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Characterization of Proteolytic and Collagenolytic Enzymes from the Larvae of *Lucilia cuprina*, the Sheep Blowfly

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Abstract
Isoelectric focusing was used to characterize proteolytic enzymes in homogenate and excretory–secretory preparations of the larvae of *L. cuprina*, the sheep blowfly. Zymogram overlays showed that the larvae produce a number of highly active proteases which have a wide range of isoelectric points and molecular weights. The alkaline and neutral pI proteases were inhibited by phenylmethyl-sulfonylfluoride, leupeptin and aprotinin; this indicated the presence of serine in the active site. Pepstatin and the metal chelating agent ethylenediaminetetraacetic acid had no effect on the activity of any of the proteases. Optimal pH for activity of the proteases was between 7 and 8. In addition, the proteases were found to be heat labile. Digestion of collagen fibrils confirmed the existence of collagenolytic activity in the excretory–secretory enzyme preparations. It is suggested that these enzymes may be involved in the nutrition of the larvae and in the pathogenesis of the lesion on the skin.

Introduction

The parasitic larvae of *Lucilia cuprina* induce a large oedematous lesion on the skin of sheep which is thought to provide their main nutrient source (Merritt and Watts 1978). However, the mechanism(s) by which this occurs has not been extensively analysed. Unlike second and third instar larvae, first instar larvae do not possess abrasive mouthhooks. However, skin damage occurs in the first 24 hours after hatching (Sandeman *et al.* 1987); this suggests that mechanisms other than mechanical disruption might be operating to cause lesion development.

Hobson (1931) demonstrated the presence of enzymes with some collagenolytic activity in larvae of *L. sericata*, and Waterhouse and Irzykiewicz (1957) showed the presence of a similar enzyme secreted by the larvae of *L. cuprina*. Such enzymes may be important in the pathogenesis of the skin wound and in the nutrition of the larvae. However, an understanding of the enzymes produced, their substrates and their specificities is required before their role in the mechanism of wound formation can be determined.

Proteolytic enzymes have been reported in a number of tissue-penetrating helminths (Mathews 1977; Dresden *et al.* 1985) and endoparasitic insects (Reich and Zorzopulos 1978; Hotez and Cerami 1983). In addition, a collagenase has been isolated from the larvae of *Hypoderma lineatum*, the warble fly which is endoparasitic on cattle (Lecroisey and Keil 1985). This collagenase is thought to assist with penetration through the skin (Lecroisey and Keil 1985). The present study describes the presence of similar enzymes produced by the larvae of *L. cuprina*.

Materials and Methods

Culture Preparations

The enzyme preparations used were obtained aseptically according to a modification of the method of Lennox (1939). *L. cuprina* eggs were incubated for 5 h in a humid box at 37°C, placed in 0·1 M NaOH for 3 min to separate the egg masses, and then sterilized in 1% sodium hypochlorite for 5 min. The eggs were then placed in sterile cultures containing 10% sodium caseinate, 2% agar, 5% brewer's yeast (Difco) and 0·5% glucose in 25 ml of phosphate-buffered saline (PBS), pH 7·4. The conical flasks were plugged

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with cotton wool, then placed in the dark for 24 h at 25°C. All cultures were set up in a sterile hood (Clemco). First instar homogenates (L_1H) and excretory–secretory (L_1ES) preparations were collected after 24 h, second instar homogenates (L_2H) and excretory–secretory (L_2ES) preparations after 48 h and third instar homogenates (L_3H) and excretory–secretory (L_3ES) preparations after 72 h.

Excretory–secretory products were obtained by adding 5 ml of deionized distilled water to the surface of the medium and gently swirling before filtering off the liquid. The extract was brought to 0·01 M phosphate, pH 7·4, by passage through a Sephadex G 50 column (Pharmacia) (2·0 × 15 cm) at 50 ml h^{-1} at room temperature. The extract was monitored at an absorbance of 280 nm and the first peak collected, freeze-dried and stored at −70°C. Homogenates were prepared by removing the larvae from the culture medium using a spatula and then immediately rinsing them with distilled water. After several washes, the whole larvae were ground up in a 25-ml mechanical tissue homogenizer with a teflon pestle (Wheaton U.S.A.) on ice in the presence of 0·05% Triton X-100 for 2 min. The extracts were centrifuged at 16 000 *g* for 30 min at 4°C, the supernatant was then removed and brought to 0·01 M phosphate, pH 7·4, freeze-dried and stored as above.

Protein concentration of the preparations was determined by the method of Bradford (1976) with bovine serum albumin as a standard. For isoelectric focusing, 20 μg protein of excretory–secretory preparation or 40 μg protein of the homogenates was applied in 2 μl of buffer to the gel.

Isoelectric Focusing Gels (IEF)

The gels were prepared using a plastic template (Corning Immunoelectrophoresis plate) according to the method of McLachlan and Cornell (1978). Each IEF gel consisted of 3·8 ml distilled water, 0·4 g D-sorbitol, 35 mg IEF agarose (Pharmacia) and 0·2 ml ampholine (Pharmacia) (pH range 3–10), giving a gel thickness of 0·4 mm. The water, sorbitol (Sigma) and agarose were boiled, then placed in a 56°C water bath. The ampholines were then added, the solution poured onto the template and a piece of Gelbond (FMC Pharmacia) overlayed. The template was placed in a plastic bag and stored at 4°C for at least 2 h before use.

Samples were applied and the gels were run at 1 watt for 400 volt-hours. They were then fixed or a zymogram gel was overlayed. All IEF gels and zymograms were fixed in a solution of 11% trichloroacetic acid and 3·5% sulfosalicylic acid in distilled water and then stained with Coomassie Brilliant Blue R-250 (Bio-Rad) in 30% methanol plus 10% acetic acid and destained in the same solution without the stain.

With each IEF run, one lane was used to establish a pH gradient by cutting it into 0·5 cm pieces, placing these in 1 ml deionised distilled water and reading the pH. The gradient curve was constructed by plotting pH values versus distance from the cathode.

Zymograms

Enzyme activity of focused protein bands was detected by overlaying the IEF gel with a substrate-containing gel (zymogram). These were made with the same plastic templates used for the IEF gels. The method was a modification of that used by Every (1981). All zymograms were photographed using incident light on a black background immediately after fixing, and/or after staining with Coomassie blue.

Collagen zymograms were made at 37°C, with an agarose of low solidifying temperature, so as to minimize denaturation of the collagen. Sheep type 1 collagen was prepared by the methods of Hong *et al.* (1979) and Trelstad *et al.* (1976). The zymogram gel consisted of 4·45 ml 0·1 M Na_2HPO_4(pH 8·6) containing 40 mg agarose of low gelling temperature (Bio-Rad) which was dissolved by boiling. Collagen (25 mg) was dissolved in 0·55 ml 0·1 M citric acid. The two solutions were mixed at approximately 38°C and poured on to the template, giving a final pH of 7·4. A piece of Gelbond was overlayed and the gel stored at 4°C. $CaCl_2$ (5 mM) and $MgCl_2$ (5 mM) were added to the zymogram mixture when clostridial collagenase was used as a standard.

Gelatin zymograms were prepared as for collagen zymograms, with 0·5% gelatin and 0·8% agarose and a water bath temperature of 42°C.

Casein zymograms were prepared as for collagen, with 0·5% casein and 0·8% agarose at 42°C.

pH Dependence

pH dependence of the proteases was measured with 2 mM *N*-α-benzoyl-D-L-arginine *p*-nitroanilide (BAPNA) (Sigma) in various buffers (phosphate buffers; pH 2·7, 5·5, 6·0, 6·5, 7·0 and 7·5; borate buffers; pH 8·0, 8·5, 9·0 and 10·0). BAPNA was firstly dissolved in the buffers containing 5% *N*-*N*-dimethylformamide (Ajax) before being incubated for 5 h with either L_2ES (0·4 mg ml^{-1}) or trypsin (0·02 mg ml^{-1}) in a Mitrotitre® plate (Dynatech Laboratories Inc., Virginia, U.S.A.) at 22°C. The plate was read at 440 nm using a Titertek Multiskan® MC reader. This method was a modification of that used by Erlanger *et al.* (1961).

Inhibitors

The activity of the enzymes was tested with various inhibitors included in the zymograms. These were EDTA (Ajax) (25 mM); pepstatin A (5 mM); aprotinin (5 mM); phenylmethylsulfonyl fluoride (PMSF; 5 mM) and leupeptin (0·1mM), which were all purchased from Sigma.

All were tested using the sodium phosphate citrate buffer system at pH 7·4, without the addition of $CaCl_2$ or $MgCl_2$.

Molecular Weight Determination

Aliquots, each comprising 20 μl of a 10 mg ml^{-1} L_2ES sample or of a 5 mg ml^{-1} L_2H sample, were run on a 10% polyacrylamide gel according to the method by Laemmli (1970). Samples were diluted 1:1 with sample buffer, with or without mercaptoethanol, and boiled or not boiled. After electrophoresis, the gel was silver-stained by the method of Morrissey (1981) or transferred to nitrocellulose (Bio-Rad) (Kerlero de Rosbo *et al.* 1984) using a Trans-Blot Cell apparatus.

Collagen Fibril Assay

A collagen fibril assay was set up to determine if the larval proteases were able to degrade insoluble collagen fibrils *in vitro*. Collagen fibrils were made by dissolving 5 mg collagen (sheep type 1) in 2 ml of 0·1 M citric acid. Aliquots of 100 μl were placed in microfuge tubes which were warmed to 37°C for 5 min. One ml of 0·2 M Na_2HPO_4 with 0·15 M NaCl was added to each tube which was then incubated for 1 h at 37°C. The fibrils were centrifuged at 10 000 *g* for 5 min, the supernatant was removed and 100 μl of 0·1 M phosphate buffer (pH 7·0) with 5 mM $CaCl_2$ was added.

The fibrils were warmed to 35°C before the addition of 100 μl of either clostridial collagenase (Type VII; EC 3.4.24.3) (10 μg ml^{-1}), L_2ES (10 or 1 μg ml^{-1}) or buffer alone, 0·1 M phosphate (pH 7·0) with 5 mM $CaCl_2$. Tubes without collagen fibrils but with aliquots of the enzymes were also included. After mixing, all tubes were incubated at 35°C for 6 h. Aliquots of 10 μl were then removed and diluted 1:1 with sample buffer containing 5% mercaptoethanol, boiled and then run on a 10% polyacrylamide gel.

Results

Collagen Zymograms

Soluble collagen as a substrate produced excellent zymograms with high sensitivity and resolution when incubated with IEF gels for 25 min at 37°C (Fig. 1). The clostridial collagenase

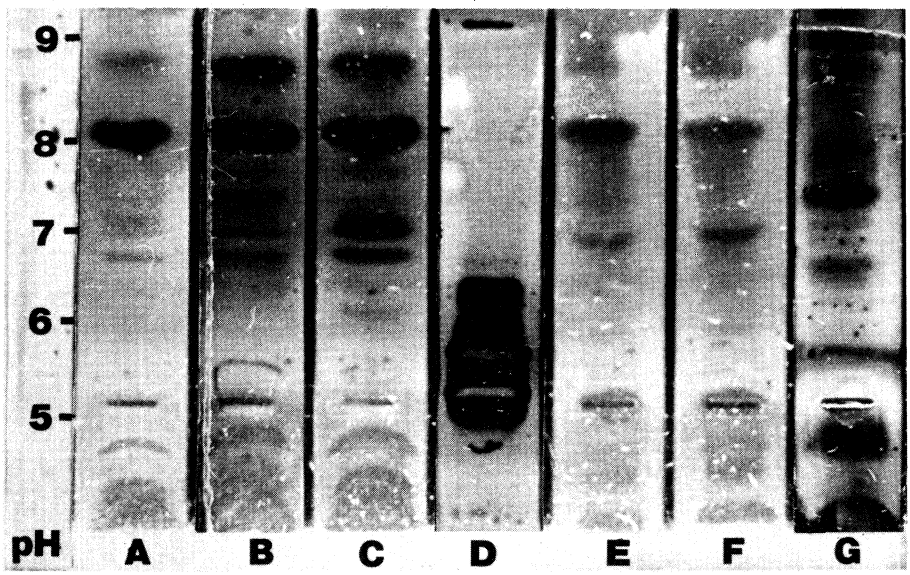

Fig. 1. Collagen zymogram after incubation for 25 min at 37°C with an isoelectric focusing gel. The pH gradient is given to the left of the picture and the lanes contained (A) L_1ES; (B) L_2ES; (C) L_3ES; (D) *Clostridium histolyticum* collagenase; (E) L_1H; (F) L_2H and (G) L_3H. (Photograph of fixed gel.)

gave three strong plaques at pIs of 5·0, 6·0 and 6·5 when Ca^{2+} and Mg^{2+} were added to the sample buffer. Activity was much reduced without these ions (data no shown). In contrast, the excretory–secretory and homogenate preparations had no such requirement and were usually dissolved in distilled water for IEF gels. Excretory–secretory products have a large number of plaques with a distinctive pattern which was reproducible in tests between and within different batches of these products. The major plaques of L_1ES products had pIs of 4·5, 6·8 8·0, 8·6 and one at the position of the basic electrode. The pattern at second stage was virtually identical, except that the plaque at pI 7·0 and 8·6 was more pronounced. L_3ES products gave similar plaques to the previous stages, though a doublet at pI 7·0 was much stronger. In addition to these major plaques, many minor bands were seen; however, these varied in intensity from preparation to preparation, suggesting that they arose as a result of the isolation procedures. The homogenate preparations gave patterns very similar to their respective instar excretory–secretory products, though only the major plaques are seen. The most noticeable difference observed was the lack of the major plaque at pI 6·8 in L_2H compared to L_2ES products, and a plaque present at pI 7·4 in the L_3H preparation when run on either collagen or gelatin which was not evident in the casein gel.

Gelatin Zymograms

Gelatin zymograms gave almost identical plaque patterns to collagen (Fig. 1). Casein zymograms also gave a similar pattern, though resolution was improved (Fig. 2). The minor variation in the pattern (pI 8·0 band) may be due to the method of measuring pH. The optimal incubation time for gelatin was 25 min at 37°C, while casein required 90 min. Gelatin was therefore used for further testing.

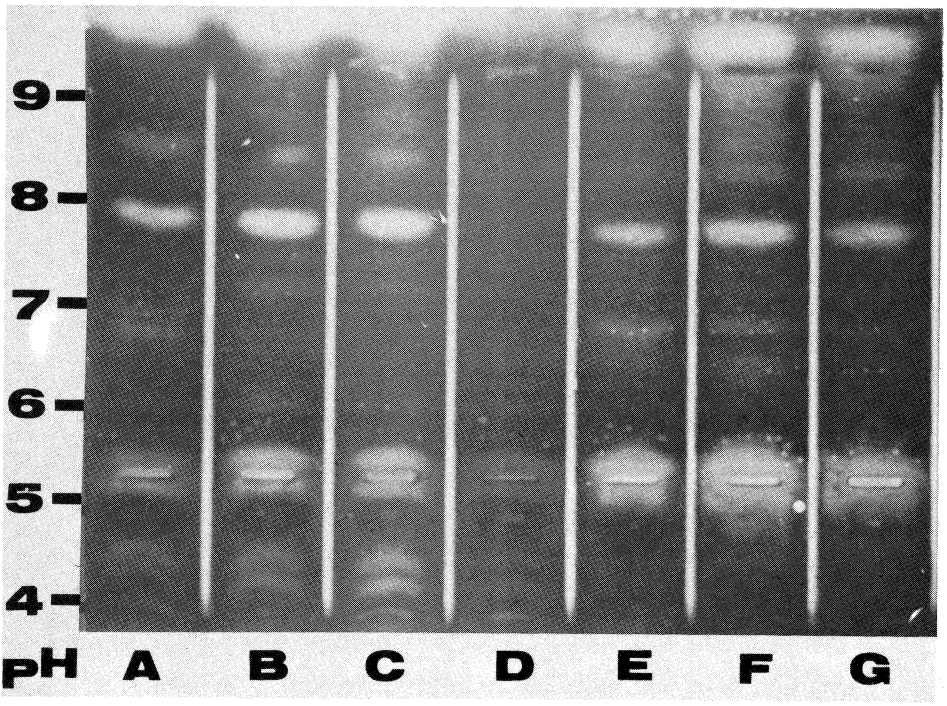

Fig. 2. Casein zymogram after incubation for 90 min at 37°C with an isoelectric focusing gel. The pH gradient is given to the left of the picture and the lanes contained (A) L_1ES; (B) L_2ES; (C) L_3ES; (D) *Clostridium histolyticum* collagenase; (E) L_3H; (F) L_2H and (G) L_1H. (Photograph of stained gel.)

pH Dependence

The effect of pH on activity of the proteases is illustrated in Fig. 3. The proteases contained in L_2ES show maximal activity in the pH range 7·0–8·0; for trypsin, maximal activity was recorded at pH 7·0.

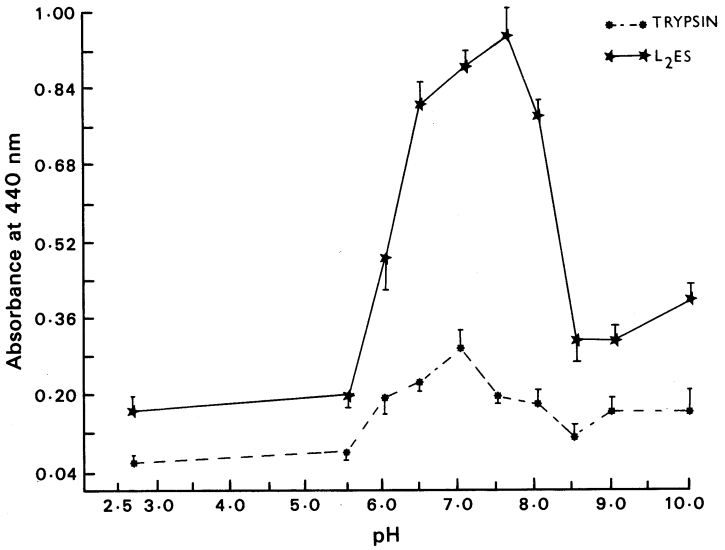

Fig. 3. pH dependence of trypsin and of the proteases produced by larvae of *L. cuprina*.

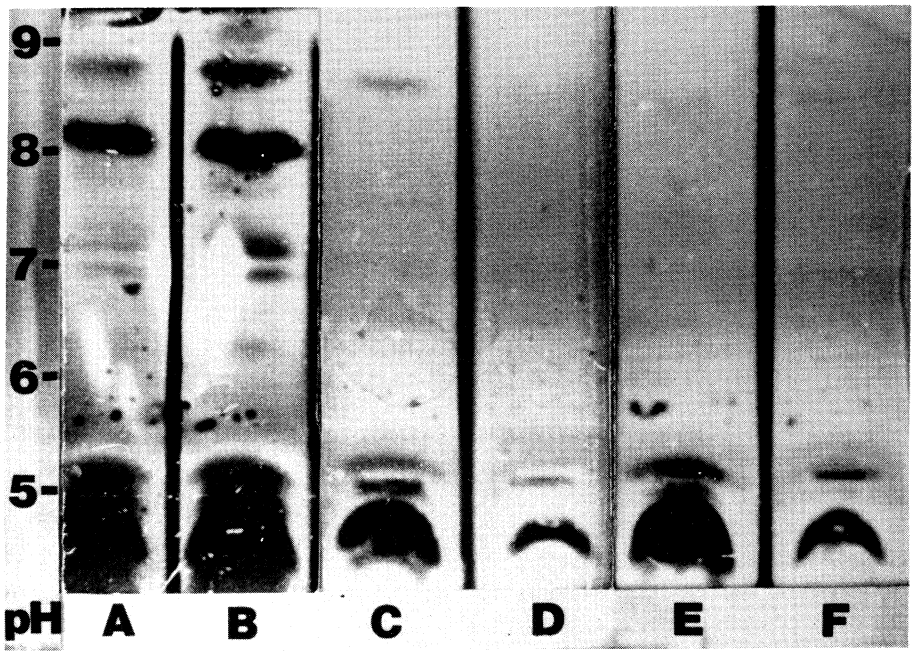

Fig. 4. Gelatin zymogram with the addition of protease inhibitors: (A) and (B) no inhibitor; (C) and (D) leupeptin (0·1 mM); (E) and (F) PMSF (5 mM). The zymogram was incubated for 25 min at 37°C with an isoelectric focusing gel which contained L_2ES in lanes (B), (C) and (E) and L_2H in lanes (A), (D) and (F). (Photograph of fixed gel.)

Inhibitors

The effect of various enzyme inhibitors on enzyme activity was determined on gelatin zymograms. EDTA had no effect on the excretory–secretory or homogenate plaque patterns but abolished the clostridial collagenase patterns (data not shown). Likewise, pepstatin did not inhibit any of the larval preparations (data not shown). Plaque formation in the neutral and basic range of the IEF gel was inhibited by leupeptin, PMSF and aprotinin and there were lesser effects on the enzymes with acidic pI plaques (Fig. 4).

Molecular Weight Determination

The molecular weights of the major proteins in both the L_2ES and L_2H preparations are shown in Fig. 5. L_2ES contains approximately 10 major protein bands; L_2H has approximately 8 major and many minor bands. These protein bands ranged in molecular weight from 25 000 to 45 000.

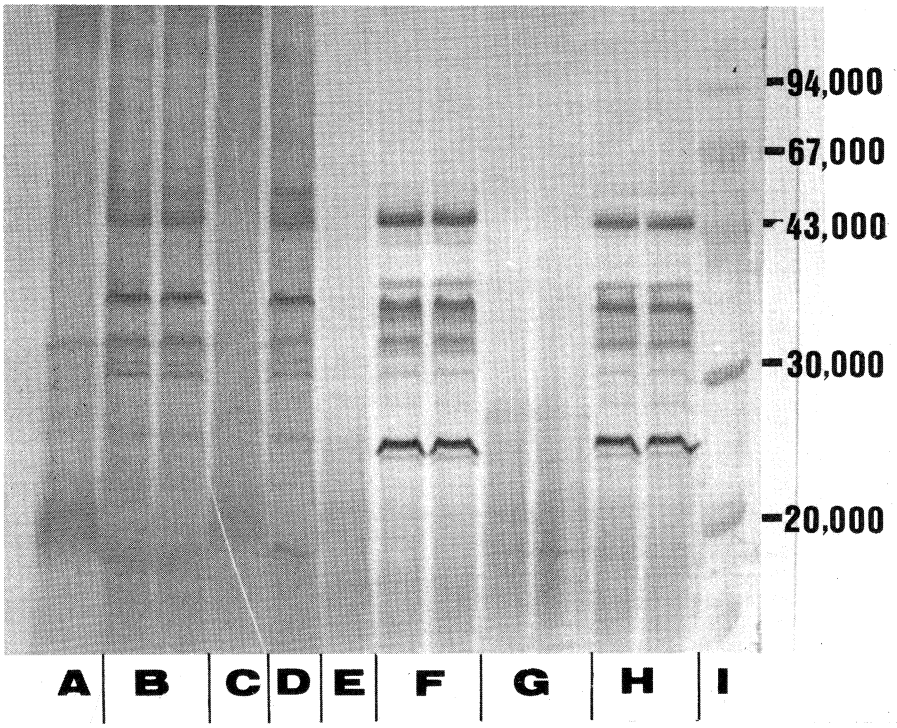

Fig. 5. Silver-stained gel of proteins in the larval preparations separated by SDS-Page. (A) L_2H with mercaptoethanol (2ME) and boiled; (B) L_2H with 2ME and not boiled; (C) L_2H with 2ME and boiled; (D) L_2H without 2ME and not boiled; (E) L_2ES without 2ME and boiled; (F) L_2ES without 2ME and not boiled; (G) L_2ES without 2ME and boiled; (H) L_2ES with 2ME and not boiled and (I) molecular weight standards.

Collagen fibril assay

SDS polyacrylamide electrophoresis (SDS—Page) analyses of breakdown products from incubations of collagen fibrils with enzyme preparations are shown in Fig. 6. Incubation with clostridial collagenase resulted in the loss of both the α and β chains indicating the almost complete breakdown of collagen in this incubation. The L_2ES preparation gave major collagen products at approximate molecular weights of 150 000 and 85 000, and a number of minor bands below these.

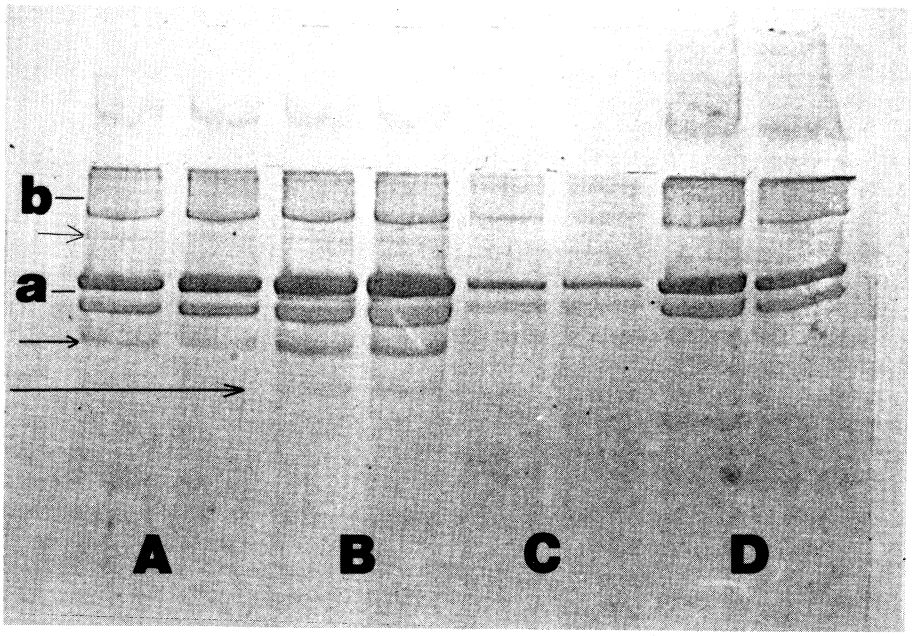

Fig. 6. SDS-Page analysis of the products from the incubation of collagen fibrils with different enzyme preparations. In the duplicate control incubation of collagen fibrils (D) only α (*a*) and β (*b*) collagen chains are seen. The addition of *Clostridium histolyticum* collagenase (10 μg ml^{-1}) resulted in an almost complete breakdown of both the α and β chains (C). The addition of either 1 or 10 μg L_2ES ml^{-1} to (A) and (B) respectively resulted in the appearance of breakdown products (arrowed).

Discussion

The results show that the larvae of *L. cuprina* produce a number of highly active proteases which have a wide range of isoelectric points and molecular weights. In addition, they produce an enzyme capable of degrading sheep type 1 collagen fibrils. Enzyme plaques formed with all three substrates. Only minor differences were observed in the number and charge of plaques in first, second and third instar execretory–secretory and homogenate preparations. The plaque present at pI 7·4 on gelatin and collagen zymograms which was not apparent in the casein zymogram may indicate the presence of a protease with greater specificity for collagen and gelatin. Overall, L_2ES consistently showed the greater number of plaques. However, it is probable that many of the plaques may be isoenzymes — for example, the doublet present at pI 7·0 in the L_2ES (Fig. 1). In addition, it is also possible that limited proteolysis (autolysis) of the enzymes may be occurring which could lead to different mobilities without the loss of proteolytic activity. Despite the existence of these minor bands, the results suggest that there are at least two major enzymes present in both preparations, i.e. the group around pI 4·5, and around pI 8·0.

SDS-Page analysis revealed a range of proteins with molecular weights between 25 000 and 45 000 in both the L_2ES and L_2H preparations. These bands have been found to correspond both with plaques on nitrocellulose transfers off SDS gels which were then incubated with casein and stained, and with lines on SDS gels incubated with chromogenic substrates (unpublished data). Overall protease activity seems to be associated with a number of these protein bands. Unfortunately, it is not possible to determine the exact number of proteases in the larval preparations because the samples were undissociated and dimers or fragments of the native

molecules may have activity. However, as in the IEF gels, a number of proteases appear to be present.

The presence of enzymes with apparently similar functions might be of advantage in maximizing the range of nutrient substrates and/or in avoiding host responses. Thus, if an enzyme is being inhibited by the host, a second enzyme may be able to assume the role of the first.

The results of the pH-dependence study indicated that the enzymes had maximal activity between pH 7 and 8. This is in accordance with other insect gut proteases which usually have pH optima in the neutral and alkaline region (House 1974). There is little information on the normal pH values of sheep skin. However, Lipson (1978) found that suint pH varied between 4·0 and 8·0 in Merino sheep with an average pH of 7·0, and that lower pH values tended to correlate with resistance to strike. It is possible therefore that suint pH values below 7·0 may cause a reduction in enzyme activity and thus explain the increased level of resistance (Lipson 1983).

The results of the inhibitory studies showed that PMSF, which is known to react specifically with functional serine residues (Ahmad *et al.* 1980) and aprotinin, inhibited all of the neutral and alkaline proteases of *L. cuprina*, suggesting an essential role for serine in the catalytic function of these enzymes (Dixon *et al.* 1956). PMSF had a lesser effect on the protease in the acidic region, though this may be due to the amount of enzyme in this region. Leupeptin which is known to inhibit thiol-containing proteases and certain serine proteases (Umezawa 1975) also inhibited the neutral and alkaline pI proteases, which would be consistent with the results for PMSF and aprotinin. Pepstatin, an inhibitor of carboxyl proteases (Umezawa and Aoyagi 1977), did not cause any inhibition. Based on these results, it is suggested that at least the basic and neutral enzymes are serine proteases. This conclusion is consistent with studies on similar enzymes produced by the fiddler crab (Eisen *et al.* 1973) and by larvae of other dipterans; the warble fly *Hypoderma lineatum* (Boulard and Garrone 1978; Lecroisey *et al.* 1979; Lecroisey *et al.* 1980), the mosquito *Aedes aegypti* (Kunz 1978) and the Hessian fly *Mayetiola destructor* (Shukle *et al.* 1985). In addition, there was little effect of EDTA or Ca^{2+} and Mg^{2+} ions at the concentrations studied on the activity of the collagenase. The finding of collagenase activity in *L. cuprina* larvae similar to that described in *L. lineatum* by Boulard and Garrone (1978) suggests a similar role in the breakdown of skin collagen. It would be expected that this enzyme would not act as a metallo-collagenase but as other serine proteases with collagenolytic activity (Eisen *et al.* 1973).

The results confirm the existence of collagenolytic activity in both the homogenate and excretory–secretory preparations of *L. cuprina* supporting the observations of Hobson (1931) and Waterhouse and Irzykiewicz (1957). The activities of these collagenase and protease enzymes are consistent with a role not only in nutrition of the larvae but also in digestion of structural components of the skin, thus assisting with lesion formation and exudate production.

Acknowledgments

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