PI) were gifts from Athens Research and Technology, Athens, Ga. Eglin C was obtained from Dr. Hans Schnelli of Ciba Geigy. Hen ovomucoid (OM), Canadian goose OM, and domestic goose OM were graciously provided by Dr. Michael Laskowski (Purdue University, West Lafayette, IN). Potato inhibitors I and II were obtained from Dr. Clarence Ryan (Washington State University, Pullman, WA). All other pNA substrates and synthetic inhibitors were provided by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA).

**Methods**

**Cultivation of Larvae**—The colonies of S. invicta were maintained according to Petralia and Vinson (5), with the 4th instar larvae being collected by manual separation from the rest of the colony with an aspirator. Enzyme Purification—Larvae were extracted in a 1:10 (w/v) ratio of 20 mM Tris-HCl, pH 7.4 (Buffer A). The mixture was stirred at 4 °C for 24 h and centrifuged at 10,000 g, for 15 min at 4 °C, with the lipid layer on top being removed physically by pipette and the supernatant retained. Chilled acetone was added at a 2:1 (v/v) ratio over a period of 15 min at 4 °C. This produced a precipitate that was collected by centrifugation (10,000 g, 15 min, 4 °C). The precipitate was redissolved in 0.1 of its original volume in buffer A, and this was applied to a Q Sepharose (Sigma) column (2.5 × 15 cm, 75 ml) previously equilibrated with buffer A. The column was washed with 2 column volumes of buffer A at a flow rate of 50 ml/hr. A gradient from 0 to 500 mM NaCl in buffer A was then applied to the column over 750 ml at this rate. At three peaks of activity were seen (see Fig. 1A). The first (peak I) was present in the flow-through and had activity against MeOSuc-Ala-Ala-Pro-Val-pNA. Peak II also had activity on this substrate but was present in fractions eluted by the salt gradient. Peak III, also eluted in the salt gradient, had activity against N-Suc-Ala-Ala-Pro-Phe-pNa.

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¶To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Life Sciences Bldg., University of Georgia, Athens, GA 30602. Tel.: 706-542-1711; Fax: 706-542-3719; E-mail: jtravis@uga.cc.uga.edu.

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**Proteinases of S. invicta**

### Purification of S. invicta 4th instar larval proteinases

| Proteinase | Fraction step | Total activity<sup>a</sup> | Total protein<sup>a</sup> | Specific activity | Purification Yield<sup>b</sup> |
|------------|---------------|-----------------------------|---------------------------|------------------|-------------------------------|
| Soli E1    | Crude extract | 26,100                      | 186                       | 140              | 1 100                         |
|            | Acetone          | 23,700                     | 110                       | 215              | 1.5 91                        |
|            | Peak I from Q-Sepharose | 10,900   | 41.5                     | 260              | 1.8 42                       |
|            | Mono S, FPLC    | 6,840                      | 0.586                     | 11,700           | 84 26                        |
| Soli E2    | Crude extract   | 26,100                      | 186                       | 140              | 1 100                         |
|            | Acetone          | 23,700                     | 110                       | 215              | 1.5 91                        |
|            | Peak II from Q-Sepharose | 9,380     | 22.7                     | 410              | 2.9 35                       |
|            | Mono Q, FPLC    | 4,190                      | 0.859                     | 4,900            | 35 16                        |
|            | Superdex 75, FPLC | 1,500             | 0.132                     | 11,400           | 81 6                         |
| Soli C1    | Crude extract   | 41,600                      | 186                       | 220              | 1 100                         |
|            | Acetone          | 38,700                     | 110                       | 350              | 1.6 93                        |
|            | Peak III from Q-Sepharose | 19,400  | 6.4                        | 3,030           | 13.8 47                       |
|            | Mono Q, FPLC    | 11,700                     | 0.725                     | 16,100           | 73 28                        |
| Soli C2    | Crude extract   | 41,600                      | 186                       | 220              | 1 100                         |
|            | Acetone          | 38,700                     | 110                       | 350              | 1.6 93                        |
|            | Peak III from Q-Sepharose | 19,400  | 6.4                        | 3,030           | 13.8 47                       |
|            | Mono Q, FPLC    | 1,010                      | 0.130                     | 7,770            | 35.3 2.4                     |
|            | Superdex 75, FPLC | 720                | 0.030                     | 24,000           | 109 1.7                      |

<sup>a</sup> Based on enzymatic activity using MeOSuc-Ala-Ala-Pro-Val-pNA or Suc-Ala-Ala-Pro-Phe-pNA, where 1 unit = 1 nmol of pNA released per min.

<sup>b</sup> 3 g of larvae extracted.

<sup>c</sup> Based on initial activity in crude extract.

Peak I, whose activity was designated E1, was pooled, concentrated by ultrafiltration to 10 ml, using a 3K membrane (Filtron), and applied to a Mono S FPLC column HR 5/5 (Amersham Pharmacia Biotech), previously equilibrated with buffer A. The column was first washed with 5 column volumes of buffer A at 1.0 ml/min followed by a gradient from 0 to 500 mM NaCl in buffer A, to elute bound proteins. Fractions of 1 ml were collected and assayed for activity against MeOSuc-Ala-Ala-Pro-Val-pNA. Those with this activity were then applied to and separated on a Superdex<sup>TM</sup> 75 gel filtration column HR 10/30, previously equilibrated with 150 mM NaCl in buffer A. Active fractions were pooled and used for further analysis.

The purification of the enzymes present in peaks II and III were essentially identical. Fractions from each peak were pooled, dialyzed (MWCO 3,500, Spectrum Medical Industries, Inc.) for 4 h at 4 °C against buffer A, and concentrated to 10 ml by ultrafiltration, using a 3K membrane (Filtron). The samples were then separately applied to a Mono Q FPLC column HR 5/5 (Amersham Pharmacia Biotech), previously equilibrated with buffer A, and the column was then washed with 5 column volumes of buffer A. Bound proteins were eluted from the column with a gradient from 0 to 500 mM NaCl in buffer A. All fractions were assayed for activity against either MeOSuc-Ala-Ala-Pro-Val-pNA (peak II) or Suc-Ala-Ala-Pro-Phe-pNA (peak III), as described above. The enhanced resolution offered by Mono Q separated the activity in peak III into two separate proteins both of which hydrolyzed Suc-Ala-Ala-Pro-Phe-pNA (see Fig. 1B). These are, therefore, referred to as C1 and C2, while a single activity was recovered from peak II (E2). All active fractions from this purification step were individually pooled and concentrated to 0.5 ml, by ultrafiltration, using a 3K membrane (Filtron). For final purification, the samples were then separately applied to a Superdex<sup>TM</sup> 75 gel filtration column HR 10/30, previously equilibrated with buffer A. Active fractions were pooled and used for further analysis.

**Enzyme Assay**—Generally, the amidolytic activities of each proteinase were measured with the substrates MeOSuc-Ala-Ala-Pro-Val-pNA and N-Suc-Ala-Ala-Pro-Phe-pNA, in 0.05 M Tris-HCl, 100 mM NaCl, pH 7.4, (assay buffer). Enzyme inhibition assays by protein inhibitors were conducted in the same assay buffer using an inhibitor concentration ranging from 0.02–50 nM with a 10-min preincubation time and with the final enzyme concentration being 1.0 nM. All kinetic studies with substrates and inhibitors were performed at the optimum pH of the individual proteinases, utilizing the following buffers: 150 mM NaCl, 50 mM Tris-HCl, pH 8.0 (E1); 150 mM NaCl, 50 mM CHES, pH 9.5 (E2 and C2); and 150 mM NaCl, 50 mM CHAPS, pH 10.0 (C1).

**SDS-PAGE**—The SDS-PAGE method devised by Shagger and von Jagow (6), using a Tris-HCl/Tricine buffer system, was used throughout this study.

**pH Analysis**—The determination of pH optimum and stability for each enzyme was performed using a Universal Phosphate Buffer system.

**Enzyme Kinetics and Specificity**—**V<sub>max</sub>** and **K<sub>m</sub>** values were determined using substrates or synthetic inhibitors at concentrations ranging from 10 nM to 5 mM, with the final concentration of each enzyme being 10 nM in the specific optimum buffers utilized. **V<sub>max</sub>** and **K<sub>m</sub>** values were measured by using Hyperbolic Regression Analysis.<sup>2</sup>

**IEF**—Isoelectrofocusing of the purified proteinases was performed on Servalyt Precote<sup>TM</sup> precast isoelectric focusing gels (pH 3–10). Sample preparations and isoelectrofocusing were carried out following instructions provided by Pharmacia and using the Pharmacia Flatbed Electrophoresis Unit from Pharmacia.

**Sequence Analysis**—Purified proteins were subjected to SDS-PAGE and electrophoresed on polyvinylidene difluoride membranes using 10 mM CHAPS, pH 11, 10% methanol (7). The membrane was washed thoroughly with water and stained with Coomassie Blue G250. The blot was air-dried, and the bands containing enzymes were cut out and subjected to N-terminal sequence analysis with an Applied Biosystems Procise Protein Sequencer, using the program designed by the manufacturer.

**RESULTS**

**Enzyme Purification**—Acetone precipitation proved to be an excellent first step, as opposed to ammonium sulfate or acid precipitation, both of which caused drastic losses of all enzymatic activities. Indeed, the utilization of acetone effectively removed 40% of the unwanted proteins from the crude extract, while it had little negative effect on the activity of Soli C and limited loss of activity for both Soli E1 and Soli E2 (10–20%). Significantly, the use of Q Sepharose as a second step then limited the activity of Soli E1 and Soli E2 (10–20%). Thus, the sample from this step was well overloaded on an SDS-PAGE gel. Therefore, gel filtration chromatography was utilized to obtain >99% purity.

Anion-exchange chromatography on Mono Q increased the specific activity of Soli E2 about 12-fold and, more significantly, separated Soli C1 and Soli C2 into distinct activities (Fig. 1B). After Mono Q, the level of purity for both Soli E2 and Soli C2 was only near 65% while Soli C1 was judged to be 90% pure. Thus, gel filtration was again utilized which again achieved 99% purity for Soli E2, Soli C1, and Soli C2.

<sup>2</sup>The hyperbolic Regression Analysis program written by J. S. Easterby (University of Liverpool, United Kingdom) was obtained as a shareware program.
Soli E2 had narrow specificity toward P1 Ala or Val, with Soli also affected by residues in the P2 to P4 positions of the sub-

E2 being particularly more active with the latter amino acid.

Details are as described under “Methods,” with the separation of individual activities of crude extracts on Q Sepharose. B, rechromatography of peak III on Mono Q to separate Soli C1 and Soli C2.

**SDS-PAGE Analysis**—SDS-PAGE of all four purified enzymes gave single bands with molecular masses of 23, 24, 25, and 28 kDa, respectively, for Soli E1, Soli E2, Soli C1, and Soli C2 (Fig. 2). A duplicate gel of that shown in Fig. 1, in which all of the samples were treated with 3H-DFP, was exposed to x-ray film with the radiolabeled proteins, confirming the fact that the purified products were the target enzymes for this serine proteinase inhibitor.

**Enzyme Specificity**—The testing of numerous synthetic para-nitroanilide substrates against each of the four isolated proteinases supported the hypothesis that two were chymotrypsin-like proteins, while the other two pairs of proteinases (Table IV), with members of the ovomucoid family being particularly specific toward Soli E1 and Soli E2 but showing no activity against Soli C1 or Soli C2. In contrast, Trasylol was only active against Soli C1 and LBTI against Soli C1 and Soli C2. All other inhibitors examined were generally inhibitory toward all four enzymes.

**pH Optimum**—The pH optimum for each enzyme, as determined by plotting the V_max of substrate hydrolysis, at room temperature, in a pH range from 3.0 to 12.0, gave values of 8.0 for Soli E1, 8.5–10 for Soli E2, from 9.0 to 10.5 for Soli C1, and 9.0 for Soli C2.

**Stability**—Soli C1 was found to be stable for several days between the pH range of 7 to 9 at 37 °C. Soli C2, Soli E1, and Soli E2 on the other hand, lost their activities fairly quickly over all pHs but were most stable at pH 8.0, 7.0, and 8.5, respectively.
### TABLE III
Effect of peptide inhibitors on the amidolytic activity of *S. invicta*

| Inhibitor                          | IC₅₀ (nM) |
|------------------------------------|-----------|
| Z-Phe-Val-Pro-Val-p(OPh)₂          | 29.41     |
| Z-Phe-Leu-Pro-Val-p(OPh)₂          | 246.7     |
| Z-Leu-Ile-Pro-Val-p(OPh)₂          | 145.8     |
| Z-Phe-Leu-Leu-p(OPh)₂              | 1116.8    |
| Z-Phe-Val-p(OPh)₂                  | 0.10      |
| Z-Phe-Leu-p(OPh)₂                  | 0.10      |
| Z-Leu-Ile-p(OPh)₂                  | 0.10      |
| Z-Pro-Val-p(OPh)₂                  | 0.00      |
| Boc-Ala-Ser-Pro-Val-p(OPh)₂        | 0.00      |
| Boc-Pro-Val-Pro-Val-p(OPh)₂        | 0.00      |
| DCIC                               | 0.24      |

### TABLE IV
Effect of proteinase inhibitors on the amidolytic activity of *S. invicta*

| Inhibitor                          | IC₅₀ (nM) |
|------------------------------------|-----------|
| Canadian goose OM                  | 0.137     |
| Domestic goose OM                  | 1.642     |
| Domestic goose OM domain III       | 0.102     |
| Ethyl chloroformate (Eglin C)      | 0.442     |
| Trasylol                           | 5.967     |
| Soy bean trypsin inhibitor I-S     | 1.210     |
| Lima bean trypsin inhibitor α₁-PI  | 3.380     |
| Potato inhibitor I                 | 2.626     |
| Potato inhibitor II                | 14.138    |

### TABLE V
Proteinases of *S. invicta* 14433

### TABLE VI
Amidolytic activity of developmental stages of *S. invicta*

| Inhibitor | IC₅₀ (nM) |
|-----------|-----------|
| Egg       | 0.00      |
| 1st Instar| 24.00     |
| 2nd Instar| 42.00     |
| 3rd Instar| 36.00     |
| 4th Instar| 100.00    |
| Prepuce   | 0.00      |
| Pupae     | 0.00      |
| Adult     | 0.00      |

* Percent activity relative to that found in 4th instar larvae.

#### DISCUSSION

Solenopsis invicta is indigenous to the Guayu and Paraguay regions of South America. It is thought to have been accidentally introduced into the United States from dirt used in the ballast of ships, which was taken from the riverbanks of these regions (8). Due to suitable climate and conditions, this species quickly spread over and infested 13 Southeastern and Southwestern states, easily out competing the indigenous species because of their aggressive behavior (9). As *S. invicta* has invaded the Southern states, it has virtually eliminated *S. xyloni,* and greatly reduced the populations of *Pogonomyrmex badius* (harvesting ant) and *S. geminata* (1). The venom of fire ants is 95% 2,6-dialkylpyridine, and this compound is responsible for the wheel-and-flare reaction that occurs after stinging (10, 11). Aside from this alkaloid, 0.1% of the venom is comprised of four highly allergenic proteins, which in 0.5% of sting victims result in an immune response that can lead to anaphylactic shock (12, 13).

Currently, the most common methods of treating fire ant infestation are with toxicants (amidohydrozones), juvenile hormone analogs, or avermectin B₁a (1, 3). However, in order to eliminate the colony completely, it is necessary to kill the queen and with the emergence of polygyne (multiple queen) colonies, this becomes more difficult (14, 15). Juvenile hormone analogs do interfere with the development of the brood but the entire colony brood must be reached, or it will quickly rebound (16, 17). It has been observed that sometimes the colony will relocate in response to physical disturbance or stress if members of the colony suddenly die. Therefore a more subtle and less invasive means of control would be useful.
The stages of development for ants are as follows: egg, 1st, 2nd, 3rd, and 4th instar larvae, prepupa, pupae, and adult. As in other social insects, each caste has a particular role. Foragers locate and bring foods to the colony. Boluses of food are then given to nursemates which are responsible for caretaking of the brood. These ants, in turn, place the bolus on the anteriores opening of the 4th instar larvae which digest the solid food and thus play an important role in the survival of the colony (1). By tropholaxis, which is the sharing of food among social insects, digested food is distributed throughout the colony. This provides essential free amino acids to all members and, in particular, to the queen (4). Thus, attempts to interfere with the digestive enzymes from the 4th instar larvae could be a useful mechanism for developing a more effective means of controlling this pest.

Previously, there has been very little description and certainly no purification of proteinases from the 4th instar larvae of S. invicta, aside from that reported by Sorensen in which general proteolytic activity was ascribed based on radial diffusion assays (18). In the current study, we were able to purify and characterize four distinctly different enzymes. Since secreted fluids from the anteriores opening of the 4th instar larvae possessed the same amidolytic activity as the proteinases, it is likely that they are involved in the digestion of food for the colony. However, it cannot be ruled out that any of the proteinases might also play a role in the larval development. In the same context, it is also important to point out that egg, prepupa, pupa, and adults when analyzed for these same putative digestive enzymes were found to be devoid of these proteinases, while the 1st, 2nd, and 3rd instar larvae possessed significantly lower levels of enzyme activity relative to the 4th instar larvae (Table VI). Although it has been documented that adult ants do have a small amount of digestive enzymes in their midguts (18), it is thought that their presence is likely due to tropholaxis.

In the current study we were able to identify and purify four major proteinases, each of which could be easily localized to the 4th instar larvae. Characterization of these enzymes indicated a strong relationship toward other members of the pancreatic proteinase family, including $M_{r}$, substrate specificity, inhibition profile, and N-terminal sequence. Curiously, we did not identify any trypsin-like activity in larvae extracts, only enzymes that could be classified as related to elastase and chymotrypsin. While each of the purified enzymes was identified, primarily through the use of synthetic substrates, all were also able to degrade both azo-casein and gelatin, thereby supporting the fact that they are, indeed, proteinases. Perhaps of most importance is the fact that all of the larvae-derived proteinases could be regulated by both synthetic and natural inhibitors, with the latter being from both plant and animal sources. Whether either class of inhibitor (natural or synthetic) could be utilized in proper bait to control S. invicta remains to be established, and experiments to examine such possibilities are currently in progress.

REFERENCES

1. Holldobler, B., and Wilson, E. O. (1990) The Ants. Harvard University Press, Cambridge
2. Lockley, R. F. (1979) Advances in Allergology and Clinical Immunology, pp. 441–448. Pergamon Press, New York
3. Lofgren, C. S. (1986) Economic Impact and Control of Social Insects, pp. 227–256. Praeger Publishers, New York
4. Vinson, S. B., and Sorensen, A. A. (1986) Imported Fire Ants: Life History and Impact. Texas Department of Agriculture, Austin, Texas
5. Petralia, R. S., and Vinson, S. B. (1978) Anal. Entomol. Soc. Am. 71, 643–648
6. Shaggar, H., and von Jagow, G. (1987) Anal. Biochem. 166, 168–379
7. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
8. Buren, W. F. (1972) J. Ga. Entomol. Soc. Am. 7, 1–27
9. Tschinkel, W. R. (1987) Entomol. Soc. Am. 81, 76–81
10. MacConnell, J. G., Blum, M. S., and Fales, H. M. (1971) Tetrahedron 26, 129
11. Baer, H., Lui, T.-Y., Anderson, M. C., Blum, M., Schmidt, W. H., and James, F. J. (1979) Toxicon 17, 397–405
12. Hoffman, D. R., Dove, D. E., and Jacobson, R. S. (1988) J. Clin. Immunol. 82, 818–827
13. Glancey, B. M., Craig, C. H., Stringer, C. E., and Bishop, P. M. (1973) J. Ga. Entomol. Soc. Am. 8, 237–238
14. Hung, A. C. F., Vinson, S. B., and Summerlin, J. W. (1974) Anal. Entomol. Soc. Am. 67, 909–912
15. Porter, S. D., Bhatker, A. W., Mueller, R., Vinson, S. B., and Clair, D. J. (1991) J. Econ. Entomol. 84, 866–874
16. Drees, B. M., and Vinson, S. B. (1990) Toxicon 25, 317–324
17. Sorensen, A. A., Kamas, R. S., and Vinson, S. B. (1983) J. Insect Physiol. 29, 163–168