BBA 66043

PURIFICATION OF AMINOPEPTIDASE A IN HUMAN SERUM AND DEGRADATION OF ANGIOTENSIN II BY THE PURIFIED ENZYME

IKUKO NAGATSU*, TOSHIHARU NAGATSU, TOMIKO YAMAMOTO, GEORGE G. GLENNER AND JOHN W. MEHL**

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Chikusa-ku, Nagoya (Japan); Section on Histochemistry, Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.); and Department of Biochemistry, School of Medicine, University of Southern California, Los Angeles, Calif. (U.S.A.)

(Received August 11th, 1969)

SUMMARY

1. Aminopeptidase A, which is activated by Ca^{2+} and specifically hydrolyzes N-terminal dicarboxylic amino acids, was purified from human serum approx. 700-fold. α-L-Glutamyl β-naphthylamide was used as substrate throughout the purification.

2. The purified enzyme hydrolyzed α-L-glutamyl β-naphthylamide, α-L-aspartyl β-naphthylamide, and aspartylalanine, which indicated that this enzyme preferentially hydrolyzes N-terminal dicarboxylic amino acids. Leucyl β-naphthylamidase activity was decreased during the purification, but complete removal of leucyl β-naphthylamidase activity from glutamyl β-naphthylamidase activity was not possible.

3. Purified aminopeptidase A removed the N-terminal aspartic acid residue from natural angiotensin II, *i.e.* α-L-Asp^1-Val^5-angiotensin II. Thus aminopeptidase A was proved to be an "angiotensinase". Leucyl β-naphthylamidase hydrolyzed mainly synthetic asparaginyl angiotensin II.

INTRODUCTION

GLENNER *et al.*[1] first reported the presence of an aminopeptidase that specifically hydrolyzes N-terminal dicarboxylic amino acids and is activated by Ca^{2+}, and they designated the enzyme aminopeptidase A (acid α-aminopeptidase). Either α-L-

* Present address: Department of Anatomy and Physiology, Aichi Prefectural College of Nursing, Moriyama-ku, Nagoya, Japan.

** Present address: Division of Biological and Medical Sciences, National Science Foundation, Washington, D.C. 20550, U.S.A.

glutamyl β-naphthylamide or α-L-aspartyl β-naphthylamide was used as substrate. It was subsequently found that this enzyme in human serum can hydrolyze N-terminal aspartic acid from natural α-L-Asp[1]-Val[5]-angiotensin II[2,3]. Aminopeptidase activity with these properties has been reported in human sera or plasma[2-7].

Although the properties of aminopeptidase A were found to be different in some respects from those of the enzyme that hydrolyzes L-leucyl β-naphthylamide, separation of α-L-glutamyl β-naphthylamidase or α-L-aspartyl β-naphthylamidase, *i.e.* aminopeptidase A, from L-leucyl β-naphthylamidase has not previously been reported. Based on the kinetic analysis of Ca^{2+} activation, ROTH[8] suggested that glutamyl β-naphthylamidase or α-L-aspartyl β-naphthylamidase may be the same enzyme as leucyl β-naphthylamidase. However, we found that in column chromatography on Sephadex G-200 and DEAE-cellulose of human plasma the distribution of activities toward naphthylamides of aspartic acid and glutamic acid was identical but different from that toward leucyl naphthylamide[9]. Starting with these observations, we attempted further purification of aminopeptidase A from human serum.

This communication describes a method for the purification of aminopeptidase A from human serum. α-L-Glutamyl β-naphthylamide was used as substrate throughout the purification. Since leucyl β-naphthylamidase activity was difficult to distinguish from glutamyl β-naphthylamidase activity, leucyl β-naphthylamidase activity was also checked in each purification step. Purified aminopeptidase A hydrolyzed glutamyl naphthylamide, aspartyl naphthylamide and aspartylalanine, and removed the N-terminal aspartic acid residue from natural angiotensin II, *i.e.* α-L-Asp[1]-Val[5]-angiotensin II. A preliminary report was presented at the Seventh International Congress of Biochemistry[10].

MATERIALS AND METHODS

α-L-Glutamyl β-naphthylamide, α-L-aspartyl β-naphthylamide and L-aspartyl-L-alanine were synthesized by the method of GLENNER *et al.*[11]. L-Leucyl β-naphthylamide was obtained from Mann Research Laboratories; α-L-Asp[1]-Val[5]-angiotensin II, β-L-Asp[1]-Val[5]-angiotensin II, D-Asp[1]-Val[5]-angiotensin II and α-L-Asn[1]-Val[5]-angiotensin II (angiotensin II amide, Hypertensin, CIBA) were obtained through the generosity of Dr. R. Schwyzer, Research Laboratories, CIBA, Basle, Switzerland. Sephadex G-200, Sephadex G-150, and DEAE-Sephadex A-50 were obtained from Pharmacia Ab; DEAE-cellulose from Serva Entwicklungslab and Brown Co.; CM-cellulose, TEAE-cellulose and ECTEOLA-cellulose from Serva Entwicklungslab; alumina Cγ and hydroxylapatite from Calbiochem; stabilized diazonium salt Fast Garnet GBC from Sigma. Calcium phosphate gel was prepared by the method of KEILIN AND HARTREE[12].

The incubation mixture for the hydrolysis of amino acid β-naphthylamide contained (in μmoles): Tris-maleate buffer (pH 7.0), 90; amino acid β-naphthylamide, 0.45; $CaCl_2$, 0.9; an appropriate amount of enzyme; and water to 0.90 ml. $CaCl_2$ was added only in the glutamyl β-naphthylamidase assays. Incubation was carried out at 37° for 30 min. Incubation was stopped by the addition of 0.3 ml of 10% Tween 20 in 1 M acetate buffer (pH 4.2) containing 0.45 mg of stabilized diazonium salt Fast Garnet GBC. After 30 min $A_{530\ m\mu}$ was measured.

The enzyme activity was also measured by a fluorimetric method[13], used

especially to measure low leucyl β-naphthylamidase activities contaminating glutamyl β-naphthylamidase preparations. The fluorescence intensity of β-naphthylamine released by enzymic hydrolysis of the substrate was determined at 410 mμ with the excitation light at 335 mμ in an Aminco–Bowman spectrophotofluorimeter. All values were corrected for spontaneous hydrolysis of amino acid β-naphthylamide[11] by subtracting the value in a control solution containing water instead of the enzyme sample.

1 unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 mμmole of β-naphthylamine per min at 37° under the assay conditions. Specific activity was expressed as units/mg protein. Ca^{2+} (1 mM) was always included in the standard assay mixture for glutamyl β-naphthylamidase but omitted for the assay of leucyl β-naphthylamidase. When a column eluate of a high concentration of phosphate buffer was used as enzyme without dialysis, Ca^{2+} was omitted from the incubation mixture. In this event, the pooled enzyme fraction was dialyzed against 3 mM phosphate buffer (pH 7.0) to give a constant phosphate effect in glutamyl β-naphthylamidase assay.

Incubation mixtures employed for the detection by paper chromatography of degradation of angiotensin octapeptide or dipeptide aspartylalanine contained (in μmoles): substrate, 0.2; $CaCl_2$, 1.5; Tris–maleate buffer (pH 7.0), 24; an appropriate amount of the purified enzyme; and water to 0.235 ml. Incubation was carried out at 37° for 4 h. The reaction was stopped by the addition of 0.02 ml of 3 M trichloroacetic acid, and the reaction mixture was centrifuged at 2000 rev./min for 20 min. An aliquot (20 μl) of the supernatant was applied to Toyo Roshi No. 51 paper. One-dimensional ascending chromatography was performed with the solvent system acetonitrile–urea–water–triethylamine (60:1:40:2, v/w/v/v) for octapeptide, and 80% pyridine water for aspartylalanine, equilibrated for 14 h before use. The chromatograms were developed with 0.2% ninhydrin in ethanol–glacial acetic acid (100:1, v/v).

When the hydrolysis of angiotensin octapeptide was assayed by using an amino acid analyzer (Hitachi KLA-3B), the same incubation mixture as in the assay of amino acid β-naphthylamide hydrolysis was used. The substrate concentration was 0.5 mM in a total volume of 0.9 ml. Incubation was carried out at 37° for 4 h. The reaction was stopped by the addition of 0.1 ml of 3 M trichloroacetic acid, and the reaction mixture was centrifuged. The deproteinized supernatant was passed through an Amberlite IR-CG-120, H^+ column (1 ml packed volume) and eluted with 3 ml of 3 M NH_4OH. The eluate was evaporated to dryness. The residue was dissolved in 3 ml of water and evaporated. This procedure was repeated twice to remove ammonia completely. The residue was dissolved in 1.2 ml of 0.2 M citrate buffer (pH 2.2). 1 ml was used for the amino acid analysis.

Electrophoresis of purified enzyme preparations on paper or on cellulose acetate was carried out in 0.07 M veronal buffer (pH 8.6) for 4.5 h at 12.5 V/cm or for 40 min at 15 V/cm, respectively. After electrophoresis, either the paper or the cellulose acetate was cut into pieces 0.5 cm in width, and each piece was eluted with 1.5 ml of 5 mM Tris–HCl buffer (pH 7.0). Each eluate was subjected to the determination of aminopeptidase activities.

Disc electrophoresis was carried out as described by DAVIS[14]. After electrophoresis, the gel was incubated in the same incubation mixture as that for the assay of glutamyl β-naphthylamidase, and the blue fluorescence of β-naphthylamine under the ultraviolet lamp was located on the gel.

The determination of the isoelectric point of the purified enzyme was made by Ampholine electrofocusing equipment (LKB 8100) using the carrier ampholytes of pH range of 3–10 or 3–5.

The approximate molecular weight was determined by gel filtration on Sephadex G-200 according to the method of WHITAKER[15]. The molecular weights of γ-globulin, bovine plasma albumin, egg albumin, or soybean trypsin inhibitor were taken as 150 000, 70 000, 40 000, or 20 000, respectively. Protein concentrations were measured by the method of LOWRY *et al.*[16] using bovine serum albumin as a standard.

RESULTS

Preliminary studies on fractionation procedures

Purification of aminopeptidase A in human serum was performed by following the activity toward α-L-glutamyl β-naphthylamide. Since the activity of the enzyme hydrolyzing L-leucyl β-naphthylamide was about 4 times higher than that of glutamyl β-naphthylamidase, removal of leucyl β-naphthylamidase activity in the early steps of purification was found to be essential for the purification of glutamyl β-naphthylamidase. Preliminary studies were therefore made on fractionation procedures to remove leucyl β-naphthylamidase from glutamyl β-naphthylamidase.

Both glutamyl β-naphthylamidase and leucyl β-naphthylamidase were stable between pH 5 and 9 in acetate, phosphate, or Tris–HCl buffer. Leucyl β-naphthylamidase activity was completely stable after freezing and thawing repeated 10 times, but glutamyl β-naphthylamidase activity was decreased to 34% of its initial activity by the same treatment. Both glutamyl β-naphthylamidase and leucyl β-naphthylamidase were stable at 50° for 10 min. At 60°, leucyl β-naphthylamidase activity was decreased slowly but glutamyl β-naphthylamidase activity more rapidly. Addition of Ca^{2+} stabilized glutamyl β-naphthylamidase activity, but Ca^{2+} had no effect on the stability of leucyl β-naphthylamidase. However, selective inactivation of leucyl β-naphthylamidase by heat treatment in the presence of Ca^{2+} was not successful.

When serum was fractionated with $(NH_4)_2SO_4$, both glutamyl β-naphthyl-

TABLE I

FRACTIONAL PRECIPITATION OF GLUTAMYL β-NAPHTHYLAMIDASE AND LEUCYL β-NAPHTHYLAMIDASE IN HUMAN SERUM BY $(NH_4)_2SO_4$

Enzyme activity		*Serum*	*Fraction precipitated by $(NH_4)_2SO_4$ (% satn.)*			
			0–40	40–50	50–60	60–80
Glutamyl β-naphthylamidase activity,	units/ml	10.0	0.68	0.69	2.43	4.28
	(%)	(100)	(6.8)	(6.9)	(24.3)	(42.8)
	units/mg	0.10	0.02	0.09	0.16	0.16
Leucyl β-naphthylamidase activity,	units/ml	41.0	4.5	5.8	15.4	6.8
	(%)	(100)	(11.0)	(14.1)	(37.6)	(16.6)
	units/mg	0.40	0.13	0.66	0.86	0.25
Leucyl to glutamyl β-naphthylamidase	ratio	4.1	6.6	8.4	6.3	1.6

amidase and leucyl β-naphthylamidase precipitated mainly between 50 and 80% of saturation, leucyl β-naphthylamidase precipitating slightly earlier than glutamyl β-naphthylamidase (Table I). The ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase was the lowest in the 60–80% fraction. This fraction was therefore used for further purification of glutamyl β-naphthylamidase.

Fractional adsorption with calcium phosphate gel was tried. The $(NH_4)_2SO_4$ fraction (60–80% satn.) was dissolved in 3 mM potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer. To 1 mg of protein was added 1 mg (dry wt.) of calcium phosphate gel. When the mixture was centrifuged, both glutamyl β-naphthylamidase and leucyl β-naphthylamidase activities remained in the supernatant. The yield of glutamyl β-naphthylamidase was 80% with a 4-fold purification. The ratio of glutamyl β-naphthylamidase:leucyl β-naphthylamidase did not change. However, this fractionation procedure was not used, since DEAE-cellulose was more effective for the removal of inactive protein.

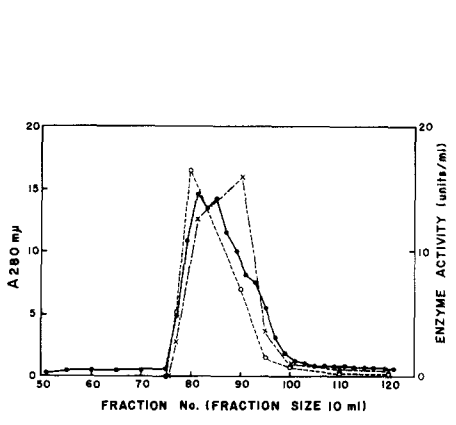
Initial purification of glutamyl β-naphthylamidase (Steps 1–3)

The following purification steps were established for the initial purification of glutamyl β-naphthylamidase. All steps were performed at 2–5°. The enzyme was concentrated by adding $(NH_4)_2SO_4$ to 80% saturation. Precipitates were collected by centrifugation for 30 min at 9000 × *g* or by filtration. When enzyme was to be concentrated from a volume of less than 50 ml, ultrafiltration was used under reduced pressure through a collodion bag (Membranfiltergesellschaft, Göttingen, Germany). All buffers were prepared by dilution of 1 M potassium phosphate (pH 7.0) to the desired concentration.

Step 1. $(NH_4)_2SO_4$ fractionation. 390 g of solid $(NH_4)_2SO_4$ (60% satn.) were added to 1 l of human serum. The solution was centrifuged and the precipitate discarded. To the supernatant were added 140 g of $(NH_4)_2SO_4$ (80% satn.), and the precipitate obtained by centrifugation was dissolved in 3 mM buffer and dialyzed against 4 l of the buffer. The buffer was changed 3 times during 24 h.

Step 2. DEAE-cellulose. Dialyzed enzyme was passed through a DEAE-cellulose column (5 cm × 46 cm) previously equilibrated with 3 mM buffer. The column was washed with 30 mM buffer until the $A_{280\ m\mu}$ of the eluate reached 0.5. The enzyme was subsequently eluted with 100 mM buffer. An example of the elution is shown in Fig. 1. Glutamyl β-naphthylamidase was eluted slightly earlier than leucyl β-naphthylamidase. The glutamyl β-naphthylamidase fractions were combined, and the enzyme was concentrated by precipitation with $(NH_4)_2SO_4$, dissolved in 3 mM buffer, and dialyzed against the same buffer.

Step 3. Hydroxylapatite. The dialyzed enzyme solution was subjected to hydroxylapatite chromatography. Hydroxylapatite (20 g) was mixed with powdered cellulose (40 g), equilibrated with 3 mM buffer, and packed into a column (2.5 cm × 50 cm). Glutamyl β-naphthylamidase was eluted with 3 mM buffer (Fig. 2). Glutamyl β-naphthylamidase activity was eluted slightly earlier than leucyl β-naphthylamidase activity. The initial portion of the glutamyl β-naphthylamidase peak, therefore, contained little leucyl β-naphthylamidase activity. The yield of glutamyl β-naphthylamidase was 100%, or a small increase in the total activity was observed in some experiments. Typical results of the initial purification procedure are shown in Steps 1–3 of Table II.



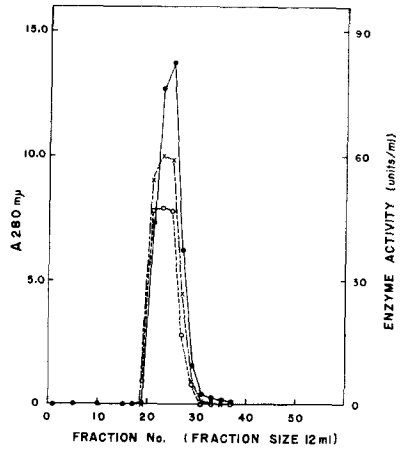


Fig. 1. Chromatography of human serum glutamyl β-naphthylamidase on DEAE-cellulose. The column (5 cm × 46 cm) was equilibrated with phosphate buffer (3 mM, pH 7.0). The sample applied was 60–80% $(NH_4)_2SO_4$ fraction containing 29.6 g protein in 550 ml. The column was washed with 2 l of 3 mM buffer and 2 l of 30 mM buffer. The active fraction was eluted with 0.1 M buffer. ●—●, A 280 mμ; ○- - -○, glutamyl β-naphthylamidase; ×—×, leucyl β-naphthylamidase.

Fig. 2. Chromatography of human serum glutamyl β-naphthylamidase on hydroxylapatite. Hydroxylapatite (20 g) was mixed with powdered cellulose (40 g) in phosphate buffer (3 mM, pH 7.0) and packed in a column (2.5 cm × 50 cm). The sample applied was DEAE-cellulose eluate containing 2.22 g of protein in 96 ml. The sample was eluted with the same buffer. Curves: see Fig. 1.

Separation of glutamyl β-naphthylamidase from leucyl β-naphthylamidase

The semipurified glutamyl β-naphthylamidase preparation still contained an almost equal activity of leucyl β-naphthylamidase. Separation of glutamyl β-naphthylamidase from leucyl β-naphthylamidase was tried, starting with this partially purified enzyme preparation (hydroxylapatite eluate).

TABLE II

PURIFICATION OF GLUTAMYL β-NAPHTHYLAMIDASE (AMINOPEPTIDASE A) FROM HUMAN SERUM

Step	*Volume (ml)*	*Protein (mg)*	*Glutamyl β-naphthylamidase*				*Leucyl β-naphthylamidase*		
			Total activity (units)	*Specific activity (units/mg)*	*Yield (%)*	*Purification*	*Total activity (units)*	*Specific activity (units/mg)*	*Leucyl to glutamyl β-naphthylamidase ratio*
Serum	1000	75 400	11 000	0.146	100	1	42 000	0.557	3.8
1. 60–80% $(NH_4)_2SO_4$	669	20 400	4 970	0.244	45.2	1.7	6 940	0.340	1.4
2. DEAE-cellulose	96	2 220	3 880	1.75	35.3	12	5 150	2.32	1.3
3. Hydroxylapatite	47	1 370	4 410	3.22	40.1	22	5 030	3.67	1.1
4. CM-cellulose	18.8	489	2 800	5.72	25.4	39	3 110	6.36	1.1
5. Sephadex G-150	25.8	76	2 940	38.7	26.7	265	2 400	31.6	0.82
6. DEAE-cellulose	14.3	15.3	1 120	73.2	10.2	501	347	22.7	0.31
7. DEAE-cellulose	11.3	4.7	471	100	4.3	685	52	11.1	0.11

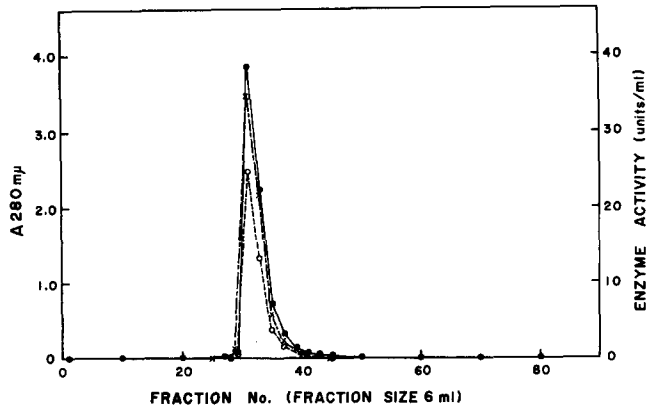


Fig. 3. Chromatography of human serum glutamyl β-naphthylamidase on CM-cellulose. The column (2.8 cm × 40 cm) was equilibrated with acetate buffer (5 mM, pH 5.5). The sample applied was hydroxylapatite eluate containing 174 mg of protein in 6.7 ml. The sample was eluted with the same buffer. Curves: see Fig. 1.

Fractional precipitation with ethanol. By ethanol fractionation, 31% of the glutamyl β-naphthylamidase activity was recovered in the 25–40% fraction. The purification was only 1.4-fold. Leucyl β-naphthylamidase precipitated in the same fraction.

Negative adsorption with alumina Cγ. To 2 parts of protein in the enzyme preparation was added 1 part (dry wt.) of alumina Cγ in 3 mM phosphate buffer. Glutamyl β-naphthylamidase and leucyl β-naphthylamidase activities were not adsorbed. After centrifugation, 75% of glutamyl β-naphthylamidase activity was recovered in the supernatant with 2-fold purification, but the ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase was not decreased.

CM-cellulose. CM-cellulose was equilibrated with 5 mM acetate buffer (pH 5.5). The enzyme solution was dialyzed against the same buffer and subjected to CM-

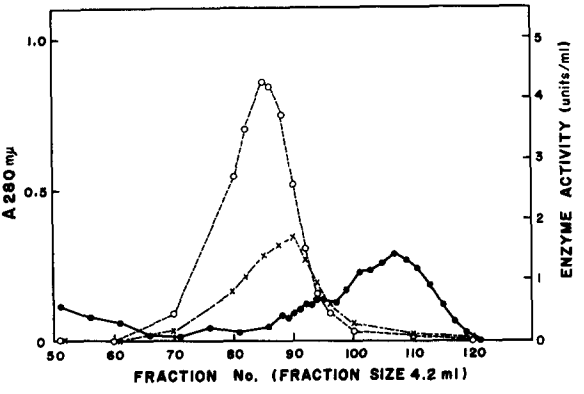


Fig. 4. Chromatography of human serum glutamyl β-naphthylamidase on Sephadex G-150. The column (4 cm × 51 cm) was equilibrated with 10 mM Tris–HCl buffer (pH 7.0) containing 0.1 M NaCl. The sample applied was hydroxylapatite eluate containing 74.5 mg of protein in 5 ml. The sample was eluted with the same buffer. Curves: see Fig. 1.

cellulose column chromatography. Both glutamyl β-naphthylamidase and leucyl β-naphthylamidase were eluted with the same buffer (Fig. 3). Recovery of glutamyl β-naphthylamidase was about 85% with a two-fold purification in this experiment.

TEAE-cellulose and ECTEOLA-cellulose. TEAE-cellulose or ECTEOLA-cellulose was equilibrated with 3 mM phosphate buffer. Stepwise elution with increased concentration of phosphate buffer was carried out. In TEAE-cellulose, inactive protein was removed with 60 mM buffer, and both glutamyl β-naphthylamidase and leucyl β-naphthylamidase were eluted with 0.1 M phosphate buffer. Separation of glutamyl β-naphthylamidase from leucyl β-naphthylamidase was not observed. In ECTEOLA-cellulose chromatography, inactive protein was removed with 3 mM buffer, and both glutamyl β-naphthylamidase and leucyl β-naphthylamidase were eluted with 30 and 60 mM phosphate buffers in the same fractions.

Sephadex G-150 and Sephadex G-200. Tris–HCl buffer (10 mM, pH 7.0) containing 0.1 M NaCl was used as the eluent. The enzyme solution was dialyzed against the same buffer. Either in Sephadex G-150 (Fig. 4) or in Sephadex G-200, glutamyl β-naphthylamidase was eluted earlier than leucyl β-naphthylamidase, but complete separation of both peaks was not possible.

DEAE-Sephadex A-50. DEAE-Sephadex A-50 was equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, and packed in a column (1.4 cm × 78 cm). The enzyme solution was dialyzed against the same buffer. The protein applied was 100 mg. A linear gradient of NaCl from 0.1 to 0.3 M NaCl was made for the elution. Glutamyl β-naphthylamidase was eluted earlier than leucyl β-naphthylamidase. Both peaks were separated but overlapped (Fig. 5).

DEAE-cellulose, stepwise elution. The eluate from DEAE-cellulose (Step 2) was subjected to rechromatography on DEAE-cellulose. Stepwise elution by increasing concentrations of phosphate buffer was carried out as in the first DEAE-cellulose

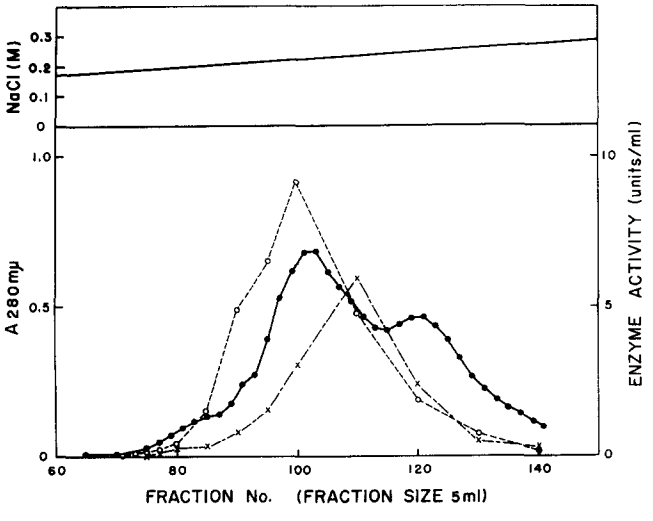


Fig. 5. Chromatography of human serum glutamyl β-naphthylamidase on DEAE-Sephadex A-50. The column (1.4 cm × 78 cm) was equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. The sample applied was hydroxylapatite eluate containing 159 mg of protein in 7 ml. The sample was eluted with the same buffer with a linear NaCl gradient from 0.1 to 0.3 M. Curves: see Fig. 1.

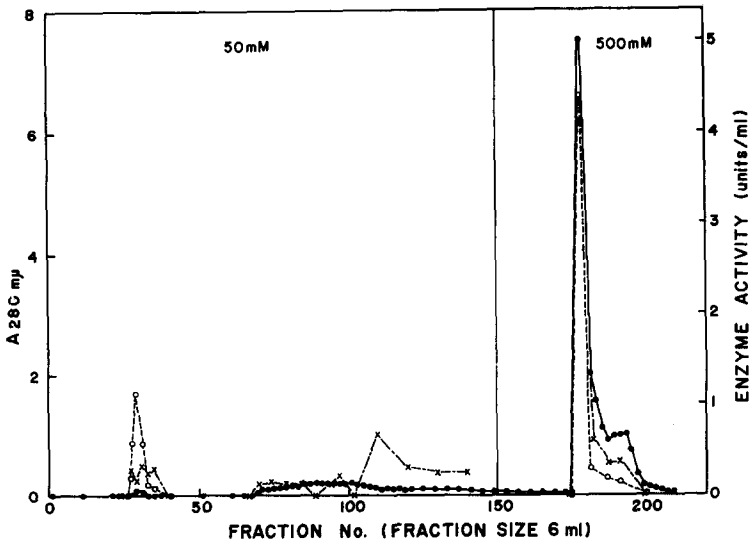


Fig. 6. Chromatography of human serum glutamyl β-naphthylamidase on DEAE-cellulose (2nd). The column (2.5 cm × 38 cm) was equilibrated with phosphate buffer (3 mM, pH 7.0). The sample applied was DEAE-cellulose eluate containing 370 mg of protein in 10 ml. The column was washed with 3 mM buffer, and the sample was eluted with 50 mM and 0.5 M buffer. Curves: see Fig. 1.

chromatography in Step 2. A portion of glutamyl β-naphthylamidase and leucyl β-naphthylamidase was eluted with 50 mM buffer. The activity retained by the column was then eluted with a higher concentration (0.1–0.5 M) of phosphate buffer. The first peak usually showed high glutamyl β-naphthylamidase activity and low leucyl β-naphthylamidase activity, whereas the relative activities were reversed in the second peak. The distribution of glutamyl β-naphthylamidase and leucyl β-naphthylamidase activities in these two peaks differed with DEAE-cellulose of different lots and from different suppliers. Fig. 6 illustrates a result obtained with a lot from Serva. The first peak contained about 10% of total activity, and the ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase was 0.241. When this fraction was pooled and rechromatographed on the third DEAE-cellulose, 38% of the glutamyl β-naphthylamidase activity was eluted with 50 mM buffer (Fig. 7). The ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase was 0.032, indicating that the contamination of leucyl β-naphthylamidase was only 3.2%. The specific activity was 60.3 units/mg. Therefore, the purification of glutamyl β-naphthylamidase was about 400-fold from serum. Because of the low yield, rechromatography on DEAE-cellulose could not be repeated more than 3 times. Another difficulty with this purification procedure was poor reproducibility. However, it was the most effective for removal of leucyl β-naphthylamidase from glutamyl β-naphthylamidase, although complete removal of leucyl β-naphthylamidase from glutamyl β-naphthylamidase was not possible.

DEAE-cellulose, gradient elution. The efficacy of gradient elution from a DEAE-cellulose column for the separation of leucyl β-naphthylamidase from glutamyl β-naphthylamidase was reported in an earlier paper[9]. The enzyme was dialyzed against 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. The column size used was

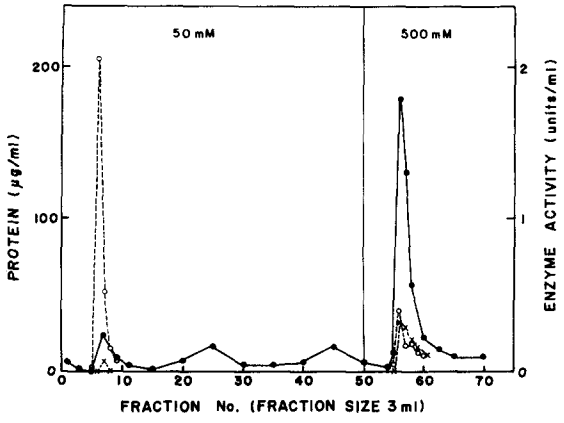


Fig. 7. Chromatography of human serum glutamyl β-naphthylamidase on DEAE-cellulose (3rd). The column (1 cm × 19 cm) was equilibrated with phosphate buffer (3 mM ,pH 7.0). The sample applied was DEAE-cellulose eluate (the first peak in Fig. 6) containing 1.21 mg protein in 1.1 ml. The column was washed with 3 mM buffer, and the sample was eluted with 50 mM and 0.5 M buffer. Curves: see Fig. 1.

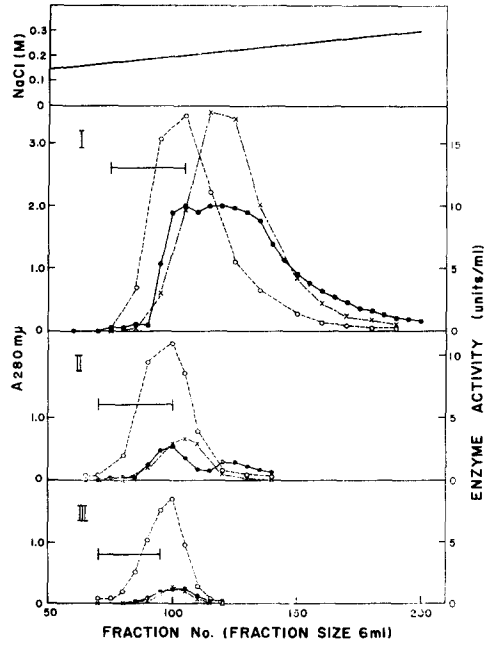


Fig. 8. Separation of human serum glutamyl β-naphthylamidase from leucyl β-naphthylamidase by repeated chromatography on DEAE-cellulose. The column (2.5 cm × 91 cm) was equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. Sample applied to the first column (I) was hydroxylapatite eluate (Step 3) containing 1.37 g protein in 47 ml. The sample was eluted with the same buffer with a linear NaCl gradient from 0.1 to 0.3 M. I, First DEAE; II, second DEAE; III, third DEAE; |—|, fractions pooled and concentrated. Curves: see Fig. 1.

2.5 cm × 91 cm. A linear concentration gradient from 0.1 M to 0.3 M of NaCl was set up by mixing 600 ml of Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl and 600 ml of the same buffer containing 0.3 M NaCl. As shown in Fig. 8 (I), glutamyl β-naphthylamidase was eluted earlier than leucyl β-naphthylamidase. The first half of the glutamyl β-naphthylamidase peak, which contained less leucyl β-naphthylamidase, was pooled and subjected to the second DEAE-cellulose chromatography (Fig. 8, II). The first half of the glutamyl β-naphthylamidase peak was again subjected to the third chromatography (Fig. 8, III). A glutamyl β-naphthylamidase fraction with a ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase of 0.11 was finally obtained. The yield decreased in each rechromatographic step but less than in stepwise elution.

Higher purification of glutamyl β-naphthylamidase

Based on the results above, a method for higher purification of glutamyl β-naphthylamidase was established, as shown in Table II.

Step 4. CM-cellulose. The eluate from hydroxylapatite (Step 3) was concentrated and dialyzed against 5 mM acetate buffer (pH 5.5). The dialyzed solution was divided into seven portions, each containing about 200 mg of protein. 20 g of CM-cellulose were equilibrated with 5 mM acetate buffer (pH 5.5) and packed in a column (2.8 cm × 40 cm). Each portion of solution was passed through a CM-cellulose column. The column was subsequently eluted with 5 mM buffer. The active fractions were combined, concentrated, and dialyzed against 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl.

Step 5. Sephadex G-150. The enzyme solution was passed through a column of Sephadex G-150 previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. For 150 mg of protein in 6 ml, a 2.8 cm × 78 cm column was used. The glutamyl β-naphthylamidase fractions were combined and concentrated.

Step 6. 2nd DEAE-cellulose. The enzyme solution was passed through a column (2.5 cm × 90 cm) of DEAE-cellulose previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. Elution was performed by a gradient from the original buffer to 20 mM Tris–HCl buffer containing 0.3 M NaCl. The first half of the protein peak which contained mostly glutamyl β-naphthylamidase was combined, concentrated, and dialyzed against the original buffer.

Step 7. 3rd DEAE-cellulose. Gradient elution from the DEAE-cellulose column was repeated as described in Step 6. Since only a portion of the protein peak was used in the DEAE-cellulose chromatographies at Steps 6 and 7, yields decreased, but much of the leucyl β-naphthylamidase activity could be separated from that of glutamyl β-naphthylamidase.

An example of the entire purification procedures is shown in Table II. Purification was 700-fold with a yield of 5%. The final preparation contained 10% of leucyl β-naphthylamidase.

Characteristics of purified glutamyl β-naphthylamidase

The approximate molecular weight determined by gel filtration on Sephadex G-200 (2.5 cm × 90 cm) according to the method of WHITAKER[15] was 190 000.

The absorption spectrum showed a peak at 280 mμ.

Electrophoresis on paper or on cellulose acetate in veronal buffer (pH 8.6,

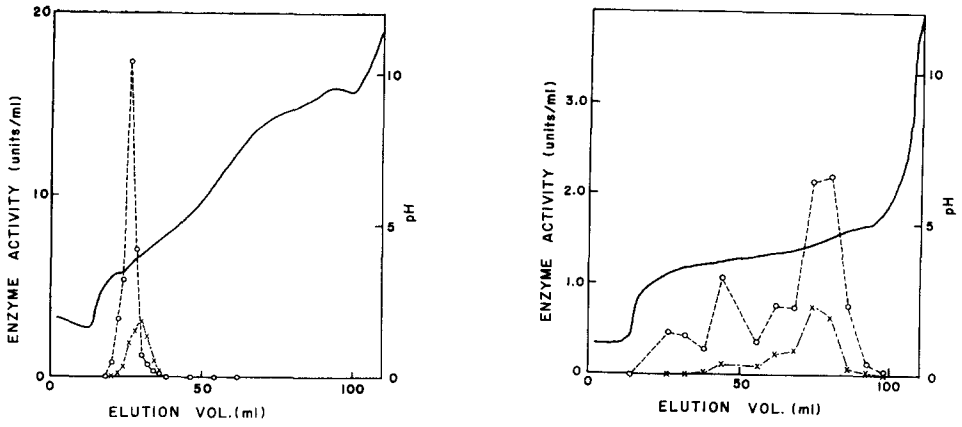


Fig. 9. Determination of the isoelectric point of human serum glutamyl β-naphthylamidase and leucyl β-naphthylamidase by Ampholine electrofocusing equipment (LKB 8100) in the pH range of 3–10. The sample applied was DEAE-cellulose eluate containing 13.1 mg protein in 2.5 ml (glutamyl β-naphthylamidase, 10.1 units/mg; leucyl β-naphthylamidase, 2.2 units/mg). Electrofocusing was carried out for 44 h at 300 V at 4°. ○— — —○, glutamyl β-naphthylamidase; ×— — —×, leucyl β-naphthylamidase; —, pH.

Fig. 10. Determination of the isoelectric point of humanserum glutamyl β-naphthylamidase and leucyl β-naphthylamidase by Ampholine electrofocusing equipment (LKB 8100) in the pH range of 3–5. The sample applied was DEAE-cellulose eluate containing 3.3 mg protein in 2.4 ml (glutamyl β-naphthylamidase, 77 units/mg; leucyl β-naphthylamidase, 24 units/mg). Electrofocusing was carried out for 41 h at 700 V at 4°. Curves: see Fig. 9.

0.07 M) showed one main band and one light band without the enzyme activity. Polyacrylamide-gel electrophoresis showed a faintly stained band in addition to a main intensely stained band which had glutamyl β-naphthylamidase activity. Leucyl β-naphthylamidase activity coincided with glutamyl β-naphthylamidase activity.

Isoelectric points of glutamyl β-naphthylamidase and leucyl β-naphthylamidase were determined by an Ampholine electrofocusing equipment. When pH 3–10 carrier Ampholine was used, the p*I* of glutamyl β-naphthylamidase was 3.6 and that of leucyl β-naphthylamidase 4.0 (Fig. 9). When pH 3–5 carrier Ampholine was used, two peaks of glutamyl β-naphthylamidase were observed (Fig. 10) with p*I* values of 3.6 and 4.3. The peak at pH 3.6 had only a small contamination of leucyl β-naphthylamidase, but that at pH 4.3 coincided with leucyl β-naphthylamidase.

TABLE III

SUBSTRATE SPECIFICITY OF PURIFIED AMINOPEPTIDASE A

Amino acid β-naphthylamide	*Activity (units/mg)*	
	minus Ca^{2+}	*plus* Ca^{2+}*
α-L-Glutamyl	86	100
α-L-Aspartyl	20	48
L-Leucyl	11	9

* Ca^{2+}: 1 mM.

The purified preparation hydrolyzed α-L-aspartyl β-naphthylamide (Table III). Ca^{2+} markedly activated the hydrolysis. Leucyl β-naphthylamidase activity was slightly decreased by Ca^{2+}. Both Sr^{2+} and Ba^{2+} also activated glutamyl β-naphthylamidase. EDTA at 1 mM inhibited glutamyl β-naphthylamidase completely. Hydrolysis of aspartylalanine by the purified enzyme was proved by paper chromatography.

The optimal pH for glutamyl β-naphthylamidase activity was 7.5. The K_m value using glutamyl β-naphthylamide was calculated as 1.2 mM from a Lineweaver–Burk plot.

Degradation of angiotensin II and its analogs by purified aminopeptidase A

Degradation of angiotensin II and its analogs by glutamyl β-naphthylamidase was first examined by paper chromatography. When α-L-Asp^1-Val^5-angiotensin II was incubated with glutamyl β-naphthylamidase, aspartic acid appeared on the paper (Fig. 11). This proved that the N-terminal aspartic acid was cloven from naturally

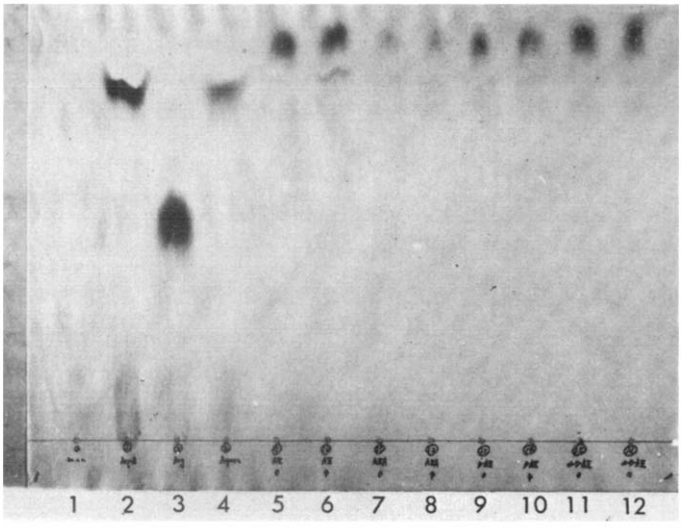


Fig. 11. Paper chromatograph of hydrolysis products of angiotensin II and its analogs after incubation with glutamyl β-naphthylamidase. Incubation was for 4 h at 37°. 1, Incubation without substrate; 2, with aspartic acid; 3, with arginine; 4, with asparagine; 5, incubation with angiotensin II, 0 time; 6, incubation with angiotensin II, 4 h; 7, incubation with angiotensin II amide, 0 time; 8, incubation with angiotensin II amide, 4 h; 9, incubation with β-L-Asp-angiotensin II, 0 time; 10, incubation with β-L-Asp-angiotensin II, 4 h; 11, incubation with α-D-Asp-angiotensin II, 0 time; 12, incubation with α-D-Asp-angiotensin II, 4 h.

occurring angiotensin II by glutamyl β-naphthylamidase. Other synthetic angiotensin II derivatives, α-D-Asp^1-Val^5-angiotensin II and β-L-Asp^1-Val^5-angiotensin II, were not hydrolyzed. Degradation of α-L-Asn^1-Val^5-angiotensin II was not observed on this chromatogram. When α-L-Asn^1-Val^5-angiotensin II was incubated with a leucyl β-naphthylamidase preparation (leucyl β-naphthylamidase:glutamyl β-naphthylamidase = 7) purified by $(NH_4)_2SO_4$ fractionation (40–50%) and on a hydroxylapatite column, an arginine spot appeared on the paper chromatogram (Fig. 12). Although the asparagine spot did not show up convincingly on the chromatogram,

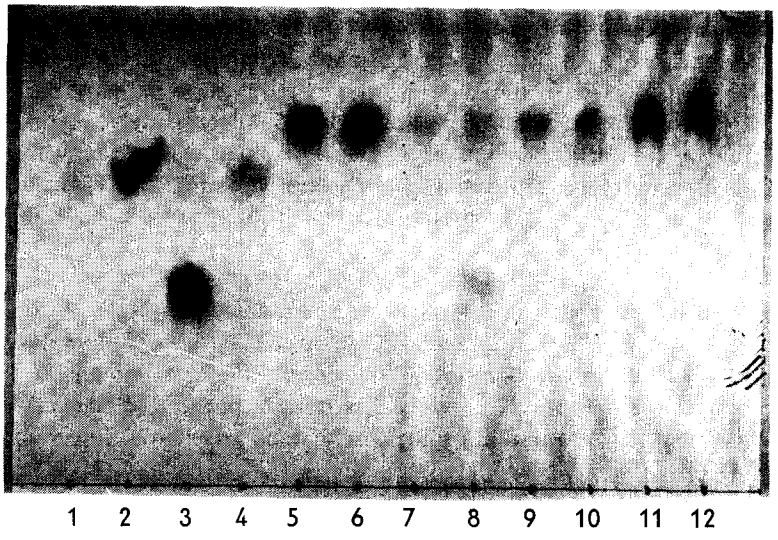


Fig. 12. Paper chromatograph of hydrolysis products of angiotensin II and its analogs after incubation with leucyl β-naphthylamidase. Incubation was for 4 h at 37°. 1, Incubation without substrate; 2, with aspartic acid; 3, with arginine; 4, with asparagine; 5, incubation with angiotensin II, 0 time; 6, incubation with angiotensin II, 4 h; 7, incubation with angiotensin II amide, 0 time; 8, incubation with angiotensin II amide, 4 h; 9, incubation with β-L-Asp-angiotensin II, 0 time; 10, incubation with β-L-Asp-angiotensin II, 4 h; 11, incubation with α-D-Asp-angiotensin II, 0 time; 12, incubation with α-D-Asp-angiotensin II, 4 h.

arginyl color indicated cleavage of the N-terminal asparagine. Incubation of α-L-Asp[1]-Val[5]-angiotensin II with this leucyl β-naphthylamidase preparation did not cause cleavage of aspartic acid as judged by paper chromatography.

Since the sensitivity of paper chromatography is low, a quantitative study on the degradation of angiotensin II and its analogs by purified aminopeptidase A was performed by using an amino acid analyzer. The results are shown in Table IV.

TABLE IV

DEGRADATION OF ANGIOTENSIN II AND ITS DERIVATIVES BY PURIFIED AMINOPEPTIDASE A IN HUMAN SERUM

The enzyme used (513 μg of protein) was purified by repeating DEAE-cellulose gradient chromatography 3 times after Step 3 (hydroxylapatite). Specific activities were: glutamyl β-naphthylamidase, 23.9 units/mg and leucyl β-naphthylamidase, 3.16 units/mg. Total activities were: glutamyl β-naphthylamidase, 2940 mμmoles per 4 h and leucyl β-naphthylamidase, 389 mμmoles per 4 h. Substrate concentration was 0.5 mM. Standard incubation mixture was used. Incubation was carried out for 4 h at 37°. Amino acids liberated were assayed by using an amino acid analyzer as described in MATERIALS AND METHODS.

Substrate	*Amino acids liberated (mμmoles)*							
	Asp	*Asn*	*Arg*	*Val*	*Tyr*	*His*	*Pro*	*Phe*
α-L-Asp[1]-Val[5]-angiotensin II	260	–	57	30	0	0	0	0
α-L-Asn[1]-Val[5]-angiotensin II	–	98	43	28	0	0	0	0
α-D-Asp[1]-Val[5]-angiotensin II	0	–	0	0	0	0	0	0
β-L-Asp[1]-Val[5]-angiotensin II	0		0	0	0	0	0	0

Purified aminopeptidase A (leucyl β-naphthylamidase:glutamyl β-naphthylamidase = 0.13) clove N-terminal aspartic acid. Arginine and valine were also cloven to lesser extents. Neither α-D-Asp[1]-Val[5]-angiotensin II nor β-L-Asp[1]-Val[5]-angiotensin II was hydrolyzed. α-L-Asn[1]-Val[5]-angiotensin II was slowly hydrolyzed by the enzyme preparation. The ratio of velocities toward glutamyl β-naphthylamide and toward α-L-Asp[1]-Val[5]-angiotensin II was constant at 11.3 in two experiments. Carboxypeptidase activity was not observed in the purified enzyme.

A leucyl β-naphthylamidase preparation (leucyl β-naphthylamidase:glutamyl β-naphthylamidase = 8.8) was prepared by Sephadex G-200 and DEAE-cellulose chromatographies, as described previously[9], and incubated with natural α-L-Asp[1]-Val[5]-angiotensin II or synthetic α-L-Asn[1]-Val[5]-angiotensin II. The result obtained by an amino acid analyzer also confirmed that leucyl β-naphthylamidase mainly hydrolyzed angiotensin II amide.

DISCUSSION

Glutamyl β-naphthylamide was used as substrate to purify aminopeptidase A from human serum. About 700-fold purification was achieved from serum. During the purification procedure, the ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase decreased. This indicates that leucyl β-naphthylamidase is a different enzyme from glutamyl β-naphthylamidase and agrees with our previous result[10]. However, complete removal of leucyl β-naphthylamidase activity from glutamyl β-naphthylamidase was very difficult. The most effective method of removing leucyl β-naphthylamidase from glutamyl β-naphthylamidase was to repeat DEAE-cellulose chromatography. However, since the yield decreased in each chromatography, the numbers of chromatographies were limited. The highest ratio of leucyl β-naphthylamidase:glutamyl β-naphthylamidase obtained was 1:31. Removal of leucyl β-naphthylamidase from glutamyl β-naphthylamidase to a ratio of 1:10 was usually possible. Extreme difficulty in the separation of leucyl β-naphthylamidase from glutamyl β-naphthylamidase may mean that they are very closely related structurally.

The enzyme which hydrolyzes aspartyl β-naphthylamide is the same enzyme as glutamyl β-naphthylamidase, since the elution patterns of both enzymes were identical on Sephadex G-200 and DEAE-cellulose. Besides arylamidase activity, the purified enzyme hydrolyzed aspartylalanine.

The cleavage of N-terminal aspartic acid from natural α-L-Asp[1]-Val[5]-angiotensin II proved that the octapeptide is a natural substrate of aminopeptidase A. Since human angiotensin II has been proved to be α-L-Asp[1]-Ile[5]-angiotensin II[17], aminopeptidase A can be expected to hydrolyze this octapeptide. Therefore, aminopeptidase A in human blood is an "angiotensinase". Angiotensin II amide was only slowly hydrolyzed by the purified enzyme. However, since leucyl β-naphthylamidase was still present in the glutamyl β-naphthylamidase preparation, the removal of N-terminal asparagine from angiotensin II amide is most probably due to leucyl β-naphthylamidase. The observation that the ratio of the activities of glutamyl β-naphthylamide hydrolysis:angiotensin II hydrolysis was constant supports this view. Aminopeptidase A in human blood may destroy mainly natural angiotensin II, whereas the aminopeptidase which hydrolyzes leucyl β-naphthylamide may hydrolyze synthetic Asn[1]-angiotensin II (Hypertensin, CIBA). The enzyme activity which hydrolyzes

leucyl β-naphthylamide is much higher than that of glutamyl β-naphthylamidase in human serum[18], and this enzyme could rapidly hydrolyze Asn[1]-angiotensin II. These results agree with the report by NOTARGIACOMO AND COHN[19] that aminopeptidases in human plasma destroy synthetic angiotensin amide more quickly than natural angiotensin.

Our results to date do not yet permit us to say that aminopeptidase A has no ability to split angiotensin amide or that leucyl β-naphthylamidase does not hydrolyze angiotensin. It is also conceivable that neither enzyme is entirely specific. This problem remains for further investigation.

ACKNOWLEDGMENTS

The authors are grateful to Prof. H. Umezawa, Dr. T. Takeuchi, Dr. K. Hara and Miss S. Miyata (Institute of Microbial Chemistry, Tokyo) for their kind help in the use of Ampholine electrofocusing equipment LKB 8100. This work was supported in part by National Institutes of Health grant 5R01 AM 02609 to J. W. Mehl. The Aminco–Bowman spectrophotofluorimeter was purchased by Public Health Service Grant 7R05 TW 00219 01A1 to T. Nagatsu. The capable technical assistance of Miss Yuko Nishikawa and Miss Yumiko Shibahara is gratefully acknowledged.

REFERENCES

1 G. G. GLENNER, P. J. MCMILLAN AND J. E. FOLK, *Nature*, 194 (1962) 867.
2 I. NAGATSU, L. GILLESPIE, J. E. FOLK AND G. G. GLENNER, *Biochem. Pharmacol.*, 14 (1965) 721.
3 I. NAGATSU, L. GILLESPIE, J. M. GEORGE, J. E. FOLK AND G. G. GLENNER, *Biochem. Pharmacol.*, 14 (1965) 853.
4 P. A. KHAIRALLAH, F. M. BUMPUS, I. H. PAGE AND R. R. SMEBY, *Science*, 140 (1963) 672.
5 D. REGOLI, B. RINIKER AND H. BRUNNER, *Biochem. Pharmacol.*, 12 (1963) 637.
6 D. KLAUS, H. KAFFARNIK AND H. PFEIL, *Klin. Wochschr.*, 41 (1963) 376.
7 R. HESS, *Biochim. Biophys. Acta*, 99 (1965) 316.
8 M. ROTH, *Enzymologia*, 31 (1966) 253.
9 I. NAGATSU AND J. W. MEHL, *Biochem. Pharmacol.*, 18 (1969) 2453.
10 T. NAGATSU, I. NAGATSU AND G. G. GLENNER, *Abstr. Intern. Congr. Biochem., 7th, Tokyo, 1967*, 5 (1967) 1041.
11 G. G. GLENNER, L. A. COHEN AND J. E. FOLK, *J. Histochem. Cytochem.*, 13 (1965) 57.
12 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. London, Ser. B*, 124 (1938) 397.
13 L. J. GREENBERG, *Biochem. Biophys. Res. Commun.*, 9 (1962) 430.
14 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
15 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
17 K. ARAKAWA, M. NAKATANI, A. MINOHARA AND M. NAKAMURA, *Biochem. J.*, 104 (1967) 900.
18 I. NAGATSU, T. NAGATSU AND G. G. GLENNER, *Enzymologia*, 34 (1968) 73.
19 A. V. NOTARGIACOMO AND J. N. COHN, *Federation Proc.*, 27 (1968) 533.