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Aminopeptidases in Webbing Clothes Moth Larvae. Properties and Specificities of Enzymes of Highest Electrophoretic Mobility

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Abstract

The group of aminopeptidase bands from *Tineola bisselliella* larvae with highest electrophoretic mobility in polyacrylamide gels were purified further and partially separated by ion exchange chromatography. Three aminopeptidase bands were present in this material and were very similar with respect to their pH optima (7·7), their molecular weight of 94 000, their responses to metal ions and enzyme inhibitors and in their substrate specificity requirements.

Kinetic constants were obtained for the hydrolysis of 17 different α-aminoacyl-β-naphthylamides by these aminopeptidases, the most favoured substrates being the derivatives of alanine, methionine, proline, leucine, glycine, glutamic acid, lysine and arginine. The enzymes also hydrolyse amino acid amides, dipeptides, dipeptide amides, tripeptides and oligopeptides at the *N*-terminal end. These enzymes differ from the other aminopeptidases in *T. bisselliella* in being able to hydrolyse bonds involving proline.

Introduction

The larvae of the webbing clothes moth, *Tineola bisselliella*, have been shown to contain a variety of proteinases, carboxypeptidases and aminopeptidases which are associated with its digestive tract (Duspiva 1936; Powning *et al.* 1951; Ward 1972, 1975*a*). Acrylamide gel zymograms indicated at least 16 different aminopeptidase bands in both bulk extracts and extracts of individual larvae, and showed that some of these bands had different substrate specificity requirements. Using Leu-NA* as substrate, two major regions of activity could be seen on the gels. The first and most active occurred at a position between relative mobilities 0·19 and 0·25 and the second in the region 0·42–0·50. Both regions contained several aminopeptidase bands with only slight differences in electrophoretic mobility. The two groups of activity could be well separated by gel filtration on Sephadex G200 but no resolution of the individual components within each group was observed (Ward 1975).

This paper describes the further purification of the fraction (C2) containing most of the activity due to the aminopeptidase bands of relative mobility 0·42–0·50 as well as the effect of pH, various inhibitors and several L-aminoacyl-β-naphthylamide and peptide substrates on the activity of the aminopeptidase components so obtained.

* 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; pCMB, *p*-chloromercuribenzoate; Z, *N*-benzyloxycarbonyl derivative.

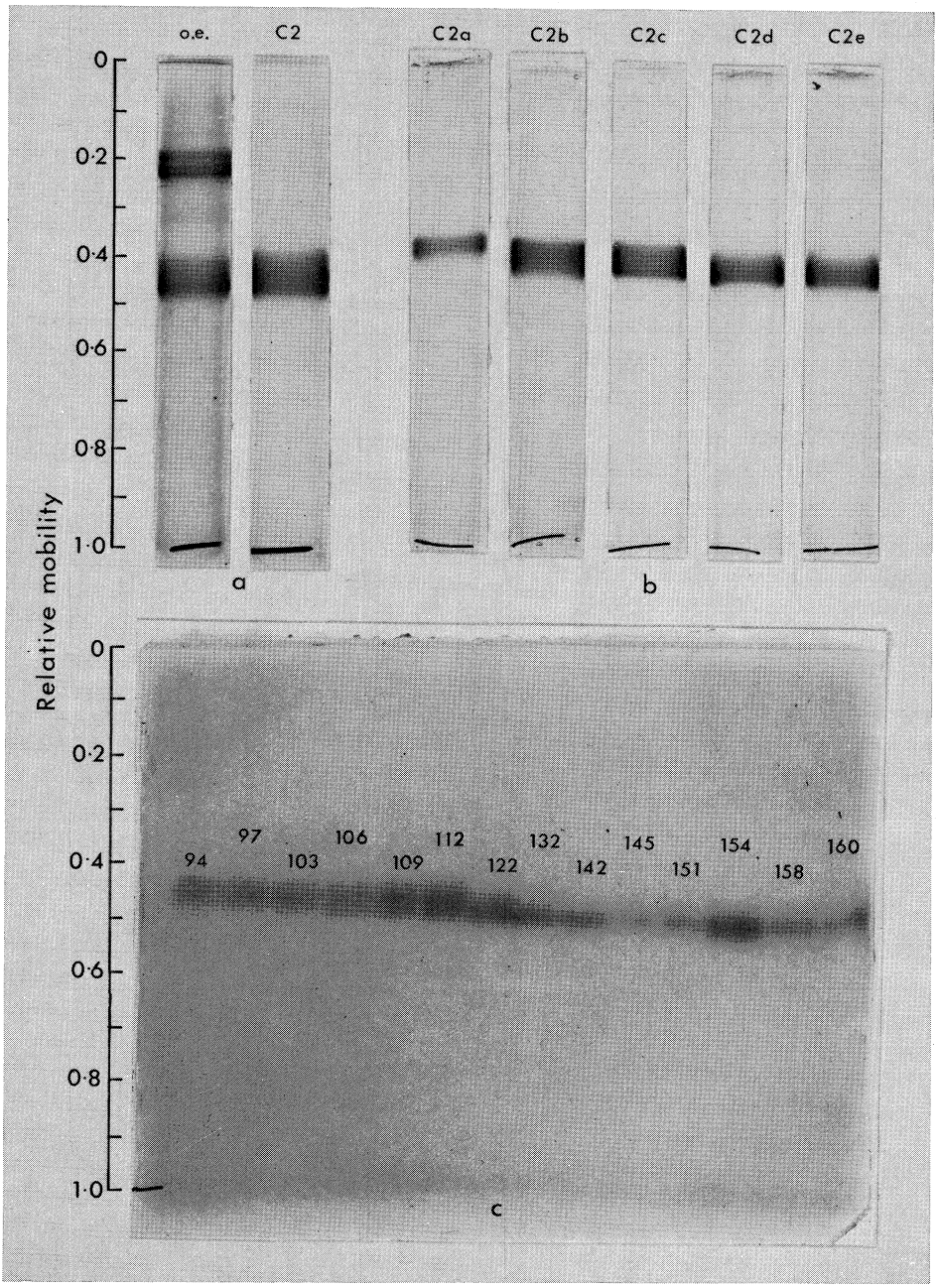

Fig. 1. (*a*) Aminopeptidase bands in original extract (o.e.) and fraction C2. Electrophoresis was in 7½% gels, and the detection was by incubating the gels at pH 9·0 for 5 min at room temperature with 0·5 mM Leu-NA and staining for 15 min with fast garnet GBC as previously described (Ward 1975*a*). Migration is towards the anode and mobility is expressed relative to that of bromphenol blue. (*b*) Aminopeptidase bands in fractions C2a–C2e from DEAE-cellulose column. (*c*) Aminopeptidase band composition of fractions taken across the enzyme peak eluted from DEAE-cellulose as shown in Fig. 2. Samples (20 μl) were taken from the tube numbers as shown. Electrophoresis and detection of bands was as described but with a 30-min incubation period in the presence of 0·5 mM Leu-NA.

Materials and Methods

Chemicals

Unless otherwise indicated, all peptides contained only L-amino acids. The β-naphthylamides of alanine, methionine, isoleucine, threonine and tryptophan and the peptide Leu-Leu were from Cyclo Chemical Co. Other L-aminoacyl-β-naphthylamides, AT, ATEE, BAPA, Z-Gly-Leu and Leu-amide were from Schwarz–Mann Research Laboratories. L-Leu-D-Leu, D-Leu-L-Leu, Leu-Pro, Pro-Leu, and Leu-Gly-amide were from Bachem Fine Chemicals. Leu-Leu-amide, Leu-Gly-Val and Leu-Gly-Ala-Ala-Ala were kindly prepared by Dr F. H. C. Stewart of this laboratory. BANA and Leu-Gly were from Calbiochem; DFP from Aldrich Chemical Co.; bovine serum albumin from Sigma Chemical Co.; fast garnet GBC from G.T. Gurr Ltd, England; Hammarsten casein from E. Merck, Germany, and DEAE-cellulose (DE-52) from Reeve Angel and Co., England.

Enzyme

The starting material used for this study was fraction C2 obtained as described previously (Ward 1975*a*) and stored in 50% glycerol at −20°C until used.

Enzyme assays, column chromatography, ultrafiltration and acrylamide gel electrophoresis were as described previously (Ward 1975*a*).

Enzyme Kinetic Determinations

The kinetic parameters K_m and V_{max} for the hydrolysis of L-aminoacyl-β-naphthylamides were determined by the method of Lee and Wilson (1971) where the instantaneous velocity is replaced by the average velocity over a fixed time period and the initial substrate concentration is replaced by the arithmetic average substrate concentration during that time interval. Initial substrate concentrations employed were 0·02, 0·05, 0·1, 0·2, 0·5, 1·0, 2·0 and 5·0 mM. Reaction conditions were 37°C and pH 7·7, and incubation time was 10·0 min. To overcome solubility problems, dimethyl sulphoxide was present at a final concentration (v/v) of 5, 15, 20 and 20% for Ile-NA, Trp-NA, Phe-NA and Tyr-NA respectively. Regression analyses of $1/\bar{v}$ versus $1/\bar{s}$, $\bar{s}/\bar{v}$ versus $\bar{s}$ and $\bar{v}/\bar{s}$ versus $\bar{v}$ were carried out on a GE terminal computer. The K_m and V_{max} values from these three regression analyses were averaged.

Hydrolysis of Peptide Substrates

The hydrolysis of peptide substrates by the purified aminopeptidase fractions C2a and C2e was carried out at 37°C in 0·22 ml of 0·02M Diol buffer (pH 8·0) containing 0·5 μmol peptide substrate. The extent of hydrolysis after 4 h was determined qualitatively by high-voltage electrophoresis at pH 3·5 on Whatman 3 MM paper (Naughton *et al.* 1960; Bennet 1967) and quantitatively by amino acid analysis on a Beckman Spinco amino acid analyser.

Protein Determination

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Further Purification of Aminopeptidases

As shown in Fig. 1*a*, the starting material (fraction C2) contained the fastest moving group of aminopeptidase bands which had electrophoretic mobilities between 0·42 and 0·50. It contained none of the other aminopeptidase bands present in crude extracts, nor any of the chymotrypsin-like proteinase activity. It did contain most of the carboxypeptidase activity of *T. bisselliella* and also small amounts of the trypsin-like and metal chelator-sensitive proteinase activities.

The fractionation of C2 on DEAE-cellulose is shown in Fig. 2. Aminopeptidase activity was assayed with both Leu-NA and Pro-NA and identical peaks of relative activity were obtained. This irregular peak of aminopeptidase activity was well

separated from the bulk of the protein and from the peak of proteinase activity. The carboxypeptidase activity in fraction C2 was lost during the dialysis against starting buffer before chromatography and this activity could not be recovered by treatment with metal ions or reducing agents.

Acrylamide gels, stained for activity with Leu-NA (Fig. 1*c*) or Pro-NA, of 14 tubes taken across the aminopeptidase peak indicated partial separation of the aminopeptidase components of fraction C2. Three distinct bands of aminopeptidase activity can be seen across the gel slab and the tubes from the central part of the peak (tubes 104–131) contained all three components. The small shoulder at the front of the aminopeptidase peak (tubes 94 and 97) contained only the two slowest aminopeptidase bands, while the trailing edge of the aminopeptidase peak (tubes 132–160) contained the single fastest moving component. Tube contents were pooled to give fractions C2a, C2b, C2c, C2d and C2e, as shown in Fig. 2, and each fraction concentrated by ultrafiltration and stored in 50% glycerol at −20°C. The aminopeptidase bands present in these pooled fractions were as shown in Fig. 1*b*.

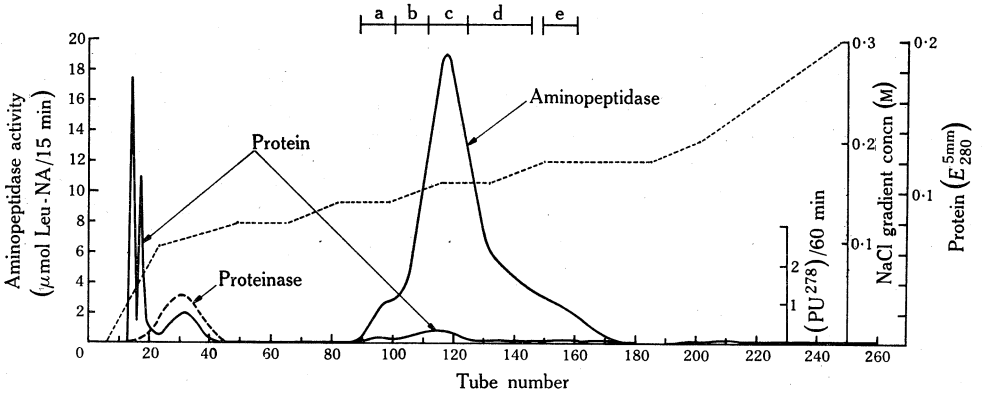

Fig. 2. Chromatography of fraction C2 on DEAE-cellulose (column 0·9 × 15 cm). Flow rate was 13·2 ml/h, fraction volumes were 4·4 ml. Sample (19·0 ml, 17·5 mg protein) was pumped on followed by 4·2 ml starting buffer (0·02M tris–HCl, pH 7·5). At tube 6 the adapted gradient shown in the figure was commenced. The gradient eluant was NaCl in starting buffer. Every second tube was assayed for total proteinase activity (measured as PU^{278}), aminopeptidase activity and carboxypeptidase activity and the results are expressed as activities per fraction. No carboxypeptidase was recovered from the column. Tube contents were pooled to give fractions C2a, C2b, C2c, C2d and C2e, and concentrated by ultrafiltration.

In earlier studies (Ward 1975*a*) it was thought there may be four bands migrating in the region of relative mobility 0·42–0·50, but from these gels it appears that there are only three. Attempts to separate the two bands present in fraction C2a from each other have proved unsuccessful, so the properties of the mixture of these two bands are compared with those of the single band in fraction C2e.

Properties of Aminopeptidases in Fractions C2a and C2e

pH optima. As shown in Fig. 3, the effect of pH on the hydrolysis of Leu-NA by both fractions C2a and C2e is virtually the same. The pH optima for both fractions was 7·7 and activity fell away rapidly below pH 6·0 and above pH 10·0. The activity of both fractions was depressed approximately 30% in tris–HCl buffers.

Molecular weight. When run on a Sephadex G200 column calibrated with γ-globulin, bovine serum albumin dimer and monomer, human transferrin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome C (Andrews 1965), the C2 aminopeptidases eluted at a position corresponding to a molecular weight of 94 000.

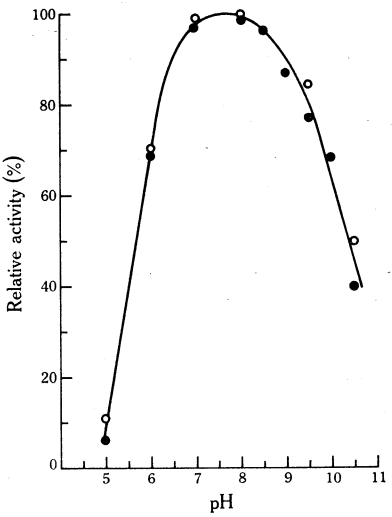

Fig. 3. Effect of pH on the hydrolysis of Leu-NA by aminopeptidase fractions C2a (○) and C2e (●). Buffers used at 0·05M were: sodium acetate–acetic acid, pH 5·0; sodium phosphate, pH 6·0 and pH 7·0; Diol buffer, pH 8·0–10·5.

Inhibitors. The effect of inhibitors on the aminopeptidase activities are shown in Table 1. Mercury, copper and zinc ions caused strong inhibition of both fractions and cobalt ions caused partial inhibition. The other ions tested were essentially

Table 1. Effect of inhibitors and metal ions on activities of aminopeptidase fractions C2a and C2e

The enzyme was pre-incubated with inhibitor for 30 min at 20°C and pH 8·0, then Leu-NA was added and the residual activity determined as described (Ward 1975*a*). pCMB was present at a final concentration of 0·1 mM, all other inhibitors were present at 1·0 mM

Addition	Relative activity (%) of C2a	C2e	Addition	Relative activity (%) of C2a	C2e
Puromycin	100	100	$MnSO_4$	100	100
DFP	100	100	$FeSO_4$	100	91
pCMB	100	100	$FeCl_3$	100	90
Iodoacetate	100	100	$CoCl_3$	83	59
EDTA	100	100	$ZnCl_2$	16	6
1,10-phenanthroline	60	26	$CuCl_2$	5	5
$CaCl_2$	100	100	$HgCl_2$	1	4
$MgCl_2$	100	100			

without effect on either aminopeptidase fraction. As reported earlier for the total aminopeptidase activity in crude extracts (Ward 1975*a*), pCMB, iodoacetate, DFP and EDTA caused no inhibition of activity in either fraction C2a or C2e, but 1,10-phenanthroline did. It inhibited fraction C2a by 40% and fraction C2e by 70%. Puromycin at a concentration of 1 mM had no effect on either aminopeptidase fraction.

Cleavage specificity with aminoacyl-β-naphthylamides. The kinetic parameters for the hydrolysis of 17 L-aminoacyl-β-naphthylamides at pH 7·7 by both aminopeptidase fractions are shown in Table 2, and these further demonstrate the similarity between the enzyme activities. For fraction C2a, Ala-NA and Met-NA gave the highest V_{max} values followed by Leu-NA, Pro-NA, Gly-NA, Glu-NA, Lys-NA and Arg-NA. For fraction C2e the order was the same with the exception that Pro-NA was most rapidly hydrolysed. For both aminopeptidase fractions the β-naphthylamides of tryptophan, tyrosine, threonine, isoleucine, valine, serine and histidine were poorly hydrolysed. As shown in Table 2, the K_m values for most substrates were similar for each aminopeptidase fraction, Val-NA having the lowest K_m and Glu-NA the highest.

Table 2. Kinetic constants for hydrolysis of L-aminoacyl β-naphthylamides by aminopeptidase fractions C2a and C2e

V_{max} values are expressed relative to those of 13·5 and 68·4 μmol (10 min)$^{-1}$ (mg enzyme)$^{-1}$ obtained for the hydrolysis of Ala-Na by aminopeptidase fractions C2a and C2e respectively. The reaction conditions and determination of the kinetic constants are as described in Methods

L-aminoacyl-β-naphthalamide	K_m (mM)		V_{max} (relative %)		V_{max}/K_m (relative %)	
	C2a	C2e	C2a	C2e	C2a	C2e
Alanyl	0·21	0·12	100	100	100	100
Leucyl	0·11	0·17	56	59	91	42
Arginyl	0·06	0·08	17	14	54	19
Lysyl	0·09	0·15	23	20	43	14
Glycyl	0·50	0·35	32	45	8·4	14
Prolyl	1·30	1·24	36	108	4·9	9·5
Methionyl	0·87	1·75	100	70	23	4·4
Phenyl[B]	0·59	0·45	16	17	5·2	4·0
Valyl	0·05	0·07	2·6	1·4	8·0	2·3
Isoleucyl[B]	0·13	0·39	2·2	3·2	3·2	0·9
Seryl	NH[A]	0·80	NH	1·9	NH	0·3
Threonyl	0·20	0·30	3·1	3·8	2·6	1·4
Tyrosyl[B]	0·53	0·73	3·6	6·4	1·2	1·1
Tryptophanyl[B]	0·39	0·90	5·6	6·6	2·5	0·8
Histidyl	0·40	1·27	0·7	0·5	0·3	0·05
Glutamyl	4·65	4·18	32·0	42	1·2	1·1
Aspartyl	0·48	2·62	5·6	14	2·0	0·6

[A] NH = negligible hydrolysis.
[B] Determinations carried out in presence of 5–20% dimethyl sulphoxide, as described in Methods.

Hydrolysis of peptide substrates. The data concerning the hydrolysis of various peptide substrates by aminopeptideas fractions C2a and C2e are summarized in Table 3. Leu-amide, dipeptides, dipeptide amides, tripeptides and oligopeptides were all cleaved extensively by both fractions, the *N*-terminal peptide bond being the bond hydrolysed initially. Pro-Leu was cleaved 5–10 times more rapidly than Leu-Pro. The peptides D-Leu-L-Leu and L-Leu-D-Leu were hydrolysed by both enzyme preparations, but at rates markedly slower than L-Leu-L-Leu.

Discussion

Three aminopeptidase bands have been identified in the aminopeptidase fraction C2, which migrate on 7½% acrylamide gels in the region of relative mobility 0·42–0·50. They have been partially resolved on DEAE-cellulose to produce one fraction containing the two slowest migrating bands and another fraction containing the fastest band. The aminopeptidase activities in both of these fractions are very similar as judged by their molecular weight, the effect of pH and several inhibitors, and from the kinetic parameters obtained for the hydrolysis of various L-aminoacyl-β-naphthylamide and peptide substrates.

Table 3. Hydrolysis of peptides by aminopeptidase fractions C2a and C2e

Values indicated are the percentage hydrolysis of the *N*-terminal peptide bond in 4 h at 37°C by 50 µl of preparations C2a (7 µg) and C2e (21 µg) under the conditions described in the text

Peptide	Extent of hydrolysis by C2a	C2e	Peptide	Extent of hydrolysis by C2a	C2e
Leu-NH_2	60·4	100	Leu-Gly-NH_2	30·9	35·7
L-Leu-D-Leu	4·0	1·2	Leu-Gly-Val	72·5	81·5
D-Leu-L-Leu	3·1	2·5	Leu-Gly-Ala-Ala-Ala	33·2	44·1
Leu-Leu	91·4	75·1	Leu-Pro	0·2	2·3
Leu-Leu-NH_2	91·1	100	Pro-Leu	3·8	10·3
Leu-Gly	100	97			

The aminopeptidases resemble those from some other sources in their pH optima of 7·5–8·0 (Prescott and Wilkes 1966; Marks *et al.* 1968; Himmelhoch 1970; Roncari and Zuber 1970), their inhibition by copper and mercuric ions (Marks and Lajtha 1970; Suszkiw and Brecher 1970) and in their lack of inhibition by thiol reagents, EDTA, magnesium, manganese and calcium ions (Smith and Spackman 1955; Marks and Lajtha 1970; Palmer 1974). The inhibition by zinc ions is similar to that reported for hog brain arylamidase (House 1973). Neither aminopeptidase fraction C2a or C2e was affected by puromycin, a good inhibitor of some but not all aminopeptidases (Behal *et al.* 1966; Ellis and Perry 1966; Marks *et al.* 1968; Suszkiw and Brecher 1970).

These C2 aminopeptidases are the smallest of the aminopeptidases in *T. bisselliella* (Ward 1975*b*, 1975*c*) and their molecular weight of 94000 is much lower than that of 300000 and 326000 reported for vertebrate leucine aminopeptidases (Smith and Spackman 1955; Himmelhoch and Peterson 1968; Melbye and Carpenter 1971).

The aminopeptidases from fractions C2a and C2e show a broad substrate specificity as judged by hydrolysis of a series of L-aminoacyl-β-naphthylamides. They rapidly hydrolysed the derivatives of proline, lysine, arginine, glutamic acid and the neutral amino acids alanine, methionine, leucine, glycine and phenylalanine. These are the only aminopeptidases from *T. bisselliella* that can hydrolyse proline derivatives (Ward 1975*b*, 1975*c*). The specificity of these aminopeptidases is similar to that exhibited by the arylamidases from bovine heart (Brecher *et al.* 1969), bovine pituitary (Ellis and Perry 1966) and bovine brain (Suszkiw and Brecher 1970), with the marked difference that the enzymes from the latter two sources do not hydrolyse Pro-NA. The *T. bisselliella* enzymes differ from the well-characterized pig kidney leucine aminopeptidase which cannot hydrolyse proline bonds, only slowly hydrolyses

alanine and glycine bonds but rapidly hydrolyses tryptophan as well as leucine, methionine and phenylalanine bonds (McDonald *et al.* 1964; Behal *et al.* 1966; Hanson *et al.* 1967; Himmelhoch 1970).

The data relating to the hydrolysis of various peptide and peptide amide substrates demonstrate that these *T. bisselliella* arylamidases are true aminopeptidases which hydrolyse the *N*-terminal peptide bonds in dipeptides, dipeptide amides, tripeptides and oligopeptides. The data confirm their ability to hydrolyse bonds involving *N*-terminal proline. However, when proline is the penultimate *N*-terminal residue the rate of hydrolysis is considerably lower. *N*-Terminal and penultimate *N*-terminal D-amino acid residues can also be cleaved, but at a relatively low rate. These bonds are not cleaved by the other *T. bisselliella* aminopeptidases (Ward 1975*b*, 1975*c*).

Aminopeptidases have not been widely studied in invertebrates. They have been reported in the digestive diverticulae of the mollusc *Chlamys hericus* (Reid and Rauchert 1970) and in the pyloric caeca of several starfish (Elyakova and Kozlovskaya 1975; Okabe and Noma 1975). The aminopeptidases from *T. bisselliella* larvae are all associated with the digestive tract (Ward 1975*a*) and, like the mammalian intestinal aminopeptidases (Gitler 1964; Kim *et al.* 1974), are probably associated with epithelial cells lining the gut and involved in the terminal stages of digestion.

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