In Vitro Properties of Protease in the Midgut of the Adult Horn Fly, *Haematobia irritans* L. (Diptera : Muscidae)¹

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Some properties of protease in the midgut of the adult horn fly, *Haematobia irritans* L., were clarified and compared with those of the stable fly, *Stomoxys calcitrans* L. In the horn fly the protease was most active at 45°C and pH 8.5, while in the stable fly it was most active at 45°C and pH 9.5. The proteases of both species were inhibited by more cations at pH 9.5 than at pH 8.5 or 8.0. The activities of trypsin-like enzyme were suppressed only by Hg⁺⁺ and Zn⁺⁺, while those of aminopeptidase were inhibited by various cations including Mg⁺⁺, cysteine and ascorbic acid.

INTRODUCTION

There have been a number of studies on the digestive processes of haematophagous insects. Since two reviews were given by GOODING in 1972 and 1975, however, only a few investigations have been carried out for the stable fly (LEHANE, 1976, 1977; DELOACH and SPATES, 1979, 1980; LEE and DAVIES, 1979; SPATES, 1981) and for the horn fly (HORI et al., 1981), which are serious insect pests of cattle in Japan. The present work was carried out in order to elucidate some properties of protease in the midgut of the horn fly, *Haematobia irritans* L., and they were compared with those of the stable fly, *Stomoxys calcitrans* L.

MATERIALS AND METHODS

Adult flies used. Unless otherwise stated, horn flies and stable flies collected from cattle in the field and fed with citrated cow blood *ad lib* for 1 or 2 days were used for protease assays.

Preparation of enzyme solution. The midguts were dissected from flies in 0.9% NaCl solution and homogenized in a solution (10 guts/ml in the case of the horn fly and 5 guts/ml for the stable fly). The homogenates were refrigerate-centrifuged for 20 min at 26,000 *g* and the supernatants obtained were salted out by ammonium sulphate (90% of saturation point). After refrigerate-centrifugation for 20 min at 30,000 *g*, the precipitates were redissolved in 0.9% NaCl solution in a volume corresponding to 10 guts/ml and 5 guts/ml, respectively. These solutions were used for assays of protease.

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Assay for protease activity. Unless otherwise stated, protease activity was assayed by using 2% (for the horn fly) or 3% (for the stable fly) casein in Kolthoff buffer at pH 8.5 (horn fly) or 9.5 (stable fly) as the substrate. The reaction mixture was composed of 0.5 ml substrate solution and 0.5 ml enzyme solution (2 guts/ml in the horn fly and 0.83 guts/ml in the stable fly). The substrate control (0.5 ml substrate solution + 0.5 ml of 0.9% NaCl solution) and the enzyme control (0.5 ml buffer solution + 0.5 ml enzyme solution) were run at the same time. Incubation was carried out in a water incubator for 2 hr (horn fly) or 30 min (stable fly) at 45°C. Then the reaction was stopped by adding 3.0 ml of 20% trichloroacetic acid (TCA) and the mixture was centrifuged at 26,000 g for 10 min. The enzyme activity was estimated by measuring the absorbancy of the supernatant at 280 nm with a Shimadzu UV-200S spectrophotometer.

For the experiment on the effect of some inhibitors on protease activity, 2.5% denatured haemoglobin in Tris-HCl buffer was used as the substrate. The reaction mixture was composed of 0.5 ml substrate solution, 0.5 ml enzyme solution and 0.25 ml inhibitor solution in Tris-HCl buffer (adjusted to a suitable pH with 2 or 5 N NaOH). The inhibitor was contained in 10^{-2} M in the reaction mixture. Substrate, enzyme and inhibitor controls were run at the same time.

For the experiment on the effect of some inhibitors on trypsin-like enzyme (TLE) and aminopeptidase (AP) activities, the substrate was 2 mM α -N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) in 50 mM Tris-HCl buffer (pH 7.9) with 6.6% *N,N*-dimethyl formamide (DMF), and 1.66 mM L-leucine-*p*-nitroanilide (LpNA) in 50 mM Tris-HCl buffer (pH 7.9) with 10% DMF, respectively. The reaction mixture was composed of 3.0 ml substrate solution, 0.3 ml enzyme solution and 0.5 ml inhibitor solution. The controls were also run at the same time. Reaction mixtures were incubated for 15 min (for TLE of the horn fly), 20 min (for TLE of the stable fly) or 30 min (for AP of the horn fly and the stable fly) at 30°C. Then the reaction was stopped by adding 0.5 ml of 30% acetic acid. The absorbancy of the reaction mixture was measured at 410 nm. Activities were expressed in OD units.

Analysis of protein content. Protein content was determined by the method of LOWRY et al. (1951) using crystallized bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Protein content in enzyme solution

The protein content in each enzyme solution was 6.8 μ g/midgut/ml in the sucrose-fed horn fly, 47.8 μ g/midgut/ml in the blood-fed horn fly and 765.0 μ g/midgut/ml in the blood-fed stable fly.

Reaction time

The reaction pH was 7.8 for the horn fly and 7.5 for the stable fly. Activity of protease in the midguts of both flies was maintained at a nearly constant level during 30 min of the incubation. Thereafter it decreased but the decreased level remained constant for 120 min (Fig. 1). Therefore, the incubation time was limited to 30 min for the protease assay, but in the horn fly (and sometimes in the stable fly) it was prolonged up to 2 hr because the activity was too low to determine.

Fig. 1. The effect of reaction time on the protease activities in the midguts of the horn fly(H) and the stable fly(S).

Fig. 2. The effect of enzyme concentration on the protease activities in the midguts of the horn fly and the stable fly.

Enzyme concentration

The enzyme concentration was expressed as 1.0 when the enzyme solution contained 5 guts/ml in the horn fly and 0.83 guts/ml in the stable fly. The protease activity of the stable fly was assayed under two conditions: (1) for 2 hr at pH 7.5 using 3% casein and (2) for 30 min at pH 9.5 using 2% casein. In general, the activity of the midgut protease of the horn fly and the stable fly increased in proportion to enzyme concentration up to 0.25 degrees but thereafter the activity per unit concentration decreased as the concentration was raised (Fig. 2).

Substrate concentration

In the case of the horn fly, the enzyme solution of 1.5 guts/ml was used for protease

assay, the incubation time being 1 hr. The protease activity of the horn fly increased rapidly in proportion to the substrate concentration up to the concentration of 0.1% but beyond that, activity leveled off. The protease activity of the stable fly increased rapidly in proportion to the substrate concentration up to 0.25% concentration but beyond that the rate of increase dropped as the concentration increased (Fig. 3).

Reaction temperature

For the stable fly, incubation was carried out for 1 hr at pH 7.5. For the horn fly, the enzyme (2 guts/ml) extracted from sucrose-fed flies was used. The protease activity of both types of fly increased with the rise of incubation temperature from 25°C, reached maximum at 45°C and then decreased toward 60°C where the activity was very low (Fig. 4).

Hydrogen-ion concentration

The protease activity was assayed using 1% casein (for the horn fly) or 2% casein (for the stable fly) in McIlvaine buffer (at pH 5.5 to 8.0) or Kolthoff buffer (above pH 8.5) as the substrate. In the horn fly, the protease was very active in a broad range between pH 6.5 and 10.5, with optimum activity at about pH 8.7, while in the stable fly, it was also very active in a broad range between pH 7.5 and more than pH 11.0, with optimum activity at about pH 9.5 (Fig. 5). The latter was active in more alkaline range than the former.

It has been known that, in general, the digestive protease of haematophagous dipteran flies were some alkaline protease with optimum activity at pH 7.5 to 9.5, whereas those of haematophagous heteropteran bugs and ticks were some acid protease with optimum activity at pH 5.0 to 5.5 and at pH 2.5 to 3.5, respectively. The

Fig. 3. The effect of substrate concentration on the protease activities in the midguts of the horn fly and the stable fly.

Fig. 4. The effect of incubation temperature on the protease activities in the midguts of the horn fly and the stable fly.

Fig. 5. The effect of pH on the protease activities in the midguts of the horn fly and the stable fly.

pH optimum of digestive protease of *Glossina morsitans* probably varies depending on the substrate used: pH 8.0 (gelatin) (WIGGLESWORTH, 1929), pH 7.8 to 8.0 (azocasein) (LANGLEY, 1966), pH 8 to 9 (BAPNA) (GOODING and ROLSETH, 1976) and pH 9 to 10 (casein) (GOODING and ROLSETH, 1976). CHAMPLAIN and FISK (1956) reported that the pH optimum of digestive protease of *Stomoxys calcitrans* was pH 7.8 (azocasein as the substrate), which was different from the result obtained in the present study

Table 1. INHIBITORY EFFECT OF SOME SUBSTANCES ON THE PROTEASE ACTIVITIES IN THE MIDGUTS OF THE HORN FLY AND THE STABLE FLY

Substance	Activity (A 280) (Horn fly)					
	pH 8.5	Replication	Relative activity	pH 9.5	Replication	Relative activity
CaCl ₂	0.234	1	102	0.025	1	38
MgCl ₂	0.225	1	98	0.024	1	37
MnCl ₂	0.230	1	100	— ^a		—
HgCl ₂	0.020	1	9	—		—
ZnSO ₄	0.050	1	22	—		—
Cysteine	0.219	1	96	—		—
Ascorbic acid	0.229	1	100	0	1	0
Honey (20%)	0.237	1	103	—		—
Nil	0.229	2	100	0.065	1	100

Substance	Activity (A 280) (Stable fly)					
	pH 8.0	Replication	Relative activity	pH 9.5	Replication	Relative activity
CaCl ₂	0.126	1	97	0.182	2	39
MgCl ₂	0.128	1	98	0.170	2	37
MnCl ₂	0.135	1	104	0.151	2	32
HgCl ₂	—		—	0.100	1	22
ZnSO ₄	—		—	0.053	1	11
Cysteine	—		—	0.479	1	103
Ascorbic acid	0.145	1	112	0.316	2	68
Honey (20%)	—		—	0.462	1	99
Nil	0.130	1	100	0.465	2	100

^a No test.

Table 2. INHIBITORY EFFECT OF SOME SUBSTANCES ON THE TRYPSIN-LIKE ENZYME ACTIVITIES IN THE MIDGUTS OF THE HORN FLY AND THE STABLE FLY

Substance	Horn fly			Stable fly		
	Activity (A 410)	Relative activity	Replication	Activity (A 410)	Relative activity	Replication
CaCl ₂	0.500	103	2	0.357	105	2
MgCl ₂	0.484	100	1	0.346	102	2
MnCl ₂	0.488	101	1	0.240	71	1
HgCl ₂	0.058	12	2	0.040	12	1
ZnSO ₄	0.196	40	2	0.140	41	1
Cysteine	0.479	99	1	0.348	102	2
Ascorbic acid	0.493	102	1	0.343	101	2
Honey (20%)	0.500	103	1	0.342	101	2
Nil	0.484	100	4	0.340	100	2

Table 3. INHIBITORY EFFECT OF SOME SUBSTANCES ON THE AMINOPEPTIDASE ACTIVITIES IN THE MIDGUTS OF THE HORN FLY AND THE STABLE FLY

Substance	Horn fly			Stable fly		
	Activity (A 410)	Relative activity	Replication	Activity (A 410)	Relative activity	Replication
CaCl ₂	0.317	97	4	0.271	94	2
MgCl ₂	0.261	80	4	0.207	72	2
MnCl ₂	0.202	62	5	0.110	38	1
HgCl ₂	0.035	11	1	0.045	16	1
ZnSO ₄	0.005	2	1	0.013	5	1
Cysteine	0.266	81	5	0.200	69	2
Ascorbic acid	0.301	92	4	0.268	93	2
Honey (20%)	0.327	100	1	0.264	92	2
Nil	0.327	100	6	0.288	100	2

(casein as the substrate).

Inhibitors

In the horn fly, incubation was carried out for 1 hr at pH 9.5 and for 2 hr at pH 8.5; enzyme solutions used for the two incubations were different. In the stable fly, the incubation was done for 30 min at pH 8.0 and pH 9.5. The protease activity of the horn fly was clearly inhibited only by Hg⁺⁺ and Zn⁺⁺ at pH 8.5, whereas it was strongly inhibited also by Ca⁺⁺, Mg⁺⁺ and ascorbic acid at pH 9.5. The protease activity of the stable fly was strongly inhibited by Ca⁺⁺, Mg⁺⁺, Mn⁺⁺, Hg⁺⁺, Zn⁺⁺ and ascorbic acid at pH 9.5, but was not inhibited by them (excepting Hg⁺⁺ and Zn⁺⁺ which were not tested) at pH 8.0. Cysteine and honey (20%) did not inhibit the protease activities of either species (Table 1). The inhibitory function of some cations at pH 9.5 might be due to the possibility that enzymes combine easily with cations especially at higher pH to make high molecular proteins and precipitate. The activities of TLE of the horn fly and the stable fly were strongly inhibited by Hg⁺⁺ and Zn⁺⁺, and, in addition, the activity of the latter was moderately inhibited by Mn⁺⁺ (Table 2). The activities of AP of the horn fly and the stable fly were strongly inhibited by Zn⁺⁺ and Hg⁺⁺ (and Mn⁺⁺ in the latter), moderately by Mg⁺⁺, Mn⁺⁺ and cysteine, and slightly by Ca⁺⁺ and ascorbic acid (and honey in the latter) (Table 3).

WAGNER et al. (1961) found that the activity of the non-trypsin proteinase of *Aedes aegypti* was inhibited by Mg⁺⁺, Ca⁺⁺, Hg⁺⁺ and Mn⁺⁺ and that of trypsin was inhibited by Ca⁺⁺, Hg⁺⁺, Cd⁺⁺ and Zn⁺⁺ but not by Mg⁺⁺ and Mn⁺⁺. Since then, GOODING (1966) confirmed the inhibitory effect of Ca⁺⁺, Mg⁺⁺ and Mn⁺⁺ on the protease activities of *A. aegypti* and *Culex pipiens quinquefasciatus*. However, SPIRO-KERN (1974) found no inhibitory effect of Ca⁺⁺ on the protease activity of *C. pipiens* and BRIEGEL and LEA (1975) found only a slight inhibitory effect of Ca⁺⁺ on the activities of *A. aegypti*, *Anopheles quadrimaculatus* and *C. pipiens quinquefasciatus*. Cysteine is a weak activator for the non-trypsin protease of *A. aegypti* (WAGNER et al., 1961) and *Rhodnius prolixus* (GARCIA et al., 1978; HOUSEMAN, 1978), whereas it is a strong inhibitor at a high concentration (5×10^{-3} M) for the protease of the tick *Hyalomma excavatum* (BOGIN and HADANI, 1973). Ascorbic acid and honey inhibit the proteases

of *A. aegypti* and *C. pipiens quinquefasciatus* (GOODING et al., 1973). The fact that the inhibitory effect of some cations appeared only at higher pH and that various cations, cysteine and ascorbic acid inhibited only AP but not TLE in this experiment may be very suggestive for future investigations.

The clear difference between the proteases of the horn fly and stable fly found in the present study is in the pH optimum. GOODING (1966) stated that proteinases functioning at pH 9.5 might be a vestige of a phytophagous ancestor. If this be true, it might be said that the horn fly whose digestive protease has optimum activity at about pH 8.7 has adapted for blood-feeding earlier or more completely than the stable fly whose digestive protease has optimum activity at about pH 9.5. This in turn might be related to the difference in their feeding behavior.

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