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AMINOPEPTIDASES IN WEBBING CLOTHES MOTH LARVAE

PROPERTIES AND SPECIFICITIES OF THE ENZYMES OF INTERMEDIATE ELECTROPHORETIC MOBILITY

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Summary

The major group of aminopeptidases (EC 3.4.11.-), of intermediate electrophoretic mobility, from *Tineola bisselliella* larvae, have been fractionated into six bands by preparative polyacrylamide gel electrophoresis and the properties of these fractions investigated. They resemble each other in their pH optima of 8.2, their molecular weight of 240 000, their responses to various active site inhibitors and metal cations, and their specificities towards seventeen L-amino-acyl-β-naphthylamide substrates. The derivatives of methionine, leucine, alanine, lysine, arginine and glutamic acid were those most rapidly hydrolysed. They appear to be true aminopeptidases hydrolysing amino acid amides, dipeptides and oligopeptides from the N-terminal end.

Introduction

In a previous report [1] the larvae of the webbing clothes moth *Tineola bisselliella*, were shown to contain multiple aminopeptidase (EC 3.4.11.-) bands. These aminopeptidases were shown to be associated with the digestive tract and all multiple forms were present in extracts of individual larva.

The major aminopeptidase activity was associated with a group of closely-running bands with electrophoretic mobilities in 7.5% acrylamide gels of 0.19—0.25, relative to bromphenol blue. This group of bands was separated from the other aminopeptidases and proteolytic enzymes of these larvae as previously described [1].

In this report, the further fractionation of this major group of aminopep-

Abbreviations: Diol buffer, 2-amino-2-methyl-1,3-propandiol buffer; DFP, diisopropylfluorophosphate; NA, β-naphthylamide derivative.

tidase bands on discontinuous acrylamide gels is described, along with observations on some of their properties, including their cleavage specificity with L-aminoacyl-β-naphthylamide and peptide substrates.

Materials and Methods

Chemicals

Except where otherwise stated all peptide substrates contained only L-amino acids. The L-aminoacyl-β-naphthylamides of alanine, methionine, isoleucine, threonine and tryptophan and the peptide substrates, Leu-Ala, Leu-Leu, Ala-Ala-Ala, Ala-Gly-Gly and Ala-Phe-Gly were from Cyclo Chemical Corporation. The L-aminoacyl-β-naphthylamides of glycine, valine, leucine, phenylalanine, tyrosine, histidine, serine, lysine, arginine, glutamic acid, aspartic acid and proline and the peptide substrates Met-Ala-Ser and Leu-amide were from Schwarz-Mann Research Laboratories. Leu-Pro, Leu-Gly-amide, L-Leu-D-Leu, D-Leu-L-Leu and Ala-Leu were obtained from Bachem Fine Chemicals. Leu-Gly was from Calbiochem. Leu-Phe, Leu-Ile, Leu-Leu-amide, Leu-Gly-Val, Leu-Gly-Leu, Leu-Thr-Gly, Leu-Gly-Ala-Ala-Ala, Leu-Leu-Leu-Leu-Leu, Ala-Gly-Phe, Ala-Gly-Pro-Leu and Ala-Gly-Phe-Gly-Gly were kindly prepared by Dr. F.H.C. Stewart of this laboratory. Bovine serum albumin was from Sigma Chemical Co.; fast garnet GBC, from G.T. Gurr Ltd., England and DFP from Aldrich Chemical Co.

Enzyme

The starting material used in this study was fraction Cl, obtained as described previously [1] and stored at −20° C in 50% glycerol. This fraction was free of any carboxypeptidase and proteinase activity.

Enzyme assays

Assays for aminopeptidase activity were as described previously [1].

Acrylamide gel electrophoresis

Electrophoresis was carried out at 5° C as described by Davis [2], but with a bisacrylamide-acrylamide ratio of 5% for minimum pore size [3] and with no sample gel. Preparative gels were run in slabs 120 mm long by 70 mm wide and 3 mm thick in the multicell Gradipore electrophoresis apparatus [4]. The preparative gels contained a 10 mm band of stacking gel, a 10 mm band of 7.5% running gel and finally a 100 mm long section of 4.5% running gel. Aminopeptidases were located in side strips as previously described [1] and the gel containing each active band cut out. These gel sections were then macerated by extruding through an 18 gauge needle and each aminopeptidase recovered electrophoretically in special cells fitted with collecting tubes sealed off with dialysis membrane.

Enzyme kinetic determinations with β-naphthylamide substrates

The kinetic parameters K_m and V for the hydrolysis of L-aminoacyl-β-naphthylamides were determined by the method of Lee and Wilson [5]. 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 values obtained from these three regression analyses were averaged. The initial substrate concentrations employed were 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 · 10^{-3} M for alanyl, leucyl, lysyl and arginyl-β-naphthylamides; 0.1, 0.2, 0.5, 1.0 and 2.0 · 10^{-3} M for phenylalanyl, tyrosyl, tryptophanyl and isoleucyl-β-naphthylamides, and 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 · 10^{-3} M for all other β-naphthylamide substrates. To overcome solubility problems, dimethyl sulphoxide was present at a final concentration (v/v) of 5%, 15%, 20% and 20% when isoleucyl, tryptophanyl, phenylalanyl or tyrosyl-β-naphthylamides respectively were being hydrolysed. Reaction conditions were 37°C and pH 8.5 with an incubation time of 60 min, the β-naphthylamine released being detected as previously described [1].

Hydrolysis of peptide substrates

The hydrolysis of peptide substrates by the purified aminopeptidase in fraction Clf, was carried out at 37°C in 0.02 M Diol buffer pH 8.0. The reaction mixture (0.22 ml) contained 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 [6,7], or quantitatively by amino-acid analysis on a Beckman Spinco amino-acid analyzer.

Protein determination

Protein was estimated by the method of Lowry et al. [8] with bovine serum albumin as standard.

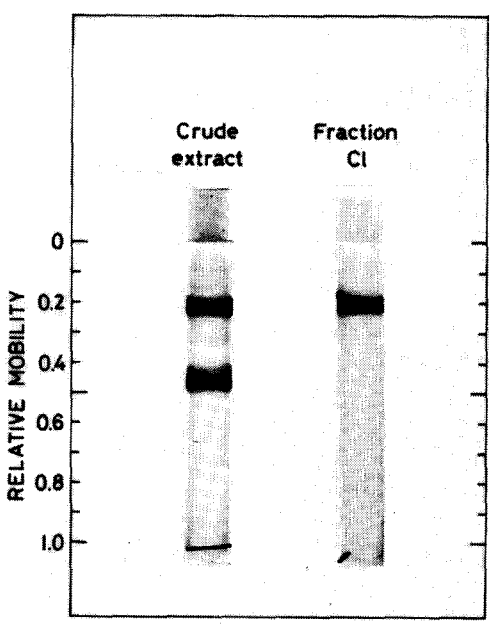

Fig. 1. Aminopeptidase bands in original crude extracts and in fraction Cl. Electrophoresis in 7.5% running gels and the detection of aminopeptidase bands by incubating at pH 9.0 for 5 min at room temperature with 0.5 mM Leu-β-naphthylamide and staining for 15 min with fast garnet GBC were as described previously [1]. Migration is towards the anode and mobility is expressed relative to that of bromphenol blue.

Results

Separation of aminopeptidase components in fraction Cl

The aminopeptidase components in fraction Cl (obtained as previously described [1]), were resolved by preparative acrylamide-gel electrophoresis. Although they have very similar electrophoretic mobilities (Fig. 1), improved separation was obtained by using a running gel which was discontinuous with respect to acrylamide concentration (7.5% layer on top of 4.5% gel), by employing long gel slabs (120 mm) and by electrophoresing for an additional time period after the bromphenol blue tracker dye had reached the bottom of the gel. Using such gels the aminopeptidase activity in fraction Cl was separated into six distinct bands which have been isolated and termed Cla to Clf (Fig. 2).

Properties of aminopeptidase components

pH Optima. All six fractions, Cla to Clf, showed almost identical pH-activity profiles with leucine-β-naphthylamide as substrate. The pH optimum for each enzyme was at pH 8.4, with no activity below pH 5.0 (Fig. 3).

Inhibitors. The effect of several inhibitors on the activity of the Cl amino-

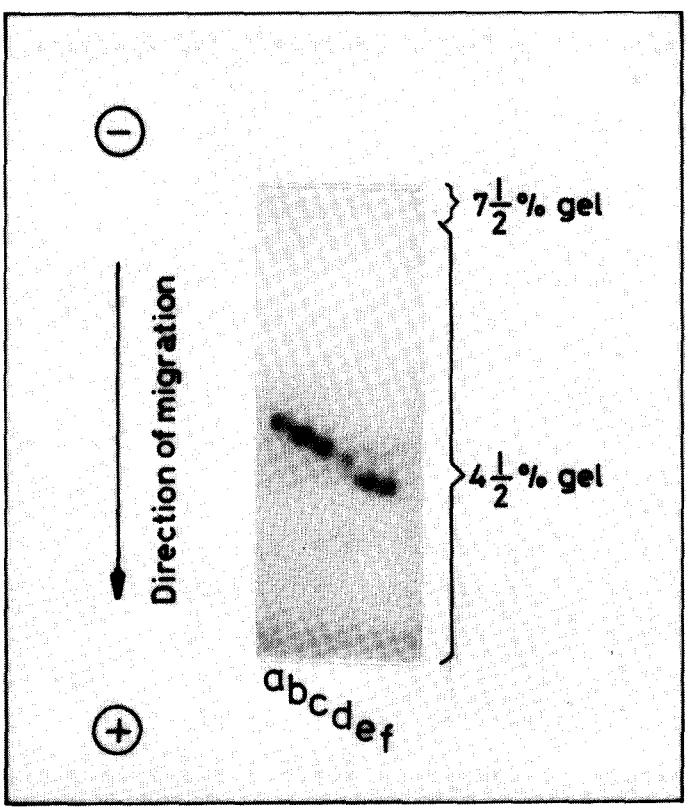

Fig. 2. Aminopeptidase bands in fractions Cla to Clf. Electrophoresis was as described in Methods. Band detection was as described in legend to Fig. 1.

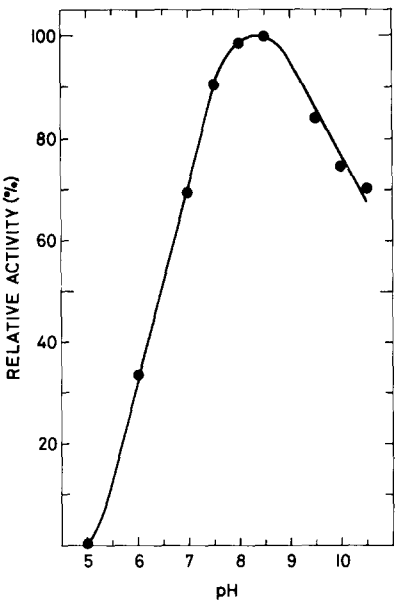

Fig. 3. Effect of pH on the hydrolysis of leycine-β-naphthylamide by the aminopeptidases in fractions Cla to Clf. Buffers used at 0.05 M were: sodium acetate/acetic acid pH 5.0; sodium phosphate pH 6.0 and 7.0; Diol buffer pH 8.0—10.5. Points plotted are the average values obtained for all six fractions.

peptidases is summarized in Table I. As reported earlier for the total aminopeptidase activity on crude extracts [1] *p*-chloromercuribenzoate, iodoacetate, DFP and EDTA had no effect on the activity of the purified *T. bisselliella* aminopeptidases, and 1 : 10 phenanthroline only partially inhibited these activities. Of the metal cations tested, only zinc, cupric and mercuric ions produced inhibition.

TABLE I

EFFECT OF INHIBITORS ON Cl AMINOPEPTIDASES

Enzyme was pre-incubated with inhibitor for 30 min at 20°C and pH 8.5, then Leu-β-naphthylamide derivative added and the residual aminopeptidase activity determined as described previously [1]. All inhibitors were present at a final concentration of $2 \cdot 10^{-3}$ M except for *p*-chloromercuribanzoate, which was $0.2 \cdot 10^{-3}$ M.

Inhibitor	Effect on aminopeptidase
1 : 10 Phenanthroline	51% inhibition
EDTA	No effect
p-Chloromercuribenzoate	No effect
Iodoacetate	No effect
DFP	No effect
$CaCl_2$	No effect
$MgCl_2$	No effect
$FeSO_4$	4% inhibition
$CoCl_3$	No effect
$ZnCl_2$	76% inhibition
$CuCl_2$	99% inhibition
$HgCl_2$	100% inhibition

TABLE II

KINETIC CONSTANTS FOR HYDROLYSIS OF AMINOACYL-β-NAPHTHYLAMIDES BY AMINOPEPTIDASES Cla-f

V values are expressed relative to those obtained for the hydrolysis of Met-NA by fractions Cla to Clf. These maximum values (expressed as μmol/60 min/mg protein) were as follows: Cla, 62.0; Clb, 125; Clc, 159; Cld, 96.3; Cle, 196 and Clf, 408. Reaction conditions were 37°C, pH 8.5 and incubation time 60 min. The parameters K_m and *V* were determined as described in the methods.

Amino acid β-naph-thylamide	K_m						*V*						V/K_m					
	Cla	Clb	Clc	Cld	Cle	Clf	Cla	Clb	Clc	Cld	Cle	Clf	Cla	Clb	Clc	Cld	Cle	Clf
Methionyl	0.27	0.20	0.25	0.27	0.24	0.18	100	100	100	100	100	100	100	100	100	100	100	100
Leucyl	0.26	0.20	0.44	0.39	0.33	0.21	73.7	76.5	95.9	87.9	76.2	81.7	74.5	76.2	54.5	60.1	56.1	68
Alanyl	0.52	0.47	0.51	0.52	0.64	0.46	61.3	72.1	65.4	68.5	68.6	74.8	31.6	30.3	32.0	34.9	26.0	28.6
Lysyl	0.35	0.28	0.45	0.34	0.39	0.24	30.4	26.0	37.3	25.6	33.5	24.1	23.2	18.4	20.7	24.4	20.8	17.6
Arginyl	0.23	0.19	0.16	0.24	0.25	0.27	20.3	17.4	17.8	19.1	20.8	22.4	24.1	18.4	27.6	21.6	20.3	15.2
α-Glutamyl	2.77	2.19	2.91	3.42	6.26	3.14	15.1	14.4	15.5	17.3	25.6	17.2	1.46	1.80	1.33	1.34	0.99	0.96
Glycyl	0.81	0.63	1.19	0.78	1.06	1.01	5.21	4.37	6.11	4.97	5.34	5.04	1.72	1.31	1.28	1.70	1.22	0.87
Isoleucyl	N.H.*	0.77	0.30	N.H.	0.63	0.57	N.H.	2.28	1.77	N.H.	2.28	1.96	N.H.	0.59	1.47	N.H.	0.88	0.60
Valyl	0.30	0.71	0.75	0.75	0.95	1.62	3.07	3.57	3.95	3.77	4.03	3.92	2.78	0.99	1.33	1.76	1.03	0.44
Threonyl	N.H.	2.20	0.97	0.96	1.55	1.07	N.H.	2.77	1.66	1.82	2.76	2.27	N.H.	0.24	0.43	0.51	0.43	0.37
Phenylalanyl	N.H.	0.88	N.H.	N.H.	1.68	1.31	N.H.	1.13	N.H.	N.H.	1.45	0.70	N.H.	0.25	N.H.	N.H.	0.20	0.09
Tryptophanyl, Tyrosyl, Seryl, Histidyl, α-Aspartyl, Prolyl	Not hydrolysed significantly.																	

* N.H. = not hydrolysed significantly.

TABLE III

HYDROLYSIS RATES OF PEPTIDE SUBSTRATES BY AMINOPEPTIDASE Clf

Values indicated are the percentage hydrolysis of the N-terminal peptide bond in 4 h at 37°C by 0.5 μg of Clf aminopeptidase. Assay methods were as described in text.

Dipeptides		Tripeptides		Other peptides	
Leu-Gly	26.2	Leu-Gly-Val	100	Leu-NH_2	3.9
Leu-Ala	29.3	Leu-Gly-Leu	94.1	Leu-Leu-NH_2	66.3
Leu-Leu	32.9	Leu-Thr-Gly	91.0	Leu-Gly-NH_2	5.6
Leu-Ile	21.4	Met-Ala-Ser	81.0	Leu-Gly-Ala-Ala-Ala	57.9
Leu-Phe	44.6	Ala-Gly-Phe	91.9	Leu-Leu-Leu-Leu-Leu	6.1
Leu-Pro	0	Ala-Gly-Leu	73.6	Ala-Gly-Phe-Gly-Gly	28.3
L-Leu-D-Leu	0	Ala-Phe-Gly	39.2	Ala-Gly-Pro-Leu	25.8
D-Leu-L-Leu	0	Ala-Ala-Ala	34.6		
		Ala-Gly-Gly	11.8		

Molecular weight. During preliminary purification [1] all components in fraction Cl co-eluted as a single peak on Sephadex G-200 and acrylamide gels of fractions taken across this peak showed that no resolution of components had occurred. When run on a Sephadex G-200 column calibrated with γ-globulin, bovine serum albumin dimer and monomer, human transferring, ovalbumin dimer and monomer, carbonic anhydrase, myoglobin and cytochrome *c* [9], the Cl aminopeptidase eluted at a position corresponding to a molecular weight of 240 000.

Hydrolysis of β-naphthylamide substrates. The kinetic parameters determined for the hydrolysis of the β-naphthylamides of L-amino acids by aminopeptidase Cla-Clf are shown in Table II. All six enzymes show very similar specificities with these substrates. The low activities observed with the isoleucyl-, tryptophanyl-, tyrosyl- and phenylalanyl-β-naphthylamides are not due solely to the presence of dimethyl sulfoxide in the reaction mixtures. When leucyl-β-naphthylamide was used as substrate with dimethyl sulfoxide present at final concentrations (v/v) of 5%, 10% and 20%, enzyme activity was only partially inhibited (2%, 9% and 18% respectively).

Hydrolysis of peptide substrates. The relative rates of hydrolysis of several dipeptide, tripeptide and oligopeptide substrates by aminopeptidase Clf are summarized in Table III. The most rapid rates of hydrolysis were obtained with tripeptide substrates.

Discussion

The major aminopeptidase activity associated with the group of bands with relative electrophoretic mobilities between 0.19 and 0.25 has been fractionated into six components, which are very similar to each other with respect to pH optimum, response to inhibitors, molecular weight and cleavage specificity on L-aminoacyl-β-naphthylamide substrates.

They differ from the vertebrate leucine aminopeptidases in their lack of complete inhibition by both EDTA and 1 : 10 phenanthroline [10], their lower molecular weights [10,11—13] and their slightly different specificity requirements. Like the vertebrate enzyme, they hydrolyse leucyl-, methionyl-,

lysyl- and arginyl-β-naphthylamides readily, but differ from the vertebrate enzymes in their low hydrolysis of tryptophanyl-β-naphthylamide and their rapid hydrolysis of the alanyl substrate [14,15]. Since both positively charged and negatively charged side chain derivatives can be hydrolysed by *T. bisselliella* aminopeptidases, along with the uncharged aliphatic residues, the binding of substrate to enzyme must be predominantly hydrophobic [16]. The binding site for the substrate N-terminal amino acid residue must also have some space limitations since hydrolysis is not favoured if there is branching (valine, isoleucine), polar groups (serine, threonine, aspartic acid) or bulky aromatic rings (tryptophan, tyrosine, phenylalanine, histidine) at the side chain β-carbon atom, or if the aminoacyl residue is proline.

These specificity requirements are in general confirmed by the data on the hydrolysis of peptide substrates. In addition the latter demonstrates that the N-terminal and the penultimate N-terminal residue cannot be of the D-configuration, nor can they be proline. A free carboxy group is not required for the hydrolysis of dipeptide substrates, but leucine amide is a very poor substrate for these insect aminopeptidases [17].

There has been considerable discussion as to whether those enzymes hydrolysing arylamide substrates should be referred to as aminopeptidases, [17,18]. However the data presented in this report indicate that the arylamidases from *T. bisselliella* larvae are true aminopeptidases, capable of hydrolysing di, tri- and oligopeptide substrates sequentially from the N-terminal end. They are associated with the digestive tract of these larvae [1] and are probably involved in the terminal stages of digestion since they have negligible activity on large protein substrates [1], and showed maximal activity with tripeptide substrates. So far no attempt has been made to determine whether these aminopeptidases from *T. bisselliella* are secreted into the lumen, or whether, as is the case with the mammalian intestinal aminopeptidases [19,20], they are associated with the epithelial cells [21] lining the digestive tract.

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