

PROTEINASES IN MOLTING FLUID OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Abstract—*Manduca sexta* pharate pupal molting fluid contains more than 10 proteolytic enzymes that differ in relative mobility during electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and gelatin. The major gelatin digesting enzyme was an endoprotease with an apparent molecular weight of 100 kDa. Gel filtration on a Sephacryl S-300 column resolved another endoprotease of similar size that digests azocoll and [³H]casein. In addition we found an aminopeptidase-like enzyme (MW_{app} 500 kDa) and at least three carboxypeptidase-like enzymes (MW_{app} 10–60 kDa). Use of pseudosubstrates and inhibitors suggested the presence of both trypsin-like and chymotrypsin-like enzymes with the former activity approx. 10-fold greater than the latter. However, none of the proteolytic enzymes were substantially inhibited by diisopropylphosphorofluoridate or phenylmethylsulfonyl fluoride which are potent inhibitors of trypsin and chymotrypsin. No carboxyl or sulfhydryl proteases were detected. The enzymes were most active in the neutral to alkaline pH range, but they were relatively unstable during storage which precluded their purification to homogeneity. Proteolysis of *Manduca* cuticular protein appears to involve a rather complex and unique mixture of endo- and exo-cleaving proteolytic enzymes.

Key Word Index: *Manduca sexta*, tobacco hornworm, insect, molting, protein, proteinases, proteases, cuticle, proteolysis, ecdysis

INTRODUCTION

Insects are limited in size and form by the physical constraints of their exoskeleton. They undergo periodic shedding of old cuticle and synthesis of a new one to allow for continued growth and development. Degradation of the old exoskeleton between apolysis and ecdysis is well established. The enzymatic nature of this degradation was long speculated upon and was not firmly demonstrated until Passonneau and Williams (1953) detected both chitinolytic and proteolytic activities in molting fluid of pharate adult cecropia moths. As part of a study of the types and properties of hydrolytic enzymes in insect molting fluid, we previously purified and characterized the chitinolytic enzymes, chitinase and exo- β -*N*-acetylglucosaminidase, from molting fluid of the tobacco hornworm, *Manduca sexta* (Koga *et al.*, 1982, 1983; Fukamizo and Kramer, 1985; Kramer and Koga,

1986). We have now begun to characterize the proteolytic enzymes present in *M. sexta* pharate pupal molting fluid. Degradation of the old cuticle may be a sequential process in which proteinases first attack the protein matrix of the endocuticle to expose the chitin and make it available for attack by chitinases (Fukamizo and Kramer, 1985).

To our knowledge, very little information is available about the properties of proteinases that occur in insect molting fluid. The most detailed studies are those of Katzenellenbogen and Kafatos (1970, 1971a,b). Those authors found that molting fluid from pharate adults of the polyphemus moth hydrolyzes certain trypsin-selective substrates, such as benzoylarginine ethyl ester and tosylarginine methyl ester (Katzenellenbogen and Kafatos, 1970). Using sucrose density gradient centrifugation and ion-exchange chromatography, two serine proteases were partially purified (Katzenellenbogen and Kafatos, 1971a). Substrate specificities and inhibitor sensitivities of these enzymes most strongly resembled trypsin. In addition, the enzymes were found to be secreted with the molting gel as inactive forms (Katzenellenbogen and Kafatos, 1971b). However, the mechanism of activation was not determined.

A previous study of proteolytic enzymes in molting fluid of *M. sexta* suggested the presence of two types of proteinase activity, a chelator-sensitive metalloenzyme active at neutral pH and a diisopropylphosphorofluoridate sensitive activity with an alkaline pH optimum (Bade and Shoukimas, 1974). We have also identified several types of proteases in *M. sexta* molting fluid, but their properties are rather different from the enzymes previously described.

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Abbreviations used: BAEE, benzoylarginine ethyl ester; BAPNA, benzoylarginine-*p*-nitroanilide; BTEE, benzoyltyrosine ethyl ester; BTPNA, benzoyltyrosine-*p*-nitroanilide; DFP, diisopropylphosphorofluoridate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GLY-pNA, glycine-*p*-nitroanilide; kDa, kilodalton; LEU-pNA, leucine-*p*-nitroanilide; OTI, ovomucoid trypsin inhibitor; PCMS, *p*-chloromercuriphenyl sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, tosyllysine chloromethyl ketone; TPCK, tosylphenylalanine chloromethyl ketone.

MATERIALS AND METHODS

Insects

Eggs of *M. sexta* were obtained from the Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, N.D. Larvae were reared on a standard diet (Bell and Joachim, 1976) and kept at $28 \pm 2^\circ\text{C}$ during a 16 h light–8 h dark photoperiod.

Molting fluid was collected from pharate pupae by pricking the old cuticle with a pin at the terminal abdominal segment and the ventral surface of the prothoracic segment. As fluid was gently expressed through the pinhole, droplets were collected in a 0.05 ml glass capillary pipette and immediately transferred to a plastic microcentrifuge tube that was chilled on a block of dry ice. When molting fluid was quickly frozen in this manner, phenoloxidase inhibitors, such as 1-phenyl-2-thiourea, were not required to prevent melanization. Molting fluid collected in this way was colorless to a light yellow color and was easily distinguished from the bright green hemolymph. Typically, larvae yielded about 0.1 ml of molting fluid but some individuals produced as much as 0.25 ml.

Electrophoresis

Proteases present in molting fluid were analyzed electrophoretically using SDS–polyacrylamide gels containing gelatin as substrate (modified from Heussen and Dowdle, 1980). For this analysis, electrophoresis was conducted in 0.75 mm thick, 7.5% acrylamide minigels as described by Laemmli (1970) except that disulfide bonds were allowed to remain intact by omitting 2-mercaptoethanol. After electrophoresis the gels were washed briefly in 2.5% Triton X-100 to remove SDS and incubated overnight in 0.1 M Tris buffer, pH 7.8, 37°C . Gels were then stained with Coomassie Brilliant Blue and destained briefly with 40% methanol, 10% acetic acid, followed by several hours in 5% acetic acid. This method visualized proteolytic activity as a zone of clearing in a dark blue background. Molecular weights were estimated by comparison of electrophoretic mobility to those of prestained molecular weight marker proteins (Bethesda Research Laboratories, Gaithersburg, Md). Proteins of *Manduca* molting fluid were analysed under the same electrophoretic conditions except that gelatin was not included in the polyacrylamide gel and Coomassie dye staining was performed immediately after electrophoresis was completed. The mobilities of the prestained standard proteins were unaffected by the presence of gelatin in the polyacrylamide gel containing SDS. It is assumed that the migration of molting fluid proteins is likewise unaffected by gelatin. Gelatin had no effect on the relative mobilities of *Drosophila* proteins (Pino-Heiss and Schubiger, 1989).

Total protein

Protein concentration was determined by the dye binding method of Bradford (1976) using bovine serum albumin as the standard protein.

Protease assays

General proteolytic activity was assayed using an insoluble substrate, azocoll (Calbiochem, San Diego, Calif.; adapted from Chavira *et al.*, 1984) or tritiated casein (Murdock *et al.*, 1987). For the former assay azocoll (5 mg ml^{-1}) was suspended in the appropriate buffer with constant stirring. One ml of the suspension was pipetted to a 1.5 ml microcentrifuge tube placed on ice. To pipette the substrate suspension, it was necessary to enlarge the opening of the pipette tip by cutting off about 3 mm of the narrow end. The enzyme preparation and buffer were added to each tube to bring the total volume to 1.2 ml and the reaction was initiated by transferring the tubes to a shaking incubator at 37°C and 350 rpm for 1 h. The reaction was stopped by centrifuging the tubes for 2 min in a Eppendorf microfuge at room temperature. Proteolytic activity was estimated by

the release of a red dye from the substrate with an absorption maximum at 520 nm. An azocoll unit was defined as the activity required to achieve a net increase of 0.2 absorbance units per hour. Blank values typically fell in the 0.05 range.

Radiolabeled α -casein was another substrate that provided greater sensitivity than the azocoll substrate when used for determining general protease activity. Details of the radiolabeling and assay procedures have been previously described (Murdock *et al.*, 1987). Briefly, bovine α -casein was labeled with tritium by means of a reductive methylation in the presence of high specific activity [^3H]NaBH₄ (sp. act. $10\text{--}20 \text{ Ci mmol}^{-1}$, New England Nuclear, Boston, Mass). The low molecular weight radioactive byproducts of this reaction were removed by gel filtration on a Sephadex G-25 column (Pharmacia PD-10 desalting column) eluted with 100 mM Tris–HCl, pH 7.8. The G-25 fractions containing [^3H]protein were pooled and diluted to $4 \times 10^6 \text{ cpm ml}^{-1}$ and protein concentration was adjusted to 2 mg ml^{-1} with cold α -casein. The solution was divided into 0.1 ml portions and stored at -70°C . Prior to use, each aliquot of [^3H]casein was diluted with 0.9 ml of unlabeled casein (2 mg ml^{-1}). Assays were performed by incubating 0.05 ml of the [^3H]casein, plus buffer and the molting fluid or enzyme preparation to give a reaction volume of 0.1 ml contained in a 1.5 ml plastic centrifuge tube. The reaction was initiated by addition of enzyme and quenched after 20 min incubation at 37°C by addition of 0.1 ml of 10% trichloroacetic acid. After standing on ice for 20 min, the samples were centrifuged at 14,000 rpm for 5 min and 0.175 ml aliquots of the supernatant were pipetted into counting vials containing 5 ml of scintillation fluid. Background levels of TCA soluble radioactivity were determined by substituting an equal volume of buffer for the enzyme solution.

Manduca molting fluid was also screened for specific proteolytic activities using synthetic substrates. Trypsin-like activity was examined using benzoylarginine ethyl ester (BAEE; Walsh and Wilcox, 1970), benzoylarginine-*p*-nitroanilide (BAPNA; Erlanger *et al.*, 1961) and tosylarginine methyl ester (TAME; Walsh and Wilcox, 1970). Chymotrypsin-like activity was assayed for using benzoyltyrosine ethyl ester (BTEE; Walsh and Wilcox, 1970), benzoyltyrosine-*p*-nitroanilide (BTPNA) and the synthetic peptide Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (Aschstetter *et al.*, 1981). Elastase-like activity was screened for with Suc-Ala-Ala-Val-*p*-nitroanilide (Wenzel *et al.*, 1980). Aminopeptidase-like activity was determined by hydrolysis of leucine-*p*-nitroanilide (LEU-pNA; Wachsmuth *et al.*, 1966) and glycine-*p*-nitroanilide (GLY-pNA; Delange and Smith, 1971). Carboxypeptidase-like activity was measured by hydrolysis of hippuryl arginine and hippuryl phenylactic acid (Folk and Schirmer, 1963).

The specificity of *Manduca* molting fluid proteases was further analyzed by examining fragments cleaved from a synthetic hexapeptide substrate, H-Leu-Trp-Met-Arg-Phe-Ala-OH (Research Plus Inc., Bayonne, N.J.). Partially purified preparations of the putative molting fluid aminopeptidase, endopeptidase and carboxypeptidase were obtained by gel filtration of *Manduca* molting fluid on Sephacryl S-300. Fractions near the peak proteolytic activity and well resolved from other activities were incubated with the peptide substrate in 0.2 ml 0.1 M ammonium acetate pH 7 at either 37 or 23°C for 10–90 min. The reaction was quenched by addition of ice-cold acetic acid. After lyophilization to remove most of the volatile buffer, cleavage products were redissolved in 0.1 ml of methanol/water (1:1; v/v) and analyzed by thin-layer chromatography (TLC) on high-performance silica gel plates developed in a mixture of butanol/acetic acid/water (3:1:1; v/v/v) for 5–6 h. Cleaved fragments were visualized by spraying the plate with ninhydrin (Pierce Chemical Co., Rockford, Ill.) and identified by comparison with the mobilities of standard fragments and the six component amino acids supplied with the synthetic peptide kit.

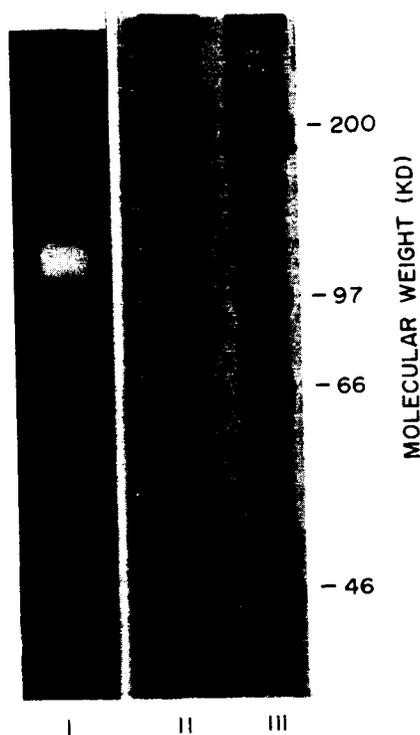


Fig. 1. Electrophoresis of molting fluid from pharate pupae of *Manduca sexta* in polyacrylamide gels containing gelatin as substrate. Molting fluid was diluted 1:10 with SDS sample buffer and applied to polyacrylamide gels (7.5% T separating gel, 4% T stacking gel) but without reducing agent. Lane I: zones of proteolysis revealed using a gelatin containing gel after rinsing and incubating 16 h at 37°C in 0.1 M Tris buffer, pH 7.8. Lane II: molting fluid electrophoresed under the same conditions as above except that gelatin was omitted from the gel. Protein bands were visualized by Coomassie Blue staining. Lane III: molecular weight markers 200 kDa, myosin; 97 kDa, phosphorylase B; 66 kDa, bovine serum albumin; 43 kDa, ovalbumin. Approximately 10–20 μ g protein was applied in each cell.

pH optimum

Optimum pH values for azocoll and [³H]casein digesting activities were determined using a universal buffer containing phosphate, acetate and borate which was designed to provide a constant ionic strength of 0.1 at all pH values (Coch Frugoni, 1957). Azocoll was suspended in buffer of appropriate pH and the assays conducted with whole molting fluid as described above. [³H]Casein was diluted to the appropriate level of radioactivity with cold casein dissolved in universal buffer at the desired pH.

Inhibition assays

Protease inhibitors were obtained from Sigma Chemical Co. and include diisopropylphosphorofluoridate (DFP), phenylmethylsulfonyl fluoride (PMSF), *N*- α -*p*-tosyllysine chloromethyl ketone (TLCK), soybean trypsin inhibitor (type II-S; STI), chicken egg white ovomucoid inhibitor (type III-O; OTI), *N*- α -*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *p*-chloromercuriphenyl sulfonic acid (PCMS), 2,2-dipyridyl-1,10-phenanthroline and ethylenediamine tetracetic acid (EDTA). The juvenile hormone esterase inhibitors, methamidophos, *O*-ethyl-*S*-phenyl phosphoramidothoate, 3-octylthio-1,1,1-trifluoro-2-propanone and 1,1,1,17,17,17-hexafluoro-4,14-dithioheptadecane were obtained from the laboratory of B. Hammock and A. Szekacs, Department of Entomology, University of California, Davis. Each inhibitor was dissolved in either methanol or water at 10 \times the desired concentration. Inhibitor solution (0.025 ml) was added to 0.225 ml of the enzyme plus buffer mixture. The mixture was vortexed and preincubated 10 min at 37°C. The remaining proteolytic activity was assayed using 0.1 ml of the preincubation mixture with LEU-pNA, azocoll, BAEE or hippuryl phenyllactic acid as substrate. For the [³H]casein substrate, 0.01 ml aliquots were assayed. An equal volume of buffer replaced the enzyme solution in blank samples.

RESULTS

Electrophoresis

Manduca molting fluid was found to contain a complex mixture of endo- and exo-cleaving proteases. Electrophoresis in polyacrylamide gels containing gelatin as substrate indicated the presence of more than 10 zones of proteolytic activity in whole molting fluid (Fig. 1). These enzymes were active at the mildly alkaline pH 7.8. The major band of gelatin digesting

activity has an apparent molecular weight of approx. 100 kDa, while the next most intense band occurred at an apparent mol. wt of 50 kDa. None of these zones of gelatin-digesting activity precisely corresponded to a dye stained protein band in companion gels lacking substrate.

pH optimum

In addition to digesting gelatin, whole molting fluid also hydrolyzed the general protease substrates, azocoll and [³H]casein. The pH optimum using azocoll as the substrate occurred at pH 6.6 with 50% of the activity remaining at pH 5.4 and at pH 9.2 (Fig. 2). On the acidic pH side activity fell sharply with little azocoll hydrolysis at pH 4.3. After fractionation of the molting fluid by chromatography on Sephacryl S-300, a single major peak of proteolytic activity was obtained [Fig. 3(A)]. The pH optimum for caseinolytic activity in the peak fraction (No. 42) was rather broad on the alkaline side from about pH 7 to 9 (Fig. 2). Increasing the pH from the optimum resulted in a rather slow decline in activity, until only about 10% relative to the maximum activity remained at pH 10.5. These results are consistent with a determination of the molting fluid pH to be slightly alkaline, about pH 7.3, a value where about 80–100% of the optimum activity was demonstrated depending on the substrate used. Proteolytic activity in 0.01 ml of whole molting fluid determined at pH 7.8 was equivalent to an azocoll digesting activity of approx. 0.1 μ g of bovine trypsin. The protein concentration of *Manduca* molting fluid collected from pharate pupae was about 12 mg ml⁻¹. Thus, the azocoll digesting specific activity of molting fluid is approximately one thousandth that of purified bovine trypsin.

Substrate specificity

To gain an understanding of the types of proteases present, whole molting fluid was screened for activity against selective substrates (Table 1). Of the three tryptic substrates tested, only BAEE was rapidly hydrolyzed. No detectable hydrolysis was observed with TAME or BAPNA. Of the chymotryptic substrates, BTEE and BTPNA were hydrolyzed, but at only one tenth the rate of hydrolysis of BAEE. The peptide substrate, Suc(Ala)₂ProPhepNA, was cleaved only a very low rate. There was no significant activity against the elastase substrate. Low activities were obtained with substrates selective for exo-cleaving carboxypeptidases and aminopeptidases. The results indicated that *Manduca* molting fluid contains several types of proteases, some with properties characteristic of trypsin, chymotrypsin, carboxypeptidase and aminopeptidase.

Fractionation of *Manduca* molting fluid by gel filtration chromatography revealed a complex but highly reproducible protein profile with the first major peak of absorbance at 280 nm co-eluting with the primary peak of azocoll digesting activity [Fig. 3(A)]. The activity peaks for BAEE hydrolysis and [³H]casein digestion, both assayed at pH 7.8, also coincided with the major peak of azocoll activity (data not shown). This peak was estimated to have a molecular weight of 100 kDa, the same as the apparent molecular weight of the gelatin digesting activity

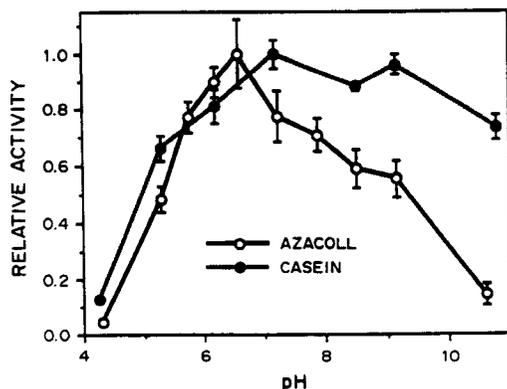


Fig. 2. pH optimum of azocoll (○) and [³H]casein (●) digesting activities in *M. sexta* molting fluid. Freshly collected molting fluid was assayed using azocoll suspended in universal buffer adjusted to the appropriate pH. The peak fraction from the Sephacryl S-300 column was assayed using [³H]casein. Mean values \pm SEM ($n = 4$).

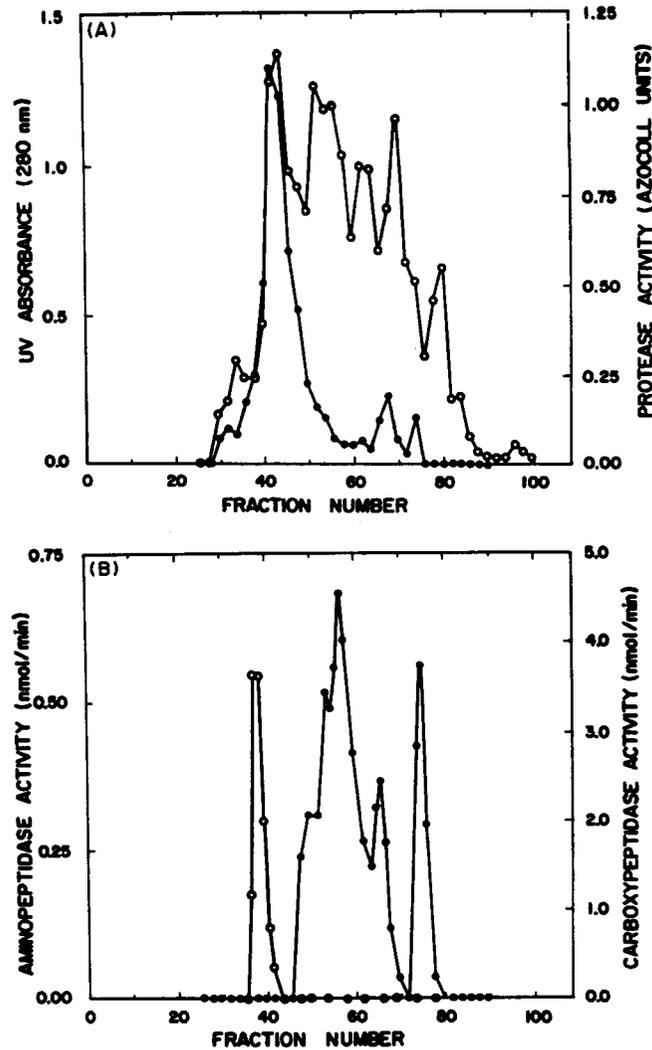


Fig. 3. Gel permeation chromatography of *M. sexta* molting fluid on Sephacryl S-300. Molting fluid (3 ml) was applied to a 1.5 cm i.d. \times 140 cm column, eluted with 0.05 M Tris pH 7.8 and 3.6 ml fractions were collected. The flow rate was 4–5 ml per h. (A) Fractions were assayed for total protein (A_{280} , ○) and general protease activity (azocoll assay, 0.1 ml/assay, ●). (B) The same fractions were also assayed for aminopeptidase activity using leucine-*p*-nitroanalide as substrate (○) and for carboxypeptidase activity using hippuryl arginine as substrate (●).

determined by using the SDS-containing substrate gels. Electrophoresis of aliquots of the Sephacryl S-300 fractions into gelatin containing polyacryl-

amide gels revealed that the major gelatin digesting fraction [Fig. 3(A), fraction 38] eluted before the peak of azocoll digesting activity (fraction 42). The elution

Table 1. Substrate specificity of *M. sexta* molting fluid proteases*

Substrate†	Substrate class	Substrate (mM)	Enzyme activity (nmol hydrolyzed/min/mg protein)
BAEE	Trypsin-like	1.0	82 \pm 38
BAPNA	Trypsin-like	1.0	<1
TAME	Trypsin-like	1.0	<1
BTEE	Chymotrypsin-like	0.1	7.4 \pm 1.0
BTPNA	Chymotrypsin-like	0.1	9.6 \pm 3.4
Suc(Ala) ₂ -ProPhe-pNA	Chymotrypsin-like	0.2	0.08 \pm 0.01
Suc(Ala) ₂ -Val-pNA	Elastase-like	10	<1
Hippuryl arginine	Carboxypeptidase-like	0.1	4.7 \pm 0.8
Hippuryl phenyllactic acid	Carboxypeptidase-like	0.1	2
Leucine-pNA	Aminopeptidase-like	1	4.0 \pm 1.1
Glycine-pNA	Carboxypeptidase-like	1	3.1 \pm 1.9

*Whole molting fluid was used. Mean values \pm SEM ($n = 3$).

†See p. 467 for list of abbreviations.

volume of gelatinase activity was nearly identical to that of the aminopeptidase activity with an apparent molecular weight in the 300–500 kDa range. Aminopeptidase activity eluted as a single peak slightly earlier than the azocoll digesting activity and the molecular weight was estimated to be 300 kDa [Fig. 3(B)]. Assay of the fractions for carboxypeptidase activity revealed three major peaks eluting after the azocoll digesting enzyme [Fig. 3(B)]. The carboxypeptidases ranged in size from <12 to about 60 kDa.

To further characterize the bond cleaving specificity of the major proteolytic enzyme activities in *Manduca* molting fluid, partially purified preparations from the gel filtration chromatography of the [³H]casein digesting activity, the leucine-*p*-nitroanilide hydrolyzing activity and the hippuryl arginine hydrolyzing activity were incubated with a model hexapeptide substrate, Leu-Trp-Met-Arg-Phe-Ala. Incubation of the model substrate with the peak fraction of the [³H]casein-azocoll-BAEE digesting activity for 10 min at 23°C produced three major fragments identified by TLC as Leu-Trp-Met-Arg, Phe-Ala and Met-Arg, as well an intense spot for the uncleaved hexapeptide (Table 2). Less intense spots were observed for Leu-Trp and the free amino acid Ala. A 10 min incubation at a higher temperature (37°C) with the same amount of enzyme produced a slightly different fragmentation with Leu-Trp, Phe-Ala and Met-Arg being the major fragments. Leu-Trp-Met-Arg was only faintly visible and Ala remained relatively weak in intensity while little uncleaved substrate remained. Increasing the incubation time to 90 min at 37°C yielded only four spots: Leu-Trp, partially resolved Phe-Ala and free Phe, Met-Arg and Ala. No uncleaved substrate was detected.

Cleavage of the model peptide substrate by the [³H]casein-azocoll-BAEE hydrolyzing activity in fractionated molting fluid was clearly consistent with the action of one or more endoproteases. However, the specificity indicated by the peptide cleavage pattern was rather general in nature. A progression in fragmentation was apparent and suggested that cleavage on the carboxyl side of Arg is preferred. However, a second cleavage occurred on the carboxyl side of Trp to yield the three depeptide fragments Leu-Trp, Met-Arg and Phe-Ala. This fragmentation

indicates a primary trypsin-like specificity and a secondary chymotrypsin-like specificity. The release of Ala upon prolonged incubation is also consistent with the action of a chymotrypsin-like enzyme cleaving the Phe-Ala fragment on the carboxyl side of Phe.

Incubation of the model substrate with the Sephacryl S-300 fraction of leucine-*p*-nitroanilide hydrolyzing (aminopeptidase-like) activity produced a partially resolved spot that probably contained Trp-Met-Arg-Phe-Ala and free Leu (Table 2). However, a standard of the pentapeptide fragment was not available for comparison of mobility on silica gel. Uncleaved model substrate was also present. Increasing the incubation time to 90 min at 37°C resulted in further hydrolysis. In addition to the putative Trp-Met-Arg-Phe-Ala and free Leu spots, there were distinct spots for Met, Arg, Ala, Met-Arg-Phe-Ala and Arg-Phe-Ala. These results are consistent with the action of an aminopeptidase-like enzyme.

Activity against the model peptide substrate in the hippuryl arginine hydrolyzing (carboxypeptidase-like) fraction tested was rather low. Only the 90 min incubation at 37°C revealed any evidence of hydrolysis (Table 2). This treatment produced a distinct spot with low mobility corresponding to Ala, a faint indication of Leu-Trp-Met-Arg-Phe and the uncleaved model substrate. This cleavage pattern is consistent with that of a carboxypeptidase-like enzyme.

From the substrate specificities and chromatographic behavior of the enzymes, four distinct proteases were apparent: an azocoll digesting enzyme which also appeared to hydrolyze BAEE and casein, and also exhibited trypsin-like and chymotrypsin-like cleavage specificities, an aminopeptidase, several carboxypeptidases and a gelatin digesting enzyme. We attempted to classify these enzymes further by examining their sensitivity to inhibitors diagnostic of the four major categories of proteolytic enzymes: the serine proteases, the sulfhydryl proteases, the metalloproteases and the acid proteases. However, the latter proteases were precluded since all of the major activities were observed at alkaline pH and the pH of molting fluid was found to be mildly alkaline. Also, the gelatin digesting activity was not included in these experiments since a quantitative assay for that enzyme was not readily available.

Table 2. Products from the hydrolysis of Leu-Trp-Met-Arg-Phe-Ala by *M. sexta* molting fluid proteases

Proteolytic activity*	Incubation time (min)	Temperature (°C)	Products
Casein-azocoll-BAEE hydrolyzing fraction	10	23	Leu-Trp-Met-Arg, Phe-Ala, Met-Arg, Leu-Trp, Ala
	10	37	Leu-Trp, Met-Arg, Phe-Ala, Leu-Trp-Met-Arg, Ala
	90	37	Leu-Trp, Met-Arg, Phe-Ala, Phe, Ala
Aminopeptidase	10	37	Leu, Trp-Met-Arg-Phe-Ala
	90	37	Leu, Trp-Met-Arg-Phe-Ala, Met-Arg-Phe-Ala, Arg-Phe-Ala, Met, Arg, Ala
Carboxypeptidase	90	37	Ala, Leu-Trp-Met-Arg-Phe

*Fractions used were from Sephacryl S-300 chromatography.

Table 3. Inhibitor sensitivity of major protease fraction from gel filtration of *M. sexta* molting fluid

Inhibitor†	IC ₅₀ (M)*				
	Azocoll	[³ H]Casein	BAEE	Leu-pNA	Hippuryl arginine
DFP	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²	10 ⁻³	> 10 ⁻²
PMSF	> 10 ⁻²	> 10 ⁻²	—	—	—
TLCK	10 ⁻⁴	2 × 10 ⁻⁵	10 ⁻³	~ 10 ⁻²	> 10 ⁻³
STI	10 ⁻⁷	2 × 10 ⁻⁷	10 ⁻⁶	—	—
OTI	> 10 ⁻⁵	> 10 ⁻⁵	> 10 ⁻⁵	—	—
TPCK	> 10 ⁻²	> 10 ⁻²	> 10 ⁻³	> 10 ⁻²	> 10 ⁻³
PCMS	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²
EDTA	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²	> 10 ⁻²
2,2-Dipyridyl	—	—	—	5 × 10 ⁻³	—
1,10-Phenanthroline	> 10 ⁻²	> 10 ⁻²	—	4 × 10 ⁻⁴	—

*IC₅₀ = molar inhibitor concentration required to give 50% inhibition. IC₅₀ value determined from curve of activity vs inhibitor concentration using 0.5 log unit steps of inhibitor concentration. Greater than (>) value denotes concentration of inhibitor tested where < 50% inhibition was observed. Azocoll hydrolyzed in 0.1 M PIPES, pH 6.6. Casein and pseudo substrates hydrolyzed in 50 mM Tris, pH 7.8.

†See p. 467 for list of abbreviations.

Inhibitor studies

The inhibitor sensitivity profiles of the azocoll-casein-BAEE hydrolyzing enzyme fraction from gel filtration chromatography did not clearly fit the enzyme(s) into any of the major categories of proteases (Table 3). DFP and PMSF, considered diagnostic inhibitors for serine proteases (Powers and Harper, 1986a), were ineffective against these activities. Even after a 4 h preincubation with 10⁻² M DFP, there was no inhibition of azocoll digesting activity. However, azocoll, casein and BAEE hydrolyses were inhibited by the trypsin inhibitors TLCK and soybean trypsin inhibitor. With [³H]casein as substrate, TLCK and STI exhibited IC₅₀s of 300 and 1.2 μM, respectively. BAEE hydrolysis was inhibited 90% by 6 mM TLCK and 100% by 10 μM STI, whereas azocoll hydrolysis by the same enzyme fraction from gel filtration was completely inhibited by 10 mM TLCK and 10 μM STI. The chymotrypsin inhibitor, TPCK, was ineffective. Neither the sulfhydryl protease inhibitor, PCMS, nor the metal chelators were inhibitory (Rich, 1986). The different IC₅₀ values characteristic of each substrate used suggest that different enzymes may be hydrolyzing azocoll, casein and BAEE.

Aminopeptidase activity in the early eluting Sephacryl S-300 fractions, revealed by hydrolysis of leucine-*p*-nitroanalide, is best classified as that of a metalloenzyme (Powers and Harper, 1986b). The activity was most effectively inhibited by a metal chelator, 1,10-phenanthroline, with an IC₅₀ of approx. 3 × 10⁻⁴ M. Another metal chelator, 2,2'-dipyridyl, also inhibited aminopeptidase activity, but at higher concentrations than required for 1,10-phenanthroline. EDTA was ineffective at high concentration. The sensitivity of the aminopeptidase activity to moderately high concentrations of DFP was unexpected.

Carboxypeptidase activity, which eluted during chromatography on Sephacryl S-300, was not inhibited by any of the compounds tested. However, the aromatic metal chelators could not be tested since they gave strong absorbance at the wavelength used to monitor hydrolysis of hippuryl arginine and hippuryl phenyllactic acid.

The lack of inhibition by PCMS strongly suggested that sulfhydryl proteases are not making significant

contributions to the proteinase activities observed in molting fluid (Table 4). But to be certain that no latent sulfhydryl enzymes were present, we tested for stimulation of proteolysis by the reducing agents cysteine and dithiothreitol (DTT). Preincubation of molting fluid with either of these sulfhydryl reagents only decreased activity. Addition of PCMS had little or no effect on activity in the presence of either DTT or cysteine. These effects are the opposite of those expected for sulfhydryl proteases.

The search for molting fluid proteolytic enzyme inhibitors was extended to include to phosphoramidothioates (methamidophos and *O*-ethyl-*S*-phenyl phosphoramidothioate) and two fluorothioethers (3-octylthio-1,1,1-trifluoro-2-propanone and 1,1,1,17,17,17-hexafluoro-4,14-dithio-heptadecane). These compounds have proven effective against juvenile hormone esterases which are classified as serine esterases but are refractory to DFP (Sparks and Hammock, 1980; Hammock *et al.*, 1984). None of these compounds were effective at 10⁻² M against the casein digesting activity of *Manduca* molting fluid.

DISCUSSION

The present study has revealed a rather complex mixture of proteolytic enzymes present in *Manduca* molting fluid. The presence of both endo- and exocleaving proteolytic and chitinolytic enzymes in molting fluid supports the hypothesis that degradation of cuticular protein and chitin occurs either simultaneously or sequentially, such that the insoluble cuticular polymers are cleaved into soluble fragments

Table 4. Effect of PCMS and reducing agents on azocoll digestion by *M. sexta* molting fluid*

Treatment†	Relative activity (%)
Control	100 ± 12
PCMS (10 mM)	103 ± 11
DTT (10 mM)	47 ± 2
DTT + PCMS	53 ± 2
Cysteine (5 mM)	85 ± 4
Cysteine + PCMS	68 ± 3

*Samples of molting fluid were preincubated in 0.1 M PIPES, pH 6.6, for 15 min at 37°C in the presence of reducing agent or reducing agent plus 10 mM PCMS. Control samples were preincubated with water added to achieve an equivalent volume to the treated preincubations.

†See p. 467 for list of abbreviations.

which are further digested by both endo- and exohydrolases (Fukamizo *et al.*, 1986). Protein degradation may result in the unmasking of chitin fibrils that are embedded in the protein matrix which facilitates attack by the endo- and exo-cleaving chitinolytic enzymes. A sequence where proteolysis necessarily precedes chitinolysis of insect cuticle has been demonstrated with hydrolytic enzymes from entomopathogenic fungi (St Leger *et al.*, 1986).

Proteases are generally classified into one of four categories based on characteristics of the active site (Perlmann and Lorand, 1970; Barrett, 1986). These categories are serine proteases, sulfhydryl proteases, metalloproteases and carboxylic acid proteases. A proteolytic enzyme can usually be placed into one of these categories by determination of substrate specificity, pH optimum and sensitivity to diagnostic inhibitors that selectively interact with the unique active site moieties. None of the major *Manduca* molting fluid proteases exhibited properties fully characteristic of the four protease categories.

Endoproteases found in *Manduca* molting fluid differed significantly from the two proteases previously described in molting fluid of the silkworm *Antheraea polyphemus* (Katzenellenbogen and Kafatos, 1971a). Most strikingly *Manduca* endoproteases were not inhibited by high concentrations of DFP, while those of *Antheraea* were highly sensitive. In addition, the molecular weight of the *Manduca* molting fluid proteases (100 kDa) was about 3 times greater than that of the *Antheraea* enzymes (30–34 kDa). Both *Manduca* and *Antheraea* molting fluid proteases hydrolyzed the trypsin-selective substrate BAEE. However, unlike those of *Antheraea*, *Manduca* molting fluid endoproteases did not hydrolyze a second trypsin-selective substrate TAME at a detectable rate. Thus, although endoproteases with trypsin-like specificity do occur in molting fluid of both species, those of *Manduca* may not be serine proteases and their substrate specificities are less like that of trypsin than are the silkworm enzymes.

Previously, *Manduca* molting fluid was reported to contain both a DFP sensitive protease and a metal chelator (EDTA) sensitive protease (Bade and Shoukimas, 1974). In the present study, no endoprotease with either of those inhibitor sensitivities was detected. Only the exo-cleaving aminopeptidase activity was sensitive to metal chelators. At this time the major endoproteases of *Manduca* molting fluid are not well characterized. Their insensitivity to DFP and PMSF would seem to rule out their being serine proteases. Absence of significant inhibition by the phosphoramidothioates and trifluoropropyl thioethers offers further evidence that *Manduca* molting fluid proteases are not of the serine enzyme class. The lack of stimulation by sulfhydryl reducing agents and failure of PCMS to inhibit at high concentrations, is contradictory to the expected response of sulfhydryl proteases. Metal chelators had no detectable inhibitory effect on the digestion of endoprotease selective substrates, azocoll, casein and BAEE. The neutral to alkaline optimum pH would seem to eliminate carboxyl proteases from consideration. If carboxyl proteinases are present in molting fluid, the alkaline condition of the fluid would not be conducive for a significant level of this activity. Although

it is not straightforward to interpret the results of inhibitor studies when using a mixture of enzyme, attainment of nearly quantitative inhibition by specific inhibitors is reasonably good evidence for enzyme classification studies. Both molting fluid and partially purified enzyme fractions were >90% inhibited by TLCK and STI when using casein, azocoll and BAEE as substrates. None of the other inhibitors caused more than 50% inhibition. Thus, the enzymatic activity present in whole molting fluid or the gel filtration fractions that hydrolyzed casein, azocoll and BAEE may be primarily due to one or more enzymes that exhibit comparable inhibitor sensitivities, i.e. they belong to the same class of proteinases. All of these data suggest that the major endoprotease activity detected by hydrolysis of azocoll, [³H]casein and BAEE represents a rather unique class of proteases.

Endocleaving proteinases participate in the degradation of calcified cuticle of crustaceans, but these appear to quite different from insect molting fluid enzymes that degrade sclerotized cuticles of insects. Cysteine and carboxyl proteinases are active in the environment between the old and the newly deposited cuticles of the Bermuda land crab, *Gecarcinus lateralis* (O'Brien and Skinner, 1987, 1988). Proteolytic activity in *Manduca* molting fluid was neither inhibited by the cysteine protease inhibitor PCMS, nor stimulated by the presence of sulfhydryl reducing agents. Also, no carboxylic acid proteases were apparent in *Manduca* molting fluid. Therefore, we conclude that, unlike the land crab, sulfhydryl and carboxyl proteases do not play important roles in the extracellular digestion of *Manduca* cuticular proteins.

Exocleaving proteases in the form of aminopeptidases have been detected in *Drosophila* (Hall, 1986). One of the aminopeptidases is associated with *Drosophila* integument and is inducible by ecdysone (Hall, 1988a,b). Those results suggest that the *Drosophila* aminopeptidase is a molting fluid enzyme and may be similar in properties to the *M. sexta* molting fluid aminopeptidase-like enzyme. To our knowledge no carboxypeptidase-like enzyme has been associated with the molting fluid of other insect species.

Results of experiments conducted with a hexapeptide and pseudopeptide substrates revealed that the major endoprotease fraction cleaves bond susceptible to both trypsin-like and chymotrypsin-like enzymes, suggesting that either a rather nonspecific enzyme, more than one enzyme or a single enzyme with two active sites of different specificities is present in the fraction. It is possible that *M. sexta* molting fluid contains a multicatalytic proteinase like those detected in vertebrate tissues which exhibit both trypsin-like and chymotrypsin-like activities (Bond and Butler, 1987; Folco *et al.*, 1988). Further purification of the endo-cleaving proteases is necessary to resolve questions concerning multiplicity, mode of action and substrate specificity. Efforts to purify the major endoproteases of *Manduca* molting fluid have met with mixed success. The azocoll digesting activity was purified 250-fold by sequential chromatography on gel filtration, anion-exchange and hydroxylapatite columns. However, the enzyme activity became increasingly unstable during storage as purification

progressed and yields were very low. Future studies of these molting fluid proteases will be focused on their stabilization, enrichment, complete purification and more detailed characterization. Some of these enzymes appear to possess unique properties and may be good targets for insecticide development.

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REFERENCES

- Aschsetter T., Ehman C. and Wolf D. H. (1981) New proteolytic enzymes in yeast. *Arch. Biochem. Biophys.* **207**, 445–454.
- Bade M. L. and Shoukimas J. J. (1974) Neutral metal chelator-sensitive protease in insect molting fluid. *J. Insect Physiol.* **20**, 281–290.
- Barrett A. J. (1986) An introduction to the proteinases. In *Proteinase Inhibitors* (Edited by Barrett A. J. and Salvesen G.), pp. 3–22. Elsevier Science, New York.
- Bell R. A. and Joachim F. G. (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. ent. Soc. Am.* **69**, 365–373.
- Bond J. S. and Butler P. E. (1987) Intracellular proteases. *A. Rev. Biochem.* **56**, 333–364.
- Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248–254.
- Chavira R., Burnett J. T. and Hageman J. H. (1984) Assaying proteinases with azocoll. *Analyt. Biochem.* **136**, 446–450.
- Coch Frugoni J. A. (1957) Tampone universale di Britton e Robinson a forza ionica costante. *Gazz. Chem. Ital.* **87**, 403–407.
- Delange R. J. and Smith E. L. (1971) Leucine aminopeptidase and other N-terminal exopeptidases. In *The Enzymes* (Edited by Boyer P.), 3rd edn, Vol. 3, pp. 81–103. Academic Press, New York.
- Erlanger B. F., Kokowsky N. and Cohen W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **95**, 271–278.
- Folco E. J., Busconi L., Martone C. B. and Sanchez J. J. (1988) Multicatalytic proteinase in fish muscle. *Arch. Biochem. Biophys.* **267**, 599–605.
- Folk J. E. and Schirma E. W. (1963) The porcine pancreatic carboxypeptidase A system. I. Three forms of the enzyme. *J. Biol. Chem.* **238**, 3884–3894.
- Fukamizo T. and Kramer K. J. (1985) Mechanism of chitin hydrolysis by the binary chitinase system in insect molting fluid. *Insect Biochem.* **15**, 141–145.
- Fukamizo T., Kramer K. J., Mueller D. D., Schaefer J., Garbow J. and Jacob G. (1986) Analysis of chitin structure by nuclear magnetic resonance spectroscopy and chitinolytic enzyme digestion. *Arch. Biochem. Biophys.* **249**, 15–26.
- Hall N. A. (1986) Peptidase in *Drosophila melanogaster*—I. Characterisation of dipeptidase and leucine aminopeptidase activities. *Biochem. Genet.* **24**, 775–793.
- Hall N. A. (1988a) Peptidases in *Drosophila melanogaster*—II. The variation of peptidase activities during development. *Insect Biochem.* **18**, 145–155.
- Hall N. A. (1988b) Peptidases in *Drosophila melanogaster*—III. The regulation of leucine aminopeptidase P and leucine aminopeptidase G. *Insect Biochem.* **18**, 157–161.
- Hammock B. D., Abdel-Aal Y. A. I., Mullin C. A., Hanzlik T. N. and Roe R. M. (1984) Substituted thio-trifluoropropanones as potent selective inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol.* **22**, 209–223.
- Heussen C. and Dowdle E. B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analyt. Biochem.* **102**, 196–202.
- Katzenellenbogen B. S. and Kafatos F. C. (1970) Some properties of silkworm molting gel and molting fluid. *J. Insect Physiol.* **16**, 2241–2256.
- Katzenellenbogen B. S. and Kafatos F. C. (1971a) Proteinases of silkworm molting fluid: physical and catalytic properties. *J. Insect Physiol.* **17**, 775–800.
- Katzenellenbogen B. S. and Kafatos F. C. (1971b) Inactive proteinases in silkworm molting gel. *J. Insect Physiol.* **17**, 823–832.
- Koga D., Jilka J. and Kramer K. J. (1983) Insect endochitinases: glycoproteins from molting fluid, integument and pupal hemolymph. *Insect Biochem.* **13**, 295–305.
- Koga D., Mai M. S., Dziadik-Turner C. and Kramer K. J. (1982) Kinetics and mechanism of exochitinase and β -N-acetylhexosaminidase from the tobacco hornworm, *Manduca sexta* L. (Lepidoptera: Sphingidae). *Insect Biochem.* **12**, 493–499.
- Kramer K. and Koga D. (1986) Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem.* **16**, 851–877.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680–685.
- Murdock L. L., Brookhart G., Dunn P. E., Foard D. E., Kelley S., Kitch L., Shade R. E., Shukle R. H. and Wolfson J. L. (1987) Cysteine digestive proteinases in coleoptera. *Comp. Biochem. Physiol.* **87B**, 783–787.
- O'Brien J. J. and Skinner D. M. (1987) Characterization of enzymes that degrade crab exoskeleton. I. Two alkaline cysteine proteinase activities. *J. exp. Zool.* **243**, 389–400.
- O'Brien J. J. and Skinner D. M. (1988) Characterization of enzymes that degrade crab exoskeleton. II. Two acid proteinase activities. *J. exp. Zool.* **246**, 124–131.
- Passonneau J. V. and Williams C. M. (1953) The molting fluid of the *Cecropia* silkworm. *J. exp. Biol.* **30**, 540–560.
- Perlmann G. E. and Lorand L. (1970) Section of individual proteolytic enzymes. In *Methods in Enzymology*, Vol. 19, pp. 31–762. Academic Press, New York.
- Pino-Heiss S. and Schubiger G. (1989) Extracellular protease production by *Drosophila* imaginal discs. *Dev. Biol.* **132**, 282–291.
- Powers J. C. and Harper J. W. (1986a) Inhibitors of serine proteinases. In *Proteinase Inhibitors* (Edited by Barrett A. J. and Salvesen G.), pp. 55–152. Elsevier Science, New York.
- Powers J. C. and Harper J. W. (1986b) Inhibitors of metalloproteinases. In *Proteinase Inhibitors* (Edited by Barrett A. J. and Salvesen G.), pp. 219–300. Elsevier Science, New York.
- Rich D. H. (1986) Inhibitors of cysteine proteinases. In *Proteinase Inhibitors* (Edited by Barrett A. J. and Salvesen G.), pp. 153–178. Elsevier Science, New York.
- Sparks T. C. and Hammock B. D. (1980) Comparative inhibition of the juvenile hormone esterases from *Trichopoesia ni*, *Tenebrio molitor* and *Musca domestica*. *Pestic. Biochem. Physiol.* **14**, 290–302.
- St Leger R. J., Cooper R. M. and Charnley A. K. (1986) Cuticle-degrading enzymes of entomopathogenic fungi: cuticle degradation *in vitro* by enzymes from entomopathogens. *J. invertebr. Path.* **47**, 167–177.

- Wachsmuth E. D., Fritze I. and Pfeleiderer G. (1966) An aminopeptidase occurring in pig kidney. I. An improved method of preparation. Physical and enzymatic properties. *Biochemistry* **5**, 169-175.
- Walsh K. and Wilcox P. (1970) Serine proteases. In *Methods in Enzymology* (Edited by Perlmann G. E. and Lorand L.), Vol. 19, pp. 31-41. Academic Press, New York.
- Wenzel H. R., Engelbrecht S., Reich H., Mondry W. and Tschesche H. (1980) Synthesis and analytical use of 3-carboxypropionyl alanyl-alanyl-valine-4-nitroanalide: a specific substrate for human leukocyte elastase. *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1413-1416.