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Separation of Two Dipeptidyl Aminopeptidases in the Human Brain

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Abstract: Soluble dipeptidyl aminopeptidases in the human cerebral cortex were purified by CM-cellulose, Sephadex G-200, and hydroxyapatite column chromatography. With hydroxyapatite chromatography two enzymes, dipeptidyl aminopeptidases A and B (DAP-A and DAP-B), were separated. DAP-A and DAP-B were different from each other in several properties: optimum pH, substrate specificity, K_m values in 7-(Gly-Pro)-4-methylcoumarinamide, and molecular weight. They were identified as dipeptidyl aminopeptidases based on the analysis of the products by thin-layer chromatography. DAP-A was similar to dipeptidyl aminopeptidase II, but DAP-B was different from any of the previously described dipeptidyl aminopeptidases (I–IV), and may be a new dipeptidylaminopeptidase. DAP-B liberated N-terminal Arg-Pro, and subsequently Lys-Pro, from substance P as substrate. Although the physiological roles of these two enzymes in the human brain are not clear yet, they may act on regulation and degradation of biologically active peptides.

Dipeptidyl aminopeptidases are a group of intracellular enzymes that are capable of cleaving dipeptide moieties from the unsubstituted NH_2 termini of peptides and dipeptidyl arylamides. Cathepsin C (dipeptidyl aminopeptidase I, DAP-I) preferentially hydrolyzes Gly-Arg-arylamide, and dipeptidyl aminopeptidase II (DAP-II) hydrolyzes Lys-Ala-arylamide. DAP-I and -II are lysosomal enzymes and have an optimum pH between 5.0 and 6.0 (McDonald et al. 1968; 1969). Dipeptidyl aminopeptidase III (DAP-III) (Ellis and Nuenke, 1967) hydrolyzes specifically Arg-Arg-arylamide at pH 8.0–9.0. Dipeptidyl aminopeptidase IV (DAP-IV, X-prolyl dipeptidyl aminopeptidase, Gly-Pro-β-naphthylamidase, or post-proline dipeptidyl aminopeptidase) hydrolyzes X-Pro-arylamides (X = Gly, Lys, Arg, Phe, Pro) at pH 7.8–8.7 (Nagatsu et al., 1976).

DAP-IV was first discovered in rat liver and kidney as glycylproline β-naphthylamidase by Hopsu-Havu and Glenner (1966). The enzyme was purified from hog kidney (Hopsu-Havu et al., 1968), lamb kidney (Yoshimoto and Walter, 1977), and human submandibular gland (Oya et al., 1972). In our laboratory the physiological roles of DAP-IV have been studied in various tissues for several years. DAP-IV is also present in human sera (Nagatsu et al., 1967; Hino et al., 1975, 1976) and saliva (Nagatsu et al., 1968). We have recently found dipeptidyl aminopeptidase activity in human cerebrospinal fluid (Kato et al., 1979), suggesting the presence of the enzyme in the brain. The enzyme in the brain may hydrolyze X-Pro dipeptide from some biologically active peptides such as substance P and bradykinin, because DAP-IV from human submandibular gland hydrolyzed NH_2-terminal dipeptide Arg^1-Pro^2 and subsequent dipeptide Lys^3-Pro^4 from substance P (Kato et al., 1978).

Developmental changes in DAP-I and DAP-III during maturation of the brain have been studied by Marks et al. (1975). We have also found dipeptidyl aminopeptidase activity in developing rat brain by using 7-(Gly-Pro)-4-methyl-coumarinamide (Gly-Pro-MCA) as substrate (manuscript in preparation).

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Abbreviations used: DAP, Dipeptidyl aminopeptidase; MCA 4-methyl-coumarin-7-amide.

Total dipeptidyl aminopeptidase activity per rat brain increased until 4 weeks of age, and then decreased during maturation. The specific activity in the young brain (from 4 days to 4 weeks) was higher than that in the adult rat brain. Starting from these results on brain dipeptidyl aminopeptidase, we have studied the properties of this enzyme in the human brain. Enzyme activity in the human cerebral cortex was the highest in all of the brain regions. In this paper, we report purification and properties of two dipeptidyl aminopeptidases (DAP-A and DAP-B) in the human cerebral cortex.

MATERIALS AND METHODS

Substance P, Gly-Pro-MCA, *p*-nitroanilides of Arg-Pro, Lys-Pro, Gly-Pro, Gly-Ala, Ala-Gly, and Ala-Ala were synthesized at Protein Research Foundation (Minoh, Osaka, Japan), as reported previously (Nagatsu et al., 1976; Kato et al., 1978). Lys-Ala-β-naphthylamide (Lys-Ala-βNA) was purchased from Bachem Fine Chemicals, and Puromycin from Sigma. Sephadex G-200 was obtained from Pharmacia, carboxymethylcellulose from Whatman, and Bio-Gel hydroxyapatite from Bio-Rad laboratories. Human brains were obtained through the pathology departments of hospitals in Nagoya. The brains were removed between 1.5 and 13 h postmortem and frozen at −80°C before use. None of the patients had a history of neurological disorder. The enzyme activities were assayed with Gly-Pro-MCA as substrate. Incubation mixture (total volume 100 μl) contained 0.5 mM-Gly-Pro-MCA, 0.06 M-sodium acetate buffer, pH 5.2, and enzyme. As the two enzyme activities increased linearly up to each 10 μg of purified protein, we usually used an amount between 0.1 μg and 2 μg per tube for kinetic studies. Incubation was at 37°C for 30 min and the reaction was stopped by adding 1 ml of 1 M-acetate buffer, pH 4.2. The fluorescence intensity of 7-amino-4-methylcoumarin (AMC) liberated by the enzyme reaction was measured at 460 nm with excitation at 380 nm by a Shimadzu RF-500 spectrophotofluorometer. For the study on substrate specificity, *p*-nitroanilides of Arg-Pro, Lys-Pro, Gly-Pro, Gly-Ala, Ala-Ala, and Lys-Ala-β-naphthylamide were used as substrates. Incubation mixtures (total volume 250 μl) contained 1.2-mM substrate, 0.06 M-acetate buffer, pH 5.2, and enzyme. As the reaction proceeded linearly for at least 120 min, incubation was carried out at 37°C between 30 and 120 min, and stopped by adding 1 ml of 1 M-acetate buffer, pH 4.2. *p*-Nitroaniline liberated by the enzyme reaction was measured, based on the absorbance at 380 nm, and β-naphthylamine was measured by the fluorescence intensity at 410 nm with excitation at 355 nm.

The enzymes were purified from the soluble fraction of human cerebral cortex. All procedures were carried out at 0–4°C. Fifty-four grams of human cerebral cortex was homogenized in 5 vol. of water using a glass Potter homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min. The supernatant was dialyzed against a large volume of 10 mM-acetate buffer, pH 4.5, overnight. This dialyzed enzyme solution was applied to a carboxymethylcellulose column (2.6 × 5 cm) equilibrated with the same buffer. The column was washed with 400 ml of the buffer and the enzymes were eluted with 1200 ml of a linear gradient of NaCl (0–0.3 M) in 50 mM-acetate buffer, pH 5.0. The pooled fractions containing the enzymes were concentrated to 6.0 ml by immersing the solution, in a dialysis tube, into sucrose crystals. The enzyme solution was passed through a column of Sephadex G-200 (2.6 × 90 cm) previously equilibrated with 5 mM-Tris-HCl buffer, pH 7.4. Elution was carried out using the same buffer at a flow rate of 12 ml/h, and fractions of 3 ml each were pooled. The active fractions were pooled and concentrated by ultrafiltration. The enzyme solution (4.8 ml) was applied to a column of hydroxyapatite (0.8 × 2 cm) previously equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. When the column was washed with 14 ml of the same buffer, the first active fraction (DAP-A) was eluted in the last 6 ml of the washings, and the second active fraction (DAP-B) was eluted with 60 ml of a linear gradient of phosphate buffer (0.01–0.1 M), pH 7.0. The purified enzymes lost about 70% of their activity by freezing and thawing once. On the other hand, the enzyme activities were stable at 0–4°C for at least 1 week.

For the study of the effect of pH on enzyme activity, 0.5 mM-Gly-Pro-MCA and 0.6 mM-Lys-Ala-βNA were used as substrates in 0.06 M-acetate buffer at various pH's. The K_m values with purified enzymes were determined from Line weaver-Burk plots using Wilkinson's program (Wilkinson, 1961) in 0.06 M-acetate buffer, pH 5.2, with Gly-Pro-MCA as substrate and expressed as mean ± standard error of mean.

The reaction products from Ly-Pro-pNA, Arg-Pro-pNA, and substance P by DAP-B were identified by thin-layer chromatography. The chromatography was performed by use of a Merk No. 5716 cellulose plate and two solvent systems, n-butanol/acetic acid/water (2:1:1, by vol.) and phenol water (3:1, by vol.). Peptides were located with 0.2% ninhydrin. L-arginine, L-lysine, substance P-(5-11)-heptapeptide, and substance P were used as standards. Homogeneous DAP-IV purified from human submandibular gland was obtained by the method of Oya et al. (1972).

RESULTS

Purification and Properties of Dipeptidyl Aminopeptidases in Human Brain

Dipeptidyl aminopeptidase activities in the human cerebral cortex were the highest of all the brain regions studied *(unpublished observation)*. The activities in the caudate nucleus and hippocampus were also high. Therefore, we used human cerebral cortex for purification of dipeptidyl aminopeptidases. It was found that 60% of the enzyme in the cerebral cortex was soluble. After centrifuging the brain homogenate at 100,000 × *g*, the soluble fraction was applied to a carboxymethylcellulose column, and the enzymes were eluted with a linear gradient of NaCl at 0.15 M (Fig. 1). Because contaminating proteins of higher molecular weights were excluded by Sephadex G-200 gel filtration (Fig. 2), purity of the enzymes was increased about sevenfold (Table 1). Two dipeptidyl amino-

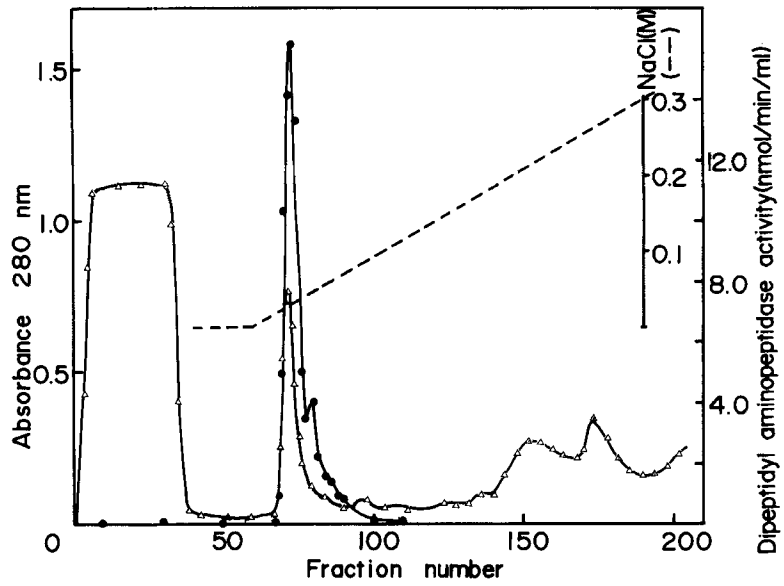

FIG. 1. Chromatography of human brain dipeptidyl aminopeptidases on a column of carboxymethyl cellulose. Absorbance was measured at 280 nm (△—△). Enzyme activity was determined using Gly-Pro-MCA (●—●). The experimental details are described in Materials and Methods.

peptidases, DAP-A and DAP-B, could be separated by hydroxyapatite column chromatography (Fig. 3). Dipeptidyl aminopeptidase A was eluted by washing with 0.01 M-phosphate buffer (pH 7.4), and dipeptidyl aminopeptidase B was eluted with a linear gradient of the same buffer (0.01–0.1 M, pH 7.4). Though the two enzyme fractions did not show a single band on polyacrylamide gel electrophoresis, these active fractions were combined and used for the following experiments. Specific activity of DAP-A was 139 nmol/min/mg protein and that of DAP-B was 173 nmol/min/mg protein, using Gly-Pro-MCA as substrate at pH 5.2. The entire purification is shown in Table 1.

The K_m values of DAP-A and DAP-B were 1.43 ± 0.068 mM and 0.89 ± 0.005 mM, respectively.

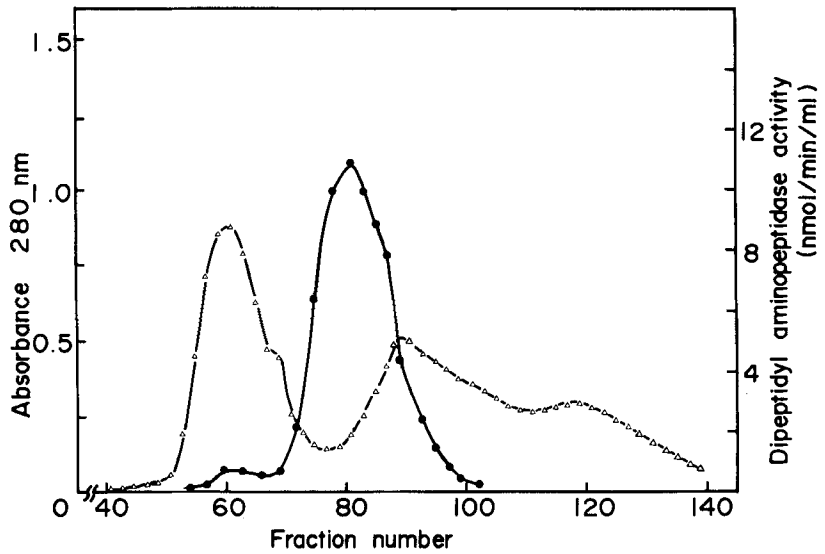

FIG. 2. Chromatography of the dipeptidyl aminopeptidases on a column of Sephadex G-200. Absorbance was measured at 280 nm (△—△). Enzyme activity was determined as described in the text using Gly-Pro-MCA (●—●). For experimental details see Materials and Methods.

TABLE 1. *Purification of dipeptidyl aminopeptidase from human cerebral cortex*

Stage	Total Protein (mg)	Activity (nmol/min/mg protein)	Yield (%)	Purification
Homogenate	3690	0.360	100	1
100,000 *g* supernatant	500	1.66	63	4.6
Carboxymethylcellulose	30.1	11.49	26	32
Sephadex G-200	1.33	72.7	7.2	200
Hydroxyapatite DAP-B	0.37	173.0		480
DAP-A	0.23	139.0		360

Molecular weights of the two enzymes were estimated by gel filtration on Sephadex G-200 according to the method of Whitaker (1963). DAP-A showed a main peak with an approximate molecular weight of 130,000, and DAP-B 160,000.

Substrate Specificities of Dipeptidyl Aminopeptidases A and B in Human Brain

Table 2 shows the substrate specificity of DAP-A and DAP-B, with various dipeptide arylamides as substrates. There were several differences between the two enzymes. The main difference was the higher activity of DAP-A towards Lys-Ala-βNA compared with the activity of DAP-B. The best substrate for DAP-B was Lys-Pro-arylamide. On the other hand, the hydrolysis rate of Gly-Pro-arylamide by DAP-A was higher than that by DAP-B. DAP-B did not hydrolyze Gly-Ala-pNA and Ala-Gly-pNA at all, but DAP-A had low activities.

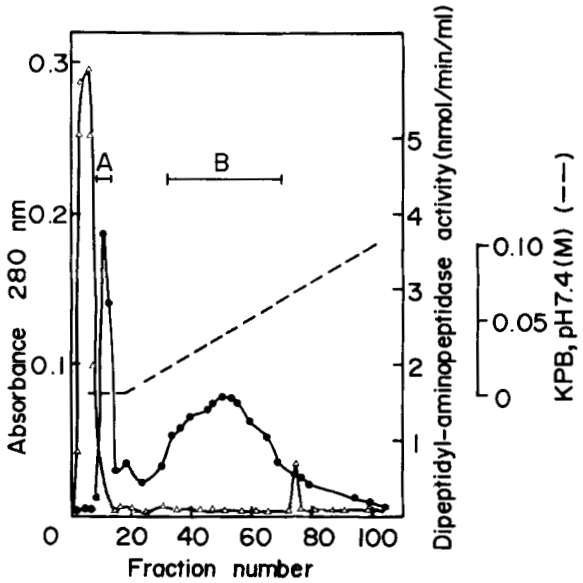

FIG. 3. Chromatography of dipeptidyl aminopeptidases A and B on a column of hydroxyapatite. The active fraction of Sephadex G-200 column chromatography was concentrated and applied to a hydroxyapatite column (0.8 × 2 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. The column was washed with 14 ml of the same buffer, and eluted with a linear gradient of the phosphate buffer (0.01–0.1 M). The active fraction (A or B) was pooled and used as dipeptidyl aminopeptidase A or B. Absorbance was measured at 280 nm (△—△). Enzyme activity was determined using Gly-Pro-MCA (●—●).

Effect of pH on the Activities of Dipeptidyl Aminopeptidases A and B in Human Brain

The pH activity curves of the two DAPs towards Lys-Ala-βNA in the acetate buffer are shown in Fig. 4. DAP-A showed the highest activity at pH 5.5, whereas that of DAP-B was at pH 4.8. On the contrary, the effect of pH on the activity towards Gly-Pro-MCA as substrate was different (Fig. 5). The optimum pH of DAP-B towards Gly-Pro-MCA in the acetate buffer was at 5.8, but that of DAP-A had two peaks at pH 4.7 and at pH 5.8.

Thin-layer Chromatography of the Products Enzymatically Released from Substance P

Hydrolysis of substance P, Arg-Pro-pNA, and Lys-Pro-pNA by homogeneous DAP-IV purified from human submandibular gland was done as described previously (Kato et al., 1978). Incubation (total volume 50 μl) was carried out at 37°C for 2 h with substrates at 2 mM (substance P, Arg-Pro-pNA, and Lys-Pro-pNA) and 3 μg of human brain DAP-B in 0.06 M-acetate buffer, pH 5.2; the reaction was terminated by heating at 95°C for 5 min. The mixture was centrifuged, and the products liberated from the substrates by the enzyme were identified on thin-layer chromatograms developed by two solvents: n-butanol/acetic acid/water (2:1:1, by vol.) and phenol/water (3:1, by vol.). The results obtained by the latter solvent are illustrated in Fig. 6.

As shown in Fig. 6, Lys-Pro and Arg-Pro were released from Lys-Pro-pNA and Arg-Pro-pNA, respectively, by incubation with DAP-B. However, neither lysine, arginine, nor proline was enzymatically liberated from these substrates. When substance P was used as substrate, Arg-Pro and Lys-Pro were detected.

TABLE 2. *Substrate specificity of dipeptidyl aminopeptidases (DAP-A and -B) in human brain*

Substrate	DAP-A		DAP-B	
	Specific activity (nmol/min/mg protein)	Ratio (%)	Specific activity (nmol/min/mg protein)	Ratio (%)
Lys-Pro-pNA[a]	257	100	784	100
Lys-Ala-βNA[b]	4580	1780	439	56
Arg-Pro-pNA	160	63	259	33
Gly-Pro-pNA	241	94	76	9.7
Ala-Ala-pNA	164	64	60	7.7
Gly-Leu-pNA	0	0	14	1.8
Gly-Ala-pNA	31	13	0	0
Ala-Gly-pNA	6.3	2.5	0	0

Incubation was carried out at 37°C. Activities were measured in 0.06 M-sodium acetate buffer, pH 5.2, and at a substrate concentration of 1.2 mM.
[a] pNA, *p*-nitroanilide; [b] βNA, β-naphthylamide.

Effect of Puromycin and Cl-on the Activities of Dipeptidyl Aminopeptidases A and B in Human Brain

The effect of puromycin on the two enzymes was examined. At 1 mM-puromycin, DAP-A was inhibited by 84%, whereas DAP-B showed a 69% inhibition; however, neither DAP-A nor DAP-B was activated by Cl^-.

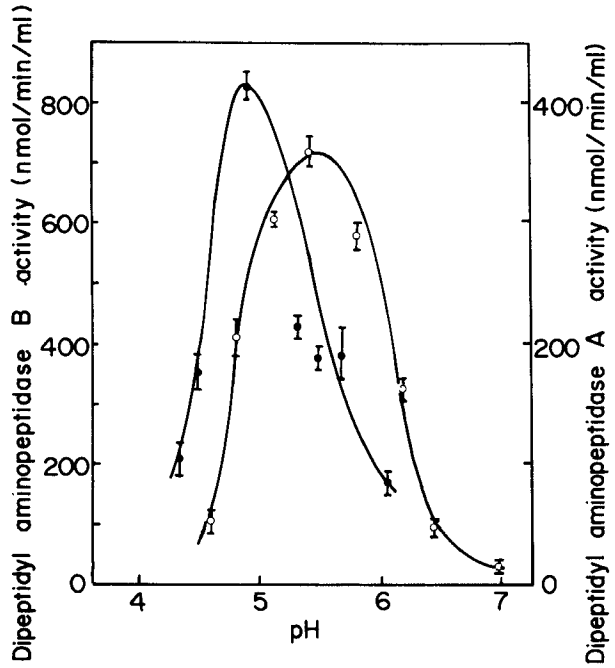

FIG. 4. Effect of pH on enzyme activity using Lys-Ala-βNA as a substrate. Activities were determined using 0.6 mM-Lys-Ala-β-naphthylamide as substrate in 0.06 M-sodium acetate buffer at various pH's and each point was expressed as mean of three experiments and standard error or mean. Dipeptidyl aminopeptidase A (○—○); Dipeptidyl aminopeptidase B (●—●).

DISCUSSION

We tried to find an enzyme to hydrolyze some natural peptides which contain an NH_2-terminal X-proline sequence such as substance P and bradykinin and found two dipeptidyl aminopeptidases (DAP-A and DAP-B) in human brain with Gly-Pro-MCA as substrate. Separation of DAP-A and DAP-B was achieved by hydroxyapatite column chromatography. Analysis of the products by thin-layer chromatography proved clearly that they are dipeptidyl aminopeptidases.

The properties of the two enzymes are different in

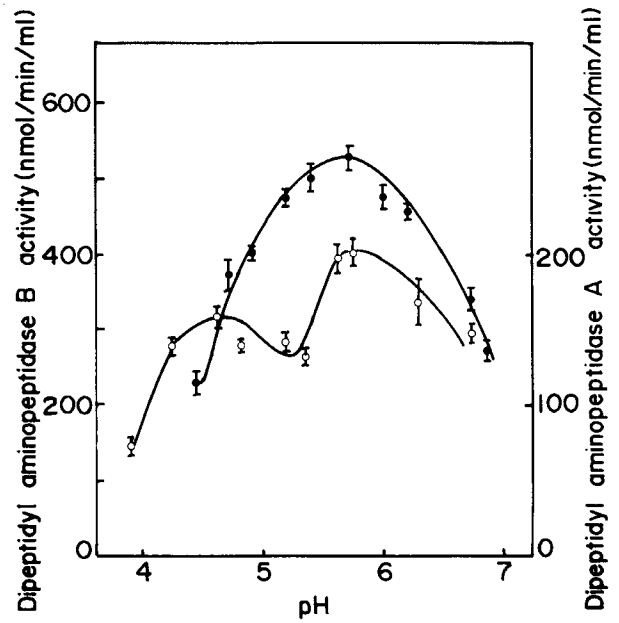

FIG. 5. Effect of pH on activity using Gly-Pro-MCA as a substrate. Enzyme activities were determined using 0.5 mM-7-(Gly-Pro)-4-methylcoumarinamide as substrate. The other procedure is the same as in Fig. 4.

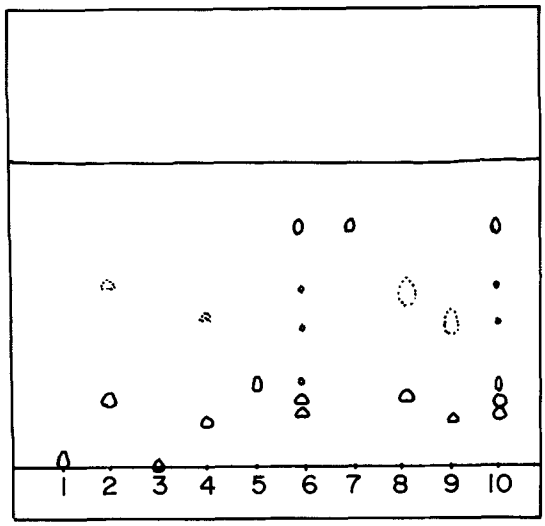

FIG. 6. Hydrolysis of substance P by dipeptidyl aminopeptidase B. Substance P was incubated with DAP-IV purified from human submandibular gland (No. 6) and human brain DAP-B (No. 10), and the reaction products were analyzed by thin-layer chromatography, as described in Materials and Methods. 1, arginine; 2, Arg-Pro (○) released from Arg-Pro-pNA (◎) by DAP-IV; 3, lysine, 4, Lys-Pro (○) released from Lys-Pro-pNA (◎) by DAP-IV; 5, substance P; 6, hydrolysis of substance P by DAP-IV; 7, substance P-(5-11)-heptapeptide; 8, 9, and 10 are the hydrolysis product(s) of Arg-Pro-pNA, Lys-Pro-pNA, and substance P, respectively, by DAP-B.

several respects. As to DAP-A, the optimum pH towards Lys-Ala-βNA was 5.5, the best substrate was Lys-Ala-βNA, and the molecular weight was 130,000. These results and the effect of the inhibitor indicate that DAP-A in human brain is similar to bovine pituitary dipeptidyl-aminopeptidase II (McDonald et al., 1968), the properties of which had been reported; the molecular weight is 130,000 and the hydrolysis rate of Lys-Ala-βNA is 20-fold faster than that of Gly-Pro-βNA. In contrast, DAP-B had an optimum pH of 4.8 towards Lys-Ala-βNA and 5.8. towards Gly-Pro-MCA, the best substrate was Lys-Pro-pNA, and the molecular weight was 160,000. These properties of human brain DAP-B do not conform to those of the previously described four DAPs (I–IV) (Ellis and Nuenke, 1967; McDonald et al., 1968; 1969; Hopsu-Havu et al., 1966). It is possible that human brain DAP-B is a new dipeptidyl aminopeptidase.

The elution pattern of DAP-B in Fig. 3 and the biphasic pH optimum for DAP-A in Fig. 5 suggest that the enzymes may be heterogeneous. Therefore, we should consider the possibility that we are not dealing with a single enzyme but with a mixture of peptidases. However, since cathepsin C (dipeptidylaminopeptidase I) in bovine pituitary gland showed a similar biphasic pH optimum (McDonald et al., 1966) as DAP-A, the biphasic pH optimum of the enzyme may not mean the presence of two enzymes.

The other possibility is that the enzyme could arise as an artifact of partial proteolysis. For example, the smaller DAP-A could arise from partial proteolysis of DAP-B. This possibility is rather unlikely since the properties of the two enzymes are very different, but this question can be answered only after complete purification of DAP-A and DAP-B and comparison of the chemical properties.

Though there are distinct differences in properties between DAP-A and DAP-B, the two enzymes were both able to hydrolyze Lys-Pro-pNA and Arg-Pro-pNA. Since NH_2-terminal tetrapeptide of substance P has a sequence of Arg^1-Pro^2-Lys^3-Pro^4 (Chang et al., 1971), we examined whether or not human brain DAPs hydrolyze substance P. Based on the analysis of the products by thin-layer chromatography, the release of Arg^1-Pro^2 and Lys^3-Pro^4 derived from substance P by DAP-B was clearly detected. Since dipeptidyl aminopeptidase IV purified from human submandibular gland hydrolyzed NH_2-terminal dipeptide Arg-Pro and subsequent dipeptide Lys-Pro from substance P (Kato et al., 1978), human brain DAP-B may also release Arg-Pro, Lys-Pro, and substance P-(5-11)-heptapeptide. It was reported that substance P-(5-11)-heptapeptide was biologically more potent than substance P itself (Yajima et al., 1973; Nakata et al., 1978). Though physiological roles of these two enzymes in the human brain are not clear yet, they could act on regulation and degradation of biologically active peptides.

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