Proteolytic activity of blowfly larvae secretions in experimental burns

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Secretions of larvae of the blowfly Calliphora erythrocephala digested experimental rat skin burn eschar in vivo and in vitro when applied topically in a vanishing cream base. Debridement was characterized by de-epithelialization and digestion of dermal collagen to a subfollicular level over a 3-day period. Analytic investigation of the secretions demonstrated the presence of enzymes with activities characteristic of trypsin, leucine aminopeptidase, and carboxypeptidases A and B. These were partially characterized. There was no evidence of chymotrypsin, elastase, or collagenase. Preparation of a suitable therapeutic form could result in a preparation useful for enzymatic debridement.

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Maggot therapy was introduced for the treatment of osteomyelitis and pyogenic infection around 1930.[1, 27] Technical difficulties in rearing and applying large quantities of sterile larvae, problems of clinical control of larvae in wounds, opposition from patients, and the discovery of antibiotics led to the diminished use of larval therapy over the next several decades, although the method is still occasionally used.[13]

Among the possible explanations for the mechanism of larval therapy, digestion of necrotic tissue by proteolytic[2, 7, 12, 16, 20] and collagenolytic[11, 26, 29] enzymes has been strongly suggested. The reports of collagenolytic activity led us to investigate the potential of larval secretions for the debridement of the largely collagenous burn eschar and other necrotic dermal tissue.

This article contains observations on the ability of topical preparations of larval secretions to debride experimental burn wounds in rats and preliminary characterization of the proteolytic enzymes contained in the secretions.

Supported in part by National Institute of General Medical Sciences grant No. RO1 GM 23379.

Accepted for publication April 30, 1981.

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MATERIAL AND METHODS

Rearing of larvae and collection of secretions. *Calliphora erythrocephala*[9] larvae* were maintained in screened cages at 20° to 25° C, 20% to 40% relative humidity, and a 14/10 hr light/dark cycle. Adult flies were fed granulated sugar mixed 5% with yeast hydrolysate (No. 2761, National Biochemical Corp.). Raw horse meat (about 50 ml) was provided for oviposition, and additional horse meat was added as larvae developed. The larval meat culture was covered with sawdust for pupation. The pH of mature meat cultures was 8.0 to 8.4.

Larvae for experimental purposes were raised either on meat cultures as previously described or on a synthetic medium.[3] Approximately 500 to 1,000 five-day-old larvae, 10 to 14 mm long, were removed from cultures, washed twice with distilled water, and placed in a 32-ounce widemouthed plastic jar containing sufficient water to cover the bottom. Water and secretions were decanted at 24-hour intervals for 1 to 3 days and stored frozen. The secretions had a pH of 7.5 and were most copious when collected at 100% relative humidity. Secretions from several collections were pooled, centrifuged at 5° C at 15,000 × g for 15 minutes, and the supernatant was lyophilized and stored at −20° C until used. When redissolved in water, this preparation is referred to as

*Supplied by Dr Leo Levenbook.

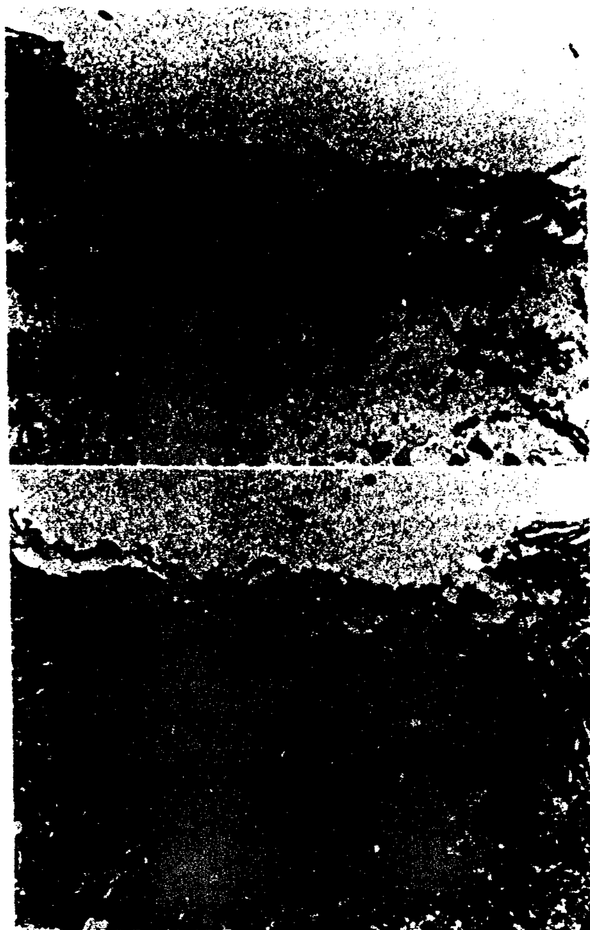

Fig. 1. Typical effect of topical application of larval enzyme to experimental eschar. **A,** Treated eschar is de-epithelialized and follicles are degenerate or lacking. **B,** Control eschar is unaffected. Enzyme (HSS fraction) was applied in three doses of 1,170 u at 24-hour intervals.

the high-speed supernatant fraction (HSS) and, unless otherwise stated, is the enzyme preparation used in these experiments.

For some experiments, enzyme activity was partially purified by ammonium sulfate precipitation. Material precipitating between 20% to 65% or between 20% to 80% ammonium sulfate saturation was collected by centrifugation at 10,000 × *g* for 15 minutes at 5° C, dialysed against distilled water for 72 hours at 4° C, and concentrated by contact desiccation or lyophilization. This is referred to as the AS fraction. Unless otherwise stated, all preparations were carried out at room temperature.

Sterile larvae were raised by sterilizing eggs and inoculating them in a sterile synthetic culture medium. Eggs were soaked in 0.01N NaOH for 5 minutes to break up clumps, rinsed in sterile distilled water, and then washed three times during 10 minutes in three changes of sodium hypochlorite (Clorox) 1:50 dilution.[21] Sterility was checked by incubating duplicate samples of eggs and culture medium in thioglycolate and trypticase soy agar and broth at 37° C for 48 hours. For the collection of secretions a sterile technique was used, but collection dishes were open to the atmosphere.

Experimental burn wounds. The backs and sides of anesthetized Sprague-Dawley rats were clipped. A 30 ml beaker, 3.5 cm in diameter, was immersed in boiling water and, while still filled with water, placed under its own weight against the rat's skin for 1 minute. Within a few hours this produced a dry leathery patch that became crusty and black in 7 to 10 days and sloughed spontaneously after 3 to 4 weeks. For in vitro use, eschar was removed surgically, diced into 3 mm cubes, washed repeatedly with distilled water, and stored at −20° C until needed.

Topical therapeutic preparations. Topical therapy was begun within 2 hours of burning. Test material was placed in the center of a 5.5 cm diameter filter paper disc (Whatman No. 2). The disc was applied to the surface of the burn, saturated with normal saline solution, and covered with a gauze sponge moistened with saline solution. This was in turn wrapped with a layer of Saran Wrap and secured to the animal's midsection with circumferential wrappings of adhesive tape. Animals were housed individually and only rarely interfered with the dressings. Halothane anesthesia was used for removal and changing of medication and dressings. Bilateral wounds were created, and one side served as the control.

Lyophilized material from larval secretions (25 to 100 mg protein) was mixed with 0.5 gm of vanishing cream (HRB Cream Base, Barnes-Hind Pharmaceuticals, Inc.) and 5 to 10 ml of distilled water. Control wounds were treated with cream only. Debridement was followed photographically and by tracing wounds on pieces of waxed paper placed over the burns. From the tracings, areas were measured by planimetry.

Enzyme assays and analytical methods. General proteolytic activity was measured by the amount of ninhydrin-positive material hydrolysed from Hammarsten casein (15 mg)[14] incubated at 37 °C for 1 hour in 0.1M phosphate buffer at pH 7.6 in a total volume of 3 ml. Reaction in an aliquot of the reaction mixture was stopped by the addition of an equal volume of 10% trichloroacetic acid. After sedimen-

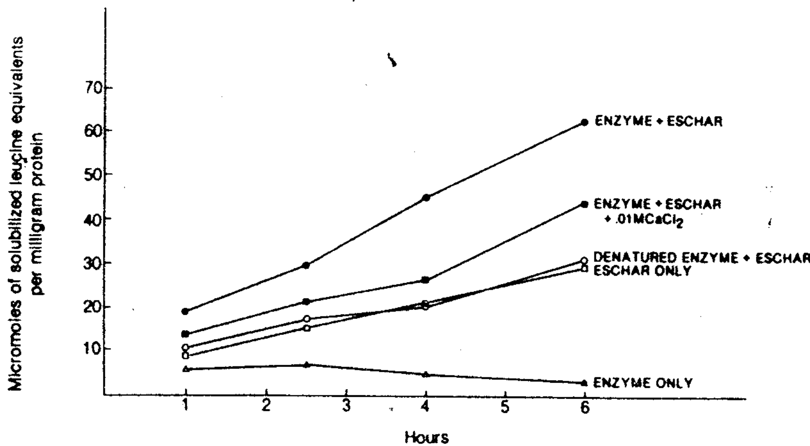

Fig. 2. In vitro hydrolysis of rat burn eschar by larval secretions. These data demonstrate that eschar is more rapidly solubilized in the presence of the larval enzymes, that the enzymes may be denatured by heating, and that $CaCl_2$ inhibits enzyme activity.

tation in a table top centrifuge, the supernatant was assayed for soluble proteinaceous material by the Ninhydrin method.[17] Eschar (130 to 150 mg) was incubated 6 hours in a total volume of 7 ml of the same buffer. Enzyme activity (units) was expressed as micromoles of leucine equivalents hydrolyzed per milligram protein per assay. In a few experiments proteolytic activity was assayed as an increase in optical density produced during incubation with 15 mg of congocol as substrate in 15 ml of buffer.[19]

Collagenase activity was measured by the appearance of soluble ninhydrin-positive material with collagen (C-9879, 15 mg, Sigma Chemical Co.) as substrate in the presence of soybean trypsin inhibitor (STI) (T-9003, Sigma Chemical Co.) at 3 mg/mg protein to eliminate activity of the predominant noncollagenolytic enzymes acting on proteinaceous impurities in the substrate preparation. Reaction conditions were as described for casein.

TRP was assayed with α-N-benzoyl-DL-arginine-p-nitroanilide (BApNA) as substrate, leucine aminopeptidase (LAP) with L-leucine-p-nitroanilide (LpNA) as substrate, carboxypeptidase A (CPA) with hippuryl-L-phenylalanine (HPA), carboxypeptidase B (CPB) with hippuryl-L-arginine (HA), and chymotrypsin with glutaryl-L-phenylalanine-p-nitroanilide (GpNA).[8] These assays were carried out in 3 ml cuvettes, and optical density changes were measured directly with a recording spectrophotometer. Elastase was assayed with elastin Congo red.[18] The assays were modified to use phosphate buffer at room temperature. Protein was determined by the method of Lowry et al.[15]

Molecular weights were determined by gel filtration on a 43 × 3 cm Sephadex G-200 column with 0.1M sodium phosphate buffer as eluant. From 6 to 60 mg of protein was applied, and 5 ml fractions were collected. There was a tendency for each detected activity to eluate near the void volume as well as in a second peak at lower molecular weight. CPA and CPB activity were present at just detectable levels. Pooling data from several runs, however, resulted in molecular weight figures close to published values for each enzyme.

Kinetic data were determined at pH 7.6 from Lineweaver-Burk plots of duplicate assays with protein present at 0.067 mg/ml. Inhibition by salts and organic compounds were tested under the same conditions.

RESULTS

Topical applications. Burns treated with topical preparations of larval secretion in vanishing cream became significantly more debrided than did control burns treated with cream only (Fig. 1). Typically, the debrided area began as small points spread over the surface of the eschar that coalesced, usually by 48 hours, into larger areas in which the epidermis was completely removed and the dermis was thinned with follicles lacking and with necrosis of the deeper collagen. Control wounds sometimes had a few areas of intact but degenerate epidermis and follicles. Occasionally an initial de-epithelialization was present at 48 to 72 hours but was normal by 96 hours. Five animals received a total topical dose of 1,000 to 3,500 u/day for 3 days. The mean debrided area of the treated burns was 23% ± 4.6%, whereas that of the control burns was 1% ± 0.7% (t = 5.3, $P <$

Table I. Partial characterization of proteolytic enzyme activity of blowfly larvae secretions

Enzyme	*Substrate*	*Specific activity (μmoles min^{-1} mg^{-1})*	*K_m (mM)*	*V_{max} (μmoles min^{-1})*	*Molecular weight*
General protease	Casein	0.688		Not done	
Collagenase	Collagen	0		Not done	
Trypsin	BApNA	0.010	0.2	0.5	26,000 ± 2,900
Leucine aminopeptidase	LpNA	0.043	0.1	2.7	280,000 ± 37,000
Carboxypeptidase A	HPA	0.034	0.02	2.7	40,000 ± 9,400
Carboxypeptidase B	HA	0.015	0.08	1.5	42,000 ± 5,900
Elastase	Elastin Congo red	0.002		Not done	
Chymotrypsin	GPpNA	0		Not done	

Legend: BApNA, α-N-benzoyl-DL-arginine-p-nitroanilide; LpNA, leucine-p-nitroanilide; HPA, hippuryl-L-phenylalanine; HA, hippuryl-L-arginine; GPpNA, glutaryl-L-phenylalanine-p-nitroanilide.

NOTE: Specific activity and kinetic data were all determined for the same batch of HSS fraction. Molecular weights are averages of four determinations from three batches of HSS fraction.

0.005). Within this range, no relation between dose and extent of debridement was seen. One animal received a single 500 u dose left in place 5 days and the area debrided was 7%, whereas an animal receiving 5 daily 500 u doses was 16% debrided, indicating that there is some cumulative effect. It was important to keep all dressings moist.

There was usually test material still present on the dressings when they were removed for changing. In two experiments the filter papers were extracted for 1 hour with 10 ml of distilled water, and the extract was assayed for total enzymatic activity. From 8% to 50% of the original activity was recovered, and there was a tendency for the amount remaining on the dressings to be greater in later applications. In addition, up to 40% of the activity applied originally to the test side could be recovered from the filter paper carrying cream only as the control treatment. Control filter paper was often the color of the enzyme. These observations mean that (1) the enzyme is stable in such applications for 24 to 48 hr and (2) that the enzyme diffuses widely through the wet dressing so that at least some of the debridement seen in control burns may well have been caused by the enzyme.

In vitro digestion of eschar. Fig. 2 shows the result of incubating larval enzymes with rat burn eschar. The preparation was the 20% to 65% AS fraction containing 0.14 mg of protein. A second sample was denatured by heating at 100° C for 7 minutes. These data show that the larval secretions do have the ability to digest eschar, that this activity can be destroyed by heat denaturation, and that it is inhibited by $CaCl_2$.

Source of enzyme activity. Two experiments were performed to demonstrate that the enzyme activity was derived from the larval secretions rather than from contaminating bacteria. One batch of meat-reared larvae (5 gm) was allowed to secrete into a collection dish in the usual way for 24 hours. Two similar batches were killed by exposure to −20° C for 30 minutes or to 54° C for 10 minutes before being placed in the collection dish. The live larvae secreted a total of 9 ml of liquid in 24 hours, and this amount of sterile water was used to wash the otherwise dry collecting dishes containing the killed larvae. Aliquots of the liquid in each dish were assayed for hydrolytic activity against congocol. The optical density of the color reaction of the aliquots from killed larvae was 0.06 and 0.03 AU, whereas that of the aliquot from the living larvae was 1.36 AU. There was, therefore, no activity from larvae which had not actively secreted. This eliminated surface bacteria carried over from the meat cultures as the possible source of the activity.

In a second experiment, a set of cultures was raised on a sterile synthetic medium. Secretions prepared from larvae raised in these sterile cultures showed enzyme activity similar to that of the usual unsterile cultures. The casein activity of six sterile cultures was 54.6 ± 6.6 μmole/mg whereas that of five unsterile cultures was 50.4 ± 7.2 μmole/mg. This eliminates any external and internal bacteria acquired during development of unsterile larvae as the source of the enzyme activity.

Characterization of proteolytic activity. All enzymatic activity could be stopped by heating in solution at 100° C for 5 minutes. Lyophilized material

Table II. Effect of organic compounds and salts on activity of each enzyme (data are percent remaining activity)

Substance	Concentration*	Enzyme			
		TRP	LAP	CPA	CPB
STI	5	0.8	97.5	107.8	99.8
TLCK	1	29.7	85.3	136.3	218.7
1-IOP	1	113.3	100.0	0	0
EDTA	1	99.9	97.5	94.6	99.2
	10	103.1	92.5	73.8	—
DTT	1	49.0	78.7	55.5	46.8
LiCl	1	—	—	84.6	95.6
NaCl	1	101.0	101.6	104.3	91.7
KCl	1	106.5	105.7	97.6	87.8
$ZnCl_2$	1	81.5	99.7	61.2	95.2
$MgCl_2$	1	101.4	101.9	100.6	91.2
$CaCl_2$	1	—	93.1	108.3	93.9
$MnCl_2$	1	66.1	—	106.7	100.3
CuC_2	1	—	17.6	—	—
	.1	82.9	—	82.0	148.1
Na_2SO_4	1	99.3	97.9	100.0†	77.4
Na Citrate	1	102.0	98.1	100.0†	89.9†

Legend: STI, soybean trypsin inhibitor TLCK, tosyl-lysine-cloro-methyl-ketone; 1-10 P, 1-10 phenanthroline; EDTA, ethylenediaminetetraacetate; DTT di-thio-thrietol.
*mM except STI which is 5 mg/mg protein.
†Based on single measurement. All other data based on duplicate observations.

stored frozen retained activity up to 12 months. Maximum activity occurred between 45° and 50° C.

The ability of the HSS fraction to hydrolyze defined substrates is shown in Table I. There is evidence that enzymes with activities characteristic of TRP, LAP, CPA, and CPB contribute to the general proteolytic activity of the larval secretions. Apparent molecular weights of the enzymes are also shown. There was possibly a trace of elastase activity present, and there was no evidence of chymotrypsin. All activity against collagen was stopped in the presence of STI. The pH optimum for LAP, CPA, and CPB was at 7.6 whereas TRP activity was greatest at 8.0. Table II shows the effect of various organic compounds and salts on the activity of the major defined enzymes.

DISCUSSION

The in vivo and in vitro experiments confirm that larval secretions are a potentially useful agent for enzymatic removal of burned tissue, since the eschar is preferentially digested. Development of an appropriate preparation and delivery system is required before the effectiveness of larval enzymes can be brought to a clinically useful level comparable to available agents. One approach would involve purification and concentration of the most active components to achieve a higher dose per application. Another involves incorporation of the enzymes into a slow-release vehicle since the activity appears relatively stable, as shown by the recovery of enzyme activity from used dressings.

The identity of at least some of the enzymes present in larval secretions is defined by the existence of activity against the specific synthetic substrates. Enzymes with specificities characteristic of TRP, LAP, CPA, and CPB are definitely present. The observed apparent molecular weights are close to those recorded for each enzyme,[5, 6, 24, 25] as are the pH optima.[4, 6, 24, 28] The inhibition of activity against BApNA by STI and tosyl-lysine-cloromethyl-ketone (TLCK) confirms the presence of a TRP-like enzyme of the serine protease class. The sensitivity of the activity against HPA and HA to 1-10 phenanthroline is characteristic of CPA and CPB.[5, 6] The activation of CPA and CPB by TLCK is not explained, but may be the result of reduced TRP proteolysis. The TRP-like activity is not accelerated by Ca^{++}, which is unusual.[22] It is also unusual

that the LAP activity is not inhibited by 1-10 phenanthroline.[10] These observations suggest that the occurrence of these enzymes as externally secreted products may involve modification so that active forms rather than inactive zymogens are secreted. These results confirm and extend earlier reports of proteolytic activity in blowfly larva secretions.[7, 12, 16, 26, 27] Brookes[2] has reported TRP, carboxypeptidase, and aminopeptidase activity in secretions of *Phormia* larva. Pendola and Greenberg[20] found TRP in the gut of *Calliphora vomitoria.*

The present data do not provide evidence for the presence of chymotrypsin or elastin. The former has been reported in the gut of *Calliphora v.*[20] Hobson[12] has reported partial solubilization of an elastin preparation, but this could have represented proteolytic digestion of nonelastin impurities. Absence of elastase is consistent with the observation by Vistnes and Hogg[23] that elastic fibers are the most stable structures binding eschar to deep tissue.

The question of the presence of collagenase is complex. The burned dermal tissues in the in vivo experiments were more or less removed by the action of the larvae, which would seem to implicate collagenase; yet the intact skin was less extensively attacked. The burned tissue was to a large extent denatured, so that the collagenous components were more susceptible to attack by noncollagenase proteolytic activity. The in vitro studies showed that activity against a collagen substrate resulted from general proteolytic activity against noncollagenous contaminants in the substrate used, as shown by the lack of activity in the presence of STI. The same consideration probably also applies to earlier reports of purported collagenolytic activity.[11, 26, 29] Messer and McClellan[16] did not detect collagenase activity in secretions of *Lucilia* larvae.

The control experiments with killed larvae and with sterile larvae ruled out the possibility that the enzymatic activity of the secretions is due to contaminating bacteria on the surface or in the gut of the larvae. Hobson[11] and Ziffren et al.[29] were also able to exclude bacterial contamination as the source of enzyme activity.

This investigation has provided evidence that suggests that the further development of a suitable larval enzyme therapeutic system may be of value.

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