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STUDIES ON AMINOPEPTIDASES IN RAT LIVER AND PLASMA

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SUMMARY

It was found that aminopeptidases (EC 3.4.1.2) were easily solubilized from rat liver by bromelain (EC 3.4.4.24) treatment. The solubilized form of the enzymes was subjected to TEAE-cellulose column chromatography resulting in separation of five types of aminopeptidases. Rat plasma also contained some aminopeptidases, which were separated by gel filtration on a Sephadex G-200 column or TEAE-cellulose column chromatography. These enzymes showed different substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide. It was found that these enzymes increased in the plasma after CCl_4 injection or ligation of the common bile duct.

INTRODUCTION

Aminopeptidases are known to be widely distributed in tissues[1–3] and are present in blood plasma and urine[4,5].

In clinical biochemistry, the activity of aminopeptidase has usually been estimated with L-leucyl-β-naphthylamide as substrate[6,7]. However, the hydrolyses of L-leucyl-β-naphthylamide and L-leucine amide have been suggested to be due to different enzymes[8–10]. Thus it is questionable whether L-leucyl-β-naphthylamide or L-leucine amide is the better substrate to use in determination of aminopeptidase.

The enzyme has been solubilized from the brush border fraction of rat kidney[11] by treatment with papain (EC 3.4.4.10). This paper reports that aminopeptidase activities were solubilized from normal rat liver by bromelain treatment, and that the soluble fraction contained some aminopeptidases which differed in substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide.

MATERIALS AND METHODS

L-Leucine amide was obtained from the Protein Research Foundation, Osaka, Japan, L-leucyl-β-naphthylamide, TEAE-cellulose powder and papain from Wako

Pure Chemical Industries, Ltd, Osaka, Japan, bromelain from Shiratori Pharmaceutical Co. Ltd, Japan, and Sephadex G-200 from Seikagaku Kogyo, Tokyo, Japan.

Ion-exchange chromatography

TEAE-cellulose was thoroughly washed with distilled water and after each washing the supernatant fluid, containing fine particles, was decanted until the cellulose settled completely within 30 min. The cellulose was then washed successively with 0.5 M NaOH, 0.5 M HCl, 0.5 M NaOH, and finally with 10 mM sodium phosphate buffer (pH 7.5). It was then packed into chromatographic tubes and equilibrated by allowing the starting buffer, 10 mM sodium phosphate buffer (pH 7.5), to run through the packed column until the pH and conductivity of the effluent were the same as those of the starting buffer.

Preparation of enzyme solution from rat liver

Male albino rats of the Wistar–King strain, weighing 150–200 g were used. 5 g of rat liver were homogenized in 15 ml of 10 mM sodium phosphate buffer (pH 7.5) with a Teflon homogenizer. The homogenate was sonicated at 20 kcycles for a total of 3 min in periods of 30 s alternating with cooling periods of 1 min.

Enzyme treatment

The sonicated suspension of rat liver was incubated with various amounts of bromelain in 10 mM sodium phosphate buffer at 37 °C for 30 min. After incubation each mixture was centrifuged at 105 000 × *g* for 60 min and the resultant supernatant solution was used as the preparation of solubilized aminopeptidases.

Preparation of blood plasma from rats after hepatobiliary damage

CCl_4 (0.16 ml per 100 g of body weight) was injected subcutaneously into rats and plasma was collected 24 h later. In other rats the common bile duct was ligated and plasma was collected 2 or 7 days later.

Enzyme assays

Assay of aminopeptidase with L-leucine amide as substrate was carried out by direct colorimetric determination of ammonia liberation as reported by Fujii and Okuda[12–14]. The reaction mixture consisted of 4 mM of L-leucine amide, 5 mM of sodium phosphate buffer (pH 7.5) and enzyme solution in a final volume of 0.5 ml. The mixture was incubated at 37 °C for 30 min. Then 2 ml of 10% sodium tungstate solution (pH 1.8) were added and the mixture was centrifuged at 3000 rev./min for 5 min. To 1 ml of the supernatant solution, Reagent A (1.5 ml) and Reagent B (1.5 ml) were added. Reagent A consisted of phenol (5 g) and sodium nitroprusside (25 mg) dissolved in water in a final volume of 375 ml. Reagent B consisted of NaOH (4.2 g), $Na_2HPO_4 \cdot 12H_2O$ (44.6 g) and 5 ml of antiformin, containing 10% Cl, dissolved in water in a final volume of 375 ml. The mixture was stood at 37 °C for 30 min, and then its absorbance was read at 625 nm.

Assay of aminopeptidase with L-leucyl-β-naphthylamide as substrate was carried out by the method of Goldbarg and Rutenburg[6,7].

Protein concentration was determined by measuring the absorbance at 750 nm by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

RESULTS

Solubilization of aminopeptidases from normal rat liver

Aminopeptidases have been solubilized from the brush-border fraction of rat kidney[11] by treatment with papain.

We examined the solubilization of aminopeptidases from normal rat liver homogenate by treatment with bromelain or papain. Papain treatment caused solubilization of 75% of the aminopeptidases from a rat liver homogenate while bromelain treatment resulted in solubilization of more than 90% (Table I). It was

TABLE I

SOLUBILIZATION OF AMINOPEPTIDASES FROM RAT LIVER BY BROMELAIN TREATMENT

Rat liver homogenate was sonicated as described in Materials and Methods, treated with enzyme as indicated, and centrifuged for 60 min (105 000 × *g* at 4 °C). The resultant supernatant and precipitate were used as enzyme solutions. Bromelain and papain were used at concentrations of 100 μg per mg tissue protein.

Treatment	*Fraction*	*Total activity (μmoles·ml⁻¹·h⁻¹)*	
		NH_3 formed	*Naphthylamine formed*
None	Whole homogenate	38.8	16.3
	105 000 × *g* supernatant	21.7	8.3
	105 000 × *g* precipitate	14.0	6.1
Bromelain	Whole homogenate	182.7	15.7
	105 000 × *g* supernatant	171.5	15.0
	105 000 × *g* precipitate	16.3	0.9
Papain	Whole homogenate	46.5	—
	105 000 × *g* supernatant	35.4	—
	105 000 × *g* precipitate	14.1	—

found that bromelain treatment remarkably activated the activity with L-leucine amide but not with L-leucyl-β-naphthylamide as substrate. The greatest solubilization of aminopeptidases were achieved when bromelain was used in a ratio to tissue protein of 1:10. Bromelain itself did not hydrolyze L-leucine amide or L-leucyl-β-naphthylamide.

Solubilization of aminopeptidases by bromelain was clearly demonstrated in the following experiment. Rat liver homogenates, which had been sonicated as described in Materials and Methods, was applied to a column of Sephadex G-200 equilibrated with 10 mM sodium phosphate buffer (pH 7.5). The column was eluted with the same buffer (Fig. 1a). The fractions eluted in the void volume were collected and concentrated about 10-fold. The concentrated solution was divided into two parts. One part was treated with bromelain (100 μg/mg protein of sonicated rat liver homogenate). The treated and untreated parts were then each applied to a Sephadex G-200 column. Without bromelain treatment aminopeptidases were again eluted in the void volume, as shown in Fig. 1b. On the other hand, after bromelain treatment most of the activity was retarded, as shown in Fig. 1c, indicating that the fraction in the void volume was degraded to an enzyme of lower molecular weight by bromelain treatment.

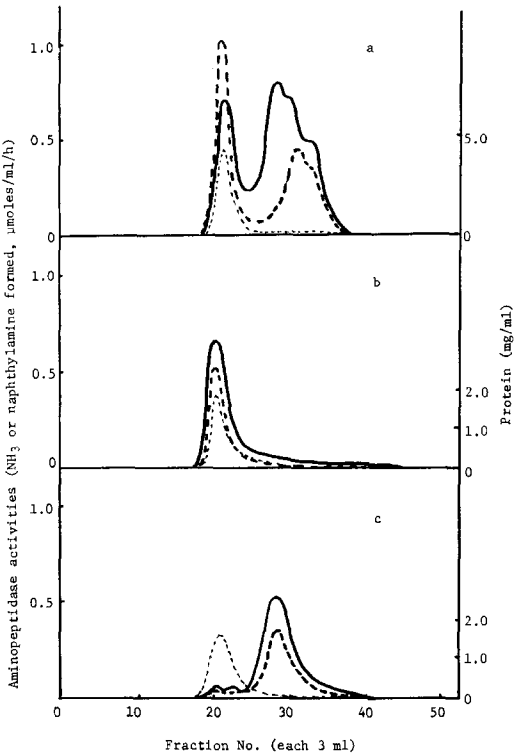

Fig. 1. Effect of bromelain treatment on patterns of gel filtration of rat liver aminopeptidases on Sephadex G-200. Column: Sephadex G-200, 2 cm × 50 cm. Elution buffer: 10 mM sodium phosphate buffer (pH 7.5). (a) Normal rat liver homogenate, sonicated as described in Materials and Methods. (b) The fractions eluted in the void volume in (a) concentrated about 10-fold. (c) Preparation (b) after bromelain treatment. ———, activity with L-leucine amide; — — —, activity with L-leucyl-β-naphthylamide; · · · · · ·, protein.

TEAE-cellulose column chromatography of rat liver aminopeptidases

5 g of rat liver were homogenized in 15 ml of 10 mM sodium phosphate buffer (pH 7.5). Two methods were employed for preparing aminopeptidase fractions from the homogenate. In one method, an aliquot of the homogenate of rat liver was sonicated, as described in Materials and Methods, and centrifuged at 105 000 × *g* for 60 min. The resultant supernatant was used as aminopeptidase Fraction I. In the other method, the homogenate was subjected to sonication and bromelain treatment as described in Materials and Methods and the legend to Fig. 2. The mixture was centrifuged at 105 000 × *g* for 60 min and the resultant supernatant was used as aminopeptidase fraction II. The aminopeptidase fraction thus prepared, was applied to a column of Sephadex G-25 equilibrated with 10 mM sodium phosphate buffer (pH 7.5) and the column was developed with the same buffer. An aliquot of 6 ml of eluate containing activity was applied to a TEAE-cellulose column (2 cm × 10 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.5) and linear gradient elution was carried out as described in the legend to Fig. 2. As shown in Fig. 2a, when the aminopeptidase fraction I was applied to the TEAE-cellulose column, most of the activity was eluted in unadsorbed fractions and only small

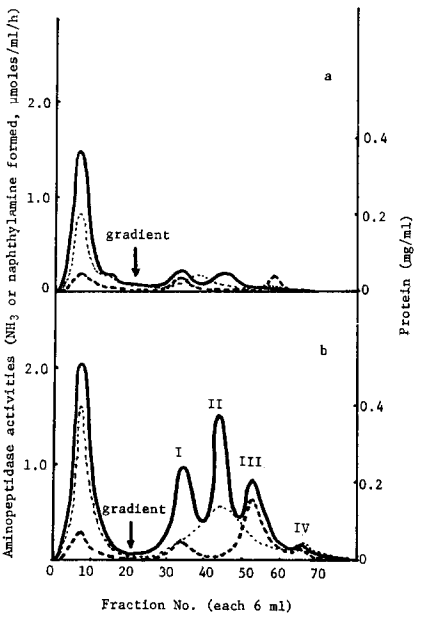

Fig. 2. TEAE-cellulose column chromatography of aminopeptidases from rat liver. A TEAE-cellulose column (2 cm × 10 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 7.5). The column was eluted with a linear gradient of 0–0.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.5) achieved with 250 ml of each buffer. The flow rate was 40 ml/h and fractions of 6 ml were collected. (a) Rat liver homogenate was sonicated as described in Materials and Methods and centrifuged at 105 000 × *g* for 60 min. The resultant supernatant was desalted by passage through a Sephadex G-25 column and 6 ml of the eluate containing activity were applied to a TEAE-cellulose column. (b) Rat liver homogenate was sonicated as described in Materials and Methods, treated with bromelain (100 μg/mg protein of the sonicated rat liver homogenate) and centrifuged at 105 000 × *g* for 60 min. The resultant supernatant was desalted as described in (a) and 6 ml of the eluate containing activity were applied to a TEAE-cellulose column. ———, activity with L-leucine amide; — — —, activity with L-leucyl-β-naphthylamide; ·····, protein.

peaks of activity were found in adsorbed fractions. On the other hand, much activity of Fraction II was adsorbed on the column. Four peaks (I, II, III and IV) of aminopeptidase activities were found in the adsorbed fraction of fraction II on TEAE-cellulose column chromatography, as shown in Fig. 2b. Peaks I and II hydrolyzed L-leucine amide preferentially to L-leucyl-β-naphthylamide, while Peaks III and IV hydrolyzed L-leucine amide and L-leucyl-β-naphthylamide equally well.

Aminopeptidase activities of rat plasma after hepatobiliary damage

Rats were treated with CCl_4 or their common bile duct was ligated as described in the Materials and Methods. Plasma was collected from these rats and control rats, and these aminopeptidase activities were estimated. As shown in Table II, when aminopeptidase activities were estimated with L-leucine amide as substrate, the activities increased after either CCl_4 treatment or ligation of the common bile duct. However, when activity was determined with L-leucyl-β-naphthylamide as substrate, little increase in aminopeptidase activities were found in either group. This suggests that the enzymes in the plasma of rats after CCl_4 treatment or ligation of the common bile duct have different substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide, respectively.

TABLE II

AMINOPEPTIDASE ACTIVITIES OF PLASMA OF RATS WITH HEPATOBILIARY DAMAGE

The procedures were as described in Materials and Methods.

Treatment	*Aminopeptidase activities (NH_3 or naphthylamine formed, $\mu moles \cdot ml^{-1} \cdot h^{-1}$)*	
	L-*leucine amide*	L-*leucyl-β-naphthylamide*
None	5.61	1.10
CCl_4-treated	39.58	1.88
Ligation of bile duct		
2 days previously	14.51	1.31
7 days previously	15.52	1.78

Sephadex G-200 gel filtration of plasma of rats with hepatobiliary damage

Samples of plasma from rats after various treatments were applied to columns of Sephadex G-200 equilibrated with 10 mM sodium phosphate buffer (pH 7.5), and columns were developed with the same buffer. The plasma of control rats gave two

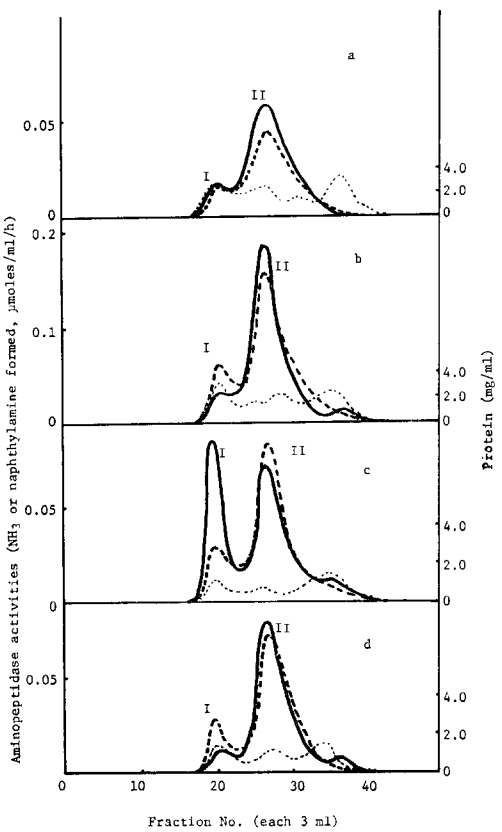

Fig. 3. Sephadex G-200 column chromatography of aminopeptidases from plasma of control rats and those with hepatobiliary damage. Column: Sephadex G-200, 2 cm × 50 cm. Elution buffer: 10 mM sodium phosphate buffer (pH 7.5). (a) Control rat plasma. (b) CCl_4-treated rat plasma. (c) Rat plasma 2 days after ligation of the common bile duct. (d) Rat plasma 7 days after ligation of the common bile duct ———, activity with L-leucine amide; — — —, activity with L-leucyl-β-naphthylamide; · · · · · ·, protein.

peaks (I and II) of aminopeptidase activities, as shown in Fig. 3a. Both peaks hydrolyzed L-leucine amide and L-leucyl-β-naphthylamide. The amount of Peak II was greatly increased in the plasma of rats after CCl_4 treatment, as shown in Fig. 3b. In contrast, the activity of Peak I was increased in the plasma of rats 2 days after ligation of the common bile duct, as shown in Fig. 3c. However, 7 days after this operation in Peak II was considerably increased, as shown in Fig. 3d, so that the pattern on gel filtration had become like that of the plasma of rats after CCl_4 treatment.

TEAE-cellulose column chromatography of plasma aminopeptidases

Plasma samples from control and CCl_4-treated rats were desalted by passage through a Sephadex G-25 column. A 10-ml aliquot of the eluate containing activity was applied to a TEAE-cellulose column equilibrated with 10 mM sodium phosphate buffer (pH 7.5) and linear gradient elution was carried out as described in Materials and Methods. The control plasma gave two peaks (I and II), as shown in Fig. 4a.

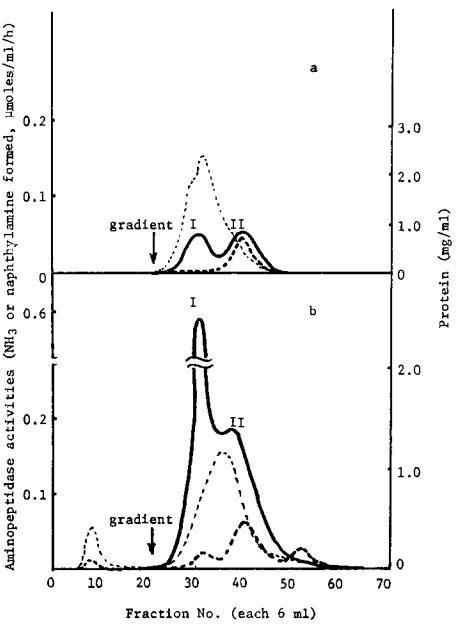

Fig. 4. TEAE-cellulose column chromatography of aminopeptidases from control and CCl_4-treated rat plasma. (a) Control rat plasma. (b) CCl_4-treated rat plasma. ———, activity with L-leucine amide; — — —, activity with L-leucyl-β-naphthylamide; ·····, protein.

Peak I hydrolyzed L-leucine amide but not L-leucyl-β-naphthylamide, while peak II hydrolyzed L-leucine amide and L-leucyl-β-naphthylamide equally well. The plasma of CCl_4-treated rats also gave two peaks (I and II) of adsorbed material on TEAE-cellulose column chromatography, as shown in Fig. 4b. As with the control plasma, Peak I hydrolyzed L-leucine amide preferentially to L-leucyl-β-naphthylamide. On the other hand, Peak II hydrolyzed L-leucine amide more readily than L-leucyl-β-naphthylamide.

DISCUSSION

The present study showed that rat liver aminopeptidases easily solubilized by bromelain treatment. Solubilization of the enzyme was confirmed by gel filtration on the Sephadex G-200 column. On gel filtration of a sonicated suspension of rat liver homogenate a large amount of aminopeptidases was found in the void volume. The gel filtration pattern of the fraction in the void volume was retarded after treating the fraction with bromelain, indicating that bromelain degraded aminopeptidases to small molecules.

The solubilized enzyme obtained by treatment of a liver homogenate with bromelain was applied to a TEAE-cellulose column. In this way five types of aminopeptidases were separated, one in the unadsorbed fraction and four in the adsorbed fraction. It is very interesting that these fractions showed different substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide (Fig. 2b). For example, Peak II in the adsorbed fraction exclusively hydrolyzed L-leucine amide, while Peaks I, III and IV hydrolyzed both L-leucine amide and L-leucyl-β-naphthylamide. This clearly indicates that rat liver contains some aminopeptidases with different substrate specificities.

Behal *et al.*[16] also reported chromatographic separation of two types of aminopeptidase from human liver, one Mn^{2+}-dependent and active on dipeptides and the other with no metal ion requirement and active on amino acid β-naphthylamides. In contrast to their results, Panveliwalla *et al.*[2] reported that the aminoacyl-β-naphthylamide-hydrolase activity of extracts of human liver gave only one peak in chromatography on DEAE-Sephadex.

It is well known that the concentration of plasma aminopeptidases increases in various liver diseases[17–22]. The present study showed that plasma aminopeptidases in rats increased considerably after CCl_4 treatment or ligation of the common bile duct. The gel filtration patterns on Sephadex G-200 of plasma from control rats and those with liver damage were compared. Plasma aminopeptidases gave two peaks (I and II) on gel filtration (Fig. 3). In plasma of CCl_4-treated rats, Peak II increased remarkably. It is of interest that the gel-filtration pattern of rat plasma changed with time after ligation of the common bile duct. On the second day after the operation, Peak I was increased, while on the seventh day Peak II was remarkably elevated, and the gel filtration pattern had become like that after CCl_4 treatment.

The present study also showed that rat plasma contained some aminopeptidases which showed different substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide. Control plasma gave two peaks (I and II) on a TEAE-cellulose column. Peak I preferentially hydrolyzed L-leucine amide while Peak II hydrolyzed L-leucine amide and L-leucyl-β-naphthylamide equally well. After CCl_4 treatment the adsorbed fraction of plasma aminopeptidases was also resolved into two peaks, and these peaks also showed different substrate specificities toward L-leucine amide and L-leucyl-β-naphthylamide. Peak I exclusively hydrolyzed L-leucine amide, while Peak II hydrolyzed both L-leucine amide and L-leucyl-β-naphthylamide.

From their behavior on the chromatography, it seems possible to conclude that Peaks I and II of the CCl_4-treated rat plasma correspond to Peaks I and II of the control rat plasma. This conclusion is supported by the substrate specificities of Peaks I and II of CCl_4-treated rat plasma and control rat plasma.

Experiments are now in progress on the characters of these fractions of plasma aminopeptidases.

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