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A Sensitive and Specific Assay for Dipeptidyl-aminopeptidase II in Serum and Tissues by Liquid Chromatography–Fluorometry

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A highly sensitive and specific method for the assay of dipeptidyl-aminopeptidase II (DAP II) in crude enzyme preparations such as serum and tissue homogenates has been established by using a newly synthesized fluorogenic substrate, 7-Lys–Ala-4-methylcoumarinamide. The enzymatically formed 7-amino-4-methylcoumarin was determined by high-performance liquid chromatography with fluorescence detection. The activities of other aminopeptidases in human serum and rat brain homogenates were completely inhibited by *o*-phenanthroline without any effect on DAP II activity to permit specific determination of DAP II. The limit of sensitivity for DAP II activity was about 300 fmol/30 min. DAP II activity was found to be increased in sera from cancer patients, in contrast to the decrease in serum DAP IV activity. DAP II activity was found to be unequally distributed in rat brain regions, and the highest activity was found in the hypothalamus.

Dipeptidyl-aminopeptidase II (DAP II[1]; dipeptidyl-peptide hydrolase II; EC 3.4.14.2), which cleaves dipeptide from the unsubstituted NH_2 termini of dipeptide derivatives, most notably from Lys–Ala-2-naphthylamide, was first identified in bovine anterior pituitary extracts by McDonald and co-workers (1,2). DAP II differs from DAP I [cathepsin C] (3) in its substrate specificity and in its lack of sulfhydryl and halide requirements, and from DAP III (4) and DAP IV (5) in its subcellular localization and pH optimum. DAP II is a serine peptidase with a restricted substrate specificity. The enzyme preferentially hydrolyzes Lys–Ala-2-naphthylamide at pH 5.5, but also cleaves N-terminal dipeptide from dipeptide 2-naphthylamides and tripeptides with a penultimate alanyl or prolyl residue. Its pH optimum is 5.5 with Lys–Ala-2-naphthylamide. The enzyme is not metal dependent. It is inhibited by diisopropyl fluorophosphate and by cations, e.g., puromycin and Tris. The inhibition rate depends on the size of the cation; puromycin and Tris are more inhibitory than are sodium ions. In the pituitary the greater part of DAP II activity was found in the lysosomal fraction.

The activity of DAP II was assayed on dipeptidyl-2-naphthylamides as substrates. The standard substrate was Lys–Ala-2-naphthylamide and reaction rates were measured at pH 5.5 by a direct and continuous fluorometric method of monitoring the rate of 2-naphthylamine formation (1). We have used a new fluorogenic substrate, 7-Lys–Ala-4-methylcoumarinamide (Lys–Ala-MCA) to avoid the carcinogenic naphthylamine derivative, and measured the activity of DAP II

[1] Abbreviations used: DAP II, dipeptidyl-aminopeptidase II (EC 3.4.14.2); Lys–Ala-MCA, 7-Lys–Ala-4-methylcoumarinamide; AMC, 7-amino-4-methylcoumarin; Ala-MCA, 7-Ala-4-methylcoumarinamide; DAP IV, dipeptidyl-aminopeptidase IV.

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fluorometrically by monitoring the rate of 7-amino-4-methylcoumarin (AMC) formation at 460 nm with excitation at 380 nm. However, the assay using Lys–Ala-2-naphthylamide or Lys–Ala-MCA at pH 5.5 or 5.3 may not be specific for DAP II with crude enzyme preparations due to contamination of aminopeptidase. Aminopeptidase, which has an optimum pH at 7.0–7.5, may cleave successively Lys–Ala bond and 7-Ala-4-methylcoumarinamide (Ala-MCA) bond to form AMC even at pH 5.3. In the case of an aqueous extract of bovine anterior pituitary, this possibility was excluded by means of thin-layer chromatography by McDonald *et al.* (1). We have examined whether Lys–Ala-MCA is hydrolyzed by both DAP II and aminopeptidase in rat brain homogenate and in human serum by means of high-performance liquid chromatography with fluorescence detection. In all assays, not only Lys–Ala-MCA and AMC, but also Ala-MCA was detected, indicating that Lys–Ala-MCA at pH 5.3 was hydrolyzed to form AMC either directly by DAP II or by aminopeptidase via Ala-MCA.

Fukasawa *et al.* (6) reported several inhibitors on DAP II purified from rat kidney. We have found that *o*-phenanthroline at 1 mM can inhibit aminopeptidase activity completely without inhibiting DAP II activity. Based on this finding, we have established a specific and highly sensitive HPLC–fluorescence assay for DAP II by adding *o*-phenanthroline in the reaction mixture for DAP II.

MATERIALS AND METHODS

Lys–Ala-MCA was newly synthesized, and Gly–Pro-MCA was also synthesized as described previously (7) at the Peptide Institute, Protein Research Foundation (Minoh, Osaka 562, Japan). Normal human blood samples were collected by venipuncture and, after clotting, the serum was separated by centrifuging at 2800 rpm for 15 min. Sera were stored at −80°C until assay. Rat brain was homogenized with 0.32 M sucrose, and the homogenate was used as the enzyme preparation. DAP II was purified from rat kidney by the method of Fukasawa *et al.* (6).

Protein was determined by the method of Bradford (8) using a Bio-Rad protein assay kit and bovine serum albumin as a standard.

Enzyme activity using Lys–Ala-MCA as a substrate was assayed by HPLC–fluorometry of the rate of AMC formation. The experimental incubation mixture (total volume, 100 μl) contained 40 μl of an 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), 25 μl of 8 mM Lys–Ala-MCA, 10 μl of 10 mM *o*-phenanthroline, 10 μl of enzyme solution, and 15 μl of water. The control tubes without enzyme were run with each sample. The reaction was carried out at 37°C for 30 min, and was stopped by adding 1.0 ml of 1 M sodium acetate buffer, pH 3.6. An identical amount of enzyme was added to the control tubes after stopping the reaction. After centrifugation at 2800 rpm for 10 min, an aliquot (10 μl) of the supernatant was injected into the column of HPLC. A high-performance liquid chromatograph (Model LC 5A, Shimadzu, Kyoto, Japan) was equipped with a column of Nucleosil $7C_{18}$ (particle size 7.0 μm, 250 × 4.0 mm i.d.) and a fluorescence detector (Shimadzu RF-530 with a 12-μl square flow cell). Fluorescence was monitored at 440 nm by excitation at 340 nm to measure enzymatically formed AMC. The mobile phase was 10 mM potassium phosphate buffer, pH 3.0, containing 20% acetonitrile. The flow rate was 0.7 ml/min.

DAP IV activity was measured fluorometrically using Gly–Pro-MCA as a substrate, as described previously (7).

RESULTS

Ala-MCA and AMC were measured with very high sensitivity by HPLC with fluorescence detection. Figure 1 shows a calibration curve, indicating the linear response of the peak height of the fluorescence intensity for

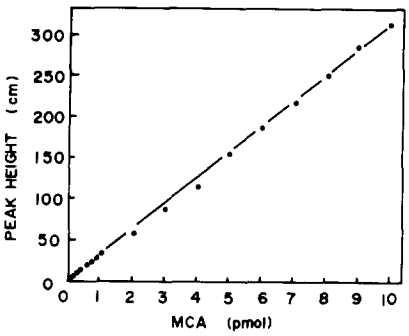

FIG. 1. Standard curve of the concentration of AMC against the peak height of fluorescence by HPLC with fluorescence detection. The conditions of HPLC are described under Materials and Methods.

the injected amounts of AMC from 10 fmol to 10 pmol.

The chromatographic pattern of the DAP II reaction with human serum as enzyme is shown in Fig. 2. Part (A) shows standards of 10 pmol each of Lys-Ala-MCA and Ala-MCA, and 500 fmol of AMC. Blank incubation without serum (B) showed only the substrate (Lys-Ala-MCA) peak. The experimental incubation without *o*-phenanthroline (C) formed both Ala-MCA and AMC. In contrast, the experimental incubation with 1 mM *o*-phenanthroline (D) produced only AMC. The retention times were: Lys-Ala-MCA, 4.2 min; Ala-MCA, 6.0 min; and AMC, 19.5 min.

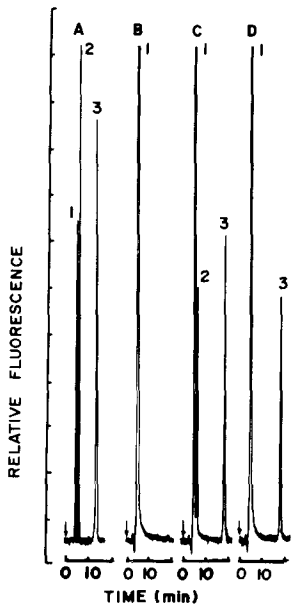

FIG. 2. The chromatographic patterns of the reaction mixture for DAP II with human serum as enzyme. (A) Standards, 10 pmol each of Lys-Ala-MCA (peak 1) and Ala-MCA (peak 2), and 500 fmol of AMC (peak 3). (B) Blank incubation without serum. (C) Experimental incubation without *o*-phenanthroline. (D) Experimental incubation with *o*-phenanthroline.

The rate of AMC formation using normal human serum as enzyme proceeded linearly up to 45 min is shown in Fig. 3. DAP II activity in human serum as a function of enzyme concentration is shown in Fig. 4.

From a Lineweaver-Burk plot illustrating the effect of Lys-Ala-MCA concentrations on DAP II activity catalyzed by a sample of normal human serum, the K_m towards AMC and the maximum velocity (V_{max}) were calculated to be 2.0 mM and 2.8 nmol/min/ml serum, respectively. The optimum pH was 5.3.

The effect of *o*-phenanthroline on enzymatic formation of Ala-MCA and AMC using human serum is shown in Fig. 5. In the presence of 1 mM *o*-phenanthroline, the peak of Ala-MCA completely disappeared, and the peak of AMC decreased to about 60% as

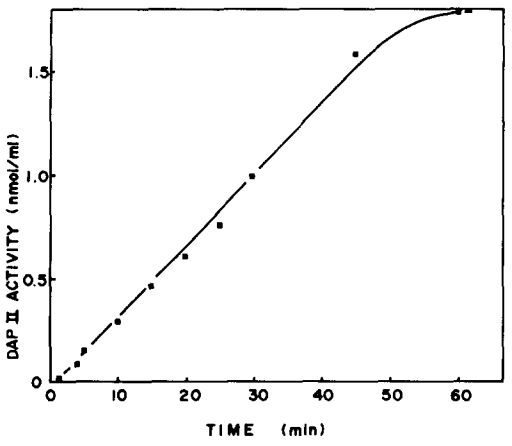

FIG. 3. Rate of AMC formation by DAP II reaction using human serum (10 μl) as enzyme. DAP II activity is expressed as nmol/ml serum.

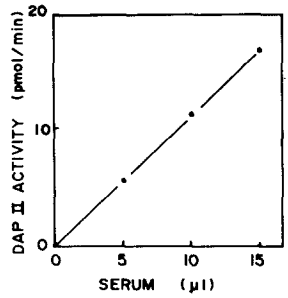

FIG. 4. DAP II activity in human serum as a function of enzyme concentration.

compared with the peak height of AMC without *o*-phenanthroline.

The effect of *o*-phenanthroline on the activity of DAP II purified from rat kidney dissolved in 10 μl of water or serum is shown in Fig. 6. DAP II dissolved in water was inhibited by about 40% with 1 mM *o*-phenanthroline, but DAP II added into serum was not inhibited.

It was also shown that after chromatographic separation of aminopeptidase and DAP II from human serum by a Gilson HPLC system on a SynChropak AX-300 column (250 × 4.1 mm i.d., 300 Å) aminopeptidase activity with Lys-Ala-MCA as a substrate was completely inhibited by 1 mM

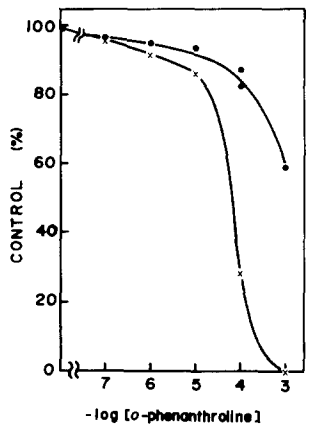

FIG. 5. Effect of *o*-phenanthroline concentration on the formation of Ala-MCA (×) and AMC (●) using human serum as enzyme. The peak height of Ala-MCA or AMC from the incubation mixture without *o*-phenanthroline is taken as 100%.

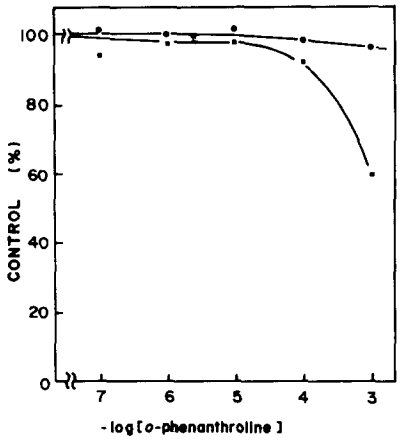

FIG. 6. Inhibition of DAP II activity by *o*-phenanthroline with DAP II purified from rat kidney and dissolved in water (■) or in serum (●). 10 μl of pure DAP II solution with an activity of 8.4 nmol/min/ml enzyme was dissolved in 90 μl of water or human serum containing 1.04 nmol/min/ml serum of DAP II activity. Ten microliters of this enzyme solution was used for the assay. The peak height of AMC from the incubation mixture without *o*-phenanthroline is taken as 100%.

o-phenanthroline but DAP II activity with Lys-Ala-MCA as a substrate was not inhibited at all (dat not shown).

The activities of DAP II and DAP IV in human serum from normal subjects and cancer patients are shown in Table 1.

The distribution of the activities of DAP II and DAP IV in rat brain regions is shown in Table 2. DAP II activity was distributed unevenly in various brain regions, and the hypothalamus had the highest activity. In contrast, DAP IV activity was distributed nearly evenly in the brain regions.

DISCUSSION

A new assay of DAP II in crude enzyme preparations such as serum or tissue homogenates has been established with Lys-Ala-MCA as a substrate. AMC enzymatically released from Lys-Ala-MCA by DAP II was measured by HPLC with fluorescence detection. By direct fluorometry using a fluorospectrometer without HPLC separation, only

TABLE 1

DAP II ACTIVITY IN HUMAN SERUM

Serum	No. of samples	Activity (nmol/min/ml, mean ± SE) DAP II	DAP IV	$\frac{\text{DAP II} \times 100}{\text{DAP IV}}$
Controls	20	1.06 ± 0.11	44.2 ± 1.3	2.4
Cancer patients	10	1.55 ± 0.15*	33.8 ± 10.8*	4.6*

* Differs significantly from controls, $P < 0.01$.

AMC is detected, but neither Ala-MCA nor Lys–Ala-MCA can be detected. When crude enzyme preparations such as serum contain both DAP II and aminopeptidase, aminopeptidase also releases AMC by stepwise reactions even at pH 5.3; it cleaves Lys–Ala bond of Lys–Ala-MCA to form Ala-MCA, and subsequently cleaves Ala-MCA bond to form AMC. Thus DAP II activity in serum measured with Lys–Ala-MCA as substrate also includes the activity of aminopeptidase. In fact, when Lys–Ala-MCA was incubated with serum, Ala-MCA was also detected by HPLC with fluorescence detection (Fig. 2). This result indicates that aminopeptidase hydrolyzed Lys–Ala-MCA to form Ala-MCA and that the peak of AMC could be produced by both DAP II and aminopeptidase activities. In an incubation mixture for DAP II including 1 mM *o*-phenanthroline, aminopeptidase activity was completely inhibited and only the activity of DAP II was measured specifically.

TABLE 2

DISTRIBUTION OF DAP II AND DAP IV ACTIVITIES IN RAT BRAIN REGIONS

Brain region (n = 5)	Enzyme activity (nmol/min/mg protein, mean ± SD) DAP II	DAP IV
Cerebellum	2.22 ± 0.21	1.03 ± 0.08
Pons–medulla	2.42 ± 0.23	1.15 ± 0.11
Midbrain	1.85 ± 0.14	0.90 ± 0.08
Hippocampus	1.87 ± 0.16	0.94 ± 0.20
Striatum	2.00 ± 0.18	0.89 ± 0.09
Hypothalamus	3.00 ± 0.62	0.96 ± 0.28
Cerebral cortex	1.31 ± 0.27	1.06 ± 0.31

This HPLC–fluorometry method is highly sensitive. The limit of sensitivity for AMC was 10 fmol. The AMC peak in the blank incubation without enzyme is supposed to be derived from Lys–Ala-MCA by nonenzymatic hydrolysis, but the rate of nonenzymatic degradation is so low that it does not affect the estimation of DAP II activity. The activity of DAP II in 10 μl of normal human serum is about 3000-fold higher than the limit of the sensitivity. Vanha-Perttula and Kalliomäki (9) reported that DAP II activity in human serum is 10.27 ± 7.24 nmol/min/ml using Lys–Ala-2-naphthylamide as a substrate. This value should also include the activity of aminopeptidase. Activity of DAP II in normal human serum using Lys–Ala-MCA in this study was 1.06 ± 0.11 nmol/min/ml (n = 20) and was lower than their values. This difference may be due not only to the difference in substrates, but also to the activity of aminopeptidase in their method (9).

As an application of this DAP II assay, we measured DAP II activity and DAP IV activity in human serum from normal subjects and from cancer patients (Table 1). As reported previously (9,10,11), DAP IV activity was reduced in serum from patients with cancer. In contrast, DAP II activity in serum from patients with cancer was found to be higher than that in normal serum. Thus the

ratio of DAP II to DAP IV tends to increase in serum from patients with cancer. It is obvious that more data are necessary, but these preliminary data suggest the possibility that the ratio of DAP II to DAP IV in serum could be a biochemical index of cancer.

DAP II was reported to be localized in peptidergic neurons in the brain (12). The high activity in the hypothalamus is of interest, since the hypothalamus is rich in neuropeptides.

This new assay method is highly sensitive for measuring the low activity of DAP II in tissues and fluids. This HPLC–fluorometry to measure AMC may also be applicable to the assay of any peptidase activity resulting in liberated AMC from fluorogenic substrates containing AMC.

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