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SEPARATION OF AMINO ACID NAPHTHYLAMIDASES IN HUMAN PLASMA ON SEPHADEX G-200 AND DEAE-CELLULOSE*

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Abstract—Enzymatic activities for the hydrolysis of β-naphthylamides of various amino acids (alanine, arginine, aspartic acid, glutamic acid, leucine, lysine, phenylalanine, tyrosine and valine) in human plasma were separated by column chromatography on Sephadex G-200 and DEAE-cellulose. The distribution of activities toward naphthylamides of aspartic acid and glutamic acid was identical, but different from that toward leucine naphthylamide. The distribution of activities toward naphthylamides of other aminoacids (alanine, arginine, lysine, phenylalanine, tyrosine and valine) was generally like that toward leucine naphthylamide. The molecular weight or aspartyl of glutamyl β-naphthylamidase was 190,000, whereas that of leucyl β-naphthylamidase was 160,000.

Glutamyl β-naphthylamidase was separated from leucyl β-naphthylamidase by Sephadex G-200 and then DEAE-cellulose. Properties of purified β-naphthylamidase directed toward the aspartyl or glutamyl substrates were different from those of leucyl β-naphthylamidase. Glutamyl β-naphthylamidase was markedly activated by Ca^{++}, had a pH optimum at 7·5, and a K_m value of $1{\cdot}2 \times 10^{-3}$ M. Ca^{++} had no effect on leucyl β-naphthylamidase. Its pH optimum was 6·6 and the K_m value was $3{\cdot}3 \times 10^{-4}$ M.

It is concluded that the enzyme in human plasma which hydrolyzes either glutamyl β-naphthylamide or aspartyl β-naphthylamide (aminopeptidase A) is a distinct enzyme.

GLENNER *et al.* first reported the presence of an aminopeptidase, i.e. aminopeptidase A, which is activated by Ca^{++} and specifically hydrolyzes *N*-terminal dicarboxylic amino acids. Either α-L-glutamyl β-naphthylamide (GluNA) or α-L-aspartyl β-naphthylamide (AspNA) was used as the substrate of aminopeptidase A. Aminopeptidase activity with these properties has been reported in human serum or plasma.[2–6] Since this enzyme can hydrolyze *N*-terminal aspartic acid from natural angiotensin II,[2] attention has been directed to the activity of this enzyme in human serum in various pathological conditions including hypertension.[7]

Although the properties of aminopeptidase A were found to be different in some respects from those of the enzyme which hydrolyzes L-leucyl β-naphthylamide (LeuNA),[1] separation of α-L-glutamyl β-naphthylamidase from LeuNA-ase has not previously been reported. Based on the kinetic analysis of Ca^{++} activation, Roth[8] suggested that GluNA-ase may be the same enzyme as LeuNA-ase. It is necessary to separate both enzymes completely in order to decide whether the aminopeptidase A of

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plasma is a distinct enzyme. Such a separation, using Sephadex G-200 and DEAE-cellulose columns, is reported in this communication.

MATERIALS AND METHODS

β-Naphthylamides of amino acids (alanine, arginine, aspartic acid, glutamic acid, leucine, lysine, phenylalanine, tyrosine and valine) were obtained from Mann Research Laboratories, Inc.; Sephadex G-200 from Pharmacia Ab; crystalline bovine plasma albumin from Armour Pharmaceutical Co.; egg albumin from Calbiochem.; γ-globulin from Mann Research Laboratories, Inc.; and soybean trypsin inhibitor from Worthington Biochemical Corp.

Gel filtration was carried out in Sephadex G-200. Ten mg of Blue Dextran 2000 in 1 ml was used to measure the void volume. Molecular weights were estimated according to the method of Whitaker,[9] using the following proteins as references: γ-globulin (150,000), plasma ablumin (70,000), egg albumin (40,000) and soybean trypsin inhibitor (20,000).

The incubation mixture for enzymatic activity (total volume, 1·0 ml) contained Tris-HCl buffer (pH 7·5), 50 μmoles; the amino acid β-naphthylamide, 0·6 μmoles;

TABLE 1. ACTIVITIES OF AMINO ACID β-NAPHTHYLAMIDASES ON SEPHADEX G-200 COLUMN CHROMATOGRAPHY*

Amino acid β-naphthylamidase	1st Protein peak	Between 1st and 2nd protein peak	Just before 2nd protein peak	Just after 2nd protein peak
Fraction (elution vol. ml)	143–148	175–180	207–212	217–222
AspNA-ase	0·003	0·002	0·04	0·02
GluNA-ase	0·03	0·02	0·38	0·22
LeuNA-ase	0·04	0·08	1·30	2·26
AlaNA-ase	0·48	0·72	4·85	6·26
ArgNA-ase	0·07	0·16	1·33	1·83
LysNA-ase	0·06	0·14	0·60	0·93
PheNA-ase			0·33	0·69
TyrNA-ase			0·13	0·22
ValNA-ase			0·15	0·17

*Values in enzyme activity; mμmoles/min/ml.

and the enzyme preparation. For aspartyl β-naphthylamidase (AspNA-ase) and GluNA-ase determination, 10 μmoles $CaCl_2$ was added to the incubation mixture. Incubations were carried out at 37°. Three-tenths mg of Fast Garnet GBC in 0·3 ml of 10%, Tween containing 1 M sodium acetate pH 4·2, was used to stop the reaction. After 30 min at room temperature, 1·5 ml *n*-butanol was added. The reaction mixture was shaken and centrifuged. The color of the *n*-butanol layer was measured at 525mμ.

Enzyme activity was also measured by the fluorometric determination of β-naphthylamine released from the substrate.[10] Excitation wave-length was 340 mμ, and fluorescence was measured at 416 mμ. Blanks with and without enzyme were taken in order to check the non-enzymatic hydrolysis.[11] Protein was estimated from the absorption at 280 mμ.

RESULTS

Separation of amino acid β-naphthylamidases in human plasma by Sephadex G-200

Normal human plasma was obtained from citrated whole blood. The plasma was dialyzed against 0·1 M Tris–1 M NaCl buffer, pH 7·5, and diluted to twice the origina volume with the same buffer. After centrifugation, the supernatant fluid, containing up to 200 mg protein, was applied on a Sephadex G-200 column (2·5 × 90 cm), and eluted with the same buffer. The flow rate was about 16·5 ml/hr, and the fraction volume was 5·5 ml. Fractions were assayed for the hydrolytic activity with fluorometry (Fig. 1) and colorimetry (Fig. 2).

Figures 1 and 2 illustrate a typical result of Sephadex G-200 column chromatography. Three protein peaks were observed. The first peak had a low activity toward β-naphthylamides. The second protein peak had high activity, but the third peak had no β-naphthylamidase activity. Enzyme activities of Sephadex G-200 fractions are shown in Table 1.

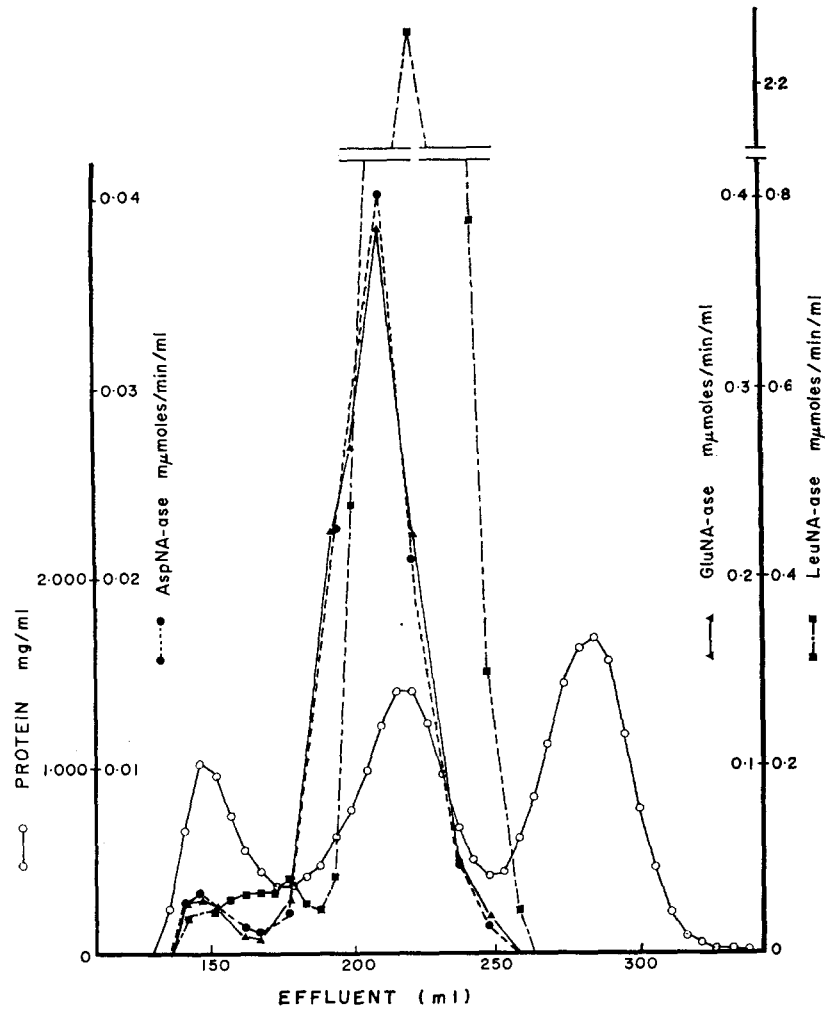

FIG. 1. Separation of amino acid β-naphthylamidases in human plasma on Sephadex G-200 column chromatography. Hydrolysis measured fluorometrically.

The enzyme which hydrolyzes AspNA (AspNA-ase) had the same distribution as that of the enzyme for the hydrolysis GluNA (GluNA-ase), both appearing as two peaks. One peak (GluNA-ase$_1$ or AspNA-ase$_1$) was in the first protein peak and the second appeared just before the second protein peak. The activity in the GluNA-ase$_2$ peak or AspNA-ase$_2$ peak was about thirteen times that in GluNA-ase$_1$ or AspNA-ase$_1$. In both peaks the ratio of AspNA-ase to GluNA-ase was 1 : 10.

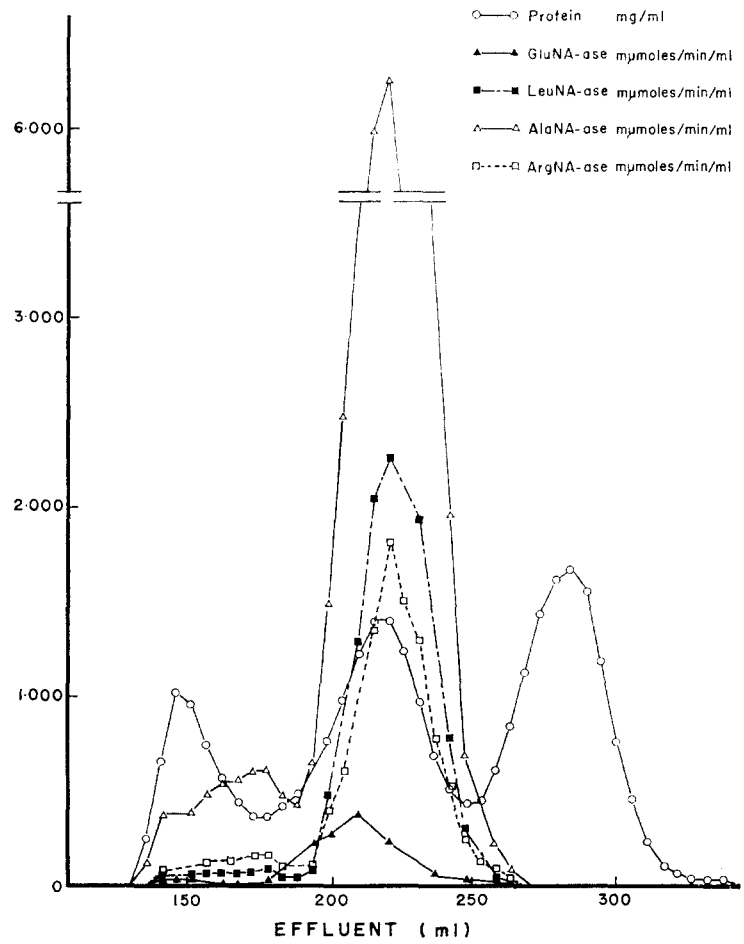

FIG. 2. Separation of amino acid β-naphthylamidases in human plasma on Sephadex G-200 column chromatography. Hydrolysis measured colorimetrically.

The enzymatic activity for the hydrolysis of LeuNA (LeuNA-ase) was also found in two peaks, but located at different positions with respect to the protein peaks. LeuNA-ase$_1$ was between the first and the second protein peaks, and LeuNA-ase$_2$ was just after the second protein peak. The activity on the LeuNA-ase$_2$ peak was much greater (28 times) than that in LeuNA-ase$_1$.

Activities toward the other β-naphthylamides were found in the LeuNA-ase peaks except in the case of phenylalanyl, tyrosyl and valyl substrates, for which activity was

detected only in the LeuNA-ase$_2$ peak. AlaNA was hydrolyzed most rapidly of any of the substrates, three times as rapidly as LeuNA. Activities toward the other substrates, relative to leucyl, were: arginyl, 0·85:1; lysyl, 0·42 : 1; phenylalanyl, 0·30 : 1; tyrosyl, 0·1 : 1; and valyl, 0·08 : 1.

Molecular weight of plasma amino acid β-naphthylamidases

The molecular weights of these amino acid β-naphthylamidases were determined by gel filtration in Sephadex G-200 (2·5 × 90 cm). Ten mg each of γ-globulin, plasma albumin, egg albumin and soybean trypsin inhibitor in 5 ml of the starting buffer were applied on the column. The elution was carried out with 0·1 M Tris-HCl buffer (pH 7·5) containing 1 M NaCl. The flow rate was about 16·5 ml per hr, and the fraction volume was 5·5 ml. The void volume of the column was 152 ml.

An excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume, *V*, to the void volume, *Vo*, of the column was found as reported by Whitaker.[9] A Whitaker plot of the present data is shown in Fig. 3. The molecular weights of α-globulin, bovine plasma albumin, egg albumin or soybean trypsin inhibitor were taken as 150,000, 70,000, 40,000, or 20,000 respectively.

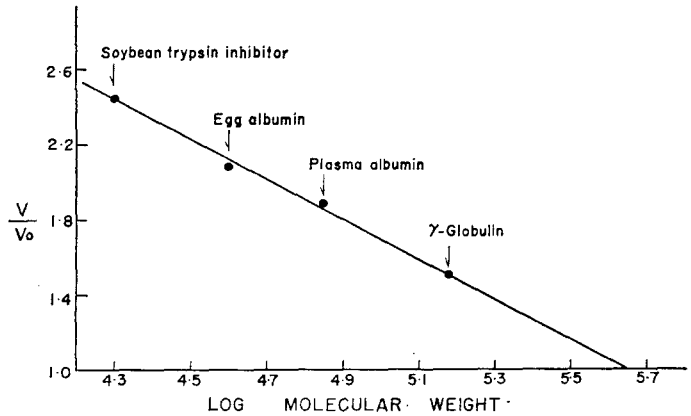

FIG. 3. Relationship between elution volume and molecular weight of α-globulin, plasma albumin, egg albumin and soybean trypsin inhibitor.

The fractionation of human plasma by gel filtration on Sephadex G-200 leads to the separation of four peaks of enzymatic activity for the hydrolysis of β-naphthylamides of amino acids. These appear in the excluded volume and at positions corresponding to molecular weights of approximately 290,000, 190,000 and 160,000.

From Fig. 3, molecular weights of β-naphthylamidases can be obtained as shown in Table 2. The variation in different experiments was about ± 2000. Although each peak exhibits some activity toward AspNA-ase, GluNA-ase and LeuNA-ase, the molecular weights of their major activities were 190,000 for AspNA-ase$_2$ and GluNA-ase$_2$, and 160,000 for LeuNA-ase$_2$.

DEAE-cellulose chromatography of amino acid β-naphthylamidases

Normal human plasma, which was dialyzed against 0·02 M Tris, pH 7·5, containing

up to 200 mg protein, was applied to a DEAE-cellulose column (2·5 × 77 cm) and eluted with the same buffer with a super-imposed linear NaCl gradient from 0 to0·4 M. One hundred and thirty fractions of 4·5 ml were collected. The flow rate was 27 ml/hr. The protein concentrations and enzyme activities against amino acid β-naphthylamides were tested. NaCl content was determined by using a conductivity bridge. The results are shown in Fig 4. The first, second, third, fourth and sixth protein peaks had no β-naphthylamidase activity. GluNA-ase or AspNA-ase was eluted in the early part of

TABLE 2. MOLECULAR WEIGHTS OF AMINO ACID β-NAPHTHYLAMIDASES FROM A SEPHADEX G-200 COLUMN

Amino acid β-naphthylamidase	1	2
AspNA-ase		190,000
GluNA-ase		190,000
LeuNA-ase	290,000	160,000
AlaNA-ase	290,000	160,000
ArgNA-ase	290,000	160,000
LysNA-ase	290,000	160,000
PheNA-ase		160,000
ValNA-ase		160,000
TyrNA-ase		160,000

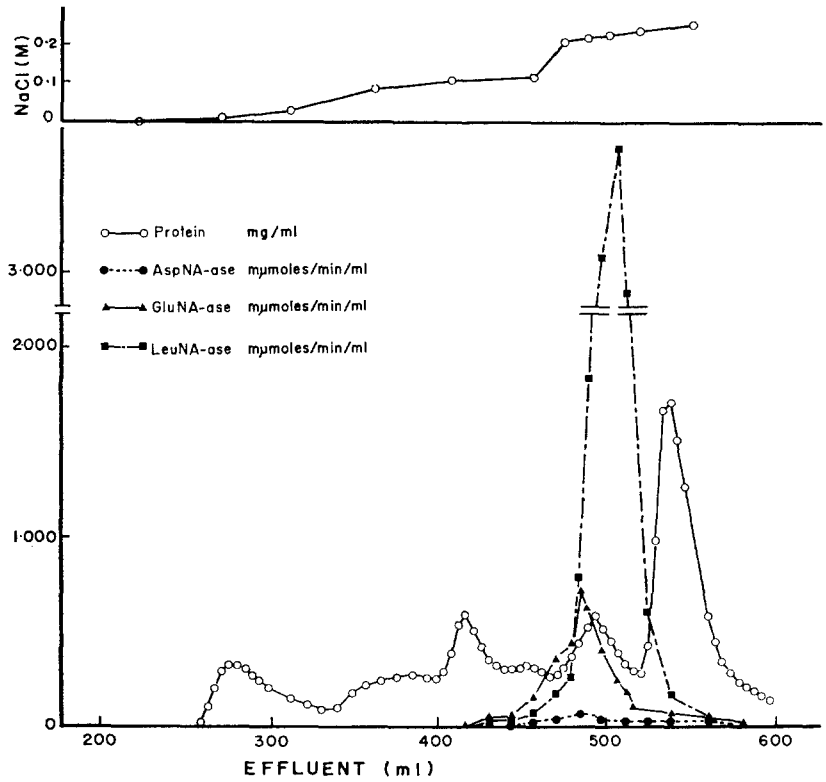

FIG. 4. Separation of amino acid β-naphthylamidases in human plasma on DEAE-cellulose chromatography.

the fifth protein peak and the other β-naphthylamidases appeared in the later part of the fifth protein peak, which was eluted by 0·1 to 0·2 M NaCl.

Combination of chromatography on Sephadex G-200 and on DEAE-cellulose for the separation of GluNA-ase and LeuNA-ase

The second protein peak was collected from five runs of Sephadex G-200 chromatography as described above, pooling fractions from the first half of the peak in one sample and from the second half of the peak in a second sample. The first sample had a ratio of GluNA-ase : LeuNA-ase of 1 : 2·3. It contained 73 mg protein and, after concentration to 10 ml by ultrafiltration, it was applied to a DEAE-cellulose column (2·5 × 80 cm) equilibrated with 0·02 M Tris-HCl buffer, pH 7·6, containing 0·1 M NaCl. Elution was performed by a gradient from the original buffer to 0·02 M Tris-HCl buffer containing 0·3 M NaCl, pH 7·7. The flow rate was about 30 ml per hr, and fraction size was 5 ml. GluNA-ase activity was eluted earlier than LeuNA-ase activity. The activity in each peak was shown in Table 3–I.

TABLE 3. PURIFICATION OF GLUNA-ASE FROM HUMAN PLASMA

Purification step	Total protein (mg)	Total act. (mμmoles/min)			Sp. act. (mμmoles/min/mg)			Yield %			Purification		
Amino acid NA-ase		Asp	Glu	Leu	Asp	Glu	Leu	Asp	Glu	Leu	Asp	Glu	Leu
Plasma	1010	10	110	405	0·01	0·1	0·36	100	100	100	1	1	1
I Sephadex G-200 2nd protein peak 1/2 ahead DEAE celulose	118	2·4	35	82	0·02	0·3	0·7	24	32	20	2	3	2
I_1 GluNA-ase peak	10	2	23		0·2	2·3		20	21		20	23	
I_2 LeuNA-ase peak	10			48			5			12			13
II Sephadex G-200 2nd protein peak, 1/2 behind DEAE-cellulose	139	1·2	17	128	0·01	0·12	0·9	15	19	38	2	2	4
II_1 GluNA-ase peak	11		16			1			17			25	
II_2 LeuNA-ase peak	11			104			9			31			43

The sample from the second half of the peak from pooled runs on Sephadex showed a ratio of GluNA-ase : LeuNA-ase of 1 : 7·5. After concentration by ultrafiltration, the sample containing 127 mg protein in 4·5 ml was applied to a DEAE-cellulose column (2·5 × 75·5 cm) equilibrated with 800 ml of 0·02 M Tris-HCl buffer, pH 7·6, containing 0·1 M NaCl. Elution was performed by a gradient from the original buffer to 0·02 M Tris-HCl buffer containing 0·3 M NaCl, pH 7·7. The flow rate was about 56·4 ml per hr and fraction size was 4·7 ml. Protein concentrations and the enzyme activities for the hydrolysis of β-naphthylamides of amino acids in each fraction were tested (Fig. 5). The GluNA-ase peak came about 80 ml (19 fraction tubes) earlier than the LeuNA-ase peak. The AspNA-ase peak was identical with that of GluNA-ase peak. The AspNA-ase peak was identical with that of GluNA-ase by fluorometry. The peak of AlaNA-ase or ArgNA-ase had the same location with LeuNA-ase peak. The GluNA-ase and AspNA-ase peak was eluted with 0·16 to 0·17 M NaCl and that of LeuNA-ase with 0·18 to 0·19 M. The activities in each peak are shown in Table 3–II.

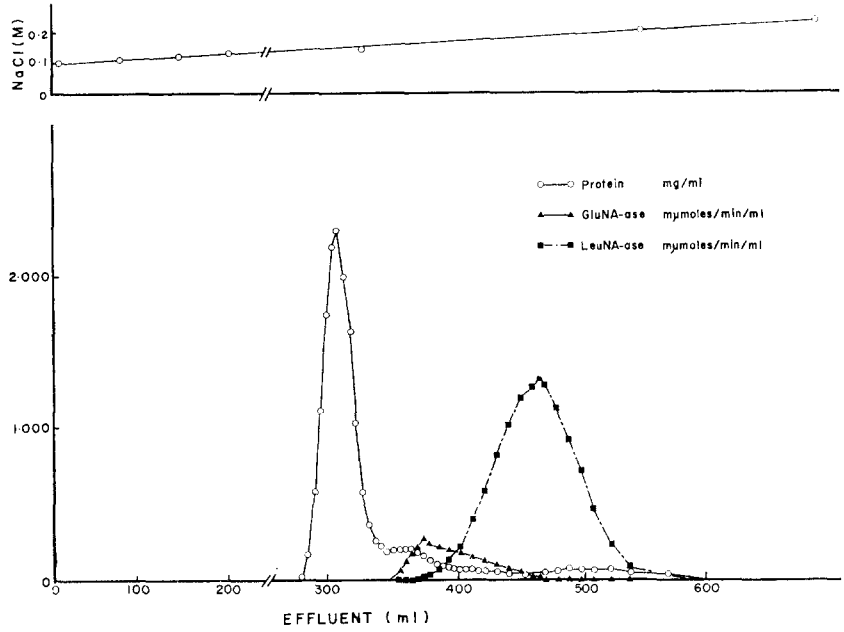

FIG. 5. Separation of amino acid β-naphthylamidases in human plasma on DEAE-cellulose after Sephadex G-200 chromatography.

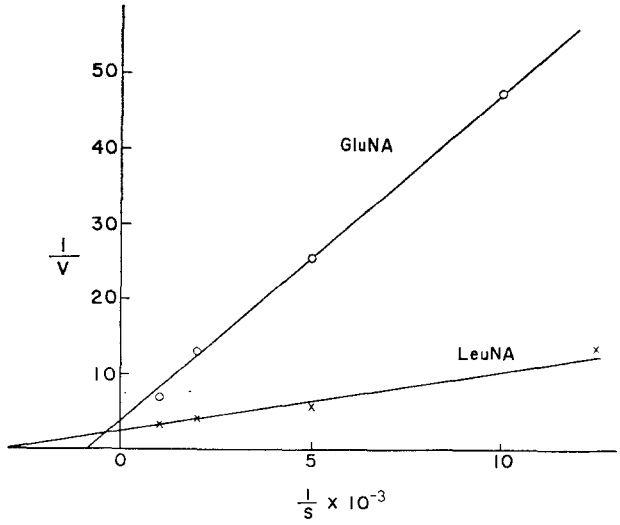

FIG. 6. Lineweaver–Burk plots of GluNA-ase and LeuNA-ase.

Characteristics of purified GluNA-ase and LeuNA-ase

Fractions II_1 and II_2 in the second DEAE-cellulose column chromatography in Table 3 were used to compare the properties of GluNA-ase and LeuNA-ase. The pH optimum of GluNA-ase was 7·5 and that of LeuNA-ase was pH 6·6.

K_m values of GluNA-ase and LeuNA-ase were obtained from Lineweaver–Burk plots as shown in Fig, 6. The K_m value for GluNA-ase was $1{\cdot}2 \times 10^{-3}$ M and that for LeuNA-ase was $3{\cdot}3 \times 10^{-4}$ M.

Six divalent cations were tested under two experimental conditions: (1) metal and enzyme were preincubated at room temperature for 30 min; (2) metal and substrate were preincubated at room temperature for 30 min. As shown in Table 4, Ca^{++},

TABLE 4. METAL EFFECTS ON GLUNA-ASE AND LEUNA-ASE

Metal	Final conc. of metal (M)	Activity of GluNA-ase (preincubation, 30 min)		Activity of LeuNA-ase (preincubation, 30 min)	
		Metal & enzyme (%)	Metal & substrate (%)	Metal & enzyme (%)	Metal & substrate (%)
None	0	100	100	100	100
Sr^{++}	5×10^{-2}	133	263	57	
	1×10^{-2}	115	278	84	
	1×10^{-3}	109	158	114	
	1×10^{-4}		107		
Ca^{++}	5×10^{-2}	93	178	66	
	1×10^{-2}	122	241	91	90
	1×10^{-3}	131	165	95	100
	1×10^{-4}		91		96
Mg^{++}	5×10^{-2}	84	91	84	76
	1×10^{-2}	73	108	110	89
	1×10^{-3}	67	89	91	89
	1×10^{-4}		97		96
Ba^{++}	5×10^{-2}	98	128	28	
	1×10^{-2}	100	175	71	
	1×10^{-3}	93	124	91	
	1×10^{-4}		89		
Mn^{++}	5×10^{-2}	0	3	0	5
	1×10^{-2}	45	91	39	54
	1×10^{-3}	45	92	83	89
	1×10^{-4}		112		93
Co^{++}	5×10^{-2}	36		27	
	1×10^{-2}	33	41	44	45
	1×10^{-3}	22	89	119	91
	1×10^{-4}		89		89

Sr^{++} and Ba^{++} activated GluNA-ase, but not LeuNA-ase when metal and substrate were preincubated. Mg^{++} had almost no effect on either enzyme. Mn^{++} or Co^{++} had slight inhibitory effect on both enzymes, when more than 10^{-2} M of metals was used.

LeuNA-ase was inhibited by *o*-phenanthroline (1×10^{-4} M), and the inhibition was reversed by Co^{++} (1×10^{-4} M). GluNA-ase was affected by neither *o*-phenanthroline nor Co^{++}.

p-Chloromercuribenzoate (1×10^{-4} M) and cysteine (1×10^{-4} M) had no effect on LeuNA-ase and GluNA-ase.

DISCUSSION

The chromatographic separation of GluNA-ase from LeuNA-ase by Sephadex G-200 and by DEAE-cellulose proved that these enzymes are different. Although each peak showed some overlapping, the GluNA-ase peak was reasonably well separated from the LeuNA-ase peak by either Sephadex G-200 or DEAE-cellulose. On the other hand, GluNA-ase and AspNA-ase showed identical elution patterns in both column chromatographies, suggesting that these enzymes are identical. The complete separation of both GluNA-ase and LeuNA-ase was not achieved even by combining both Sephadex G-200 and DEAE-cellulose column chromatography. However, the early part of the GluNA-ase peak after DEAE-cellulose chromatography showed very little activity toward LeuNA. The ratio of activity of GluNA-ase was about 30 : 1, as compared with a ratio of 1 : 4 in the original plasma.

These results support the original report by Glenner *et al.*[1] that there is a distinct aminopeptidase, i.e. aminopeptidase A, which is specific for *N*-terminal dicarboxylic amino acids. These results did not support the view that aminopeptidase A and LeuNA-ase are identical enzymes.[8] However, our findings in the effect of Ca^{++} on GluNA-ase agree with the results reported by Roth.[8] Preincubation of the substrate with Ca^{++}, Sr^{++} or Ba^{++} greatly increased the rate of hydrolysis of GluNA by GluNA-ase. Ca^{++} increased the activity of the AspNA-ase and GluNA-ase peak toward both the aspartyl and glutamyl substrates, but not that of LeuNA-ase. The lower GluNA-ase activities obtained when metal ions were preincubated with the enzyme indicate that the metal ions do not stabilize the enzyme and suggest that cation–enzyme complexes are less active than an uncomplexed form. The results suggest, rather, that GluNA and AspNA are better substrates when complexed with Ca^{++}, Sr^{++} or Ba^{++}.

The differences in optimum pH, K_m and the effect of metals are also consistent with the idea that GluNA-ase and LeuNA-ase are different enzymes, though such differences could be related to differences in substrate. It seems likely that the LeuNA-ase is relatively nonspecific and is responsible for the hydrolysis of the other amino acid naphthylamides. Indeed, considering the greater rate of hydrolysis of the alanyl substrate, it might more appropriately be called AlaNA-ase. It is somewhat surprising that there does not appear to be any regular relation between the rates of hydrolysis and structures of those substrates which seem to be acted upon by the same enzyme (LeuNA-ase), although it is possible that such a regularity might appear if the kinetic parameters were evaluated adequately (V_{max}, K_m for each substrate).

In addition to the major enzymatic activities found in the molecular weight range 160,000–190,000, lower levels of activity were observed in higher molecular weight fractions from gel filtration. The question of whether these represent different enzymes or polymeric forms of the major components has not been explored.

The origin of plasma LeuNA-ase or GluNA-ase is not clear. However, properties of LeuNA-ase, that is, inhibition by *o*-phenanthroline, reactivation by Co^{++}, and no effect of *p*-chloromercuribenzoate and cystein, showed that plasma LeuNA-ase is similar to the tissue-bound, sulfhydry-independent LeuNA-ase in the kidney.[12]

It is interesting to see that purified GluNA-ase may preferentially hydrolyze naturally occurring α-L-aspartyl[1]-Val[5]-angiotensin II, whereas purified LeuNA-ase may mainly hydrolyze α-L-asparaginyl[1]-Val[5]-angiotensin II (Hypertensin-CIBA) as reported in earlier observation.[2, 7] Further work on this question is in progress.

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