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<b>Autor:</b>	Vundla, R.M.W. / Whitehead, D.L.
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The International Centre of Insect Physiology and Ecology, Nairobi, Kenya

## An enterokinase in the gut of pharate adult of *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae)

R. M. W. VUNDLA, D. L. WHITEHEAD

### Summary

An enterokinase (Enteropeptidase, EC. 3.4.21.9) has been described in the pharate adult of *Glossina morsitans morsitans*. The enzyme is present in pharate adults, 21 days after pupation. It activated commercial crystalline bovine trypsinogen to trypsin. It showed affinity for concanavalin A bound to sepharose and was reversibly sensitive to boiling at pH 6.0. The apparent molecular weight, as determined by gel permeation on sepharose 6B-CL, suggests self-aggregation or an association with a large molecule ( $M.Wt. \cong 2.5 \times 10^6$ ).

**Key words:** digestion; enterokinase; *Glossina morsitans morsitans*; *Trypanosoma brucei brucei*; trypsinogen; trypsin.

### Introduction

The role of digestive physiology in vector-parasite relations has not been systematically examined although it has been suspected that the relationship could be important (Nuttal, 1908; Day and Waterhouse, 1953). Digestive enzymes may retard the establishment of *Trypanosoma brucei brucei* in the tsetse. Damaged trypanosomes have been observed *in vivo* during the first 8 h after an infected blood meal (L. H. Otieno, unpublished data). A similar effect has been observed *in vitro* where the degree of lysis appears to be directly proportional to tryptic activity (M. Vundla, unpublished data). Gass and Yeates (1979) have shown that *Aedes aegypti* trypsin is the major factor in the destruction of ookinetes of *Plasmodium gallinaceum* *in vitro*, while Vundla et al. (in preparation) have shown that the accumulation of trypsin activity in the midgut of *Glossina morsitans* is delayed in newly emerged flies infected with *Trypanosoma brucei brucei*. M. Owaga (personal communication) has observed that infected

Correspondence: Mrs. R. M. W. Vundla, International Centre of Insect Physiology and Ecology, P.O. Box 30772, Nairobi, Kenya

wild *G. pallidipes* appear to digest their blood meal more slowly than non-infected flies.

Although we have shown a delay in the accumulation of trypsin in infected *G. morsitans morsitans*, we also observed that the quantity of enzyme present at the time of maximum proteolysis (24 h after feeding) is unaffected (Vundla et al., in preparation). These observations pointed to a delay of activation of the enzyme precursor. In the present study, we have demonstrated the presence of zymogen and of an enterokinase. Enterokinase is a key enzyme in other well-known gut proteinase systems. It is hoped that this will add to our understanding of the physiology of the tsetse gut especially with regard to vector-parasite relations.

## Materials and Methods

### *Tsetse*

The *G. m. morsitans* pupae used in this study were obtained from tsetse reared as described by Denlinger and Ma (1974). For the enzyme studies, 2 whole pupae (days 1, 4, 7, 10, 14 and 17 after pupation) or 2 isolated guts (days 21, 22 and 25) were homogenized in 0.2 ml chilled (4°C) 10<sup>-1</sup> M tris-acetate buffer pH 6.0 containing 5 × 10<sup>-2</sup> M NaCl. They were then disrupted by sonication for 2 min using a Headland electrosonic H60-2 and centrifuged at 6200 g for 20 min to remove debris. Sonication and centrifugation were carried out at 4°C. The supernatant was assayed for enzyme activity. For the partial purification of enterokinase, 200 guts from day 21 pharate adults were homogenized in 1 ml chilled buffer, sonicated, centrifuged and placed on a 75 × 1.6 cm sepharose CL-6B column equilibrated with tris-acetate buffer or on a 23 × 0.6 cm concanavalin A-sepharose 4B column equilibrated with tris-acetate buffer pH 6.0. After sample application the affinity column was washed with 40% ethylene glycol to remove non-specifically bound proteins and again with buffer and eluted with 5 × 10<sup>-2</sup> M α-methyl-D-mannoside. The entire separation procedure was performed at 4°C. The protein was monitored at 278 nm on a LKB 2138 Uvicord S.

### Enzyme assays

The assays were performed using a Perkin-Elmer 402-UV spectrophotometer. Aminopeptidase (AP: EC 3.4.1.2) was assayed by the method of Wachsmuth et al. (1966) using 2 × 10<sup>-2</sup> M L-leucine-p-nitroanilide (LpNA) (Sigma) with 10% dimethyl formamide (DMF) in 5 × 10<sup>-2</sup> M phosphate buffer (pH 8.0;  $A_{410} = 8800$ ). Trypsin and/or proteinase VI activity was assayed according to Erlanger et al. (1961), using 2 × 10<sup>-3</sup> M α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BApNA, Sigma) with 6.6% DMF in 5 × 10<sup>-2</sup> M phosphate buffer, pH 7.9 ( $A_{410} = 8800$ ). Enterokinase was assayed by a modification of the method of Kunitz (1939). The assay was coupled to the trypsin assay, so that there were two steps. In step one the enzyme was incubated at 4°C with 5 × 10<sup>-2</sup> mg · ml<sup>-1</sup> trypsinogen in 5 × 10<sup>-2</sup> M sodiumcitrate-citric acid buffer (pH 5.6) for up to 2 h. The reaction was stopped by adding 0.05 ml of 1.5 M HCl at 0, 60 and 120 min. Aliquots were then assayed for BApNA hydrolysis as described above. The zymogen (trypsinogen) was assayed as follows: 20 guts from 21-day-old pharate adults were homogenized in 2 × 10<sup>-1</sup> M diisopropyl phosphofluoridate (DFP) and dialysed overnight at 4°C, against sodiumcitrate-citric acid buffer (pH 5.6). The homogenate was then centrifuged at 6200 g for 20 min. The DFP treatment irreversibly inactivated all the trypsin in the homogenate. The clear supernatant was assayed for trypsin by the method of Erlanger et al. (1961) but with the buffer adjusted to pH 6.0.

## Results

Trypsin/proteinase VI activity (BApNA hydrolysis) decreased steadily from day 1 of pupation to day 21, when it was lowest (Fig. 1) (see also Langley, 1967). This activity was entirely inhibited by  $2 \times 10^{-3}$  M N- $\alpha$ -p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK). 21-day-old pharate adults were therefore investigated for enterokinase activity. The presence of zymogen in 21-day-old pharate adults was indicated by the appearance of active enzyme (trypsin) which was monitored by its ability to hydrolyse BApNA (Fig. 2). Since the trypsin previously present in the homogenate had been inactivated, the new activity must have been due to newly activated enzyme. This is also supported by the fact that the activity increased with time. Three major protein peaks were eluted from the sepharose CL-6B column. Enterokinase activity was detected in the first peak together with AP, at a point corresponding to  $2.5 \times 10^6$  Daltons (Fig. 3). The other two peaks had neither enterokinase nor AP activity. Trypsin was detected in the second peak, having a MW of  $2.3 \times 10^4$ . There was no trypsin activity in the first peak. From the affinity column, enterokinase was eluted separately from trypsin (Fig. 4). The eluted enzyme hydrolysed commercial crystalline bovine trypsinogen to trypsin as shown by its hydrolysis of BApNA. Boiling abolished the activity of the enzyme but this appeared to be regained after 60 min, on standing at  $4^\circ\text{C}$  (Fig. 5).

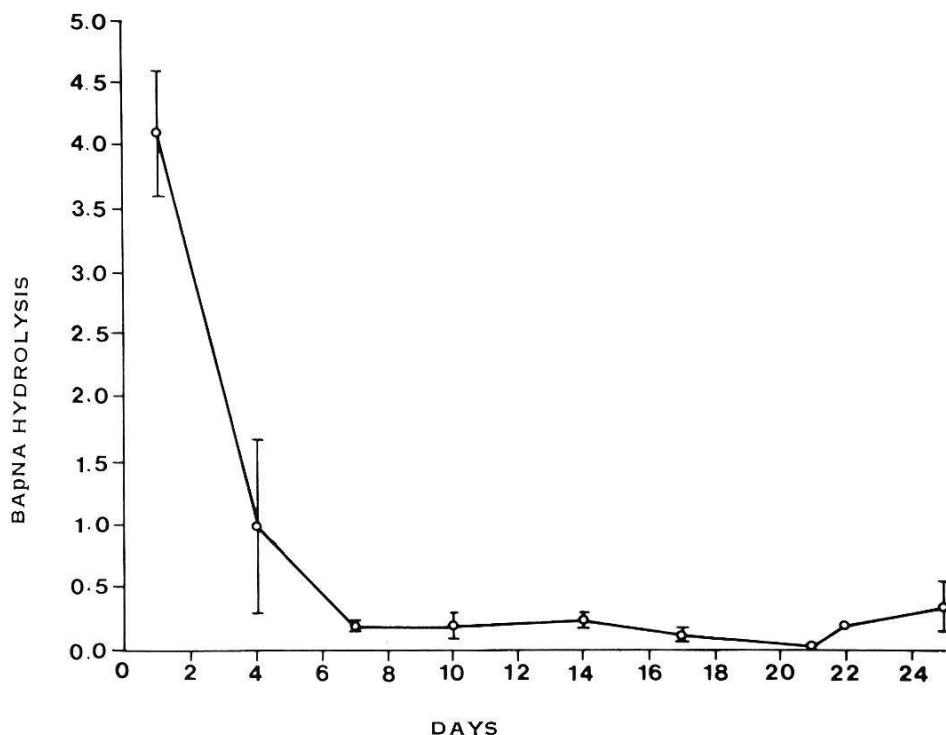


Fig. 1. Trypsin/proteinase VI activity (BApNA hydrolysis expressed in  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{gut}^{-1}$ ) in *G. m. morsitans* from day 1 to day 25 of pupation.

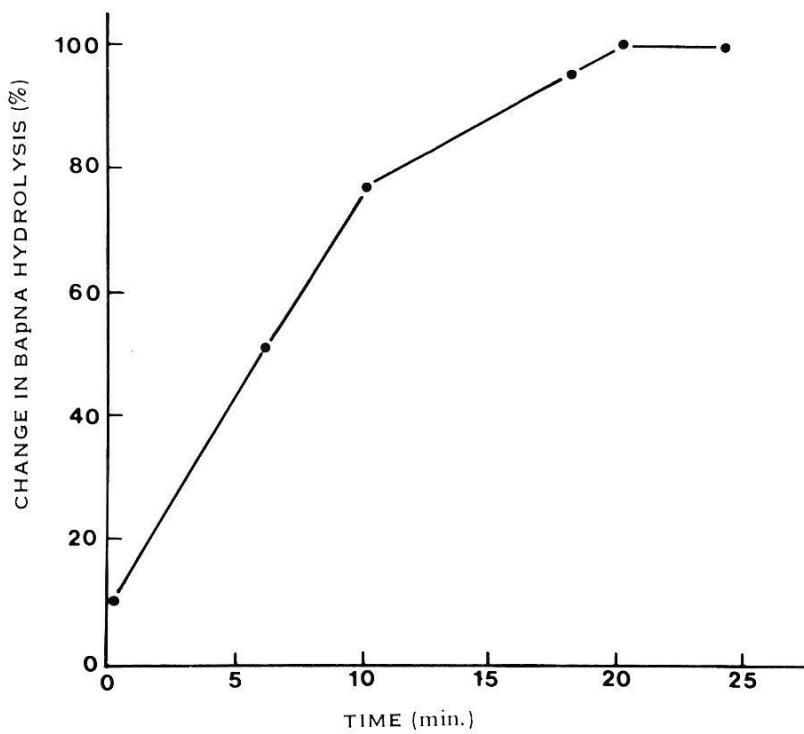


Fig. 2. The activation of trypsinogen to active enzyme. The percentage change in activity i.e. the appearance of trypsin is plotted against the time.

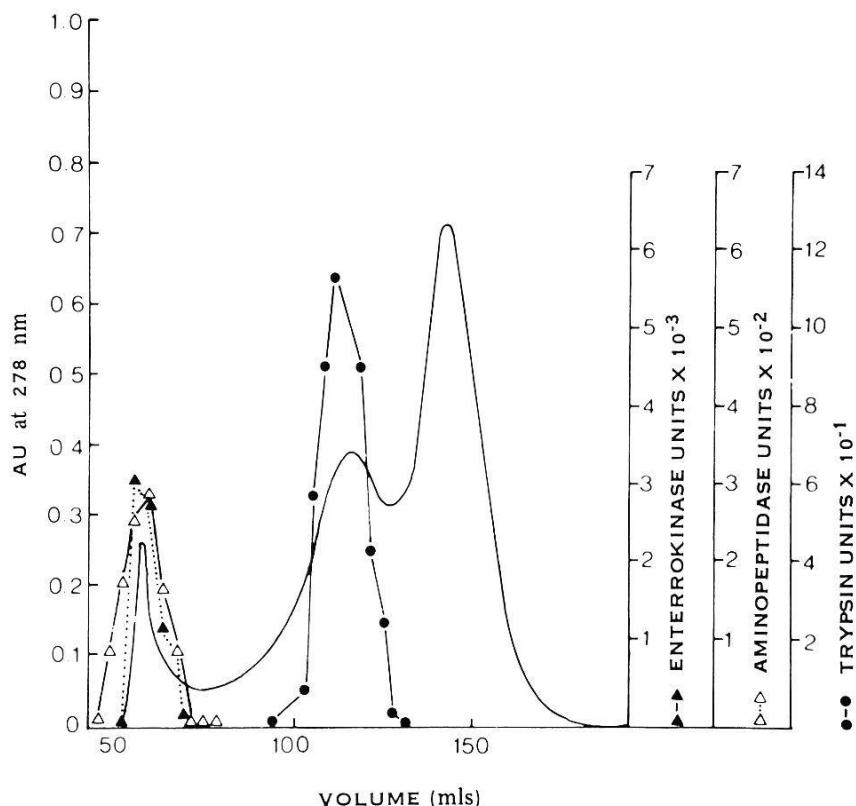


Fig. 3. Separation of *G. m. morsitans* gut enterokinase by gel permeation on sepharose CL-6B. Flow rate,  $30 \text{ ml} \cdot \text{h}^{-1}$ . Enzyme activities expressed in  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ .

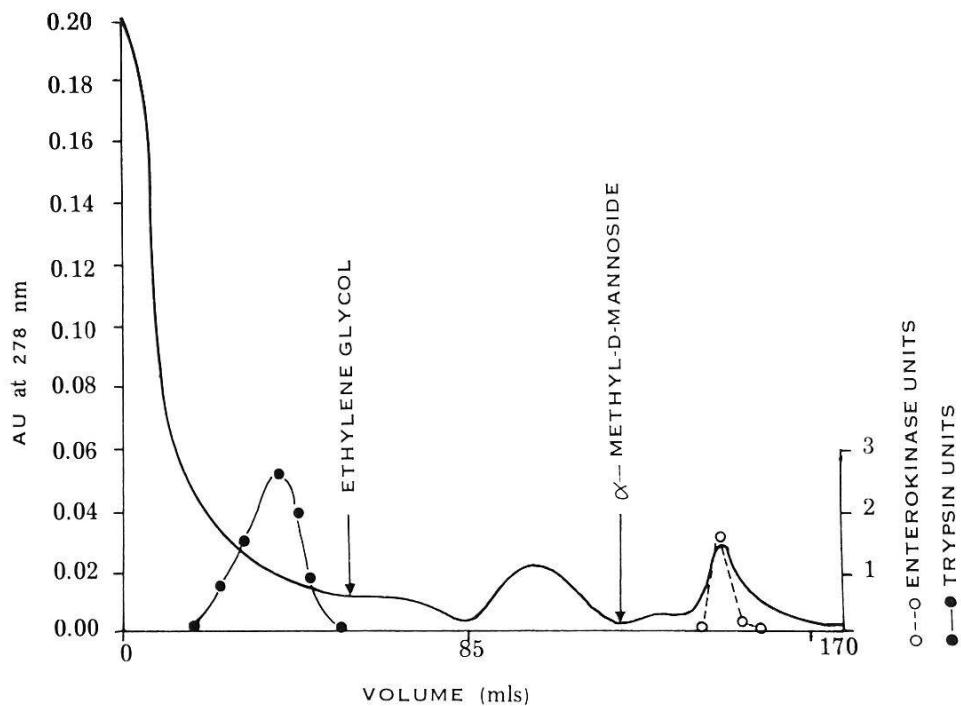


Fig. 4. Separation of *G. m. morsitans* gut enterokinase by affinity chromatography on concanavalin A-sepharose 4B. Flow rate,  $15 \text{ ml} \cdot \text{h}^{-1}$ . Enzyme activities expressed in  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ .

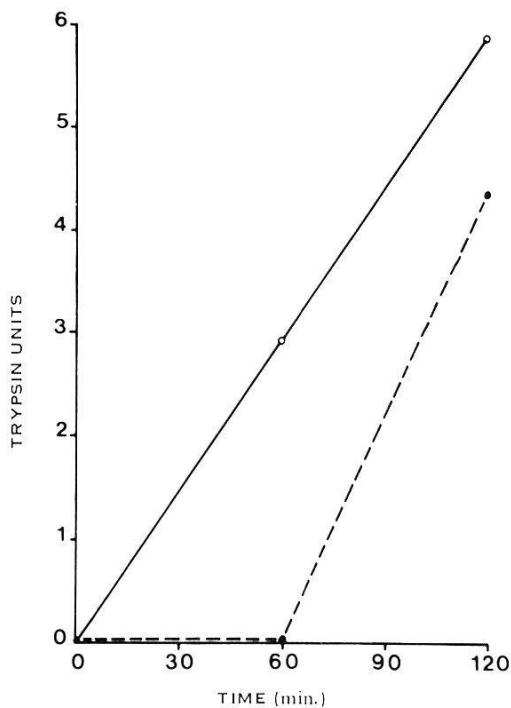


Fig. 5. Formation of trypsin from crystalline bovine trypsinogen by *G. m. morsitans* gut enterokinase before and after heat inactivation. Trypsin units are expressed in  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{gut}^{-1}$ .  $\circ - \circ$  = normal enzyme;  $\circ - - - \circ$  = boiled enzyme.

## Discussion

Trypsin activity was assayed in pupae of various ages to determine at what time during development the active enzyme is absent. The best time to assay for enterokinase would be when trypsinogen alone occurs. For days 1, 4, 7, 10, 14 and 17 whole pupae were used in the trypsin assays as it was assumed that all or most of the activity is associated with the gut as is the case in other insects such as *A. aegypti* (Kunz, 1978).

As expected, both zymogen and enterokinase activity were shown to be present in 21-day-old pharate adults. The latter appeared to have a large molecular weight suggesting either self-aggregation of the enzyme or possible association with other large molecules. A similar phenomenon has been observed in *A. aegypti* when trypsin was purified in the absence of its substrate (Kunz, 1978). The *G. m. morsitans* enterokinase regained activity after heat inactivation at pH 6.0. This phenomenon also occurs with trypsin itself, which can be reversibly heat-denatured at pHs below 8 (Anson and Mirsky, 1934). These observations agree with those made for mammalian trypsin and enterokinase which have many similarities in their properties (Maroux et al., 1971).

Enterokinase converts trypsinogen to trypsin. Once formed, trypsin can effect the specific cleavage of trypsinogen. Moreover, trypsin is the sole known activator of chymotrypsinogen and the procarboxypeptidases (Neurath, 1964). Enterokinase is therefore the key activator of the proteinases (Hadorn et al., 1969; Tarlow et al., 1970). In the tsetse, Gooding (1977) has demonstrated a correlation in the activities of trypsin and carboxypeptidase B, an indication that, as in the mammalian system, trypsin is central to the activity of the proteinases.

The elucidation of the mechanism by which trypsin is produced in the tsetse gut is important to our understanding of the digestive physiology of the fly and the vector-parasite relationship. Our results, though preliminary, contribute to our understanding of the relation between trypanosome and vector and hence the barrier to the establishment of infection by trypanosomes. Work is currently in progress to purify the enzyme by affinity chromatography so that the tsetse gut enterokinase can be characterized.

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