Journal of Chromatography, 416 (1987) 131–137
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3531

Note

Rapid chromatographic purification of dipeptidyl-aminopeptidase II from human kidney

TOKI SAKAI* and KOHICHI KOJIMA**

Department of Life Chemistry, Tokyo Institute of Technology, Yokohama 227 (Japan)

and

TOSHIHARU NAGATSU*

Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466 (Japan)

(First received June 11th, 1986; revised manuscript received November 25th, 1986)

Dipeptidyl-aminopeptidase II (DAP-II, dipeptidyl-peptide hydrolase II, EC 3.4.14.2) was first recognized as one of a family of three enzymes present in extracts of bovine pituitary glands [1,2]. All members of this class of dipeptidyl-peptide hydrolases (EC 3.4.14.–) characteristically catalyse successive release of peptide moieties from the N-termini of polypeptides. Both DAP-I and -II are lysosomal enzymes. Fukasawa et al. [3] completely purified and characterized DAP-II from rat kidney. DAP-II is a serine peptidase, removes dipeptides with a penultimate alanyl or prolyl residue from dipeptide derivatives and tripeptides; the most efficient substrates have the sequence Lys-Ala-X. Its optimum pH is 5.5 with Lys-Ala-2-naphthylamide (Lys-Ala-NA) as a substrate, which has frequently been used for measurements of DAP-II activity in various organs. The enzyme is not metal-, halide- or sulphydryl-dependent. It is inhibited by diisopropyl fluorophosphate (DFP) and by cations, e.g. puromycin and Tris; the inhibition rate depends on the size of the cation (e.g. puromycin and Tris are more potent than sodium ions).

We found that the previous assay with Lys-Ala-NA as substrate at pH 5.5 using crude tissue preparations such as serum or brain homogenates gave the additive activity of DAP-II and aminopeptidase, and we have developed a new assay spe-

*Present address: Kyowa Hakko Kogyo Co. Ltd., Shizuoka 411, Japan.
**Present address: Hatano Research Institute, Food and Drug Safety Center, Hatano 257, Japan.

0378-4347/87/$03.50

cific for DAP-II using a newly synthesized fluorogenic substrate 7-(Lys-Ala)-4-methylcoumarinamide (Lys-Ala-MCA) in the presence of 1 m*M* o-phenanthroline, an inhibitor of aminopeptidase [4]. Using this new assay method, we have developed a rapid purification procedure of DAP-II from human kidney by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Lys-Ala-MCA, Ala-Ala-*p*-nitroanilide (pNa), Ala-Gly-pNa, Arg-Pro-pNa, Asp-Pro-pNa, Glu-Pro-pNa, Gly-Gly-pNa, Gly-Leu-pNa, Gly-Pro-pNa, Lys-Pro-pNa, Ala-pNa and Pro-pNa were purchased from the Peptide Institute, Protein Research Foundation (Minoh, Osaka, Japan). o-Phenanthroline was obtained from Wako (Tokyo, Japan); TSK gel Butyl Toyopearl, TSK gel DEAE-5PW, and TSK gel G3000SW and G4000SW from Toyosoda (Yamaguchi, Japan); Lentil Lectin-Sepharose 4B from Pharmacia Fine Chemicals; standard proteins for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), a protein assay kit and a silver stain kit from Bio-Rad; and standard proteins as molecular markers from Oriental Yeast (Tokyo, Japan). All other reagents were of the highest purity commercially available.

DAP-II assay

DAP-II activity was measured by high-performance liquid chromatography (HPLC)–fluorometry using Lys-Ala-MCA as a substrate [4]. The standard incubation mixture consisted of the following components in a total volume of 100 μl: 25 μl of 8 m*M* Lys-Ala-MCA, 40 μl of a universal buffer (pH 5.3, 0.2 *M* sodium borate and 0.05 *M* citrate adjusted to pH 5.3 with 0.1 *M* sodium phosphate), 10 μl of 10 m*M* *o*-phenanthroline as an inhibitor of aminopeptidase, 10 μl of enzyme and 15 μl of water. The control and standard tubes contained water and 1 nmol of 7-amino-4-methylcoumarine (AMC), instead of enzyme, respectively. The control tube did not contain any enzyme.

Protein assay

Protein was determined by the method of Bradford [5] using a Bio-Rad protein assay kit and bovine serum albumin as a standard.

Substrate specificity

DAP-II activity with pNa derivatives of peptides as substrate was measured by a modification of a continuous photometric method reported previously [6]. The incubation mixture contained universal buffer (pH 5.3), 3 m*M* peptide pNa as a substrate and enzyme plus water to 100 μl.

Purification of DAP-II from human kidney

The following steps were conducted at 4°C. Human kidneys (ca. 50 g) were homogenized with a PTFE homogenizer in five volumes of 0.25 *M* sucrose. The homogenate was centrifuged at 7500 *g* for 20 min, then at 100 000 *g* for 60 min.

To the supernatant solid ammonium sulphate was added to 40% saturation. After centrifugation at 10 000 *g* for 20 min, the supernatant was applied to a Butyl-Toyopearl column (20×2.6 cm I.D.) that had been equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.0) containing 1.76 *M* ammonium sulphate. The column was washed with two volumes of the starting buffer and then eluted with a linear gradient of ammonium sulphate decreasing from 1.76 to 0 *M* in the buffer. The active enzyme fraction was eluted at ca. 0.8 *M* ammonium sulphate concentration. The pooled active enzyme fraction was dialysed against 10 m*M* phosphate buffer (pH 7.0), and then the enzyme solution (ca. 4 ml) was applied to a Lentil Lectin-Sepharose 4B column (2.6×1 cm I.D.) equilibrated with 10 m*M* potassium phosphate buffer (pH 7.0). The column was washed with the starting buffer and then eluted with the buffer containing 0.2 *M* α-methyl-D-mannoside. The pooled enzyme was concentrated by ammonium sulphate precipitation (80% saturation). After centrifugation the precipitated enzyme was dissolved in 1 ml of 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* dithiothreitol (DTT) and 0.2 *M* sodium chloride, and purification was carried out by gel permeation (GP) HPLC, as described below. The enzyme was eluted with 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* DDT. The enzyme was finally purified by ion-exchange (IE) HPLC. The pooled enzyme solution was desalted with PD-10 (Pharmacia), and the enzyme solution (1 ml) was applied on a DEAE-5PW column (7.5×0.75 cm I.D.), which was combined with a Gilson HPLC system, equilibrated with 10 m*M* tris–HCl (pH 7.2) and 1 m*M* DTT. The enzyme was eluted with a sodium chloride gradient from 0 to 0.5 *M* in the buffer. Enzyme fractions were pooled and stored at −80°C.

SDS slab gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli [7]. The sample was treated with hot water containing 1% 2-mercaptoethanol at 60°C for 10 min. Reduced samples were subjected to electrophoresis in 4–15% gradient gel containing 0.1% SDS and then stained for protein by the silver staining technique [8].

Molecular mass determination by GP-HPLC

GP-HPLC was carried out at 4°C and at a flow-rate of 0.5 ml/min using a high-performance liquid chromatograph (Toyo Soda HLC-803D) with three combined columns of two TSK gel G3000SW and one TSK gel G4000SW (each 60×0.75 cm I.D.) and with a UV (SPD-2A) spectrophotometer (Shimadzu, Kyoto, Japan). Potassium phosphate buffer (50 m*M*) containing 1 m*M* DTT and 0.2 *M* sodium chloride was used for the elution of the protein. The molecular masses of glutamate dehydrogenase, lactate dehydrogenase, enolase, adenylate kinase and cytochrome *c* were taken as 290 000, 140 000, 67 000, 32 000 and 12 400.

RESULTS

Purification of DAP-II from human kidney

DAP-II was efficiently purified by chromatographies by Butyl-Toyopearl (Fig. 1) and then Lentil Lectine-Sepharose 4B (Fig. 2). The enzyme fraction was

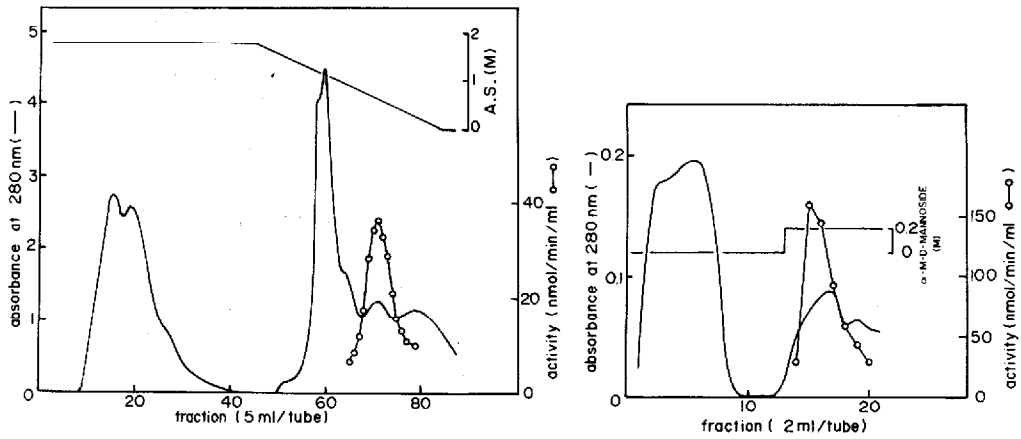

Fig. 1. Chromatography of DAP-II on a column of Butyl Toyopearl. The column (20×2.6 cm I.D.) was equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.0) containing 1.76 m*M* ammonium sulphate. The column was washed with two volumes of the starting buffer and then eluted with a linear ammonium sulphate gradient decreasing from 1.76 to 0 *M* in the buffer. Absorbance was measured at 280 nm (—). Enzyme activities were determined as described in the text using Lys-Ala-MCA as substrate (O-O).

Fig. 2. Chromatography of DAP-II on a column of Lentil Lectin-Sepharose 4B (2.6×1 cm I.D.) equilibrated with 10 m*M* potassium phosphate buffer (pH 7.0). The column was washed with the starting buffer and then eluted with the buffer containing 0.2 *M* α-methyl-D-mannoside. Other details as shown in Fig. 1.

adsorbed on a Lentil Lectin-Sepharose 4B column, most of the contaminating proteins passed through the column and the active protein was eluted with 0.2 *M* α-methyl-D-mannoside. Subsequent GP-HPLC and IE-HPLC permitted rapid and complete purification of the enzyme. A summary of the purification procedure is shown in Table I. The specific activity of DAP-II purified from human kidney was $52.8 \cdot 10^{-9}$ mol/s per mg protein using Lys-Ala-MCA as a substrate. The purification from human kidney was ca. 5000-fold with a yield of 5%.

TABLE I

PURIFICATION OF DAP-II FROM HUMAN KIDNEY

	Total activity (10^{-9} mol/s)	Total protein (mg)	Specific activity (10^{-9} mol/s per mg protein)	Purification (fold)	Recovery (%)
Homogenate*	33.67	3330	0.010	1	100
100 000 *g* Supernatant	33.50	1292	0.026	2.6	99
40% Ammonium sulphate supernatant	19.00	787	0.024	2.5	57
Butyl Toyopearl	14.67	132	0.11	11.0	44
Lentil Lectin	11.17	2.29	4.88	483	33
GP-HPLC	5.87	0.335	17.50	1730	17
DEAE-5WP-HPLC	1.78	0.0338	52.83	5220	5.3

*Human kidney, 45 g.

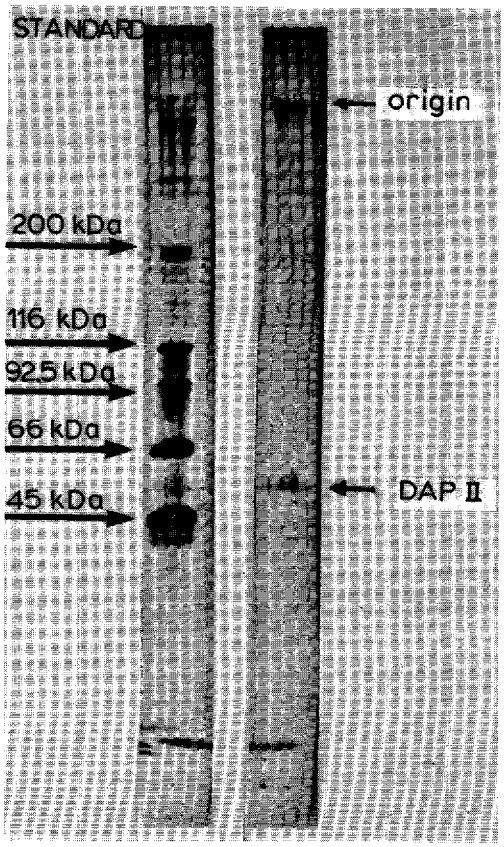

Fig. 3. SDS-PAGE of purified DAP-II from human kidney.

Properties of the purified enzyme

Purified DAP-II from human kidney showed a single band on SDS slab gel electrophoresis (4–15% gradient gel) (Fig. 3). Electrophoresis of DAP-II in SDS-PAGE gave an approximate molecular mass of 55 000. The apparent molecular mass was estimated to be 110 000 by GP-HPLC. The optimal pH for DAP-II activity with Lys-Ala-MCA as a substrate was at pH 5.3.

Various effectors on DAP-II activity

The influence of several agents such as metal chelators and inhibitors on the DAP-II activity is presented in Tabel II. Diisopropyl fluorophosphate (DFP) and phenylmethylsulphonyl fluoride (PMSF) were potent inhibitors.

Substrate specificity

DAP-II was incubated with various dipeptide pNas. The results are shown in Table III. Ala-Ala-pNa had the highest activity among the substrates and Lys-Pro-pNa and Ala-Pro-pNa had the smallest Michaelis constant (K_M).

TABLE II

EFFECT OF SEVERAL AGENTS ON ENZYME ACTIVITY

Agent	Concentration	Enzyme activity (%)
EDTA	1 m*M*	85.1
N-Ethylmaleimide	1 m*M*	91.1
Phenylmethylsulphonyl fluoride (PMSF)	1 m*M*	15.0
Diisopropyl fluorophosphate (DFP)	1 m*M*	1.0
Tris	50 m*M*	37.9
Puromycin	1 m*M*	107
Bestatin	0.5 mg/ml	46.1
Leupeptin	0.05 mg/ml	151

DISCUSSION

DAP-II was partialy purified from bovine hypothalamus by McDonald et al. [2]. Recently, Fukasawa et al. [3] homogeneously purified DAP-II from rat kidney to a specific activity of 66.2 μmol/min per mg protein using Lys-Ala-Na as a substrate.

We describe a relatively simple purification procedure including affinity chromatography and HPLC steps for human kidney DAP-II. This purification procedure by affinity chromatography and HPLC is rapid compared with conventional methods. The molecular mass of DAP-II estimated by means of GP-HPLC was 110 000, and that of the subunit by SDS-PAGE was 55 000. Therefore, DAP-II may be a homodimer. Fukusawa et al. [3] reported that the molecular masses of the enzyme and subunit purified from rat kidney were 130 000 and 65 000, respectively.

As shown in Table II, purified DAP-II from human kidney showed changes by various effectors similar to those of DAP-II from rat kidney [8]. Serine protease

TABLE III

SUBSTRATE SPECIFICITY OF DAP-II FROM HUMAN KIDNEY

Substrate	K_M (m*M*)	V_{max} (10^{-9} mol/s per mg protein)
Ala-Ala-pNa	1.22 ±0.11	286 ± 3
Ala-Pro-pNa	0.0457±0.0067	226 ± 8
Arg-Pro-pNa	0.086 ±0.012	129 ± 3
Asp-Pro-pNa	1.16 ±0.19	45.5± 4.8
Glu-Pro-pNa	0.311 ±0.033	64.0± 3.3
Gly-Pro-pNa	0.282 ±0.022	160 ± 5
Lys-Pro-pNa	0.0457±0.0057	210 ± 4
Ala-Gly-pNa	–	0.0
Gly-Gly-pNa	–	0.0
Gly-Leu-pNa	–	0.0
Lys-Ala-MCA	0.93 ±0.27	174 ±12
Ala-pNa	–	0.0
Pro-pNa	–	0.0

inhibitors (PMSF and DFP) inhibited the two enzyme activities. SH reagents such as N-ethylmaleimide did not inhibit the activity. The effect of metal chelators was also small. Cations such as Tris inhibited DAP-II, but puromycin did not inhibit the enzyme. Bestatin, an aminopeptidase inhibitor, was slightly inhibitory for DAP-II activity.

The purified enzyme hydrolysed X-Ala- or X-Pro-arylamides almost specifically (Table III). X-Leu- and X-Gly-arylamides were not hydrolysed. V_{max} values were relatively large with peptide arylamides consisting of Ala or Lys at N-terminal position, such as Ala-Ala-pNa, Ala-Pro-pNa, Lys-Pro-pNa and Lys-Ala-pNa. Neither Ala-pNa nor Pro-pNa was hydrolysed, indicating complete removal of amino peptidases during purification. K_M values of X-Pro-arylamides were smaller than those of X-Ala-arylamides.

The present purification procedure of DAP-II from human kidney using affinity chromatography and HPLC is rapid and reproducible. The purified enzyme free from amino peptidase may be useful for structural studies of peptides.

REFERENCES

1 J.K. McDonald, T.J. Reilly, B.B. Zetiman and S. Ellis, J. Biol. Chem., 243 (1968) 2028.
2 J.K. McDonald, F.H. Leibach, R.E. Grindeland and S. Ellis, J. Biol. Chem., 243 (1968) 4143.
3 K. Fukasawa, K.M. Fukasawa, B.Y. Hiraoka and M. Harada, Biochim. Biophys. Acta, 745 (1983) 6.
4 T. Nagatsu, T. Sakai, K. Kojima, E. Araki, S. Sakakibara, K. Fukasawa and M. Harada, Anal. Biochem., 147 (1985) 80.
5 M.M. Bradford, Anal. Biochem., 72 (1976) 248.
6 T. Nagatsu, H. Hino, H. Fuyamada, T. Hayakawa, S. Sakakibara, Y. Nakagawa and T. Takemoto, Anal. Biochem., 74 (1976) 466.
7 U.K. Laemmli, Nature, 227 (1970) 680.
8 B.R. Oakley, D.R. Kirsch and N.R. Morris, Anal. Biochem., 405 (1980) 361.