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INFLUENCE OF FEEDING ON MIDGUT PROTEASE ACTIVITY IN *Aedes aegypti*

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ABSTRACT

The stimulation of midgut protease activity by various diets was studied in *Aedes aegypti* females fed through membranes. All proteins tested were effective in stimulating protease, but the level of protease attained and the time of peak activity were related to the protein fed and to its concentration in the diet. Whole blood induced protease most effectively; haemoglobin in solution was more effective than intact erythrocytes; sheep serum stimulated protease, but was inhibitory for several hours after feeding. Engorging on a nutritionally inert solution stimulated a small amount of protease, but the distension of the midgut was not essential for protease production. The relation between protein feeding, protease production and ovary development is discussed.

INTRODUCTION

The fate of the blood meal in the digestive tract of the mosquito female has been studied extensively; about half of the 240 papers cited in a recent review on the digestive processes of haematophagous insects deal with mosquitoes (Gooding, 1972).

In mosquitoes, as in all blood-feeding insects, the midgut proteolytic activity increases after engorging on blood, and then decreases again. In the midguts of unfed (or sugar-fed) adult *Aedes aegy'pti* females no proteolytic activity can be detected (Fisk, 1950; Akov, 1965). In this respect mosquitoes differ from other haematophagous insects, such as tsetse flies (Langley, 1966), simuliid flies (Yang and Davis, 1968), *Rhodnius* (Persaud and Davey, 1971), and fleas (personal observation), which contain considerable proteolytic activity in their midguts prior to blood feeding. Thus, in mosquitoes, midgut proteolytic activity which appears following feeding probably indicates that protease synthesis has taken place.

There is no evidence that in insects proteases are stored in the form of an inactive proenzyme, like those of the vertebrate pancreas , and later activated by a proteolytic enzyme. In *Aedes aegypti* trypsin is synthesized *de novo* after the ingestion of a blood meal (Gooding, personal communication). Attempts to activate mosquito protease *in vitro* were unsuccessful (Shambaugh, 1954; Chen, 1969). Further evidence for the synthesis of midgut protease after blood feeding comes from studies of the ultrastructure of the mosquito midgut epithelial cells (Bertram and Bird, 1961; Stäubli *et al.,* 1966).

The mechanism that controls the synthesis of protease in insects is not known; probably no single mechanism operates in all insects (see Capps *et al.,* 1972). It has been suggested that in mosquitoes the control of protease secretion is secretogogue rather than nervous or hormonal (Fisk and Shambaugh, 1952). Attempts to demonstrate neuroendocrine regulation of mosquito midgut protease were inconclusive (Chen, 1969). Ligaturing the abdomens immediately after blood feeding decreased but did not prevent the production of protease in *Culex* (Chen, 1969) and in *A. aegypti* (Akov, unpublished observation). Results with decapitated mosquitoes were similar (Gooding, 1966a). Histological studies did not show great changes in the neurosecretory cells of the brain of *A. aegypti* in relation to blood feeding (Larsen and Broadbent, 1968). Aedine mosquitoes deprived of their median neurosecretory cells were able to utilize the nutrients from the blood meal for the synthesis of triglycerides (Lea, 1967).

The aim of this study was to determine the factors in the blood meal which are responsible for stimulating the appearance of midgut protease in *A. aegypti* mosquitoes. Using ATP as a feeding stimulant (Galun, 1967) enabled us to dispense with the erythrocytes formerly used in membrane feeding (Shambaugh, 1954). By feeding the mosquitoes on various blood fractions and other solutions we could evaluate the efficiency of each blood component separately and also study the effect of distending the midgut by solutions of little or no nutritive value.

MATERIALS AND METHODS

Aedes aegypti mosquitoes ("Ness-Ziona" strain) were reared under standard laboratory conditions at 28°C. Mated females, aged 5-12 days were fed through membranes, using 10^{-2} M ATP and 10^{-2} M $NaHCO_3$ as feeding stimulants (Galun, 1967). The diets consisted of fresh sheep blood (defibrinated by glass beads); thrice washed sheep erythrocytes; sheep serum; haemoglobin (prepared from thrice washed sheep erythrocytes that were haemolyzed, centrifuged, and dialyzed against distilled water for 24 hours) and various proteins, such as bovine serum albumin and casein. A solution of 12% dextran in 0.85% NaCl was used to suspend the erythrocytes and as the diluent for the various diets. This solution also served as the control diet. Each diet was offered for 30 minutes. Only fully engorged mosquitoes were taken for experiments and kept at 28°C until dissected. At various intervals after feeding 10-30 midguts from each group were dissected and kept frozen at -25°C until protease determination. Only midguts with visible amounts of food were taken, since empty midguts never contain protease activity.

The midguts were homogenized in distilled water and the protease activity was measured using casein (a 0.5% solution prepared by dissolving the casein in water and adding enough NaOH to bring the pH to 10.0, then neutralizing the solution with HCl) as substrate. The incubation mixture (final volume 2.4 ml) consisted of 2.0 ml of 0.05 M Tris buffer, pH 8.8, 0.3 ml casein solution and 0.1 ml midgut homogenate (containing an equivalent of 0.2 to 2.5 midguts, depending on the protease activity). All experiments, including the appropriate controls, were run in duplicate. Incubation was for 30 minutes at 39°C. The reaction was stopped with 0.6 ml of 30% trichloroacetic acid. After 20 minutes in the cold the precipitated protein was removed by centrifuging and filtering. Aliquots of 2.0 ml of the supernatant were taken for measurement of tyrosine according to the nitrosonaphthol method of Waalkes and Udenfriend (1957). The nitrosonaphthol reagent (from recrystallized nitrosonaphthol) and the nitric acid reagent were prepared daily. Since vigorous shaking (to extract the excess of nitrosonaphthol into the ethylene dichloride) is very important, we mixed the tubes for 15 seconds on a Vortex. Standards containing 0.2 to 3.2 μg/ml tyrosine in 6% trichloroacetic acid were run through the entire procedure. The fluorescence was measured on a Turner Model 111 fluorometer (with a blue lamp No. 110-853, primary and secondary filters 470 and 570 mμ, respectively).

The chemicals used were: albumin (bovine) fraction V powder, fatty acid poor, from Pentex, Miles Lab.: casein, vitamin-free and casein hydrolyzate (enzymatic, vitamin-free) from Nutritional Biochem. Corp.; dextran, clinical grade, average mol. wt. 186,000, from Mann Research Lab., Inc.

RESULTS AND DISCUSSION

Protease activity is given as μg of tyrosine released in one hour by one midgut. The fluorescence was proportional to the amount of tyrosine up to 10 μg of tyrosine per tube. The minimal amount of tyrosine that could be detected was 0.6 μg tyrosine released per tube. The amount of tyrosine released was proportional to the amount of enzyme (midgut homogenate or bovine trypsin) and to the time of incubation up to 60 minutes (at 39°C). The optimal conditions for enzyme activity (pH, temperature) with casein as substrate were similar to those obtained by Gooding (1966b), who used haemoglobin. A preliminary report on the *in vitro* properties of the protease was given (Akov and Samish, 1971).

The activity of bovine trypsin (Miles-Servac, Ltd.) as measured by our method was 25 μg tyrosine/hr/μg of enzyme. Protease activity of sugar-fed females was negligible (less than 0.2 μg tyrosine/hr/midgut), corresponding to less than 0.01 μg of bovine trypsin. Two hours after feeding on a human arm the activity was 5.5 μg tyrosine/hr/midgut, and increased to 30, 95, and 130 μg tyrosine/hr/midgut at 6, 19, and 24 hours, respectively, after feeding. The protease activity of one midgut at the time of peak activity corresponds to about 5 μg of bovine trypsin. Similar results were obtained with another method using TAME (ρ-toluenesulphonyl-L-arginine methyl ester), and will be published elsewhere.

In order to find out what blood component stimulated the protease activity, the females were fed through membranes on various dilutions of sheep blood, erythrocytes, and serum, as described in Materials and Methods. The controls were given a dextran solution, because the addition of 12% dextran to the physiological saline increased the retention time of the meal in the midgut (Akov and Sternberg, unpublished results). There was no increase in protease activity 10 to 20 minutes after feeding, but 4 and 8 hours later the differences in the level of protease among the diets were considerable (Table 1). Engorging on 12% dextran induced a small, but measurable, amount of protease. Twenty hours after feeding all the control females had empty midguts. (Females that had emptied their midguts, irrespective of the diet, had no protease activity.)

Table 1. Proteolytic activity (μg tyrosine/hr/midgut) in mosquitoes fed on various dilutions of sheep blood, resuspended sheep erythrocytes, and sheep serum.

		Hours after feeding				
Food	% of protein	0-½	4	8	20	28
Whole blood	18.0	0.2	8.9	33.7	70.0	104.0
Blood diluted 1:5	3.6	0.1	11.5	28.0	57.0	45.0
Blood diluted 1:10	1.8	0.15	7.2	24.0	25.7	28.8
Rbc (red blood cells)	18.0	0.3	1.9	4.3	16.0	26.7
Rbc diluted 1:5	3.6	0.4	2.4	4.4	9.0	14.3
Rbc diluted 1:10	1.8	0.3	3.2	4.8	7.8	8.8
Serum	4.5	0.1	0.15	4.5	42.0	–
Serum diluted 1:10	0.45	0.1	5.5	18.0	26.0	–
Controls (12% dextran)	–	0.2	1.3	4.4	–	–

The highest level of protease was obtained after feeding on whole blood; diluting the blood had no effect on the amount of protease 4 hours after feeding, but later on, the level of protease attained was related to the amount of blood in the meal. Peak protease activity was attained earlier with more dilute diets, and midguts were also emptied more rapidly. (Twenty-eight hours after feeding on whole blood 2/13 females had empties their midguts, and 2/10 and 7/17 of those fed on blood diluted 1:5 and 1:10, respectively).

Feeding on washed erythrocytes induced very little protease, and the activity appeared many hours later. One day after feeding on red blood cells the amount of protease was related to the amount of protein in the food, but it was much lower than in mosquitoes fed on serum. Haemoglobin in solution is a better inducer than equivalent amounts of intact washed red blood cells (Table 2). The level of protease is higher, and the enzyme also appears earlier after engorging.

Table 2. Proteolytic activity (μg tyrosine/hr/midgut) in mosquitoes fed on sheep haemoglobin in solution.

Percentage of haemoglobin	Hours after feeding		No. of females with full midguts 24 hrs after feeding
	4	24	
12.0	15.1	30.0	12/12
6.0	10.7	29.7	7/7
1.5	6.6	24.9	9/9
0.75	3.9	6.8	4/21
0.18	3.5	11.5	3/17
0.06	4.1	9.8	4/21

The effect of feeding serum alone on protease production could not be evaluated, because the inhibitors in the sheep serum obliterated the protease activity at 4 and 8 hours after feeding (Table 1). The inhibitory effect could be removed by diluting the serum 1:10; the small amount of protein left was sufficient to induce protease activity. Similar results were obtained with dialyzed serum, indicating that the inhibitors were non-dialysable. Sheep serum contains relatively more *A. aegypti* trypsin inhibitors than sera from other mammals (Huang, 1971). This is probably the reason why protease appeared somewhat later after feeding on sheep blood than after feeding on human blood (see above, the results of feeding on a human arm). Sheep serum inhibited the appearance of proteolytic activity only temporarily; 20 hours after feeding on serum the inhibitory effect had already been overcome (probably by inactivation of the proteins responsible), and the protease level was proportional to the amount of protein in the diet.

Haemoglobin (Table 2), bovine serum albumin and casein (Table 3), which contain no inhibitor, induced more protease than serum (which contained 4.5% protein).

In order to induce protease activity, the protein has to be in solution. Since there is no evidence that the erythrocytes are haemolyzed immediately after reaching the mosquito midgut (Gooding, 1972), the initial stimulus for protease production probably comes from the plasma proteins. It is possible that one day after blood feeding the haemoglobin, which is gradually released from the corpuscles as digestion proceeds, plays a role in sustaining protease production.

Proteins from sources other than blood are also good inducers of protease. Casein (Table 3) was very effective in stimulating protease, but casein hydrolyzates (enzymatic and acid) were ineffective.

Table 3. Proteolytic activity (μg tyrosine/hr/midgut) in mosquitoes fed on various solutions.

Food	%	Hours after feeding 4	8	20	No. of females with full midguts 20 hrs after feeding
Bovine serum albumin	5	5.5	16.5	14.0	6/15
	0.5	5.7	7.2	6.3	5/10
	0.25	3.8	7.7	6.8	6/16
Casein	5	5.8	8.5	12.4	29/47
	1	9.4	14.3	18.0	10/24
	0.25	8.9	11.2	–	none
Casein hydrolysate (acid)	2.2	2.0	3.1	–	none
Casein hydrolysate (enzymatic)	1	1.4	3.7	–	none
Controls (12% dextran)		1.7	3.3	–	none

Although the distension of the midgut with an inert solution induces the appearance of protease, it is not essential for protease production. Females fed on sugar solutions containing blood and other proteins are able to develop eggs (Clements, 1963). They feed continuously; the food first enters the crop and is gradually released into the midgut. We fed mosquitoes of both sexes on sheep serum and sheep haemoglobin with 5% sucrose as feeding stimulant. The protease activity in the midguts of the females was 19.4 and 17.6 μg tyrosine/hr/midgut after 16 hours of continuous feeding on sheep serum and haemoglobin, respectively. Results were similar after feeding on whole sheep blood with sugar. The males had full crops, but no protease was found in their midguts. Males of *A. aegypti* lack the finger-print-like structures in their midgut epithelial cells, which are probably responsible for protease synthesis (Hecker *et al.*, 1971).

Females fed on dextran alone never initiated ovary development. Females that had fed on sheep blood, sheep serum or 5% casein solution (with sugar or with ATP through membranes) initiated egg development, but those fed on diluted blood and serum, or on 1% casein did not. After feeding on 10% and 20% bovine serum albumin, more than half of the females initiated egg development (Weissman-Strum, unpublished results), but we observed no ovary development in females fed 5% albumin or less. Some proteins, such as sheep haemoglobin, did not initiate egg development when fed at any concentration. On the other hand all proteins tested so far initiated protease, often after feeding on less than 1% solutions. Clearly, there is no relation between the

ability of a diet to induce protease activity and its capacity to initiate egg development. Less protein is needed for inducing protease than for ovary development.

The role of the small amount of protease that appeared in response to engorging on a nutritionally inert solution is not clear. It is probably not essential for triggering the production of protease, because the protein in the food was a sufficient stimulus and midgut distension was not necessary. It is possible that the function of the small amount of protease releasėd after engorging is related to events which precede the digestion of the blood, such as the coagulation of the blood mass and the formation of the peritrophic membrane (Clements, 1963).

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