ISOLATION AND PARTIAL CHARACTERIZATION OF BOVINE LIVER AMINOPEPTIDASE B

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Aminopeptidase B, specifically hydrolyzing the L-lysine and L-arginine derivatives of *p*-nitroaniline and β-naphthylamine, was isolated from bovine liver. A multistep purificaton procedure involving fractionation with ammonium sulfate, gel filtration on Sephadex, ion exchange chromatography on Ecteola-cellulose, and adsorption chromatography on hydroxylapatite, afforded an enzyme whose activity was approximately 240 times higher than the activity of the original material. The molecular weight of the enzyme determined by gel filtration on Sephadex G-200 was approximately 55 000. The Michaelis constant with respect to L-lysyl-*p*-nitroanilide was $1{\cdot}2 \cdot 10^{-3}$ mol/l.

The so far very little understood role of aminopeptidases in the degradation of various compounds of peptidic character has stimulated continuous interest in studies on these enzymes. The amino peptidases (also called sometimes arylamidases[1,5]) present in various animal tissues are inhomogeneous proteins of different specificity with respect to hydrolysis of β-naphthylamine and *p*-nitroaniline amino acid derivatives. The studies on the chromatographic and electrophoretic behavior of these enzymes from some bovine tissues, carried out in this Laboratory, have shown that the cytosol moiety of bovine liver homogenate contains, like the brown rat liver homogenate[2], at least two enzymatically different fractions showing a high activity when assayed with L-lysyl-*p*-nitroanilide as substrate[1,3]. The aim of this study has been to isolate and partially characterize the enzyme catalyzing the hydrolysis of chromogenic substrates (derived from *p*-nitroaniline and β-naphthylamine) whose N-terminal moiety is represented by either L-lysine or L-arginine. A similar enzyme named aminopeptidase B (EC 3.4.11.6) has been isolated from the liver of brown rat[4], man[5], and pig[6].

EXPERIMENTAL

Material

Aminopeptidase B was isolated from mixed samples of bovine liver of clinically healthy animals obtained from the slaughterhouse and stored at −20°C. The chromogenic substrates derived

from *p*-nitroaniline were synthesized by one of us (E.K.); the amino acid derivatives of β-naphthylamine were from Koch-Light Laboratories (Great Britain). A 0·01 mol/l aqueous solution of L-lysyl-*p*-nitroanilide was used throughout the purification procedure. Hydroxylapatite for chromatography was prepared according to Bailey[7], Sephadex G-200 was from Pharmacia, (Uppsala, Sweden), Ecteola-cellulose (capacity 0·5 mequiv/g) was from Balston (Great Britain), Tris-(hydroxymethyl)amino methane was a product of Loba-Chemie (Wien-Fischamend, Austria). Protein samples used as reference samples for the determination of molecular weight were twice recrystallized myoglobin (Calbiochem. Switzerland), human serum albumin (Fluka, Switzerland), and pig IgG globulin of Czechoslovak origin. The dialysis was effected in dialyzing tubing (Kalle, Wiesbaden-Biebrich, FRG) and the Sartorius (FRG) membrane filters 13 200 were used for ultrafiltration. The remaining chemicals used were Czechoslovak products of analytical purity.

Methods

A 30% bovine liver homogenate in precooled 0·1 mol/l Tris-HCl buffer, pH 7·1, was used to start with. The homogenization was effected in a blender for 60 s. The homogenate was centrifuged 60 min at 107 000*g* and 2°C in a preparative ultracentrifuge (VAC 60, Janetzki). The supernatant fraction was used for the isolation of the enzyme.

Determination of aminopeptidase activity: The enzymatic activity was tested with different *p*-nitroaniline derivatives using essentially the procedure described elsewhere[1]. The measurements were carried out by photometric determination at 400 nm of *p*-nitroaniline released after 60 min incubation of the enzyme with the substrate in 0·1 mol/l Tris-HCl buffer at pH 7·1 at 37°C. The kinetic characteristics of purified aminopeptidase B were determined by continuous measurement of the absorbance increase using a cell thermostated at 25°C. The absorbance values were read off at 1 min intervals. The hydrolysis of chromogenic substrates derived from β-naphthylamine was measured as follows: the incubation mixture contained 0·1 ml of the enzyme solution, 0·1 ml of the substrate solution (0·02 mol/l substrate in 0·1 mol/l Tris-HCl buffer containing 25% methylcellosolve), and 0·8 ml of 0·1 mol/l Tris-HCl buffer at pH 7·1. The reaction was discontinued by the addition of 1 ml of 40% trichloroacetic acid and the β-naphthylamine released was diazotized; after coupling the color intensity of the solution was determined colorimetrically at 560 nm. The incubation period and the temperature were the same as described above. The enzymatic activity with respect to both types of substrates is expressed in μmol of chromogene released per min per mg of protein. The concentration of *p*-nitroaniline and of β-naphthylamine was read off from the calibration curves.

Determination of proteins: The initial concentrations of protein in the liver homogenate and in the supernatant were determined by a microkjeldahl method. The protein content was assayed during the purification procedure by a modification of the method of Lowry[7] using the Folin–Ciocalteau reagent; the protein concentrations were read off a standard curve prepared with human serum albumin.

Salting-out by ammonium sulfate: The precipitation of proteins of the supernatant fraction (106 000*g*) of the liver homogenate was effected by the addition of crystalline ammonium sulfate at pH 7·1. The fraction precipitated at 50% saturation was allowed to stand 2 h at 4°C, then centrifuged, and the sediment dissolved in the corresponding buffer.

Gel filtration: The 106 000*g* supernatant of the liver homogenate was passed over a Sephadex G-200 (5 × 60 cm) column equilibrated in 0·1 mol/l Tris-HCl buffer at pH 7·1. The sample was displaced by the same buffer at a rate of 12–13 ml/h. The fractions were collected at 1 h intervals.

Chromatography on Ecteola-cellulose: A 2 × 25 cm column of Ecteola cellulose ET 41 was used. The resin was equilibrated in 5 mmol/l Tris-HCl buffer at pH 7·1. The sample was eluted by 100 ml of 0·20 mol/l NaCl and 100 ml of 0·35 mol/l NaCl in 5 mmol/l Tris-HCl buffer at pH 7·1; the flow rate was 24 ml/h.

Adsorption chromatography on hydroxylapatite: The fractions from ion exchange chromatography which showed enzymatic activity were concentrated and applied onto a 2 × 6 cm column of hydroxylapatite. The latter was mixed with cellulose powder (4 : 1) and resuspended in 5 mmol/l phosphate buffer at pH 7·0 before packing of the column. The column was eluted stepwise by phosphate buffers at pH 7·0 (0·02 mol/l, 0·05 mol/l, and 0·20 mol/l) at a rate of 21 ml/h.

Dialysis and concentration: Before every chromatographic separation the samples were dialyzed in dialyzing tubing for *c.* 24 h against an approximately 200-fold volume of the buffer in which the columns were equilibrated. The buffer was replaced twice. The concentration was effected in a glass apparatus (Sartorius SM 16 311) using the Sartorius membrane filter.

Molecular weight analysis: The molecular weight of the purified enzyme was assayed on a Sephadex G-200 column (2·5 × 90 cm) according to Andrews[8]. The column was calibrated with horse myoglobin, human serum albumin, and pig IgG globulin (concentration 5 mg/ml of 0·1 mol/l Tris-HCl buffer at pH 7·1.

RESULTS

The hydrolysis of some amino acid derivatives of *p*-nitroaniline by enzymes of liver homogenate and of the supernatant fraction (106 000*g*, 60 min) showed that the rate at which these substrates are cleaved depends on the character of the N-terminal amino acid residue (Table I). It is obvious from the Table that under the experimental conditions given a larger part of the substrate-hydrolyzing activity is present in the cytosol, the hydrolysis of L-lysyl-*p*-nitroanilide being the fastest one.

When we used ammonium sulfate precipitation, a method often used for the purification of enzymes, we observed that approximately 70% of the L-lysyl-*p*-nitroanilide-

TABLE I

Rate of hydrolysis of some chromogenic substrates by aminopeptidases (arylamidases) of bovine liver expressed in nmol/l of released *p*-nitroaniline/min/mg protein

Substrate	Homogenate	Supernatant
L-Leucyl-*p*-nitroanilide	84	137
L-Lysyl-*p*-nitroanilide	118	176
Glycyl-*p*-nitroanilide	40	96
α-L-glutamyl-*p*-nitroanilide	14	37
L-Phenylalanyl-*p*-nitroanilide	36	80
S-Methyl-L-cysteinyl-*p*-nitroanilide	27	46

-hydrolyzing fraction was precipitated at an ammonium sulfate saturation lower than 50% whereas at higher saturation degrees the precipitate was richer in enzymes hydrolyzing other amino acid derivatives of *p*-nitroaniline.

Hence, we subjected to gel filtration (Fig. 1) the fraction precipitated at 50% saturation of the liver homogenate with solid ammonium sulfate. The sediment obtained after centrifugation was dissolved in *c.* 35 ml of 0·1 mol/l Tris-HCl buffer at pH 7·1, applied onto a column of Sephadex G-200 (5 × 60 cm), and eluted by the same buffer at a rate of 12–13 ml/h.

The partly purified fraction was again salted out by saturation of the solution to 50% with ammonium sulfate, the sediment was dissolved in 5 mmol/l Tris-HCl buffer at pH 7·1, and was dialyzed against the same buffer. The dialyzed solution was concentrated to about 8 ml and was then applied onto a column (2 × 25 cm) of Ecteola-cellulose ET 41 equilibrated in 5 mmol/l Tris-HCl buffer at pH 7·1. The fractions were eluted by gradually increasing the concentration of NaCl in 5 mmol/l Tris-HCl buffer at pH 7·1 (0·2 mol/l and 0·35 mol/l NaCl) at a rate of 24 ml/h. The result of chromatography shows additional purification of amino peptidase B whose majority (second peak) is eluted from the column at higher concentrations of sodium chloride.

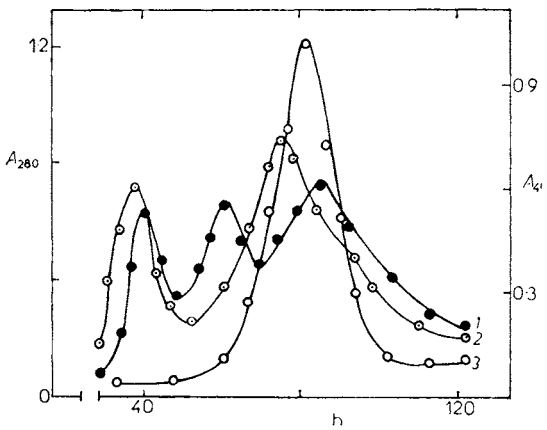

FIG. 1

Gel filtration of enzymes from the supernatant (106 000*g*) fraction of liver homogenate. After application of 35 ml of sample on the Sephadex G-200 column (3·1 × 60 cm) was eluted with 0·1 mol/l Tris-HCl buffer, pH 7·1, at a rate of 12–13 ml/h. The concentration of proteins was measured at 280 nm (curve *1*), the enzymatic activity was assayed by colorimetry at 400 nm of *p*-nitroaniline liberated. Activity assayed with L-Leucyl-*p*-nitroaniline (curve *2*) and with L-lysyl--*p*-nitroanilide (curve *3*). *n*, fraction number

The fractions showing the highest activity after chromatography on Ecteola-cellulose (second enzyme peak, Fig. 2) were pooled, dialyzed against 5 mmol/l phosphate buffer at pH 7·0, concentrated to *c.* 5 ml and applied to a column of hydroxylapatite (2 × 6 cm) equilibrated with 5 mmol/l phosphate buffer at pH 7·0. The sample was eluted by a stepwise gradient of increasing concentrations of phosphate buffers (0·02 mol/l, 0·05 mol/l, and 0·20 mol/l, pH 7·0) at a rate of 21 ml/h. The result of adsorption chromatography on hydroxylapatite, which represents the last step of the purification procedure, is shown in Fig. 3.

The entire procedure of purification of aminopeptidase B was carried out at 4–8°C and its results are summarized in Table II.

We were also able to determine some characteristics of purified aminopeptidase B. The enzyme (approximately 0·2 mg/ml) mixed with reference proteins was placed onto the Sephadex G-200 column and eluted by 0·1 mol/l Tris-HCl buffer. The molecular weight of aminopeptidase B determined from the plot of elution volume versus logarithm of molecular weight of reference proteins is approximately 55 000.

The Michaelis constant was determined by the measurement of the absorbance increase in one-min intervals during the hydrolysis of L-lysyl-*p*-nitroanilide in 0·1 mol/l Tris-HCl buffer, pH 7·1 at 25°C. The K_m-value for aminopeptidase B read off from

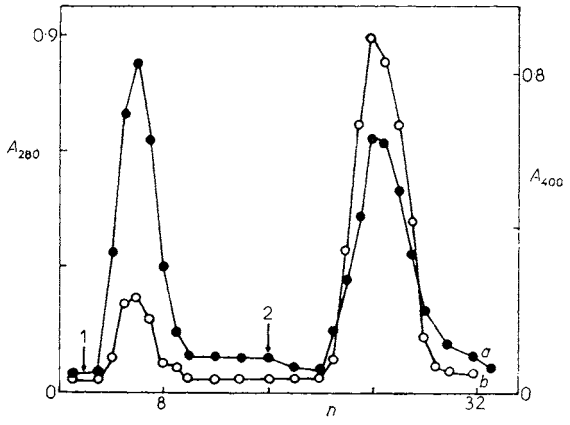

FIG. 2

Chromatographic separation of enzymes hydrolysing L-lysyl-*p*-nitroanilide on Ecteola-cellulose. The enzymatically active fraction after gel filtration was salted out with ammonium sulfate, dissolved in 5 mmol/l Tris-HCl buffer, pH 7·1, and dialyzed against the same buffer. The sample was concentrated to *c.* 8 ml and then placed on the ion exchange column (2 × 25 cm). The latter was eluted by 0·2 mol/l and 0·35 mol/l NaCl in 5 mmol/l Tris HCl buffer at pH 7·1. The application of the individual eluents is marked by an arrow. Proteins (curve *a*), enzymatic activity (curve *b*). *n*, fraction number, 1 0·20 mol l^{-1}, 2 0·35 mol l^{-1}

the Lineweaver–Burke reciprocal plot of reaction rates *versus* substrate concentrations is $1{\cdot}2 \cdot 10^{-3}$ mol.

The catalytic action of purified aminopeptidase B was tested with a number of chromogenic substrates. The enzyme did not hydrolyze the *p*-nitroaniline derivatives of the following amino acids: L-leucine, L-phenylalanine, L-proline, glycine, norleucine, D,L-benzoylarginine, ε-aminocaproic acid, ethylcysteine, N(α-tosyl-L-arginine), N(α-L-glutamic acid), N-(γ-L-glutamic acid), N-succinyl-L-phenylalanine. N-acetyl-L-phenylalanine; neither were hydrolyzed L-histidyl-β-naphthylamide and L-tryptophanyl-β-naphthylamide. Aminopeptidase B catalyzed the hydrolysis of L-lysyl-*p*-nitroanilide, L-arginyl-*p*-nitroanilide, and L-lysyl-β-naphthylamide only.

DISCUSSION

Bovine liver extracts as well as the extracts of many other organs hydrolyze chromogenic synthetic substrates at various rates dependent on the character of the side chain of the amino acid residue. The length of the chain, its aliphatic or aromatic character and, above all, its charge are the main factors affecting the rate of hydrolysis of the substrates (Table I).

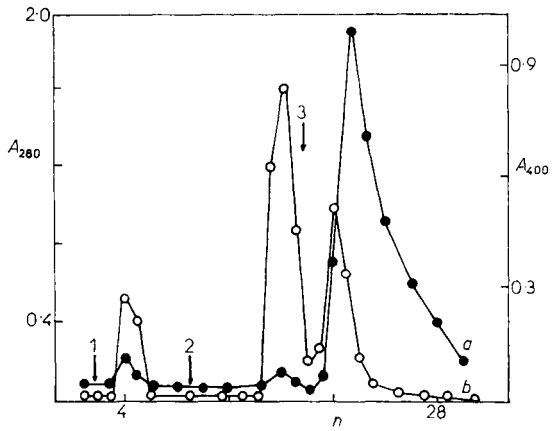

FIG. 3

Adsorption chromatography of enzymes hydrolyzing L-lysyl-*p*-nitroanilide on hydroxylapatite. The fraction purified on Ecteola-cellulose was dialyzed, concentrated to about 5 ml and applied to a column of hydroxylapatite (2 × 6 cm). The elution was effected by an increasing stepwise gradient of phosphate buffers, pH 7·0 (0·02 mol/l, 0·05 mol/l, and 0·2 mol/l, the application is marked by an arrow). Designation the same as in Fig. 2, **1** 0·02 mol l^{-1}, **2** 0·05 mol l^{-1}, **3** 0·20 mol l^{-1}

TABLE II

Purification of bovine liver aminopeptidase B

Purification step	Total protein mg	Total activity mol *p*-nitro-aniline/min	Specific activity μmol *p*-nitro-aniline/min/mg	Yield %	Purification factor
30% Homogenate	38·500	4·196	0·109	100	1
106 000*g* supernatant	27·600	4·029	0·146	96	1·3
Salting-out (50% saturation of $(NH_4)_2SO_4$)	12·000	1·920	0·160	45	1·4
Sephadex G-200	500	660	1·32	15	12
Ecteola cellulose	150	360	2·4	8·5	22
Hydroxylapatite	2	52	26·0	1·2	238

By using the five-step purification process we were able to separate and purify aminopeptidase B specifically catalyzing the hydrolysis of the basic substrate, L-lysyl--*p*-nitroanilide. The purification procedure by which our enzyme was isolated (before the report[6] on the purification of a similar enzyme from pig liver had appeared) is essentially identical to a series of chromatographic operations used by Kawata and coworkers[6] for the purification of pig aminopeptidase B. Efforts with a simpler procedure first used by Hopsu and coworkers[4] for the isolation of rat aminopeptidase B were unsuccessful.

The fractionation of the enzymes by ammonium sulfate used at the beginning of the isolation and purification procedure did not result in a marked purification effect but by this step the majority of enzymes hydrolyzing L-leucyl-, L-phenylalanyl-, and glycyl-*p*-nitroanilide were separated from the L-lysyl-*p*-nitroanilide hydrolyzing activity.

A more marked purification effect was achieved by using other procedures, above all gel filtration on Sephadex and adsorption chromatography on hydroxylapatite. Gel filtration of the enzymes of the supernatant fraction of the liver homogenate (Fig. 1) did not lead, however, (unlike in the experiments by Hopsu and coworkers[4]) to the separation of two L-lysyl-*p*-nitroanilide hydrolyzing enzymes even though it is obvious that at least two such enzymes do exist[3,6]. In addition to a marked purification effect the chromatography on hydroxylapatite resulted in the separation of traces of L-leucyl-*p*-nitroanilide-hydrolyzing activity (unpublished observation).

The specific activity of aminopeptidase B achieved by the purification procedure summarized in Table II was 240-times increased with respect to the starting homogenate. Calculating the purification degree we did not take into account the presence of two L-lysyl-*p*-nitroanilide hydrolyzing enzymes in the extract homogenate (even though it is probable). This fact considerably altered the calculation[4] of the final purification degree of rat liver aminopeptidase B.

The enzymatic activity of purified bovine liver aminopeptidase B did not change practically over 10 days of storage at 4°C; a more marked activity decrease was observed during prolonged periods of storage. The enzyme retained one quarter of its original activity after 30 days. By contrast, Hopsu and coworkers[4] have reported that a similar (or even identical) enzyme from rat liver is highly stable in the cold. More similarity with our enzyme show in this respect aminopeptidase B from pig[6] and human[5] liver which lost a significant part of their activities during long term storage or after freezing.

The molecular weights of aminopeptidase B isolated from various mammalian lissues vary over the range of 43 000–95 000. The molecular weight of our bovine tiver aminopeptidase B is approximately 55 000, a value close to the molecular weight of 58 000 reported for pig liver aminopeptidase B (ref.[6]). A different molecular weight (95 000) show aminopeptidase B from the liver of brown rat[4], human

fetus (95 000, ref.[9]), human liver (43 000, ref.[5]), and from the muscle of brown rat (66 000, ref.[10]).

The value of the Michaelis constant determined by the graphical Lineweaver–Burke plot of the hydrolysis of L-lysyl-*p*-nitroanilide in 0·1 mol/l Tris-HCl buffer, pH 7·1 at 25°C was $K_m = 1{\cdot}2 \cdot 10^{-3}$ mol/l. An approximately ten times lower value has been reported for aminopeptidase B from the liver of brown rat[11] which was tested with L-arginyl-β-naphthylamide as substrate. The value obtained with pig liver aminopeptidase B and the same substrate was $K_m = 3{\cdot}5 \cdot 10^{-5}$ mol/l whereas with L-lysyl-β-naphthylamide $K_m = 1{\cdot}2 \cdot 10^{-4}$ mol/l[12].

Aminopeptidase B from bovine liver shows a high substrate specificity. Of the wide variety of chromogenic substrates tested only those were hydrolyzed whose amino acid moiety contained L-arginine or L-lysine. The obviously higher rate of hydrolysis of L-lysyl-β-naphthylamide (39 μmol/l/min/mg) compared to the rate of hydrolysis of L-lysyl-*p*-nitroanilide (26 μmol/l/min/mg) indicates that the activity of the enzyme is affected also by the character of the second part of the substrate. The enzyme did not cleave substrates whose amino acid moiety contained apolar groups of aliphatic or aromatic character (leucine, glycine, phenylalanine); neither were hydrolyzed the substrates which were lacking a free α-amino group (benzoylarginine, N-(α-tosyl-L-arginine) and hydrolysis was also prevented by the anionic character of the amino acid residue (glutamic acid). A marked substrate specificity has also been observed with aminopeptidases B isolated from the liver of other animal species[5,11].

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