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Purification and Characterization of Dipeptidyl Aminopeptidase III from Human Placenta

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Dipeptidyl aminopeptidase III (DAP III) from a human placental post-microsomal supernatant was purified 760-fold by means of ammonium sulfate fractionation and successive chromatographies on diethylaminoethyl (DEAE)-cellulose, Sephadex G-200, Butyl-Toyopearl 650 and DEAE-Toyopearl columns. The enzyme showed a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and its molecular weight was estimated to be 84000. The enzyme was strongly inhibited by metallochelators, and the activity lost was most effectively restored by the addition of Zn^{2+}. The enzyme was also extremely inhibited by some sulfhydryl reagents such as *p*-chloromercuribenzoic acid (PCMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and the activity of the enzyme inhibited by DTNB was restored by the addition of thiol reagents. Among various β-naphthylamides examined, Arg–Arg–β-naphthylamide (Arg–Arg–βNA) was most rapidly hydrolyzed.

Keywords——dipeptidyl aminopeptidase III; metalloenzyme; thiol enzyme; human placenta

Introduction

Four types of dipeptidyl aminopeptidases have been identified in various mammalian tissues.[1] These peptidases are classified according to their specific activity for the liberation of dipeptide from the N-terminus of peptides. Dipeptidyl aminopeptidase III (DAP III) (EC 3.4.14.4) has been partially purified from the cytosolic fractions of various mammalian tissues, including bovine pituitary gland,[2] rat skin,[3] bovine lens,[4] human erythrocytes[5] and rat brain.[6] The physiological functions of DAP III are not well understood. However, the degradation by rat brain enzyme of angiotensins and enkephalins with micromolar affinities was reported.[6] Thus, DAP III was proposed to play a role in the regulation of angiotensin and/or enkephalin disposition.

On the other hand, during pregnancy, it is well known that the renin-angiotensin system undergoes an activation, and angiotensin-hydrolyzing activities are increased in the plasma.[7] In comparative studies on the activities of cysteine aminopeptidase (another name for which is oxytocinase, and the enzyme activity of which is regarded as a placental marker) and DAP III in control and retroplacental sera, we found marked increases in these activities in the latter serum (see Results). Various lines of evidence indicate that the cysteine aminopeptidase is synthesized in placental cells and is released into the maternal circulation.[8] Therefore, DAP III in the retroplacental serum also seems to be derived from placenta. The present report deals with the purification and characterization of DAP III from human placenta.

Experimental

Materials——Arg–Arg–β-naphthylamide (βNA), Leu–Gly–βNA, Ser–Tyr–βNA, Arg–βNA, phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) and N_α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma Chemical Co., Saint Louis. Ala–Arg–βNA, Ala–Ala–βNA, Gly–Phe–βNA,

Lys–Ala–βNA and Gly–Pro–βNA were from Bachem Feinchemikalien Co., Budendorf. Gly–Gly–βNA was a product of Research Organic Inc., Ohio. Iodoacetic acid (IAA), *N*-ethylmaleimide (NEM) and *o*-phenanthroline were from Wako Pure Chemicals Ind., Osaka. Chromatographic resins were purchased from the following sources: DE-52 cellulose (Whatman Biochemical Ind., Maidstone), Sephadex G-200 and PBE 94 (Pharmacia Fine Chemicals Co., Uppsala), and Butyl-Toyopearl 650 and DEAE-Toyopearl (Toyo Soda, Tokyo). Microbial protease inhibitors were from the Protein Research Foundation, Osaka. Cysteine aminopeptidase (CAP) assay kits based on the method of Watson[9] were purchased from Sankyo Co. Ltd., Tokyo. Other chemicals were of the best quality available commercially.

Assay of DAP III Activity——The activity of DAP III was assayed essentially according to the procedure for DAP III in bovine pituitary gland.[2] A standard reaction mixture (0.2 ml) contained 0.5 mM Arg–Arg–α^{NA}, 50 mM TRis–HCl, pH 9.0, and enzyme. After 30 min at 37 °C, released βNA was estimated by the method of Goldberg and Rutenburg.[10] One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of βNA from the substrate per min. The DAP III activities during purification (step 1 to 5) and of the sera were assayed in the presence of 10 μg/ml bestatin to inhibit the aminopeptidase activity. Under these conditions, negligible aminopeptidase activity was observed.

Protein Concentration——The absorbance at 280 nm was used to monitor the protein peaks on chromatography. For other samples. the protein concentration was measured by the method of Lowry *et al.*[11] with bovine serum albumin as the standard.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli to determine the homogeneity and molecular weight of the denatured enzyme.[12] After the run, the gel was stained for protein with Coomassie brilliant blue G-250. Marker proteins for the molecular weight determination were as follows: rabbit muscle phosphorylase a (M_r 94 K), bovine serum albumin (M_r 68 K), ovalbumin (M_r 45 K) and bovine pancreas chymotrypsinogen (M_r 25 K).

Molecular Weight of the Native Enzyme——The molecular weight of the native enzyme was estimated by elution through a TSK-GEL 4000 SW (0.75 × 60 cm) column as described by Fukano *et al.*[13] Elution was performed with 70 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl at a flow rate of 0.5 ml/min. The reference proteins were ferritin (M_r 440 K), bovine liver catalase (M_r 232 K), rabbit muscle aldolase (M_r 158 K), bovine serum albumin (M_r 68 K) and ovalbumin (M_r 45 K).

Determination of the Isoelectric Point——The isoelectric point of the enzyme was determined by chromatofocusing. The purified enzyme in 25 mM imidazole–HCl, pH 7.4, was applied to a Polybuffer Exchanger PBE 94 column (0.7 × 10 cm) equilibrated with the same buffer. The enzyme was eluted with 8-fold diluted Polybuffer 74, pH 4.0. Fractions of 0.5 ml were collected and each fraction was assayed for DAP III activity.

Kinetic Analysis——The K_m and V_{max} values for Arg–Arg–βNA of the purified enzyme were determined by the method of Lineweaver and Burk.[14] The substrate concentration in this study ranged from 25 μM to 4 mM.

Purification of DAP III——DAP III from human placenta was purified by a six-step procedure. All operations were performed at 0—4 °C.

Step 1. Extraction: Placenta was placed on ice immediately after delivery and the membranes and cord were dissected away. It was washed free from blood with precooled physiological saline (0.9% NaCl). The placental tissue (100 g) was chopped into small pieces and then homogenized with an Ultra-Turrax homogenizer in 8 volumes of 0.25 M sucrose for 5 min. The post-microsomal supernatant was used for the enzyme purification.

Step 2. Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to the above supernatant and the precipitate appearing between 40% and 65% saturation was collected by centrifugation at 13000 × *g* for 20 min. The precipitate was dissolved in 20 mM Tris–HCl, pH 8.0, containing 50 mM NaCl (buffer I), and then the solution was dialyzed overnight against the same buffer.

Step 3. Chromatography on DE-52 Cellulose: The dialyzed materials were applied to a column of DE-52 cellulose (1.5 × 20 cm) previously equilibrated with buffer I. After being washed with the starting buffer, the column was developed with a linear gradient of NaCl (50—400 mM) in 20 mM Tris–HCl, pH 8.0, in a volume of 600 ml. Fractions of 6 ml were collected. The enzyme that hydrolyzed Arg–Arg–βNA was eluted at 0.2—0.27 M NaCl. The pooled fractions were adjusted to 65% ammonium sulfate saturation, and then stirred for 30 min. The resulting precipitate was collected by centrifugation.

Step 4. Gel Filtration on Sephadex G-200: The precipitate was dissolved in 3 ml of buffer I and then applied to a Sephadex G-200 (2.5 × 90 cm) column previously equilibrated with buffer I. Elution was performed with the same buffer and fractions of 4 ml were collected.

Step 5. Chromatography on Butyl-Toyopearl 650: The active fractions from step 4 were pooled and solid ammonium sulfate was added to 40% saturation. The solution was applied to a column of Butyl-Toyopearl 650 (1.5 × 5 cm) previously equilibrated with 40% saturated ammonium sulfate in buffer I. The column was washed with the buffer, and the adsorbed proteins were eluted with a 200 ml gradient of ammonium sulfate, from 40% to 0%, in buffer I. Fractions of 2 ml were collected, and the active fractions were pooled. In this step, contaminating aminopeptidase was completely separated from DAP III (Fig. 1), so bestatin was omitted from the assay system for the last step.

Step 6. Chromatography on DEAE-Toyopearl: The pooled fractions from step 5 were dialyzed against buffer I. The dialyzed solution was applied to a column of DEAE-Toyopearl (1.5 × 8 cm) previously equilibrated with buffer I. The column was washed with the buffer and adsorbed proteins were eluted with a linearly increasing NaCl concentration (50—300 mM) gradient. A total volume of 400 ml of the buffer was used for the gradient elution. Fractions of 4 ml were collected. The fractions containing DAP III activity were combined, concentrated and stored −80 °C until use.

Results

DAP III and Cysteine Aminopeptidase Activities in Sera

DAP III and cysteine aminopeptidase (oxytocinase) activities were significantly higher in retroplacental serum than in control serum ($p<0.01$) (Table I). The DAP III activity was increased 11.6-fold and that of cysteine aminopeptidase was increased 7.28-fold compared to the activities in the control sera.

Enzyme Purification

The results of a typical purification of DAP III from human placenta are summarized in Table II. The enzyme was purified 760-fold from the post-microsomal extract with a yield of 9.3%, and the final specific activity of the enzyme was 11.1 units/mg protein. The human placental cytosolic fraction contains several aminopeptidases which release Arg-residues. With the addition of bestatin (10 μg/ml) to the assay system for the initial extract, the hydrolytic activity toward Arg–Arg–βNA was decreased to about 80% relative to that without the addition. Butyl-Toyopearl 650 column chromatography (step 5) was useful for eliminating the contaminating aminopeptidase which hydrolyzes Arg–βNA (Fig. 1). In this step, the contaminant was completely separated from the DAP III. The final preparation showed a single protein band on SDS-polyacrylamide gel electrophoresis (Fig. 2).

TABLE I. Cysteine Aminopeptidase and DAP III Activities of Control and Retroplacental Sera

Serum	Cysteine aminopeptidase (mU/mg protein)	DAP III (mU/mg protein)
Control serum (1)	0.184±0.0088 ($n=3$)	0.045±0.0003 ($n=3$)
Retroplacental serum (2)	1.34 ±0.082 ($n=4$)	0.524±0.066 ($n=4$)
(2)/(1)	7.28	11.6

Values are means±SEM. Serum samples from non-pregnant women were used as controls. Retroplacental sera were obtained from normal deliveries. The sera were separated after clotting by centrifugation at 600 × *g* for 10 min. DAP III activities were assayed in the presence of 10 μg/ml bestatin. Cysteine aminopeptidase activities were measured by using CAP assay-kits (Sankyo Co., Ltd.).

TABLE II. Summary of the Purification of DAP III from Human Placenta

Step	Protein (mg)	DAP III activity (total units)	Specific activity (mU/mg protein)	Fold	Yield (%)
1. Post-microsomal supernatant[a)]	3690	53.7	14.6	1	100
2. Ammonium sulfate fractionation[a)]	501	38.6	77.0	5.3	72
3. DE-52 cellulose[a)]	78.9	19.8	251	17.2	37
4. Sephadex G-200[a)]	22.0	12.2	555	38.0	23
5. Butyl-Toyopearl	2.60	8.53	3280	225	16
6. DEAE-Toyopearl	0.45	4.99	11100	760	9.3

a) DAP III activities were assayed in the presence of 10 μg/ml bestatin.

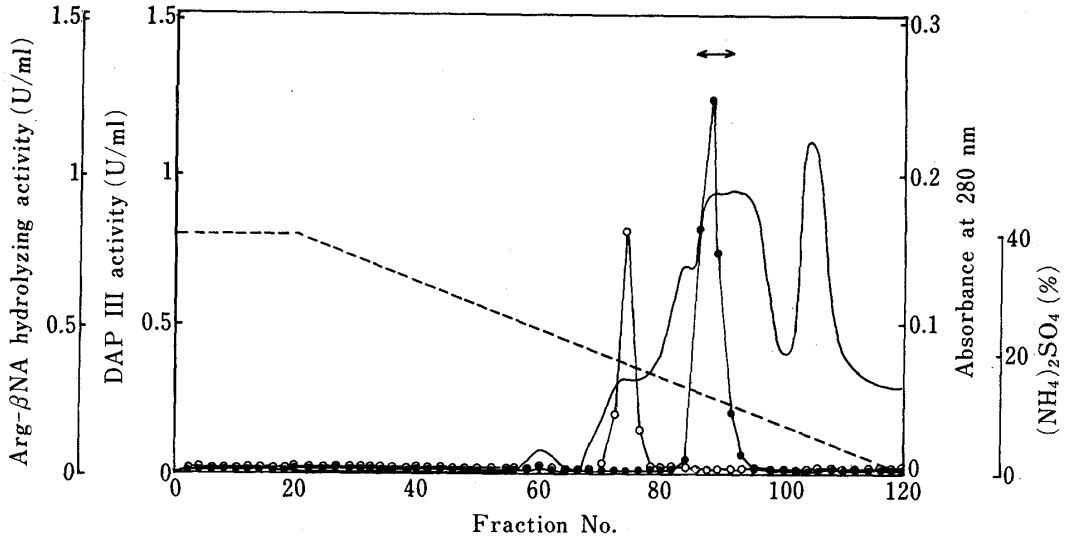

Fig. 1. Butyl-Toyopearl 650 Column Chromatography of the Fraction Obtained from Sephadex G-200

The column (1.5×5 cm) was equilibrated with buffer I containing 40% saturated ammonium sulfate. Elution was performed using a gradient of 40—0% ammonium sulfate in buffer I (----). The column of each fraction was 2 ml. Absorbance was monitored at 280 nm (——). DAP III activity was measured under the standard conditions (●—●). Aminopeptidase activity was measured with Arg-βNA as a substrate by a method similar to that used for measuring the DAP III activity (○—○). The horizontal line with arrowheads indicates the pooled fractions.

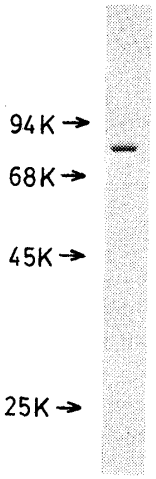

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Purified DAP III

About 10 μg of the purified enzyme was subjected to SDS-polyacrylamide gel (10%) electrophoresis. The numbers to the left of the gel indicate the molecular weights of standard proteins.

Physicochemical Properties of the Enzyme

The molecular weight of the enzyme was estimated to be 84000 by SDS-polyacrylamide gel electrophoresis (Fig. 2). A value of 84000 was also obtained by gel filtration chromatography on TSK-GEL 4000 SW. These results indicate that the enzyme consists of a single polypeptide chain. Analytical chromatofocusing of the purified enzyme gave a single peak of enzyme activity with an isoelectric pH of 4.4.

Kinetic Properties of the Enzyme

The pH dependence of the activity of the purified enzyme was determined in 50 mM sodium phosphate (pH 6.0—7.0), 50 mM Tris–HCl (pH 7.0—9.0) and 50 mM glycine–NaOH (pH 9.0—10.5) buffers. Maximum enzyme activity toward Arg–Arg–βNA was observed in the alkaline region, around pH 9.0. The K_m and V_{max} were determined in 50 mM Tris–HCl, pH 9.0, at 37 °C. Lineweaver–Burk analysis gave a K_m value of 38.2 μM and a V_{max} of 0.885 nmol/min. Substrate inhibition was observed at higher concentrations of the substrate (≧1 mM).

TABLE III. Effects of Inhibitors on the Human Placental DAP III

Inhibitor	Concn.	Activity (%)
Sulfhydryl reagents		
PCMB	1.0 μM	18
DTNB	50.0 μM	10
IAA	1.0 mM	80
NEM	1.0 mM	64
Serine protease inhibitors		
PMSF	0.5 mM	70
DFP	0.5 mM	71
TLCK	0.5 mM	84
Metallochelators		
EDTA	10.0 mM	24
o-Phenanthroline	0.5 mM	8
Microbial protease inhibitors		
Amastatin	10 μg/ml	99
Antipain	10 μg/ml	91
Bestatin	10 μg/ml	99
Chymostatin	10 μg/ml	90
Elastatinal	10 μg/ml	92
Leupeptin	10 μg/ml	93
Pepstatin	10 μg/ml	101
Phosphoramidon	10 μg/ml	83

The enzyme was preincubated with each inhibitor in 50 mM Tris–HCl, pH 9.0, at 0 °C for 15 min before assay. The activity is expressed as a percentage of the control activity.

TABLE IV. Substrate Specificity of Human Placental DAP III

Substrate	Specific activity (μmol/min/mg protein)	Relative activity (%)
Arg–Arg–βNA	11.1	100
Ala–Arg–βNA	3.36	30.3
Ala–Ala–βNA	0.20	1.80
Lys–Ala–βNA	0.18	1.62
Leu–Gly–βNA	0.10	0.90
Gly–Gly–βNA	0	0
Gly–Phe–βNA	0	0
Ser–Tyr–βNA	0	0
Gly–Pro–βNA	0	0

The enzyme was incubated at 37 °C in the standard reaction mixture with the substrates at the final concentration of 0.5 mM. The relative activity was calculated in relation to the fastest-hydrolyzed substrate, Arg–Arg–βNA (100%).

Effects of Protease Inhibitors

To determine the particular class to which the enzyme belongs, we tested a number of protease inhibitors (Table III). The enzyme was extremely inhibited by some sulfhydryl reagents such as *p*-chloromercuribenzoic acid (PCMB) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). However, alkyl reagents for –SH groups (IAA and NEM) showed little inactivation. The enzyme was also strongly inhibited by metallochelators such as *o*-phenanthroline and ethylenediaminetetraacetic acid (EDTA). Slight inhibition was observed with serine protease inhibitors. No significant inhibition was seen with some microbial protease inhibitors at 10 μg/ml concentration.

Effects of Metal Ions

After *o*-phenanthroline pretreatment, the suppressed activity of DAP III could be partially restored by Zn^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+}; 0.05 mM Zn^{2+} was the most effective for reactivation (Fig. 3A). The unmodified enzyme was strongly inhibited by Ni^{2+} and Zn^{2+} (Fig. 3B). These results suggest that the enzyme is a zinc-containing metalloenzyme.

Reversal of DTNB Inhibition by Thiol Reagents

In addition to the sensitivity of the enzyme to inhibition by some sulfhydryl reagents, further evidence for the involvement of an –SH group in the enzymic catalysis was obtained by using thiol reagents. The addition of 50 μM DTNB to the reaction mixture caused inhibition of the enzyme activity (90% inhibition) and further addition of reducing agents such as dithiothreitol, dithioerythritol, β-mercaptoethanol and cysteine almost completely restored the enzyme activity. (Fig. 4A). However, the enzyme activity was inhibited by the addition of the reducing agents alone (Fig. 4B). This shows that an –SH group in the enzyme

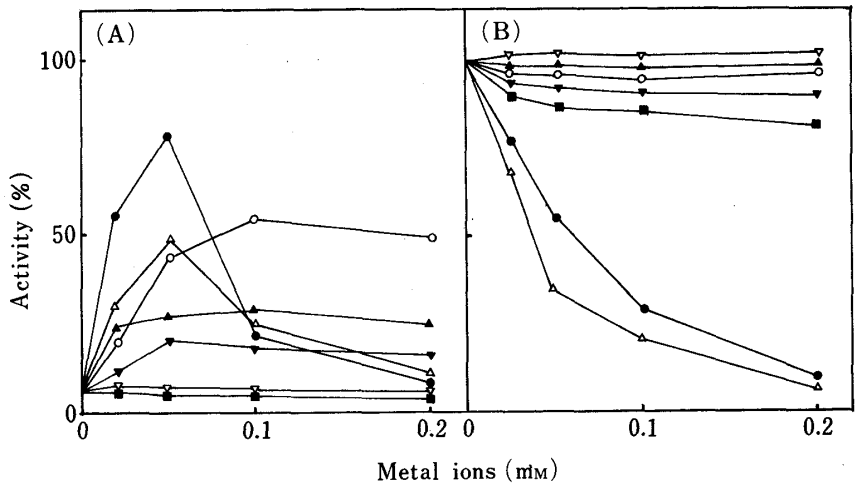

Fig. 3. Effects of the Addition of Metal Ions on the *o*-Phenanthroline-Inactivated (A) and Untreated (B) Enzymes

DAP III was inactivated with 0.5 mM *o*-phenanthroline in 50 mM Tris–HCl, pH 9.0, at 0 °C for 15 min. The inactivated and untreated enzymes were preincubated with various concentrations of metal compounds at 0 °C for 30 min before the assay. The activity is expressed as a percentage of that of the untreated enzyme in the absence of a metal compound. (●) $ZnCl_2$, (○) $CoCl_2$, (▲) $FeSO_4(NH_4)_2SO_4$, (△) $NiCl_2$, (▼) $MnCl_2$, (▽) $MgCl_2$ and (■) $CaCl_2$.

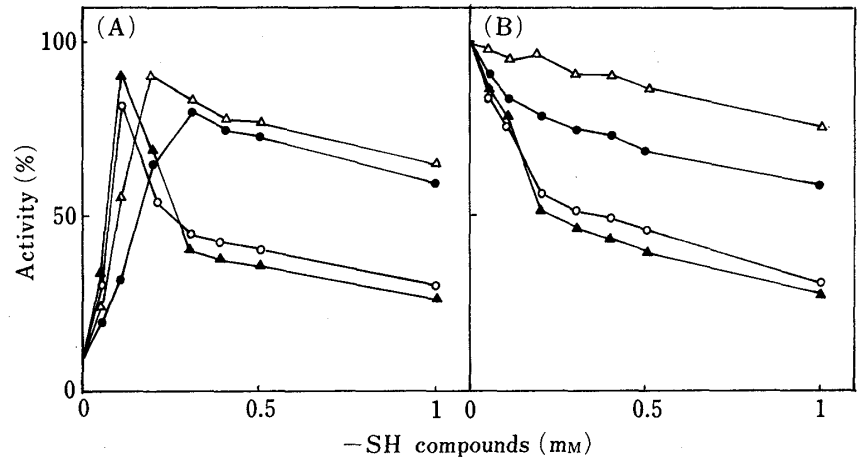

Fig. 4. Effects of the Addition of Thiol Reagents on the DTNB-Inactivated (A) and Untreated (B) Enzymes

DAP III was inactivated with 50 μM DTNB in 50 mM Tris–HCl, pH 9.0, at 0 °C for 15 min. The inactivated and untreated enzymes were assayed in the presence of various concentrations of thiol reagents. The activity is expressed as a percentage of that of the untreated enzyme in the absence of a thiol reagent. (●) Cysteine, (○) dithioerythritol, (▲) dithiothreitol and (△) β-mercaptoethanol.

is involved in the catalysis.

Substrate Specificity

The relative rates of hydrolysis of eight dipeptide β-naphthylamides by the enzyme are given in Table IV. The hydrolysis of the substrates by the enzyme was linear with respect to time and enzyme concentration. The enzyme hydrolyzed the substrate, Arg–Arg–βNA, most rapidly, and the activity with Ala–Arg–βNA was only 30% of that observed with Arg–Arg–βNA. Ala–Ala–βNA, Lys–Ala–βNA and Leu–Gly–βNA were hydrolyzed very slowly, whereas Gly–Gly–βNA, Gly–Phe–βNA, Ser–Tyr–βNA and Gly–Pro–βNA were not hydrolyzed at all.

Discussion

In the present study, we purified DAP III from human placenta in a homogeneous form. The purified enzyme consists of a single polypeptide chain with a molecular weight of 84000. This value is similar to those of the rat brain[6] and human erythrocyte[5] enymes, which have been reported to be about 80000, as determined by gel filtration. Other properties of the placental enzyme such as pI value, pH optima and K_m value were also similar to those of the rat brain enzyme.[6] The placental enzyme was strongly inactivated by some sulfhydryl reagents and metallochelators. This general characteristic of DAP III was first reported by Ellis and Nuenke for bovine pituitary gland DAP III,[2] and was also noted by others.[3,4] However, a marked difference was seen with alkylating reagents for –SH groups. The pituitary gland enzyme was completely inhibited by 0.01 mM NEM, and stimulated about 3-fold by added β-mercaptoethanol, whereas only a slight inactivation was observed of human placental DAP III with 1 mM NEM and IAA, and furthermore, no stimulation by thiol compounds was seen at any concentration. These differences in sensitivity to the reagents cannot be explained at present. There are a number of peptidases that have been shown to be metalloenzymes, and others that have a sulfhydryl group at the active site. Placental DAP III seems to require both cysteine residue(s) and metal ion(s) in its catalytic process. Certain aminopeptidases are known to be enzymes of this type.[15] Bovine lens aminopeptidase was inhibited by metallochelators, sulfhydryl reagents and heavy metals,[15a] and it was suggested that the metal ion bound through a sulfhydryl group at the active site of the enzyme.[15b] Porcine liver[15d,e] and bovine leukocyte[15f] aminopeptidases were also inhibited by the above reagents. However, the sulfhydryl group of these enzymes did not participate directly in the catalytic process and it was essential for manifestation of the full activity.[15e,f] Further studies are necessary to clarify how the cysteine residue(s) and metal ion(s) participate in the placental DAP III action.

The physiological significance of DAP III in the placenta is not clearly understood. Lee and Snyder reported that the rat brain DAP III hydrolyzes angiotensins and enkephalins, and the affinity for angiotensin II is about 17-fold higher than that for Leu-enkephalin.[6] Earlier, Kokubu *et al.* characterized an angiotensinase in rabbit red cells as an endopeptidase which releases ^{1}Asn–^{2}Arg and ^{3}Val–^{4}Tyr from angiotensin II.[16] Comparative studies on this enzyme and bovine pituitary gland DAP III have shown that they are closely similar with respect to substrate specificity, sensitivity to inhibitors and pH optima, and have similar K_m values for Arg–Arg–βNA.[1] These results indicate that the placental DAP III may participate in the regulation of the angiotensin level in placental cells. Further studies on the degradation of some biologically active peptides by the purified enzyme are in progress.

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