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PURIFICATION AND PROPERTIES OF HUMAN INTESTINE ALANINE AMINOPEPTIDASE

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Summary

Human intestinal alanine aminopeptidase has been purified to greater than 90% homogeneity. The enzyme was released from mucosal cell membranes by Triton X-100 treatment. The native enzyme had a molecular weight of 206 000 in dilute buffer and 108 000 in the presence of sodium dodecyl sulfate. The enzyme was inhibited by chelators suggesting the presence of a metal ion in the enzyme. The most potent chelator inhibitor tested, *o*-phenanthroline, gave mixed kinetics (K_i = 67 μM). Activity was restored by removal of the chelator. The enzyme was inhibited competitively by amino acids having hydrophobic side chains such as L-phenylalanine (K_i = 0.67 mM). Puromycin and methicillin also inhibited the enzyme in the competitive (K_i = 12.5 μM) and noncompetitive (K_i = 4.6 mM) manner, respectively. Kinetic analysis of several amino acid β-naphthylamides as substrates demonstrated the preference for substrates having hydrophobic or basic amino terminal residues with no β-branching. L-Methionyl-β-naphthylamide was the most tightly bound while L-alanyl-β-naphthylamide was the most rapidly hydrolyzed.

Introduction

Alanine aminopeptidases have bee isolated from several tissues from human [1—5] as well as other sources, [6—9] and are distinct from classical leucine aminopeptidases (α-aminoacyl-peptide hydrolase (cytosol), EC 3.4.11.1) [10]

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by virtue of differences in substrate specificity and metal ion requirements. Alanine aminopeptidases readily hydrolyze peptides and aminoacyl β-naphthylamides. Peptides and amides with L-alanine as the N-terminal residue are the most rapidly hydrolyzed. Alanine aminopeptidases are stimulated by cobalt ion. Two of these aminopeptidases, from human liver and kidney, have previously been purified to homogeneity [1,3] and some of their properties have been reported [11—14]. Both are zinc metalloproteins having 2 mol of zinc for each native molecule with a molecular weight of approx. 235 000. Also both are glycoproteins containing sialic acid.

In this paper the purification of human intestinal alanine aminopeptidase is reported together with certain kinetic and chemical properties. The existance of highly purified preparations makes possible the comparison of the physical, chemical and enzymatic properties of this enzyme with aminpeptidases isolated from liver and kidney in an effort to elucidate their roles in metabolism.

Materials and Methods

Materials. Common laboratory reagents, buffers and salts were purchased from usual sources at the highest grade available. Amino acid amides were purchased from Sigma Chemical Co., St. Louis, MO. Puromycin was a product of Nutritional Biochemicals, Cleveland, OH. Chelators were supplied by Eastman Kodak Co., Rochester, NY. Reagents for polyacrylamide disc gel electrophoresis and DEAE Bio-Gel-A (DEAE-agarose) were purchased from Bio-Rad Laboratories, Richmond, CA. Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) and Sephadex G-200 were supplied by Sigma Chemical Co. Water was passed through two high purity deionization columns containing activated charcoal. Methicillin was a gift of Dr. W.L. Starnes.

Assays. There were two assays of aminopeptidase activity. In the first, the amount of β-naphthylamine released during a suitable incubation was measured by diazotization and coupling to *N*-(1-naphthyl)-ethylene diamine. The absorbance of the chromophore produced was measured at 580 nm. The details have been previously published [14]. In the second, the rate of release of β-naphthylamine (ϵ = 1780, [15]) or *p*-nitroaniline (ϵ = 9600, [16]) was measured as the change in absorbance at 340 nm or 405 nm, respectively, as described [14]. One unit of enzyme activity corresponds to the release of 1 μmol of aromatic amine per min.

Kinetic constants K_m, V, and K_i were determined from plots of v vs. v/s as described by Webb [17]. Five to eight different substrate concentrations (s) were used in each experiment. V is reported as units of enzyme activity per A_{280} unit. All lines were fitted to the data by linear regression analysis. All lines had correlation coefficients of 0.95 or greater. The error estimates were based on the standard error in the abscissa and ordinate intercepts.

Protein determinations were made either by the biuret procedure [18] using serum albumin as standard or from the absorbance at 280 nm.

Neuraminidase treatment. 10 μg aminopeptidase was treated with 1 μg neuraminidase for 24 h at 37°C in 0.1 ml 0.01 M potassium phosphate (pH 7.0). Aliquots were then subjected to electrophoresis under non-denaturing conditions.

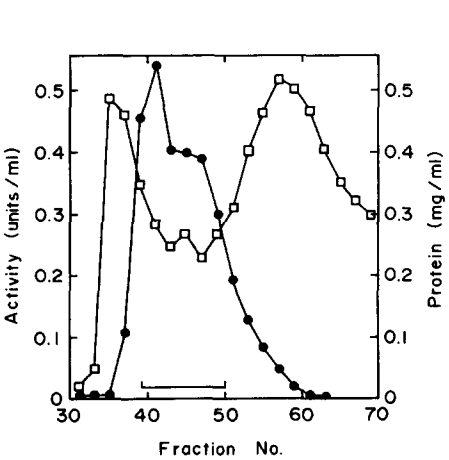
Gel electrophoresis. Polyacrylamide electrophoresis in 7% gels was performed under non-denaturing conditions according to the procedure of Davis [19] and under denaturing conditions in the presence of sodium dodecyl sulfate (SDS) according to Weber and Osborn [20]. Gels, fixed in 5% trichloroacetic acid, were stained in 0.02% Coomassie blue for 30 min at room temperature and destained in 7% acetic acid.

Results and Discussion

Purification of human intestinal alanine aminopeptidase

Human intestine alanine aminopeptidase has been isolated by Triton X-100 solubilization from intestinal mucosa cell membranes followed by chromatography on Sephadex G-200 and DEAE-agarose. This enzyme accounts for most if not all the activity against L-Ala-*p*-nitroanilide found in the intestinal mucosa.

Frozen, human small intestine, obtained at autopsy, was freed of as much surrounding fat and mesentary as possible. The partially thawed intestine was cut open and washed with distilled water. The mucosa was removed from the inside wall with a metal spatula. This material was diluted to 2 ml/g intestine with 0.01 M potassium phosphate (pH 6.9), and was homogenized using a Polytron homogenizer. This and all subsequent steps were performed at 0—5°C.



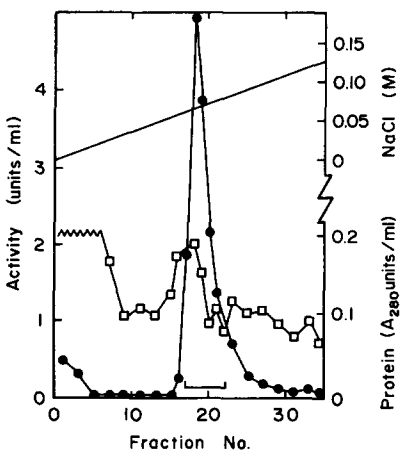


Fig. 1. Gel filtration of aminopeptidase on Sephadex G-200. The dissolved ammonium sulfate precipitate was applied to the surface of a 5 × 80 cm column of Sephadex G-200 equilibrated and developed in a buffer containing 0.5 M NaCl, 0.1 M sodium borate, pH 8, and 1% Triton X-100. Fractions of 20 ml each were collected at a flow rate of 30 ml/h. Fractions marked by a bracket were combined, concentrated by pressure dialysis and dialyzed against 0.01 M Tris-HCl (pH 8.1). Protein, measured by the biuret procedure (□———□); aminopeptidase activity (●———●).

Fig. 2. Chromatography of aminopeptidase on DEAE-agarose with a 400-ml NaCl gradient (0—0.20 M). The dialized, pooled fractions from the Sephadex G-200 column were applied to a 0.9 × 25 cm column of DEAE-agarose equilibrated and developed in a buffer containing 0.01 M Tris-HCl (pH 8.1). After the enzyme solution was placed in the column, the column was washed with 100 ml of buffer. Fractions of 12 ml each were collected at a flow rate of 10.5 ml/h. Those marked by a bracket were combined. Protein (□———□); aminopeptidase activity (●———●); NaCl concentration (———).

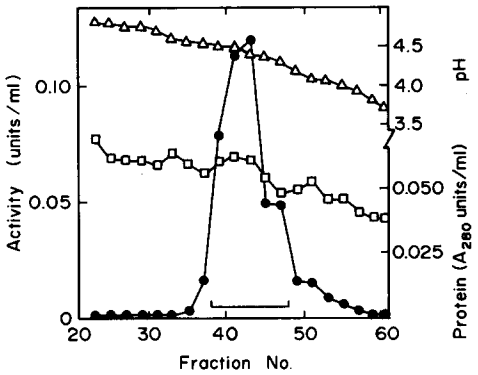


Fig. 3. Chromatography of aminopeptidase on DEAE-agarose with a pH gradient. The dialyzed, pooled, fractions from the DEAE-agarose column with the NaCl gradient was applied to a 0.9 × 25 cm column of DEAE-agarose equilibrated in 0.01 M potassium phosphate buffer, pH 8.0, at 4° C. After the enzyme was applied to the column, the column was washed with 200 ml of 0.01 M acetate buffer (pH 5.0). The column was developed with a 0.01 M acetate pH gradient (pH 5.0—3.5), 400 ml total vol. Fractions of 10 ml each were collected at a flow rate of 10.5 ml/h. Those marked by a bracket were combined. Protein (□———□); aminopeptidase activity (●———●); pH (△———△).

After centrifugation, the extract was fractionated with ammonium sulfate between 0.35 and 0.65 saturation in the presence of 1% (v/v) Triton X-100. The sediment was dissolved in 35 ml of 0.01 M potassium phosphate (pH 6.9) and chromatographed on Sephadex G-200 as shown in Fig. 1. The activity was then chromatographed twice on DEAE-agarose using a NaCl gradient (Fig. 2) and a pH gradient (Fig. 3), as summarized in Table I.

The purified aminopeptidase was subjected to polyacrylamide gel electrophoresis in the presence and absence of SDS. Scans of the gels indicated greater than 90% purity. In none of the gels was there any major contaminating protein band.

This procedure used a nonionic detergent for solubilization of the enzyme. Since the detergent was omitted from the DEAE-agarose steps, it is possible that the enzyme was isolated in an altered form which resulted from mucosal endoprotease action.

TABLE I

SUMMARY OF THE PURIFICATION OF INTESTINAL AMINOPEPTIDASE

	Step	Total protein (A_{280} units)	Total enzyme activity (units)
1	Intestinal wall extract	7752	490
2	Ammonium sulfate fractionation in Triton X-100	134 *	311
3	Sephadex G-200 chromatography in Triton X-100	53 *	229
4	DEAE-agarose chromatography (NaCl gradient)	6.5	47
5	DEAE-agarose chromatography (pH gradient)	1.3	25

* Protein was measured by the biuret method with serum albumin as standard.

Molecular weight determination on SDS-gels and Sephadex G-200 column chromatography

The molecular weight of the denatured enzyme was determined by electrophoresis on 7.5% polyacrylamide gels in SDS. Only one protein band was observed on these gels. Ovalbumin and serum albumin monomer and dimer bands were used as standards. A standard curve having a correlation coefficient of greater than 0.99 was constructed from known molecular weights and R_F values of the standard proteins. The molecular weight of the aminopeptidase was 108 000 ± 4000.

The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-200 in 0.5 M NaCl/0.1 M sodium borate, pH 8.0, in the absence of detergents. Calculation of the molecular weight from the void and elution volumes by the procedure of Determann and Michel [21] gave a molecular weight of 206 000 ± 10 000.

Evidently this enzyme like the liver form is a dimer of similar or identical subunits. However, the molecular weight of the intestinal enzyme is less than that of the liver enzyme [22], which has a molecular weight of 242 000. The molecular weights of alanine aminopeptidases from the intestine of other species are all higher in molecular weight than the human intestinal form [6,8,9].

Neuraminidase treatment

Human alanine aminopeptidases from liver and kidney contain sialic acid [22,3]. The intestinal enzyme appears to lack a substantial amount of sialic acid, treatment with neuraminidase failed to alter its electrophoretic mobility on disc gels under native conditions, $R_F = 0.18$. The identical treatment of the human liver enzyme lowered its mobility from $R_F = 0.31$ to 0.22. This enzyme contains 4% sialic acid [22]. Even after removal of sialic acid, the mobility of the liver enzyme was different from that of the intestinal enzyme showing that other differences still existed. Insufficient material was available for direct chemical analysis.

Energy of activation

The effect of incubation temperature on the reaction rate was determined between 3.9 and 55.8°C. Data obtained were plotted in an Arrhenius plot shown in Fig. 4. The data show a sharp bend in the curve at 36.1°C. This suggests a change in the nature of the rate-determining step which may be related to a thermally induced enzyme structural alteration. The energy of activation for the hydrolysis of L-Leu-*p*-nitroanilide calculated from the slope of the line between 3.9 and 36.1°C is 18.6 cal/mol and from 36.1 to 55.8°C the value is 8.5 cal/mol. The K_m did not change in value in the temperature range studied.

Substrate specificity

The substrate specificity of the intestinal aminopeptidase for the N-terminal residue was studied with 15 aminoacyl-β-naphthylamides and aminoacyl-*p*-nitroanilides. K_m, V, and V/K_m values for the appropriate substrates are recorded in Table II. Residues with straight aliphatic chains, aromatic groups, and γ-branched aliphatic chains had high V values. Low V values were associ-

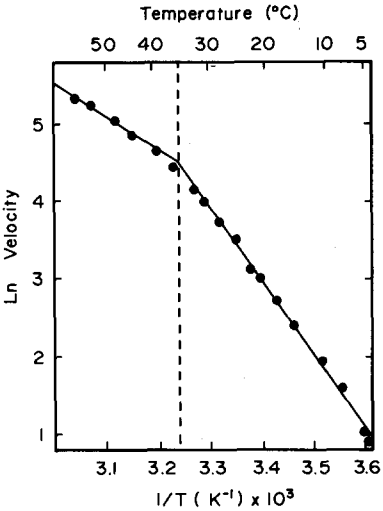


Fig. 4. Energy of activation for the enzyme-catalyzed reaction. The rate of *p*-nitroaniline release was monitored at 405 nm using 2 mM Leu-*p*-nitroanilide as substrate. The temperature of the reaction ranged from 3.9 to 55.8° C. The bend occurs at 36.1° C.

ated with residues with β-branched side chains. Low K_m values were characteristic of residues with non-polar straight chains or basic side chains, regardless of branching. The V/K_m ratio, a measure of substrate effectiveness, was highest for substrates with straight chain aliphatic amino acids and lowest for those containing polar amino acids. This enzyme had a pH optimum near pH 7.0 and was stimulated by Co^{2+} approximately 2-fold.

TABLE II

KINETIC CONSTANTS FOR INTESTINAL AMINOPEPTIDASE AND SEVERAL SUBSTRATES

Aminoacyl-β-naphthylamide	K_m (mM)	V	$\frac{V}{K_m}$
Ala	0.0913 ± 0.0035	5.02 ± 0.17	55.0
Phe	0.202 ± 0.009	3.69 ± 0.15	18.3
Met	0.0204 ± 0.0009	2.29 ± 0.11	112
Leu	0.0673 ± 0.0025	2.14 ± 0.08	31.8
Ile	0.0921 ± 0.0030	0.221 ± 0.007	2.40
Val	0.0703 ± 0.0036	0.130 ± 0.007	1.85
Arg	0.0880 ± 0.0036	0.845 ± 0.034	9.60
His	0.367 ± 0.009	0.639 ± 0.017	1.74
Lys	0.0245 ± 0.0028	0.384 ± 0.045	15.7
Asn	0.785 ± 0.007	0.676 ± 0.006	0.861
Glu	2.33 ± 0.05	0.607 ± 0.012	0.260
Thr	0.617 ± 0.025	0.628 ± 0.026	1.02
Ser	0.108 ± 0.005	0.281 ± 0.015	2.60
Aminoacyl-*p*-nitroanilide			
Leu	0.647 ± 0.026	1.78 ± 0.07	2.75
Gly	0.740 ± 0.024	0.288 ± 0.009	0.389

Chelator inhibition

Inhibition by various chelators suggests that the enzyme is a metalloprotein. At 24°C, inhibition by the chelator *o*-phenanthroline gave a mixed inhibition pattern. A K_i value of 67 ± 30 μM was calculated. The inhibition by *o*-phenanthroline was reversed by dilution or by dialysis. Metal analysis was not possible because of insufficient material.

A comparison of different chelators as inhibitors of the intestinal aminopeptidase was made. These measurements were made at 40°C in order to eliminate the time lag in inhibition observed at lower temperatures. The more potent chelators, *o*-phenanthroline (0.4 mM), 8-hydroxyquinoline (3.0 mM), and 2,2'-bipyridine (5.0 mM), which are all structurally similar, inhibited 99%, 91% and 90%, respectively. A less effective inhibitor, EDTA (1.0 mM), only inhibited 14%. This is in contrast with the liver aminopeotidase which was strongly inhibited by 1 μM EDTA [13].

Inhibition by amino acids and antibiotics

The amino acid inhibition of the intestinal aminopeptidase by nonpolar amino acids gave competitive inhibition in some cases and mixed inhibition in others. The K_i value for L-phenylalanine inhibition was 0.67 ± 0.11 mM. Human liver and kidney aminopeptidases are also inhibited by these amino acids with similar values of K_i [1].

Puromycin, a peptide antibiotic having a free amino terminus also inhibited the intestinal aminopeptidase competitively with a K_i value of 12.5 μM. Similar results were obtained with the liver aminopeptidase [23].

It has been shown that penicillin antibiotics inhibit alanine aminopeptidases, from liver and kidney (Starnes, W.L., unpublished data). Methicillin inhibited the intestinal aminopeptidase in the noncompetitive manner. A K_i value of 4.61 ± 0.95 mM was calculated.

The role of human tissue alanine aminopeptidases is not known, however the great similarities in substrate specificity and molecular properties suggest a similar role in each tissue. But in the case of the intestinal form, it is tempting to propose a role in amino acid transport or in digestion. It is known that amino acids are transported more rapidly when presented as peptides [24], even though only free amino acids are found in the blood stream. An enzyme having similar properties has been found by Maroux and Louvard [25] to be located on the intestinal surface anchored by a short hydrophobic peptide giving credence to its involvement in digestion and/or transport.

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