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Resolution of Proteases in the Keratinolytic Larvae of the Webbing Clothes Moth

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Abstract

The proteases of the larvae of the webbing clothes moth, *Tineola bisselliella*, were investigated because of this organism's phylogenetic rank as a member of the lower invertebrates, its unique position as one of the relatively few organisms that can digest keratin and its importance as a serious fabric pest. Both the number and nature of different proteolytic enzymes present were investigated and the various activities partially fractionated by ammonium sulphate precipitation and chromatography on DEAE-cellulose and Sephadex G200 columns. A complex mixture of peptidases and proteinases has been found in extracts of whole larvae and has been shown to be associated with the larval digestive tract.

The proteinases include metal-chelator-sensitive proteinases (metalloproteinases) and serine proteinases but no SH-proteinases or acid proteinases. The serine proteinases include both trypsin-like and chymotrypsin-like activities. Four major and three minor anionic trypsin-like enzymes and a single major cationic trypsin-like enzyme have been detected. Only a single anionic chymotrypsin-like enzyme appears to be present. The trypsin-like enzymes are unaffected by the naturally occurring proteinase inhibitors, chicken ovomucoid, soybean trypsin inhibitor and lima bean trypsin inhibitor, while the chymotrypsin-like enzyme is inhibited by soybean trypsin inhibitor only. The enzymes resemble the serine proteinases from microorganisms in their pH stability.

The peptidases include both aminopeptidase and carboxypeptidase activities and both are present in multiple forms. Sixteen aminopeptidase bands have been detected and all are present in individual larvae. They are not inhibited completely by reagents specific for any of the common active sites, and have different specificity requirements. Two carboxypeptidases have been detected on acrylamide gels and have been completely separated on DEAE-cellulose.

No evidence could be found for the existence of any of these proteases as inactive precursors.

Introduction

Hard keratins as a class are characterized by the presence of 8–16% of the sulphur-containing amino acid cystine. They are naturally insoluble and resistant to biological attack, presumably because of the occurrence of much of this cystine in intermolecular disulphide linkages (Fraser *et al.* 1972). Insects are the only animals in which digestion of keratin has been demonstrated, the insects involved being the larvae of some thirty species of moths (Lepidoptera), the larvae of some fifteen species of beetles (Coleoptera) and several hundred species of bird lice (Mallophaga). However, only a few of these insect species are considered serious fabric pests and the most important are the larvae of the webbing clothes moth, *Tineola bisselliella* (Waterhouse 1958). The process of wool digestion in these larvae has been reviewed by Waterhouse (1958) and McPhee (1965).

Preliminary investigations of the digestive enzymes of *T. bisselliella* larvae demonstrated a very active alkaline proteinase* in extracts of midgut epithelia. The reported pH optimum for this proteinase was pH 9·3 (Duspiva 1936*a*; Linderstrøm-Lang and Duspiva 1936) or pH 9·8 (Powning *et al.* 1951; Powning and Irzykiewicz 1962*a*), the latter corresponding to the pH in the central midgut region of these larvae (Duspiva 1936*b*; Linderstrøm-Lang and Duspiva 1936; Waterhouse 1952*a*), where the bulk of wool digestion occurs (Day 1951; Waterhouse 1952*b*).

Only a partial attempt at enzyme classification has been made. The alkaline proteinase activity in homogenates of dissected larval midguts was unaffected by thioglycollate, cyanide, *p*-chloromercuribenzoate or iodoacetate (Duspiva 1936*a*; Linderstrøm-Lang and Duspiva 1936; Powning *et al.* 1951) and thus does not belong to the class of thiol-activated proteinases (SH-proteinases) (Hartley 1960). The proteinase was also unaffected by the naturally occurring proteinase inhibitors ovomucoid and *Ascaris* inhibitor and only partially inhibited by soybean inhibitor (Powning *et al.* 1951). Later work with an enzyme sample purified, from 600 g of whole larvae, by ammonium sulphate and potassium phosphate precipitation procedures (Powning and Irzykiewicz 1962*b*) confirmed the lack of effect of cyanide and *p*-chloromercuribenzoate (Powning and Irzykiewicz 1962*a*).

There is very little information regarding the heterogeneity of the proteases present in the alimentary canal of the moth larva or in the partially purified preparations used in these studies. Powning and Irzykiewicz (1962*a*) presented evidence to suggest that their proteinase preparation may be contaminated with other proteolytic activities and Duspiva (1936*a*) demonstrated both dipeptidase activity (against alanylglycine) and aminopeptidase activity (against alanylglycylglycine) in homogenates of midgut epithelia. Powning and Irzykiewicz (1962*a*) also commented on a possible similarity between their *T. bisselliella* proteinase preparation and the protease preparation (Pronase) from the microorganism *Streptomyces griseus* (Nomoto *et al.* 1960) which has subsequently been shown to consist of a mixture of peptidases and proteinases (see Narahashi and Yoda 1973 for review).

Relatively few studies have been carried out on the proteases of the lower invertebrates. This paper reports the results of a re-investigation of the proteolytic enzymes of *T. bisselliella* larvae. A complex mixture of peptidases and proteinases have been found which are associated with the digestive tract of the larvae. The detection and partial purification of these enzymes is reported. A preliminary report of these findings has been published (Ward 1972*a*).

Materials and Methods

Chemicals

Chemicals used and their sources were: AT,† ATEE, BAPA, *N*-β-naphthyloxycarbonyl-L-phenylalanine, glycyl-NA hydrochloride, DL-alanyl-NA hydrochloride, L-leucyl-NA hydrochloride,

* The nomenclature of proteases (peptide hydrolases) used here is in accord with the Recommendations (1972) of the Commission on Biochemical Nomenclature on the nomenclature and classification of enzymes. The two recognized subgroups are peptidases (formerly termed exopeptidases) and proteinases (formerly endopeptidases).

† Abbreviations used are: AT, *N*-acetyl-DL-tyrosine; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BANA, *N*-benzoyl-DL-arginine β-naphthylamide; BAPA, *N*-benzoyl-DL-arginine *p*-nitroanilide; DFP, diisopropylfluorophosphate; Diol buffer, 2-amino-2-methyl-1,3-propandiol buffer; NA, β-naphthylamide derivative; Z, *N*-benzyloxycarbonyl derivative.

L-valyl-NA, L-phenylalanyl-NA, L-tyrosyl-NA, L-seryl-NA, L-histidyl-NA, L-prolyl-NA hydrobromide, L-α-glutamyl-NA, L-arginyl-NA hydrochloride, L-lysyl-NA carbonate, Z-glycyl-L-leucine, trypsin, lima bean trypsin inhibitor and chicken ovomucoid, Schwarz–Mann Research Laboratories; BANA, Calbiochem; DFP, Aldrich Chemical Co.; bovine serum albumin, L-amino acid oxidase (*Crotalus adamanteus* venom) and horseradish peroxidase, Sigma Chemical Co.; α-chymotrypsin and soybean trypsin inhibitor, Nutritional Biochemical Co.; *o*-dianisidine and Thiomersal, BDH Chemical Co.; fast garnet GBC and fast blue RR, G.T. Gurr Ltd, England; Hammarsten casein, Hopkins and Williams Ltd, England, or E. Merck, Germany; Haemoglobin Substrate Powder, Worthington Biochemical Co.; Whatman ion-exchange celluloses, W. R. Balston, England; Sephadex gel-filtration resins, Pharmacia, Sweden. All other chemicals used were the purest available commercially.

Enzyme Assays

Total protease activity was determined by Kunitz's casein digestion method (Laskowski 1955) using a 0·5% (w/v) casein solution in 0·05M Diol buffer, pH 9·8 (0·4 ml final volume), and 30 min digestion at 37°C. Undigested casein was precipitated with 0·6 ml of 5% trichloracetic acid and removed by centrifugation after 30 min. The solubilized digestion products were estimated either spectrophotometrically at 278 nm or colorimetrically at 660 nm with the Folin–phenol reagent (Herriott 1955). In the colorimetric determinations 0·5N NaOH was replaced by 2% Na_2CO_3–0·1N NaOH (Lowry *et al.* 1951). With the former reagent colours fade very rapidly, particularly during the first 10 min, whereas with the latter the colours reach maximum intensity after 30 min and are stable for several hours. Protease units were estimated from a standard curve of enzyme concentration against digestion products, as measured by the spectrophotometric (PU^{278}) or colorimetric (PU^{F}) procedure. The initial slope in both standard curves is equal to the production of 1 μ-equiv. of tyrosine per 30 min per protease unit under the above reaction conditions. With casein as substrate the two protease units PU^{278} and PU^{F} are not equivalent because of the unequal relative contributions of tyrosine and tryptophan to ultraviolet absorption and colour formation (Northrop *et al.* 1948).

Metalloproteinase activity was determined as for total protease activity after a 30-min preincubation at room temperature with 0·002M DFP in the absence of casein.

Trypsin-like activity was assayed with 0·001M BAPA (Erlanger *et al.* 1961) in 0·02M Diol buffer, pH 9·8. Reaction mixtures (1·0 ml) were incubated for 30 min at 37°C, the reaction was stopped by the addition of 0·2 ml of 20% (v/v) acetic acid, and the quantity of released *p*-nitroaniline was estimated spectrophotometrically at 410 nm ($\varepsilon = 8{\cdot}8 \times 10^3$ litre mol^{-1} cm^{-1}). In the studies on zymogen activation, intracellular distribution and ammonium sulphate fractionation, the presence of turbid extracts interfered with the accurate spectrophotometric estimation of *p*-nitroaniline at 410 nm. In these cases the acidified reaction mixture was extracted with an equal volume of ethyl acetate and the released *p*-nitroaniline in the ethyl acetate layer was quantitated spectrophotometrically ($\varepsilon =$ 17300 litre mol^{-1} cm^{-1}) at 363 nm (Ward 1973).

Chymotrypsin-like activity was assayed by three methods, all of which involved the hydrolysis of ATEE:

1. Method 1. Differential spectrophotometric assay, based on that described by Schwert and Takenaka (1955). At alkaline pH values the difference spectra result from the differences in the pK_a of the phenolic hydroxyl groups of AT and ATEE (Ward 1972*b*). The reaction mixture (1·0 ml) contained 20 μmol of Diol buffer (pH 9·5) and 0·5 μmol of ATEE (test cuvette) or 0·5 μmol of AT (reference cuvette). Hydrolysis of ATEE is followed by a fall in absorption at 250 nm. The molar absorption difference coefficient at pH 9·5 and 250 nm is $1{\cdot}75 \times 10^3$ litre mol^{-1} cm^{-1} (Ward 1972*b*).
2. Method 2. Potentiometric assay (Wilcox 1970) in a pH-stat (Radiometer TTA31-TTT111-ABU12-SBR2c). The reaction mixture (2·0 ml) contained 20 μmol Diol buffer (pH 8·0), 200 μmol KCl and 1·0 μmol ATEE and was maintained at 37°C in a jacketed vessel. The titrant was 0·020M NaOH and the proportional band setting was 0·1.
3. Method 3. High-voltage electrophoresis spot test used to monitor column chromatographic separations. The reaction mixtures (0·5 ml), containing 20 μmol Diol buffer (pH 9·5) and 0·5 μmol ATEE, were incubated for 30 min at 37°C. The reaction was stopped with 0·1 ml of 20% acetic acid and 0·02 ml of this acidified reaction mixture was spotted on Whatman 3MM paper and subjected to high-voltage electrophoresis at pH 3·5 for 30 min. Papers

were sprayed with 3 ml of freshly prepared Pauly reagent (Easley 1965) in 17 ml of 12% aqueous Na_2CO_3 (Bennet 1967).

Carboxypeptidase activity was assayed with Z-glycylleucine as substrate (Narahashi and Yanagita 1967), the leucine liberated being determined by the ninhydrin procedure of Rosen (1957). The reaction mixtures (0·5 ml), containing 100 μmol tris.HCl (pH 8·0) and 0·5 μmol Z-glycylleucine, were incubated for 30 or 60 min at 37°C, then the reaction was stopped with 0·1 ml of 20% acetic acid. Samples (0·2 ml) were used for leucine determinations, corrections being made for control incubations with no Z-glycylleucine present. In some cases the hydrolysis of Z-glycylleucine was monitored by high-voltage electrophoresis at pH 1·9 for 30 min, the spots being visualized with the ninhydrin–collidine reagent (Bennet 1967).

Aminopeptidase activity was assayed colorimetrically as described by Marks *et al.* (1968) with 0·001M leucyl-NA in 0·02M Diol buffer, pH 9·0. Incubation was for 10 min at 37°C and the released β-naphthylamine was determined with fast garnet GBC. In a final volume of 5·0 ml, 0·18 μmol of β-naphthylamine gave an absorption of 1·0 at 520 nm with this procedure (T. C. Elleman, personal communication). The determination of β-naphthylamine in the presence of thiol reagents was carried out as described by Barrett (1972).

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Source of Larvae

Background information on the biology and growth requirements of *T. bisselliella* are given in the review by Waterhouse (1958). The laboratory culture methods were based on the recommendations given by Whitfield and Cole (1958). Larvae were grown at 28°C in ventilated screw-top jars (1 litre) containing a diet of casein flour dust (45%), lactic casein (45%), dried yeast (9%) and cholesterol (1%). Adult moths oviposited on light flannel strips impregnated with yeast and cholesterol. These strips were placed on top of the casein mixture in the culture jars and the larvae collected approximately 32 days after oviposition. The larval cases were collected by sieving through a coarse-mesh ASTM-12 sieve. The cases were then exposed to a 60-W lamp for 60 min, the lamp being positioned approx. 6 in. (15 cm) above the larval cases. The larvae crawled through the coarse sieve away from the light and were collected over a fine-mesh ASTM-40 sieve. They were quick-frozen with liquid nitrogen and stored at −20°C until used.

Larval Dissections

Midguts of freshly collected live larvae were removed, washed in normal saline, quick-frozen with liquid nitrogen and stored at −20°C overnight. The residual larval body minus the gut portion was also retained and frozen.

Preparation of Extracts

Frozen larvae or dissected organs were homogenized in 10 vol. of 0·25M sucrose–0·005M tris.HCl (pH 7·5) containing 0·01 mM phenylthiourea by grinding in a Virtis 45 homogenizer for three 1·0-min periods (small-scale experiments) or in a Waring blender for five 1·0-min periods (large-scale experiments) at 2–4°C. The homogenate was centrifuged at 24000 *g* for 30 min at 4°C and the supernatant used for enzyme assay and purification. All buffer solutions used during purification contained 0·01 mM phenylthiourea to prevent severe blackening of protein solutions, presumably caused by a very active monophenol monooxygenase (tyrosinase) present in the insect extracts (R. F. Powning, personal communication; Katzanellenbogen and Kafatos 1971). All fractionation procedures were carried out in the cold (2–4°C).

Ammonium Sulphate Precipitation

Ammonium sulphate fractionation was carried out at 4°C and pH 6·0. Each step involved the slow addition of solid salt with continuous mechanical stirring (approximately 15 min), standing for an additional 30 min and then centrifugation of the precipitated protein at 14000 *g* for 20 min. The nomogram of Dixon (1953) was used to calculate the quantities of salt required to reach the various final concentrations.

Chromatography

Ion-exchange chromatography was carried out on DEAE-cellulose (Whatman microgranular DE52, Reeve Angel and Co.). Prior to chromatography the adsorbent was degassed, the fines

removed and the adsorbent equilibrated according to the manufacturer's instructions. Sephadex G200 (Pharmacia) was swollen and handled as described by Fischer (1969). Flow rates were controlled by a peristaltic pump. When not in use, bacterial growth on the Sephadex columns was inhibited by the presence of 0·01% Thiomersal in the column buffers. This preservative was not present when the columns were running.

Acrylamide Gel Electrophoresis

Disc gel electrophoresis was carried out at 5°C as described by Davis (1964) but with a bis-acrylamide–acrylamide ratio of 5% for minimum pore size (Gordon 1969) and no sample gel. All gels run contained 7·5% acrylamide in the running gel except where stated. Gel rods were run in glass tubes (5 mm i.d. by 80 mm) in conventional apparatus. Gel slabs (70 by 70 by 3 mm) were run in glass cells (Margolis and Kenrick 1968) in the multicell Gradipore electrophoresis apparatus, modified to keep top and bottom buffers separate. For cationic proteins the imidazole–taurine method of Racusen (1967) was modified to include only the cationic gel system in 7·5% acrylamide, no stacking gel and methyl green (0·5 μg/ml) as tracker dye in the imidazole buffer. Protein bands were fixed in 12·5% trichloracetic acid for 1 h, then stained with 0·05% amido black in 7% acetic acid (Gordon 1969). Gels were destained by diffusion in 7% acetic acid.

Trypsin-like enzymes were detected by incubating the gels at pH 9·8 with 0·5 mM BANA (Blackwood and Mandl 1961) for 30 min at room temperature and staining by immersion for 15 min in 0·33M acetate buffer (pH 4·5) containing 0·15% fast garnet GBC and 5% non-ionic detergent (Marks *et al.* 1968). Gels were stored in 0·33M acetate buffer until photographed. The bands are fairly stable, but fade over a period of months.

Aminopeptidase bands were similarly detected by incubation of gels at pH 9·0 with 5 mM aminoacyl-NA for 5–180 min depending on the substrate (see Fig. 4). Bands were stained with fast garnet GBC and stored as described for the trypsin-like enzymes.

Attempts to detect chymotrypsin-like enzymes on gels with the chromogenic substrate glutaryl-phenylalanyl-NA (Blackwood *et al.* 1965) involved incubating gels at pH 9·5 with 0·5 mM substrate for 30–120 min and staining with fast garnet GBC as described for trypsin-like enzymes.

Carboxypeptidase bands were detected in gels by the coupled L-amino acid oxidase–peroxidase method of Lewis and Harris (1967). Each gel was immersed in a reaction mixture prepared immediately before use by mixing 2 ml of a 1% aqueous Agarose solution at 55°C with 2 ml of a solution containing 150 μmol tris.HCl buffer (pH 7·5), 5 μmol $MnSO_4$, 5 μmol Z-glycylleucine, 0·4 mg L-amino acid oxidase (0·3 units/mg), 0·8 mg peroxidase (160 purpurogallin units/mg) and 0·4 mg *o*-dianisidine. They were incubated at room temperature until the dark brown band appeared (10–60 min) then photographed immediately. Control gels in which Z-glycylleucine was omitted from the reaction mixture were run to correct for any non-specific band formation that might occur with some crude extracts. The method for detection of carboxypeptidase bands with the chromogenic substrate naphthyloxycarbonylphenylalanine (Ravin and Seligman 1951; Pacaud and Uriel 1971) involved incubating the gel at pH 8·0 with 0·001M substrate for 30 min and then staining in a 0·075% aqueous solution of fast blue RR.

High-voltage Electrophoresis

High-voltage electrophoresis was performed on Whatman 3MM paper in apparatus based on that described by Michl (see Bennet 1967) with Varsol as the cooling solvent. The buffers used (Naughton *et al.* 1960) were: pyridine–acetic acid–water (25 : 1 : 225 v/v, pH 6·5, and 1 : 10 : 189 v/v, pH 3·5) and formic acid–acetic acid–water (1 : 5 : 36 v/v, pH 1·9). The usual voltage gradient was 50 V cm^{-1}.

Ultrafiltration

Ultrafiltration was carried out at 2–4°C over UM-10 membranes in Diaflo cells (Amicon Corporation, Mass., U.S.A.) at 50 lb in^{-2} (approx. $3·5 \times 10^5$ Pa).

Results

Identification of Proteases in Crude Extracts of Whole Larvae

Preliminary experiments were carried out to determine the number and nature of different proteolytic enzymes in *T. bisselliella* extracts. An initial screen of crude

Table 1. Effect of activators and inhibitors on proteolytic activities

The enzyme preparation used was the dialysed material obtained by ammonium sulphate precipitation between 1·435 and 2·255M salt. The preparation was pre-incubated for 30 min at 30°C with effector, the substrate was added, and the residual activity determined at 37°C as described in the Methods. ATEE hydrolysis was monitored by the pH-stat assay at 30°C (method 2)

Additions	Final concn (M)	Proteinase substrates[A]							Peptidase substrates[A]			
		Casein			BAPA		ATEE		Leucyl-NA		Z-glycylleucine	
		Activity (PU^{278})	Activity (PU^{F})	Inhibition (%)	Activity (μmol/30 min)	Inhibition (%)	Activity (μmol/min)	Inhibition (%)	Activity (μmol/10 min)	Inhibition (%)	Activity (μmol/30 min)	Inhibition (%)
None		10·90	3·78	0	0·229		0·135		5·66		3·88	
L-Cysteine	0·002	8·39	3·13	19·8	0·254	0	0·143	0	4·84	14·5		
Mercaptoethanol	0·002	9·16		16·0	0·252	0	0·143	0	5·81	0	3·37	13·2
p-Chloromercuri-benzoate	0·0002	10·57	4·04	0	0·204	11	0·077	43	5·46	3·5	0·42	89·2
Iodoacetate	0·002	10·49	3·85	1·2	0·211	8	0·119	12	6·26	0	0·59	84·9
KCN	0·020	5·74		47·3	0·250	0	0·148	0				
EDTA	0·002	5·01	1·70	54·5	0·229	0	0·148	13	6·25	0	4·75	0
1,10-Phenan-throline	0·002		2·85	24·5	0·231	0	0·032	76	3·48	39·0	0·44	88·8
DFP	0·002	5·73	2·02	46·0	0·017	92·4	0	100	4·61	18·6	3·49	10·0

[A] Enzyme activities are expressed per milligram of enzyme protein.

homogenates (centrifuged at 1000 *g* for 20 min) against acid-denatured haemoglobin (Herriott 1955) at pH 3·5 or heat-denatured casein at pH 5·5, 7·5 and 9·5 (Laskowski 1955) showed that the alkaline proteinase at pH 9·5, previously reported by Duspiva (1936*a*) and Powning *et al.* (1951), was the dominant protease in this organism (Fig. 1). There was negligible acid proteinase present and no acid, neutral or alkaline thiol-activated proteinases (SH-proteinases), since similar curves were also obtained when crude extracts were pre-incubated for 30 min, and then assayed, in the presence of the thiol reagents L-cysteine, cyanide, *p*-chloromercuribenzoate and iodoacetate.

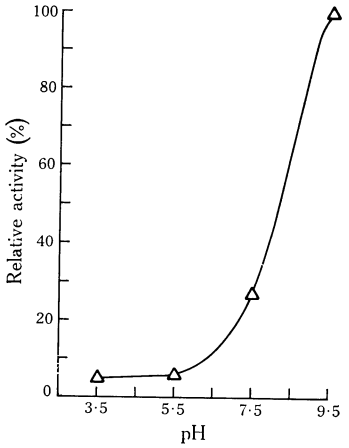

Fig. 1. Total protease activity in homogenates of whole larvae at acid, neutral and alkaline pH. Homogenates were centrifuged at 1000 *g* for 20 min and 0·02 ml was used for the protease assay (see Methods). Protein substrates were acid-denatured haemoglobin at pH 3·5 and heat-denatured casein at pH 5·5, 7·5 and 9·5. Incubation was at 37°C for 60 min and the maximum activity at pH 9·5 was 1·135 PU^F per 0·02 ml of homogenate.

The complexity of the mixture of proteolytic enzymes in *T. bisselliella* is indicated in Table 1. In addition to showing a high caseinolytic activity these extracts hydrolysed: BANA and BAPA, two chromogenic substrates used to assay for trypsin-like enzymes and for some SH-proteinases such as cathepsins and plant proteinases (see Ward 1973 for references); ATEE, the preferred substrate for chymotrypsin-like enzymes (Neurath *et al.* 1967; Wilcox 1970); Z-glycylleucine, a carboxypeptidase substrate (Narahashi and Yanagita 1967), and leucyl-NA, an aminopeptidase substrate (Patterson *et al.* 1963; Marks *et al.* 1968; Suszkiw and Brecher 1970). The activities against the trypsin substrates BANA and BAPA, and the chymotrypsin substrate ATEE, are both DFP-sensitive and presumably indicate the presence of serine proteinases (Table 1). The protease mixture also included considerable DFP-insensitive, metal-chelator-sensitive caseinolytic activity. The characterization of the peptidases is more complex. The aminopeptidase activity is not completely inhibited by any of the reagents tested. It is partially inhibited by 1,10-phenanthroline and slightly inhibited by DFP and L-cysteine. The carboxypeptidase may be a thiol enzyme, since it is strongly inhibited by *p*-chloromercuribenzoate and iodoacetate, but is also strongly inhibited by 1,10-phenanthroline.

A further degree of complexity in the *T. bisselliella* protease mixture is the existence of some enzymes in multiple forms. Disc gel electrophoresis (Fig. 2) indicates at least four trypsin-like enzymes (*m* 0·53, 0·61, 0·67 and 0·73) in these extracts. Three very minor bands (*m* 0·42, 0·47 and 0·80) have been detected in some extracts but cannot be seen in this photograph. No decision could be made regarding the multiplicity of the other proteinases because of the lack of suitable chromogenic

substrates. Glutarylphenylalanyl-NA (Blackwood *et al.* 1965) has been successfully used to detect chymotrypsin-like enzymes on acrylamide gels (Giebel *et al.* 1971) but was not hydrolysed by *T. bisselliella* extracts. However, when the gels were sliced and the slices extracted and assayed with ATEE, chymotrypsin-like activity was recovered in a very sharp peak at *m* 0·50 (Fig. 2), suggesting that only a single enzyme was present. When gels were sliced, extracted and tested for metalloproteinase activity, again only a single peak of activity was detected but this peak was spread out over several slices (Fig. 2), suggesting that there may be more than one metalloproteinase present.

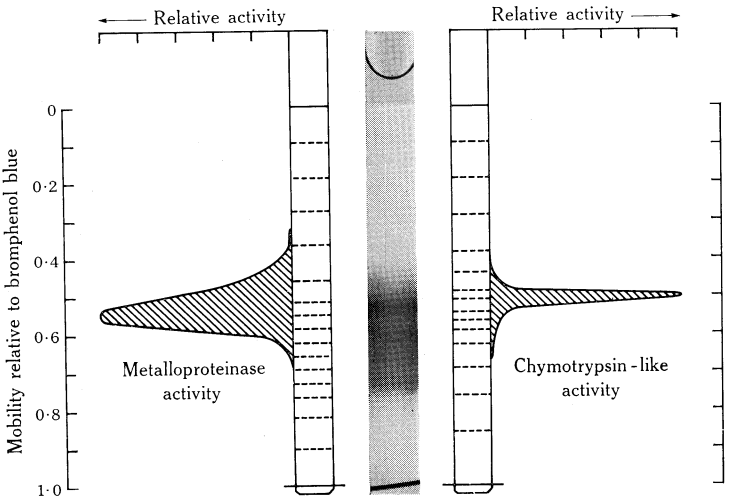

Fig. 2. Acrylamide gel zymograms of proteinases in extracts of whole larvae. The enzyme samples were partially purified by ammonium sulphate precipitation (between 1·435 and 2·255M salt) and gel filtration on Sephadex G100 (1·5 by 100 cm). Electrophoresis in 7·5% gels was as described in the Methods. Trypsin-like enzymes (central zymogram) were detected with fast garnet GBC after a 30-min incubation at 20°C, pH 9·5, with 0·5 mM BANA (Marks *et al.* 1968). For the metalloproteinase and chymotrypsin-like enzymes the gels were sliced as shown, the slices macerated and extracted with water and the extracts assayed for enzyme activity (see Methods; the differential spectrophotometric assay was used for chymotrypsin-like activity). The relative activities are plotted in the figure.

There are also multiple forms of both peptidases in homogenates of whole larvae (Fig. 3). At least 16 aminopeptidase bands can be detected on acrylamide gels, and this complex pattern is not an artefact caused by the presence of proteins that bind azo dyes (Mäkinen and Mäkinen 1971). The major activities against leucyl-NA are in bands 8–11 and 13–16. The results obtained with lighter loading (gel 2) and less acrylamide (gel 1) suggest the presence of four bands in each of these regions of major activity (Fig. 3). The multiplicity of aminopeptidases is not attributable to variations between individual members of the larval population, as found for butterfly esterases (Burns and Johnson 1971), since the same band pattern can be detected in homogenates of individual larvae (Fig. 3, gel 4). These aminopeptidases differ in their specificity requirements as shown in Fig. 4. Bands 1–7 prefer an acidic substrate such as glutamyl-NA and stain up very slightly with neutral or basic amino acid derivatives, while bands 8–16 prefer the alanyl, leucyl, lysyl or arginyl derivatives.

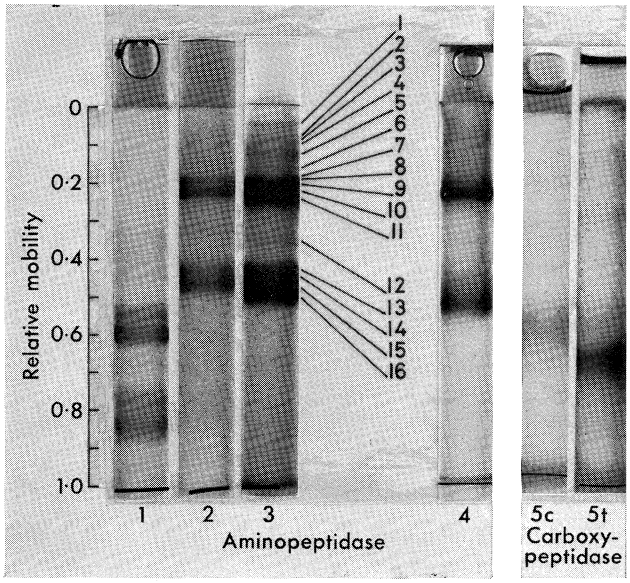

Fig. 3. Acrylamide gel zymograms of peptidases. Electrophoresis and the detection of aminopeptidase and carboxypeptidase bands, with leucyl-NA and Z-glycylleucine respectively, were as described in the Methods. Aminopeptidase bands in the dialysed 1·435–2·255M ammonium sulphate fraction are shown in gels 1–3 (gel 1, 5% acrylamide, 0·01 ml fraction, 5 min incubation; gel 2, 7·5% acrylamide, 0·01 ml fraction, 5 min incubation; gel 3, 7·5% acrylamide, 0·025 ml fraction, 10 min incubation); aminopeptidase bands in the homogenate of an individual larva are shown in gel 4 (7·5% acrylamide, 15 min incubation); carboxypeptidase bands in the 1·435–2·255M ammonium sulphate fraction are shown in gels 5c (control gel with no Z-glycylleucine present during enzyme detection) and 5t (7·5% acrylamide, 0·1 ml fraction).

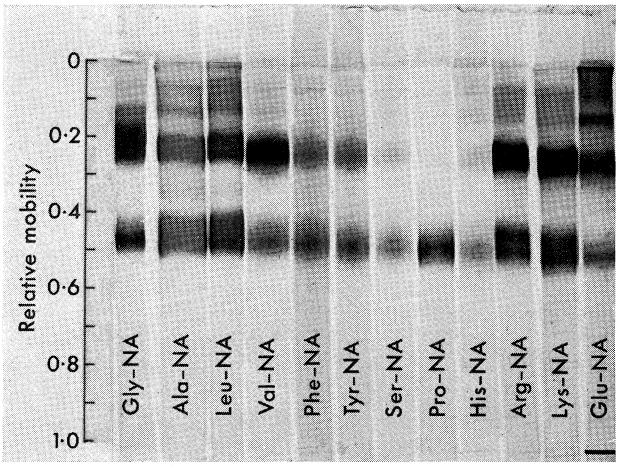

Fig. 4. Effect of different substrates on aminopeptidase zymogram pattern. Dialysed ammonium sulphate fraction (0·25 ml) was diluted to 1·0 ml and subjected to electrophoresis in a 70 by 70 by 3 mm gel slab, then the slab was sliced into 12 equal sections and incubated with different amino acyl β-naphthylamides. The substrates used and the incubation times before staining were as follows: leucyl-NA, 5 min; alanyl-NA, 7·5 min; glycyl-, arginyl- and lysyl-NA, 10 min; prolyl- and glutamyl-NA, 15 min; valyl-NA, 45 min; phenylalanyl- and tyrosyl-NA, 120 min; seryl- and histidyl-NA, 180 min. Electrophoresis and band detection were as described in the Methods.

Activity against prolyl-NA was confined to the region of the gel near bands 15 and 16. The relative rates of hydrolysis of these different substrates varied considerably. The intense patterns obtained with glutamyl-, glycyl-, alanyl-, leucyl-, lysyl- and arginyl-NA appeared after incubation for 5–10 min, while the faint patterns with seryl- and histidyl-NA required 180 min incubation.

As shown in Fig. 3, there are one major and two minor carboxypeptidases in *T. bisselliella*. The major activity occurs at *m* 0·69 with minor activities at *m* 0·14 and 0·30. A control gel (Fig. 3, gel 5c), incubated without Z-glycylleucine in the reaction mixture, indicates that the staining occurring at *m* 0·56 is an artefact, as may be some of the staining associated with the two minor carboxypeptidase activities. In the latter cases, however, the colour formation in the test gel was always stronger than that in the control, suggesting that these minor activities are real. The carboxypeptidase bands could not be detected with naphthyloxycarbonylphenylalanine (Ravin and Seligman 1951; Pacaud and Uriel 1971). This chromogenic substrate was not hydrolysed by extracts of *T. bisselliella*.

Cellular Origin of Proteolytic Activities

Since there exist several potential sources of origin for the various proteases detected in homogenates of whole larvae, dissections were carried out to establish which activities were associated with the larval digestive tract. As shown in Table 2 all six activities were found to be associated primarily with the midgut fraction. The

Table 2. Cellular location of proteolytic activities

Freshly dissected midguts and the residual gut-free larval bodies from approximately 200 larvae were each homogenized in 3·0 ml of 0·25M sucrose–0·005M tris–HCl–0·01 mM phenylthiourea (pH 7·5) and centrifuged at 24000 *g* for 30 min, then the supernatant was assayed for the various protease activities as described in the Methods

Enzyme	Enzyme units[A]	Tissue	
		Midgut	Gut-free body
Proteinases			
Total protease	PU^{278}	24·4	0·94
Metalloproteinase	PU^{278}	6·1	0·41
Trypsin-like proteinase	μmol BAPA/30 min	0·86	0·008
Chymotrypsin-like proteinase[B]		++++	+
Peptidases			
Carboxypeptidase	μmol Z-glycylleucine/60 min	3·10	0·15
Aminopeptidase	μmol leucyl-NA/10 min	3·63	0·37

[A] All activities expressed per milligram of enzyme protein.
[B] Assayed by the high-voltage electrophoresis spot test.

acrylamide gel zymogram patterns for trypsin-like, aminopeptidase and carboxypeptidase enzymes in the midgut fraction were the same as those obtained for extracts of whole larvae. Only low levels of activity were found in the residual larval body and probably arose from contamination with some midgut fragments.

These gut proteases are unlikely to be due to gut microflora, since Crewther and McQuade (1955) showed that the gut of *T. bisselliella* was essentially devoid of microorganisms. Furthermore, none of these protease activities were found to be associated with the casein–yeast diet used to culture the *T. bisselliella* larvae.

Search for Zymogens

Since many proteolytic enzymes from higher vertebrates (see Boyer 1971 for reviews), lower vertebrates (Bradshaw *et al.* 1970; Reeck and Neurath 1972), one starfish (Camacho *et al.* 1970) and one insect (Felsted *et al.* 1973) occur as inactive precursors, experiments were carried out to determine if any of the proteolytic enzymes in *T. bisselliella* also occurred as inactive zymogens. No evidence for such inactive precursors could be found. Homogenates were centrifuged (1000 *g* for 20 min) and incubated in the presence and absence of 0·05M $CaCl_2$ (McDonald and Kunitz 1941; Voytek and Gjessing 1971) at 25°C, pH 9·5, for 0, 1, 2, 3 and 4 h. They were then assayed for total caseinolytic activity, metalloproteinase activity, BAPA hydrolysis, ATEE hydrolysis, carboxypeptidase activity and aminopeptidase activity. There was no increase in any of these activities following such treatments. This confirms the previous findings of Powning *et al.* (1951) on the failure of enteropeptidase (enterokinase) to activate the total protease activity of crude homogenates of dissected midguts from *T. bisselliella* larvae.

Fractionation Procedure

The fractionation of the proteolytic activities of *T. bisselliella* is summarized in Table 3. Whole larvae were used as starting material, since it was impractical to attempt a large-scale purification on dissected midguts, owing to the small size of these larvae (5 mm by 0·7 mm diam.). Also, because of the difficulty involved in culturing large amounts of larvae, it was necessary to co-purify all six protease activities during one large-scale fractionation. This was facilitated by the finding that various subfractions obtained during the purification could be stored in 50% glycerol at −20°C for long periods of time with negligible loss of activity.

Deep-frozen larvae (350 g) were homogenized in a Waring blender with 3500 ml of buffered sucrose (0·25M sucrose – 0·005M tris.HCl – 0·01 mM phenylthiourea, pH 7·5). Grinding was carried out for five 1·0-min periods with a 1·0-min cooling period between each grinding. The homogenate was centrifuged at 24000 *g* for 30 min and the supernatant filtered through a bed of glass wool to remove floating debris. The resulting turbid solution was used for ammonium sulphate precipitation.

Ammonium Sulphate Fractionation

Pilot fractionations revealed a bimodal distribution of protease activities between the microsomal fraction (Palade and Siekevitz 1956) and the soluble protein fraction of homogenates. The microsomal material precipitated between 1·025M (26% saturation at 4°C) and 1·435M (36·4%) ammonium sulphate and appeared as a floating lipid-like layer after centrifugation. The proteinases are firmly bound to this membranous fraction and cannot be separated from it by extraction with 1M sodium chloride (Moore *et al.* 1970). However, they can be readily solubilized by extraction of this material with n-butanol (Morton 1955). The trypsin-like activity was recovered completely (98·5%) by this procedure but the other activities were recovered in lower yield. Characterization of these solubilized trypsin-like enzymes on acrylamide gels showed the same banding patterns obtained for the non-particulate enzymes (Fig. 2), with the exception that an additional band (*m* 0·795) is present. However, this paper is primarily concerned with the fractionation of the non-

Table 3. Purification of *T. bisselliella* proteases

Fraction	Total protein (mg)	Total protease (PU^{278})	Enzyme units[A] Metallo-proteinase (PU^{278})	Trypsin-like proteinase (μmol/30 min)	Chymotrypsin-like proteinase[B] (μmol/min)	Carboxy-peptidase (μmol/30 min)	Amino-peptidase (μmol/10 min)
20000-*g* supernatant	25983	87563	48069	4677	2367	21306	41391
1·435–2·255M ammonium sulphate fraction	5142	40618	22320	882	n.d.[C]	n.d.	21924
Dialysed ammonium sulphate fraction	3568	39360	21632	818	920·5	10105	19010
DEAE-cellulose fractions							
A	451	1280	320	26	0	449	161·5
B	171	7281	3952	102	20	590	432·5
C	185	5032	2376	143	175	1644	9835
D	64	1897	371	88·7	90·6	645	3404
Sephadex G200 fractions							
A1	31·3	0	0	0	0	0	78·6
A2	41·1	0	0	0	0	129	0
A3	41·0	485	80·6	6·7	0	0	0
B1	4·0	0	0	0	0	0	15·6
B2	7·1	0	0	0	0	0	146·4
B3	7·8	552	207	7·3	4·4	128·2	0
B4	17·8	2146	1380	24·1	9·5	34·7	0
B5	6·2	579	228	7·4	0	0	0
C1	10·0	0	0	0	0	0	2405
C2	34·1	225	68·6	4·1	0	408	1391·1
C3	9·8	337	76·1	12·3	13·5	281	0
C4	7·7	1493	773	17·8	106·3	82·5	0
C5	2·2	337	116	10·8	7·3	0	0
D1	4·1	0	0	0	0	0	1002
D2	5·2	18·6	0	0·3	0	101	143
D3	2·4	71·7	10·1	4·1	12·5	16·2	0
D4	2·1	138	33·3	3·4	60·6	0	0
D5	2·7	267	85·9	11·9	24·9	0	0

[A] Enzyme units are the total units per fraction. [B] pH-stat assay, pH 8·0, 37°C. [C] n.d., not determined

particulate protease activities precipitable between 1·435 and 2·255M ammonium sulphate, and the extraction and characterization of the microsomal-bound proteases will be described in a subsequent report.

The non-particulate protease activities precipitate primarily between 1·64 (42%) and 2·255M (57%) salt concentrations. Subsequent precipitations at 2·46 (62%) and 3·075M (78%) salt concentrations contain considerable protein but little proteolytic activity.

The results of the large-scale ammonium sulphate fractionation are presented in Table 4. The material precipitating between 1·435 and 2·255M salt was resuspended in 700 ml of 0·01M tris.HCl–0·01 mM phenylthiourea, pH 7·7, and dialysed over 15 h against three changes (3 litres each) of the same buffer, before ion-exchange chromatography.

Table 4. Ammonium sulphate fractionation of proteases

Fraction	Total protein (mg)	Total enzyme activity in fraction: Total protease (PU^{278})	Metallo-proteinase (PU^{278})	Trypsin-like proteinase (μmol/30 min)	Chymotrypsin-like proteinase (μmol/min)	Carboxy-peptidase (μmol/30 min)	Amino-peptidase (μmol/10 min)
20000-*g* supernatant	25983	87563	48069	4677	2367	21306	41391
Ammonium sulphate fractions							
0–1·435M	7964	45600	18609	2658	+++[A]	+++[A]	14575
1·435–2·255M	5142	40618	22320	882	+++[A]	+++[A]	21924
2·255–2·460M	1281	1082	1040	112	0	0	1562
2·460M-soluble	12570	0	0	0	0	0	1115

[A] Assayed by the high-voltage electrophoresis spot test as described in the Methods.

Ion-exchange Chromatography

Small-scale batch adsorption tests showed that both the protein and total protease activity of a 1·435–2·255M ammonium sulphate fraction were almost completely adsorbed onto CM-cellulose at pH 5·0 and DEAE-cellulose at pH 6·0, 7·0, 8·0 and 9·0. They were only partially adsorbed on CM-cellulose at pH 6·0 and negligibly adsorbed on this resin at pH 7·0 and 8·0. Preliminary experiments also indicated that the proteinases of *T. bisselliella*, though quite stable under neutral and alkaline conditions, were unstable at pH values below 6·0. Thus investigations were confined to chromatography on DEAE-cellulose at pH values above 7·0.

Fig. 5 shows the pattern obtained when the dialysed material (approx. 750 ml) obtained by ammonium sulphate precipitation was chromatographed on a column of microgranular DEAE-cellulose at pH 7·7. Much of the protein was eluted from the column first and was not associated with any of the protease activities. Total protease activity and the metalloproteinase activity showed similar elution profiles, with maximum activity at tube 180. The trypsin-like enzymes were poorly resolved on DEAE-cellulose. Activity was spread over a very large number of fractions and there was little separation of the multiple enzyme forms. All four major trypsin-like bands were present in fractions B and C and the two slowest bands (*m* 0·53 and 0·61) were present in fraction D. The chymotrypsin-like activity was quite well separated from the metalloproteinase activity. The spot test assays indicated that the bulk of this activity was distributed between tubes 200 and 300, with a probable peak around tube 250.

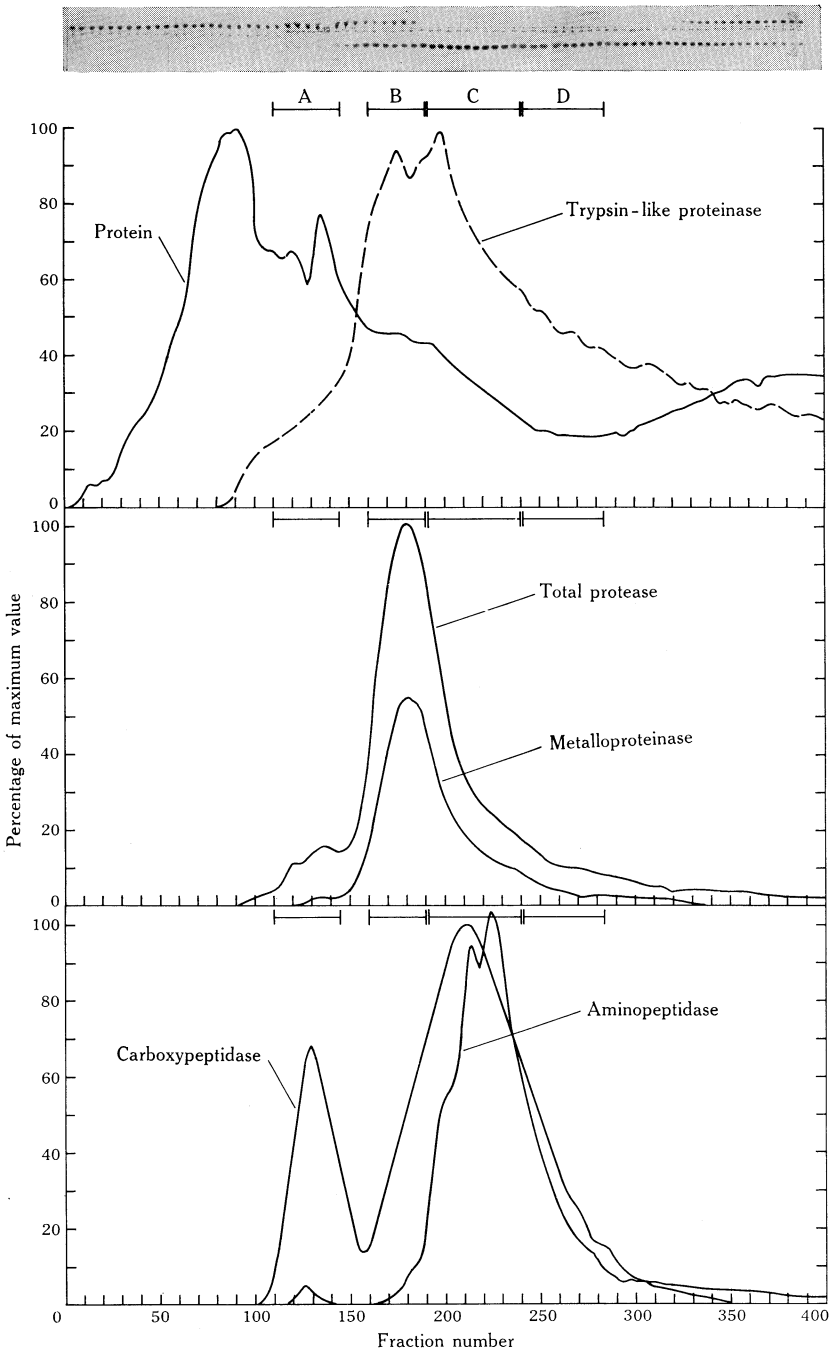

Fig. 5. Chromatography of ammonium sulphate fraction on DEAE-cellulose column (2·5 by 40 cm). Flow rate 69 ml/h, fraction volumes 11·5 ml. At fraction 71 a linear gradient was established from 0·01M tris–HCl–0·01 mM phenylthiourea, pH 7·7 (2 litres) to 0·4M NaCl in the same buffer (2 litres). Starting with tube 8 every fifth tube was monitored for all six protease activities and the results were expressed as a percentage of the maximum activity per tube obtained during the fractionation. These maximum values were: total protease, 294 PU^{278}; trypsin-like activity, 4·12 μmol BAPA/30 min;

Two peaks of carboxypeptidase were obtained, the first at tube 130, the second at tube 210. The first peak (fraction A) is the carboxypeptidase with *m* 0·3 while the second, larger peak, spread out over fractions B, C and D, is the major carboxypeptidase component *m* 0·69 (Fig. 6). The control gel for fraction A, incubated with no Z-glycylleucine in the reaction mixture, showed that the faint staining at *m* 0·56 is an artefact. No artefacts were observed in the control gels for fractions B, C and D. The complex mixture of aminopeptidases was only partially resolved on the DEAE-cellulose column. A minor peak, tubes 120–140, corresponded to band 5 (*m* 0·13) in the original zymogram pattern (Fig. 3) and was well separated from the rest of the aminopeptidases. The latter were eluted as a series of overlapping peaks between fractions 150 and 280 and not well resolved as shown by gel zymograms.

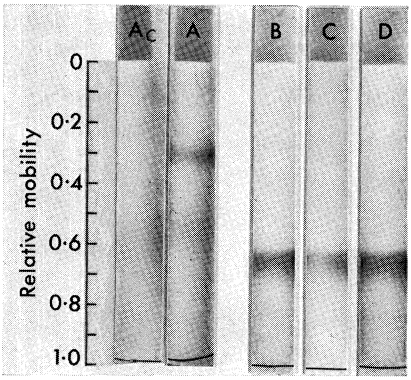

Fig. 6. Acrylamide gel zymograms of carboxypeptidase activities in fractions A–D. Electrophoresis in 7·5% gels and detection of enzyme activity was as described in the Methods. Migration is towards the anode and is expressed relative to the mobility of bromphenol blue. The control gel for fraction A (gel A_C) was incubated with no Z-glycylleucine in the reaction mixture (see Methods).

The pooled fractions A (tubes 110–145), B (160–190), C (191–240) and D (241–285) were concentrated (to 5·0 ml approx.) by ultrafiltration and stored with an equal volume of glycerol at −20°C.

Gel Filtration on Sephadex G200

Fractions A, B, C and D were further fractionated by downward-flow gel filtration on Sephadex G200 (2·5 by 90 cm). To avoid distortions due to viscosity effects (Fischer 1969) the glycerol was removed from these samples before gel filtration, by ultrafiltration of diluted samples in Diaflo cells. The samples (5·0 ml) were loaded by direct application to the drained surface of the column bed.

The resolution of fraction A by gel filtration is shown in Fig. 7. The original material contained a single aminopeptidase, a single carboxypeptidase and a minor amount of proteinase activity. These components were effectively resolved on Sephadex G200. Fractions A1 (tubes 46–50), A2 (56–62) and A3 (69–76) were pooled and concentrated and stored in 50% glycerol.

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aminopeptidase, 255·8 μmol leucyl-NA/10 min; carboxypeptidase, 83 μmol Z-glycylleucine/60 min. Metalloproteinase activity is expressed as a percentage of the maximum total protease activity. Chymotrypsin-like activity was not quantitated, but was monitored by the high-voltage electrophoresis spot test (electrophoretograms at top of figure; enzyme hydrolyses ATEE, upper row of spots, to AT, lower row). The pooled fractions A–D were concentrated by ultrafiltration.

The resolution of fraction C on Sephadex G200 is shown in Fig. 8. The elution profiles for the fractionation of fractions B and D were basically similar, but there were differences in both the relative peak heights of the various enzyme activities in each fraction and in the total activities present in each peak. In each fractionation two aminopeptidase peaks were eluted first followed closely by a single carboxypeptidase peak and then the proteinases. With fraction B the first aminopeptidase

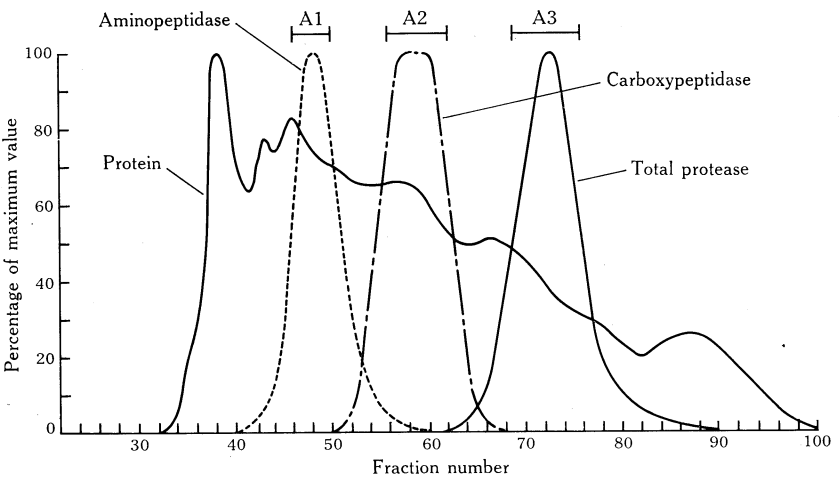

Fig. 7. Gel filtration of fraction A on Sephadex G200 (2·5 by 90 cm). Downward flow rate 15·3 ml/hr fraction volumes 5·1 ml; the buffer used was 0·1M tris–HCl–0·01 mM phenylthiourea, pH 7·5- Starting with tube 30 every second tube was assayed for total protease, aminopeptidase and carboxyl peptidase activities. The results are expressed as a percentage of the maximum activity per tube obtained during the fractionation, these being: total protease, 78·9 PU^{278}; aminopeptidase, 19·1 μmol leucyl-NA/10 min; carboxypeptidase, 25·7 μmol Z-glycylleucine/60 min. The pooled fractions A1–A3 were concentrated by ultrafiltration.

peak was a very small one and consisted of multiple minor enzyme bands, while the first peak from fractions C and D were major peaks and both contained aminopeptidase bands 8–11. The second aminopeptidase peak consisted of just two bands (13 and 14) in the case of fraction B, but contained all four bands 13–16 in the fraction C and D separations (Figs 3 and 9). The relative total activities of the various peaks are indicated in Table 3.

The carboxypeptidase components of all three fractions B, C and D appear to be identical. They were eluted as a single peak following the second aminopeptidase and contained a single active band on acrylamide gels. They were distinct from the single carboxypeptidase in fraction A (Fig. 6).

The proteinases in fractions B, C and D were poorly resolved on Sephadex G200 and were eluted together during these fractionations. Four overlapping peaks of

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peptidase, 73·6 μmol Z-glycylleucine/60 min. Metalloproteinase activity is expressed as a percentage of the maximum total protease activity. Chymotrypsin-like activity was not quantitated, but was monitored by the high-voltage electrophoresis test (electrophoretograms at top of figure; enzyme hydrolyses ATEE, upper row of spots, to AT, lower row). The pooled fractions C1–C5 were concentrated by ultrafiltration.

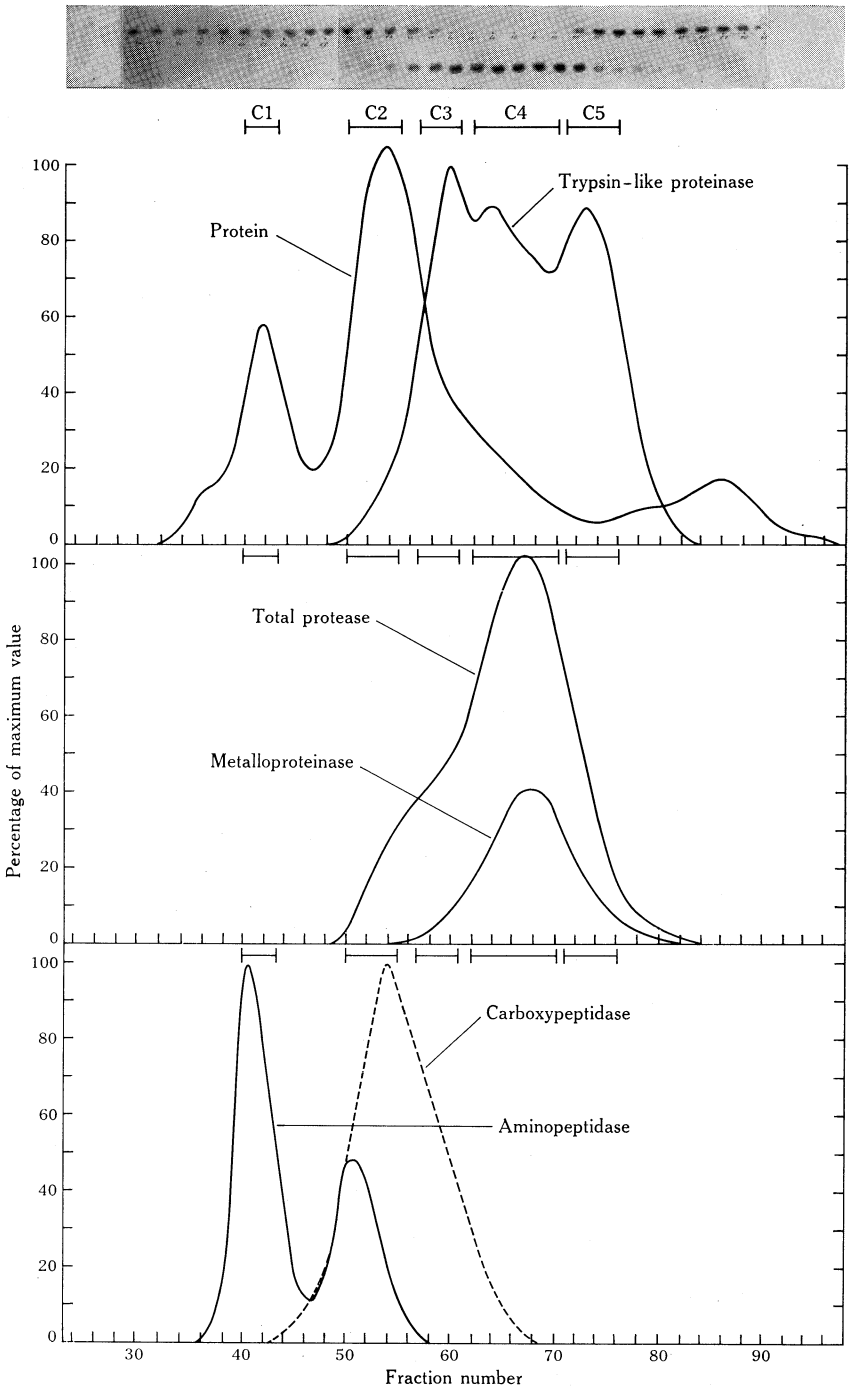

Fig. 8. Gel filtration of fraction C on Sephadex G200 (2·5 by 90 cm). Downward flow rate 16·5 ml/h, fraction volumes 5·5 ml; the buffer used was 0·1 M tris–HCl–0·01 mM phenylthiourea, pH 7·5. Starting with tube 30 every second tube was assayed for all six protease activities as described in the legend to Fig. 5. The maximum tube activities were: total protease, 163·9 PU^{278}; trypsin-like activity, 9·0 μmol BAPA/60 min; aminopeptidase, 653·4 μmol leucyl-NA/10 min, carboxy-

trypsin-like activity can be seen in Fig. 8, but acrylamide gels of the pooled fractions C3, C4 and C5 (Fig. 10) and similarly B3–B5 and D3–D5 indicated only partial resolution of the multiple anionic trypsin-like bands. The relatively faint banding pattern in fractions C5 and D5 compared to the other peaks suggested that much of their BAPA-hydrolysing activity may be due to a cationic trypsin-like enzyme.

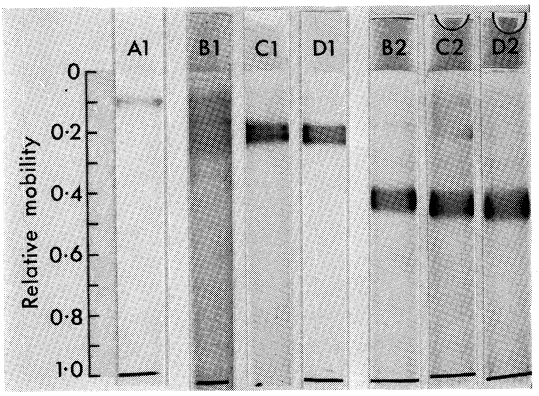

Fig. 9. Acrylamide gel zymograms of amino-peptidase activities in fractions from Sephadex G200 columns. Electrophoresis in 7·5% gels and detection of enzyme activity was as described in the Methods. Migration is towards the anode and is expressed relative to the mobility of bromphenol blue. For bands in original extract see Fig. 3.

Several methods were employed to detect such cationic trypsin-like bands on acrylamide gels. The methods of Reisfeld *et al.* (1962) and Sakai and Gross (1967) were unsuccessful because the acid pH of their running gels (pH 4·5 and 5·5 respectively) made detection of activities with alkaline pH optima difficult. However, the procedure of Racusen (1967) for cationic proteins was successful and a single cationic trypsin-like enzyme with an *m* value (relative to methyl green) of 0·32 was demonstrated in fractions B5, C5 and D5. Only traces of this enzyme were present in fractions B4 and C4 and none was present in fractions B3, C3, D3 or D4.

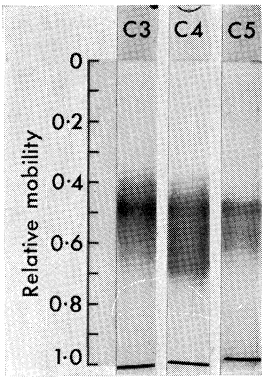

Fig. 10. Acrylamide gel zymograms of trypsin-like activities in fractions C3, C4 and C5. Electrophoresis in 7·5% gels and detection of enzyme activity was as described in the Methods. Migration is towards the anode and is expressed relative to the mobility of bromphenol blue.

The metalloproteinase was eluted as a single symmetrical peak in the gel filtration of both fractions C and D, but as a peak with a shoulder in fraction B, suggesting that more than a single component may be present.

The chymotrypsin-like activity profiles for the Sephadex separations of fractions B, C and D were not quantitated, but the spot test assays showed that this enzyme was eluted at a similar position in all three separations.

Discussion

In all organisms the process of digestion is extracellular, although the nature of the extracellular region in which digestion occurs and the control of the digestion process has become increasingly specialized with evolution. Unicellular organisms such as bacteria and fungi secrete hydrolytic enzymes into their surrounding medium; amoeba and paramecia digest material within a temporary vacuole which is kept separate from the cytoplasm until digestion is completed; in higher organisms digestion occurs in a highly specialized extracellular environment, the gastro-intestinal tract. Most of the detailed information on proteolytic enzymes has been on those from either a few mammals or a few microorganisms, two groups which represent the two extremes of evolutionary development. There is much less information on the proteases from intermediate phyla, and very few studies have been aimed at resolving all the proteases present in lower organisms (De Villez 1968).

In this study an attempt has been made to detect and characterize all the proteolytic enzymes in the larvae of the insect *T. bisselliella.* A complex mixture of peptidases and proteinases has been found in extracts of whole larvae and these have been shown to originate from the larval midguts. They are unlikely to be the products of gut microflora, since Crewther and McQuade (1955) showed that the digestive tract of *T. bisselliella* larvae, grown on either a casein–yeast diet (as in this study) or on wool, contained negligible numbers of microorganisms. It has been shown further that these proteases do not originate from the casein–yeast diet used to culture the larvae.

The peptidases include both aminopeptidase and carboxypeptidase activities and both are present in multiple forms. Sixteen aminopeptidase bands have been detected and all are present in individual larvae. They are not inhibited completely by reagents specific for any of the common active sites and have different specificity requirements. Duspiva (1936*a*) reported activity against alanylglycylglycine and alanylglycine in homogenates of *T. bisselliella* and it is interesting to note that alanyl-NA is very rapidly hydrolysed by several of these aminopeptidase bands. Two carboxypeptidases have been detected and separated and are probably thiol enzymes. Little is known so far of their specificity requirements. Both hydrolyse Z-glycyl-leucine but differ from pancreatic carboxypeptidase A in not being able to hydrolyse naphthyloxycarbonylphenylalanine (Ravin and Seligman 1951).

Of the proteinases, both trypsin-like and chymotrypsin-like enzymes were identified, based on their hydrolysis of the specific substrates BANA and BAPA (trypsin) and ATEE (chymotrypsin) and their complete inhibition by DFP. Multiple forms of the trypsin-like enzymes were detected on acrylamide gel zymograms and include four major and three very minor anionic enzymes and a single cationic enzyme. The trypsin-like enzymes from most invertebrates studied have been anionic proteins (Neurath *et al.* 1967; Gates and Travis 1969; Camacho *et al.* 1970; Winter and Neurath 1970; De Villez and Reid 1971; Eisen *et al.* 1973) but there are reports of cationic trypsin-like enzymes in the insects *Pieris brassicae* (Lecadet and Dedonder 1966), *Apis mellifica* (Giebel *et al.* 1971) and *Tenebrio molitor* (Zwilling *et al.* 1972). Only a single chymotrypsin-like enzyme appears to be present. These enzymes differed from the pancreatic proteinases in their response to the naturally occurring proteinase inhibitors. The trypsin-like enzymes were unaffected by pre-incubation

with excess chicken ovomucoid, lima bean trypsin inhibitor or soybean trypsin inhibitor, while the chymotrypsin-like activity was inhibited 88 % by soybean inhibitor, but not affected by either ovomucoid or lima bean inhibitor. They resembled serine proteinases from microorganisms (Belew and Porath 1970; Danno 1970; Yoshimoto *et al.* 1971; van Heyningen 1972) and other invertebrates (Gates and Travis 1969; Camacho *et al.* 1970; Winter and Neurath 1970; De Villez and Reid 1971; Peng and Williams 1973) in their pH stability, being quite stable at alkaline pH values but unstable at acid pH, especially below pH 4·0.

Trypsin-like enzymes, and to a lesser extent chymotrypsin-like enzymes, amino-peptidases and carboxypeptidases, have been reported in a variety of microorganisms (Morihara and Tsuzuki 1968; Narahashi *et al.* 1968; Lesk and Blackburn 1971; Merkel and Sipos 1971; Pacaud and Uriel 1971; Hayashi and Hata 1972; Turkova *et al.* 1972), insects (see Giebel *et al.* 1971 for review; Hagenmaier 1971; Yang and Davies 1971; Zwilling *et al.* 1972), other invertebrates (Winter and Neurath 1970 for review; De Villez and Reid 1971; Peng and Williams 1973) and lower vertebrates (Bradshaw *et al.* 1970; Reeck and Neurath 1972), and their detection in *T. bisselliella* larvae was to be expected.

Quite unexpected was the finding of considerable proteinase activity that was unaffected by DFP or thiol reagents but was inhibited by EDTA. It is presumably due to a metalloproteinase (Hartley 1960; Vallee and Wacker 1970), although the term must be used with caution since neither the absolute requirement for a metal nor the nature of the metal involved has been established. With the exception of the collagenases, a proteinase from rat kidney microsomes (Maruyama *et al.* 1970) and some snake venom proteinases (Satake *et al.* 1963; Pfleiderer and Kraus 1965; Takahashi and Ohsaka 1970), metalloproteinase activity has been demonstrated only in microorganisms (Morihara *et al.* 1967, 1968; Narahashi *et al.* 1968; Matsubara and Feder 1971; Arvidson 1973). Such enzymes may occur in other invertebrates, however, since a DFP-insensitive alkaline proteinase has been reported in the gastric juice of the crayfish *Orconectes virilis* (De Villez 1968; De Villez and Lau 1970).

The proteases of *T. bisselliella* also differ from those of higher organisms in the apparent absence of any of these enzymes in an inactive form. Activation experiments (McDonald and Kunitz 1941; Voytek and Gjessing 1971) were negative and the data on the bimodal distribution of these activities between the microsomal fraction (Palade and Siekevitz 1956) and the soluble fraction of crude homogenates further supports this view. Proteins destined for extracellular function are synthesized on membrane-bound polyribosomes (Redman and Sabatini 1966; Hicks *et al.* 1969; Both *et al.* 1972) and the presence of high protease activity in the microsomal fraction of *T. bisselliella* suggests that they are synthesized in an active form. At one time it was thought that the lack of protease zymogens may be a general characteristic of all invertebrates (Neurath *et al.* 1967; Winter and Neurath 1970) but Camacho *et al.* (1970) have subsequently reported an inactive trypsinogen in the starfish *Dermasterias imbricata.* The special trypsin-like enzyme, cocoonase, also exists as an inactive zymogen (Felsted *et al.* 1973) and zymogens for the trypsin-like enzymes in the lower vertebrates *Squalus acanthias* (Bradshaw *et al.* 1970) and *Protopterus aethiopicus* (Reeck and Neurath 1972) have been reported.

In this report the detection and partial resolution of proteolytic enzymes in larvae of *T. bisselliella* have been described. Their terminal purification and characterization

will be dealt with in subsequent reports and will indicate their relative importance in the rather unique process of keratin digestion (McPhee 1965).

Acknowledgments

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