complicated and time consuming, finally involving preparative electrophoresis[9] or isoelectric focussing[5]. In an attempt to devise an alternative chromatographic procedure, we have looked at the behaviour of octopine dehydrogenase on blue Sepharose[10].

Purification of octopine dehydrogenase. Cibacron blue F3G-A Sepharose derivatives bind enzymes having a 'dinucleotide-fold' common to most NAD^+ and $NADP^+$-dependent dehydrogenases[11,12]. Cross-linked Sepharose 4-B was coupled with Cibacron blue F3G-A[13] and a 4× 1.5 cm column of the blue Sepharose was equilibrated with 10 mM Tris HCl buffer, pH 7.5 containing 100 mM NaCl. An aliquot of a preparation of octopine dehydrogenase made by the method of Gäde and Grieshaber[3] from *P. maximus* adductor muscle was added to the column (2 mg protein/ml gel). Under these conditions, octopine dehydrogenase was surprisingly not bound by the blue Sepharose. The eluate was collected together with about 5 ml of washings. The table shows that the final preparation contained all of the octopine dehydrogenase but none of the malate dehydrogenase activity. The column was regenerated by washing with 3M NaCl (3 column volumes) and 10 mM Tris HCl buffer, pH 7.5 containing 100 mM NaCl. The eluates from several batches were pooled and the octopine dehydrogenase activity concentrated by precipitation with 80% saturated ammonium sulphate and then resuspended in 10 mM Tris HCl buffer, pH 7.5 containing 100 mM NaCl. This rapid 2-step procedure resulted in a preparation of octopine dehydrogenase which was free from malate dehydrogenase and had a sp. act. of 65 units/mg protein.

Contaminating activities. A prerequisite for the use of octopine dehydrogenase to determine metabolite levels in tissue extracts is the absence of contaminating NAD^+-oxidoreductase activities. The mol.wt of octopine dehydrogenase (40,000 daltons) is one of the lowest known for NAD^+-dependent dehydrogenases, so that high mol.wt (100,000 daltons) contaminating enzymes will be removed by gelfiltration on Sephadex G-100. Also Cibacron blue F3G-A is a group specific adsorbent for NAD^+ and $NADP^+$-dependent dehydrogenases.

Purification of octopine dehydrogenase from adductor muscle of *Pecten maximus*. Specific activities: Units/mg protein

Fraction	Lactate dehydrogenase	Octopine dehydrogenase	Malate dehydrogenase
Crude homogenate	0.12	1.7	2.3
Sephadex G-100 preparation	0	19.5	9.2
Blue Sepharose preparation	0	65.0	0

Unexpectedly, octopine dehydrogenase does not bind to blue Sepharose and this has been exploited to separate octopine dehydrogenase from other NAD-dependent dehydrogenases, although we have only assayed for malate dehydrogenase (mol.wt 70,000 daltons). There is still a 10-fold difference in the specific activity of the final preparation and the pure enzyme from *P. maximus* (1000 units/mg protein[9]). The most likely contaminating proteins are soluble sarcoplasmic proteins which may constitute up to 25% of the total protein in the adductor muscle[14].

An experimental check on the possible interference of contaminating activities in the enzymatic assay for octopine was carried out, using extracts of the adductor muscle from *Ostrea edulis* which does not contain octopine[15]. Under standard assay conditions[16], no increase in absorbance at 340 nm was recorded on addition of the octopine dehydrogenase preparation to the oyster extracts. This preparation has been used successfully for the measurement of changes in metabolite levels in the adductor muscle of *P. maximus*, during the escape response[16].

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Proteolytic enzymes in the *Rhodnius prolixus* midgut[1]

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Summary. Sequential chromatographic fractionation of *Rhodnius prolixus* midgut homogenate yielded only one endopeptidase, but revealed the presence of carboxypeptidase-A and B-like enzymes, di- and tripeptidases, as well as aminopeptidase activities.

R. prolixus is a blood-sucking insect and one of the known transmissors of *Trypanosoma cruzii*, the agent causing Chagas disease. The bug requires a single and very large blood meal for ecdysis and oogenesis[2]. In a previous report we have described a SH-dependent endopeptidase[3], which seems to initiate the digestion of proteins in the insect's midgut. This enzyme is confined to the midgut and is present in all instars of the insect[4,5]; its production seems to be controlled by a secretagogue mechanism[6]. The present paper describes other midgut enzymes related to the proteolytic hydrolysis of ingested proteins.

In order to characterize different proteolytic activities, the

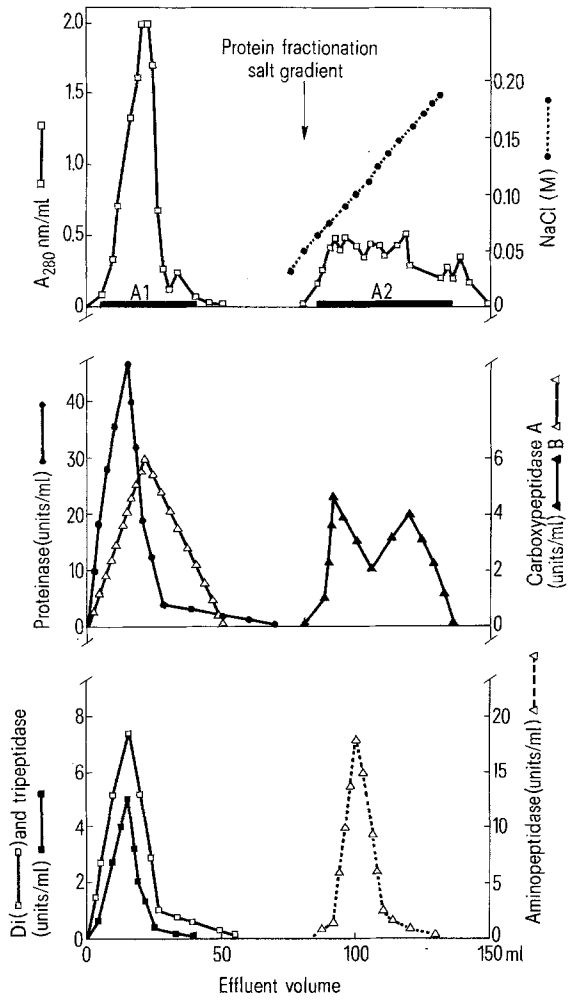

Fig. 1. DEAE-cellulose chromatography of *R. prolixus* midgut homogenate. Fractionation of proteolytic enzymes was performed at r.t. on a column (0.29×18.7 cm) previously equilibrated with 0.05 M Tris·HCl–0.03 M NaCl–10 mM tetrathionate, pH 7.5. A 5-ml sample of 10-fold concentrated crude preparation was applied to the column and then washed with 45 ml of the same buffer. Elution of adsorbed protein was performed by the use of a linear gradient (0.03–0.2 M NaCl in the above buffer). Fractions of 2 ml were collected at a flow rate of 15 ml/h.

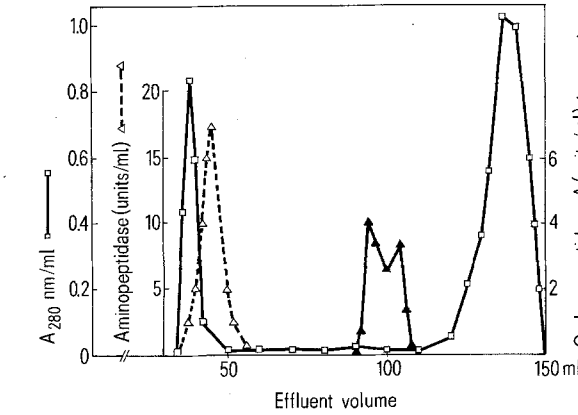

Fig. 2. BioGel P-150 gel-filtration of pool A2. A column (0.71×88 cm) was previously equilibrated with buffer (0.01 M phosphate – 1 M NaCl, pH 6.0). Pool A2 was dialyzed, lyophilized, dissolved in 1 ml of the equilibrating buffer and applied to the column. Fractions of 2 ml were collected at 20 ml/h flow rate.

midguts from 100 adult insects were collected 8 days following the last blood meal, and were homogenized in 0.15 M NaCl–10 mM tetrathionate (2 intestines/ml). The homogenate was centrifuged at 15,000 rpm, for 15 min at 4 °C. The clear supernatant was collected and concentrated 10-fold by pressure dialysis using Visking tubing under a pressure of 1.25 kg/cm^2 at 4 °C and dialyzed at the same time against several changes of 0.05 M Tris · HCl–0.03 M NaCl–10 mM tetrathionate pH 7.5 (crude preparation). The crude preparation was fractionated by DEAE-cellulose chromatography. A major protein peak (pool A1) was not adsorbed to DEAE-cellulose column (figure 1). It contains an endopeptidase, the *R. prolixus* proteinase, a sulfhydryl-enzyme[3] assayed by Kunitz' method[7]. It also contains di- and tripeptidases (assayed by the ninhydrin method[8]), and carboxypeptidase B-like enzyme (determined by the method of Folk et al.[9]). The adsorbed proteins were eluted by application of a linear gradient (0.03–0.2 M) of NaCl in 0.05 M Tris · HCl–10 mM tetrathionate pH 7.5 (figure 1). These fractions (figure 1, pool A2) contained an aminopeptidase (assayed by the method of Goldbarg and Rutenburg[10]) as well as a carboxypeptidase A-like activity (determination was based upon the method of Folk and Schirmer[11]). Pool A1 was then chromatographed in a Sepharose-mercurial affinity column (not shown) which is a convenient method used for fractionation of this endopeptidase[3]. The di- and tripeptidases as well as the carboxypeptidase B appeared in the filtrated fractions, which were then combined, giving pool A3. The thiol-proteinase was then eluted from the column by percolation with 2-mercaptoetanol. Pool A2 (figure 1) as well as pool A3 were separately dialyzed at 4 °C against several changes of distilled water followed by lyophilization. The materials were stored at –20 °C until used. Each sample was dissolved in 1 ml of 0.01 M phosphate buffer – 1 M NaCl, pH 6.0, and chromatographed in a column of BioGel P-150. Di- and tripeptidases could not be separated from each other, and they were not separated either from carboxypeptidases B (not shown). However, this column can be used as a final step for separating aminopeptidase from carboxypeptidase-A activities (figure 2).

These data support the suggestion that these are auxiliary enzymes, important for further digestion of proteins in the intestine of this insect, thus completing the proteolytic hydrolysis started by the proteinase[3]. The thiol-endopeptidase is an essential enzyme for the insect's survival, since its in vivo inhibition clearly limits the ecdysis in the larvae, and egg production in the insect female[12].

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