Cloning and functional expression of rat kidney dipeptidyl peptidase II

Kayoko M. FUKASAWA^{*1}, Katsuhiko FUKASAWA^{*}, Koichi HIGAKI[†], Naoki SHIINA[†], Michiaki OHNO[†], Shigeki ITO[†], Junichi OTOGOTO[†] and Norio OTA[†]

*Department of Oral Biochemistry, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan, and †Department of Periodontology, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan

Dipeptidyl peptidase II (DPP II; EC 3.4.14.2) from rat kidney was purified to a specific activity of 65.4 μ mol/min per mg of protein for Lys-Ala- β -naphthylamide. The N-terminal and partial amino acid sequences of the enzyme were determined. The peptide sequences were used to identify expressed sequence tag (EST) clones. By using the cDNA fragment of one of the EST clones as a probe, we isolated a cDNA clone with 1710 bp encoding DPP II from a rat kidney cDNA library. The cDNA of rat DPP II contained an open reading frame of 1500 bp, coding for a protein of 500 amino acids. The first 10 residues of the purified enzyme matched the deduced protein sequence starting with residue 37, suggesting the presence of a signal peptide. The mature enzyme (464 residues) had a calculated molecular mass of 51400 Da, which was lower than the value (about 60000 Da)

INTRODUCTION

Mammalian dipeptidyl peptidases (DPPs) sequentially release dipeptides from polypeptides, except those with a proline or hydroxyproline at the third position (P1') from their N-terminus. Among those enzymes, DPP II and DPP IV willingly cause the release of the dipeptide containing a penultimate proline or alanine residue. They are different with respect to their pH profiles based on their locations in the cell [1]. DPP II, first identified in bovine anterior pituitary extracts by McDonald et al. [2,3], is a serine-class enzyme and exists in the lysosomal fraction obtained by sucrose-density centrifugation. Histochemical [4–7] and immunohistochemical [8] studies on DPP II also showed it to be found exclusively within lysosomes. Its activity is affected by cations such as Tris and puromycin [2,3,9] and by anions [10].

The possible function of DPP II was proposed to be tripeptidyl peptidase I (EC 3.4.14.9)-coupled degradation of collagen in reproductive organs [11]. On the other hand, rat brain DPP II also hydrolysed the Pro–Pro bond of Xaa-Pro-Pro and released N-terminal Xaa-Pro dipeptides from casomorphin and substance P. So, this enzyme is also involved in the degradation of proline-containing neuropeptides [12]. However, the physiological function of DPP II has been obscure for a long time. In 1992, based on the results of a cytochemical study, Smid et al. [13] proposed that DPP II may have a role in programmed cell death in rat incisor tooth ameloblasts. There are several reports on mammalian enzymes having DPP activities [14–19], but they have not been classified by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.

Huang et al. [20] determined the amino acid sequence of the first 41 residues of the enzyme from pig seminal plasma and

determined by SDS/PAGE; and the deduced amino acid sequence showed six potential N-glycosylation sites. The deduced amino acid sequence of rat DPP II shared high similarity with quiescent-cell proline dipeptidase (78% identity) and prolyl carboxypeptidase (38% identity) and bore the putative catalytic triad (Ser, Asp, His) conserved in serine peptidase families. We transiently transfected COS-7 cells with pcDNA3.1 containing the cloned cDNA and obtained the overexpression of an immuno-reactive protein (of about 60000 Da). The transfected cells showed Lys-Ala-methylcoumarinamide-hydrolysing activity that was 50 times higher than the control cells.

Key words: lysosomal peptidase, pH profile, serine enzyme, signal peptide.

concluded that DPP II is related to granzymes. On the basis of the N-terminal amino acid sequence from pig seminal plasma and a number of biochemical similarities between both enzymes, Rawlings and Barrett [21] showed that DPP II is homologous with lysosomal Pro-X-carboxypeptidase [22], and thus belongs to peptidase family S 28 of the clan SC [23]. For verification of the above prediction, we cloned the enzyme from rat kidney. During the course of our experiments, Underwood et al. [24] isolated and cloned an enzyme (quiescent-cell proline dipeptidase, QPP) related to the cell death of quiescent lymphocytes, and showed it to belong to family S 28 of the clan SC. However, the pH profile of QPP covered a broad pH range, from acidic to neutral pH.

Here, we describe for the first time the full-length cDNA of DPP II cloned from rat kidney and expression of the enzyme in COS-7 cells. Moreover, we discuss the similarity in pH profile between rat DPP II and QPP.

EXPERIMENTAL PROCEDURES

Materials

Ala-β-naphthylamide (-NA), Pro-NA, Lys-Ala-NA, bestatin and Fast Garnet GBC were obtained from Sigma (St. Louis, MO, U.S.A.); Arg-methylcoumarinamide (-MCA), Gly-Pro-MCA and Lys-Ala-MCA were from Peptide Institute (Osaka, Japan); Gly-Pro-NA and benzyloxycarbonyl (Cbz)-Gly-Pro-NA were from Bachem Biosocience (Philadelphia, PA, U.S.A.); and Gly-NA and Lys-NA were from Koch–Light (Colnbrook, Bucks., U.K.). Polybuffer exchanger 94 was from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.) or Toyobo Co. (Osaka, Japan). Medium,

Abbreviations used: NA, β -naphthylamide; MCA, methylcoumarinamide; DPP, dipeptidyl peptidase; QPP, quiescent-cell proline dipeptidase; PCP, prolyl carboxypeptidase; Cbz, benzyloxycarbonyl; EST, expressed sequence tag.

¹ To whom correspondence should be addressed (e-mail kmf@po.mdu.ac.jp).

The nucleotide sequence data in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB038232.

buffers and several reagents for cell culture were purchased from Life Technologies (Gaithersburg, MD, U.S.A.).

Protein and enzyme assays, purification of the enzyme from rat kidney

Protein content was determined according to Bradford with BSA as the standard [25]. Enzyme assays and purification procedure were performed as described previously [9,26,27] except that polybuffer exchanger 94 (the isoelectric point of DPP II, pH 4.76) was added to the purification steps (see Table 1 below). The concanavalin A–Sepharose column fraction was dialysed against 25 mM imidazole/HCl buffer, pH 7.4 (start buffer), and applied on to the polybuffer exchanger 94 column (0.9 cm \times 16 cm). After the column had been washed with 25.5 ml of the start buffer, the elution was carried out with 117.3 ml of the gradient buffer (polybuffer 74/HCl, pH 4.0).

Antibody production

The purified DPP II (200 μ g) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutanously into a 2 kg male rabbit. After 10 days, 100 μ g of the antigen was injected three times at 2 week intervals in the same manner. After the last injection (10 days), the animal was bled from the carotid artery. The selective reactivity between the enzyme and the antiserum was verified by Western-blot analysis. The antiserum was stored at 4 °C.

SDS/PAGE and immunoblotting

Electrophoresis of proteins was performed under denaturing and reducing conditions on either 5.0 % (w/v) polyacrylamide gels for determination of molecular mass or on 10% (w/v) gels for other purposes by the method of Laemmli [28]. The proteins were stained with Coomassie Brilliant Blue R-250 and blotted on to nitrocellulose membranes (Bio-Rad, Hercules, CA, U.S.A.) in a Bio-Rad apparatus (1.5 h, 40 V). The membranes were blocked at room temperature in Tris-buffered saline [TBS, 50 mM Tris/ HCl buffer (pH 8.0)/0.15 M NaCl] containing 3.0 % (w/v) BSA and 0.05% Tween-20. Rabbit anti-rat DPP II antiserum (described above) for detection of the purified enzyme or the protein expressed in COS-7 cells was diluted 1:3000 with the same buffer, and the membranes were incubated in it for 1 h at room temperature. The membranes were then washed twice with TBS containing 0.05% Tween-20 and incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat antirabbit IgG (Bio-Rad), diluted 1:2000 with blocking solution. After the membranes had been washed twice with TBS containing 0.05 % Tween-20, the bands were visualized by use of an alkaline phosphatase colour development kit (Bio-Rad). For determination of molecular mass, the following marker proteins were used: myosin (200000 Da), Escherichia coli β -galactosidase (116250 Da), rabbit muscle phosphorylase b (97400 Da), BSA (66200 Da), hen egg-white ovalbumin (45000 Da) and bovine carbonic anhydrase (31000 Da). SDS/PAGE for molecular-mass determination was done five times for the purified enzyme, and the molecular-mass value was calculated by a logarithmic regression program (mean \pm S.D.).

Protein sequencing

To obtain the N-terminal amino acid sequence of the enzyme, we resolved the purified protein by SDS/PAGE on a 10% gel (described in the previous section), blotted it on to a PVDF membrane (Bio-Rad), visualized it by Coomassie Brilliant Blue

R-250 staining and excised the band. For determination of the partial amino acid sequence, the purified enzyme (200 μ g) was digested with 2% (w/w) trypsin in 100 mM ammonium bicarbonate buffer, pH 8.0, for 16 h at 37 °C. The digested material was fractionated on a cation-exchange HPLC column (Pepkat 300; 5 μ m, 4.0 mm × 125 mm) and also on a reversed-phase HPLC column (Shim-pack CLC-ODS; 6.0 mm × 150 mm). The preparation conditions were performed as described previously [27]. The protein and the purified peptides were sequenced by Edman degradation using an Applied Biosystems Procise Sequencer (PROCISE-cLC).

Enzyme kinetics

For curve fitting by non-linear regression and calculation of $V_{\rm max}$ and $K_{\rm m}$, the program KaleidoGraph (Hulinks, Tokyo, Japan) was used. The amounts of substrates used in these kinetic studies ranged from 0.1 to 1.0 mM for Lys-Ala-NA, from 0.05 to 0.4 mM for Gly-Pro-NA, from 0.025 to 0.4 mM for Lys-Ala-MCA and from 0.00625 to 0.1 mM for Gly-Pro-MCA. Concentrations of enzyme tested were 0.0267 μ g/ml for Lys-Ala-NA, 0.2177 μ g/ml for Gly-Pro-NA, 0.0964 μ g/ml for Lys-Ala-MCA and 0.0653 μ g/ml for Gly-Pro-MCA. The $k_{\rm cat}$ values were calculated based on a molecular mass of 120000 Da determined by Sephadex G-150 superfine column chromatography according to the methods described previously [9].

pH profile

The following buffers substituted for the standard assay buffer for determination of the purified enzyme's pH activity profile: 170 mM cacodylate buffer (pH range 4.0–7.0, increments of 0.5) and 50 mM Hepes (pH range 7.0–8.5, increments of 0.5). Results are expressed as a percentage of the maximum activity.

Datebase searches

The sequences of the three tryptic peptides of the enzyme were compared with those of the predicted translation products from the expressed sequence tag (EST) databases by use of the BLAST program at DDBJ. The search detected a rat clone encoding a sequence containing the peptide 3 sequence (accession number AI716264).

Cloning and sequencing of rat DPP II cDNA

For isolation of the rat kidney cDNA, mRNA from female Wistar rat kidney was obtained by use of a mRNA isolation kit (Stratagene, La Jolla, CA, U.S.A.). The cDNA was constructed and ligated into λ ZAP II vector digested with *Eco*RI/*Xho*I by use of a Uni-Zap synthesis kit from Stratagene.

Double-stranded cDNA was amplified by PCR using the two primers based on the nucleotide sequence of the rat EST clone (accession number AI716264). The sequence of the 5'-oligomer was 5'-GCAACATCATTTTCTCCAACGG-3', and that of the 3'-oligomer was 5'-TGTCACACGCGGCTCACATAG-3'. The reaction was carried out on a Quick Thermo Personal cycler (Nippon Genetics, Tokyo, Japan). The following amplification protocol was employed: 30 cycles of denaturing at 94 °C (1 min), primer annealing at 58.5 °C (1 min) and primer extension at 72 °C (1 min). Initial amplification of the cDNA revealed the presence of a 300 bp amplified product. This PCR product was sequenced and used as a probe to screen the rat kidney cDNA library. pBluescript phagemids containing DPP II cDNA were excised from the Uni-Zap clones by use of ExAssist helper phage for subcloning the cDNA.

Table 1 Purification of DPP II from rat kidney (244 g, wet weight)

ND, not determined.

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (-fold)
(NH ₄) ₂ SO ₄ fraction (40-70%)	440	15172	0.029	100	1
DEAE-TOYO	365	2807	0.13	82.9	4.48
Concanavalin A-Sepharose	117	20	5.85	26.5	202
Polybuffer exchanger 94	75.7	ND	ND	17.2	ND
Phenyl-Sepharose	70.3	1.3	54.0	15.9	1862
Sephadex G-150 Superfine	65.4	1.0	65.4	14.8	2255

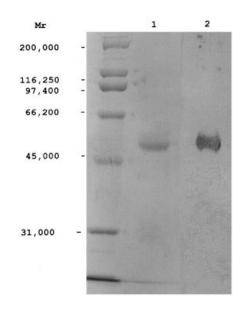


Figure 1 SDS/PAGE of purified rat kidney DPP II

A 5 μ g amount of enzyme purified from rat kidney (lane 1) was loaded on to a standard 10% gel and stained with Coomassie Brilliant Blue following electrophoresis. Western-blot analysis of the purified enzyme was also done (lane 2, 0.5 μ g).

The nucleotide sequence was determined by the dideoxynucleotide chain-termination method [29] using the singlestranded pBluescript SK(-) phagemid excised from the Uni-Zap DPP II clone by use of VCSM13 helper phage. Appropriate nucleotide oligomers were synthesized by Sawaday Co. (Tokyo, Japan) for sequencing primers.

Functional expression of DPP II in COS-7 cells

A DNA fragment containing the entire coding region of the rat DPP II was inserted at an *Eco*RI/*Xho*I site into pcDNA3.1/HisA (Invitrogen, SanDiego, CA, U.S.A.). The constructed plasmid, named pcDrdpII, was purified by using a Qiagen plasmid-purification kit and used for transfection of COS-7 cells (obtained from Riken Cell Bank, Tsukuba, Japan) with LIPOFECTAMINE[®] (Life Technologies, Gaithersburg, MD, U.S.A.). COS-7 cells (2×10^5 cells/35 mm dish) grown in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal calf serum were washed twice with serum-free DMEM, and transfection medium containing 1.5 µg of pcDrdp II and 12.5 µl of LIPOFECTAMINE[®] in 1 ml of serum-free DMEM was then added. A negative control was performed under the same conditions with 1.5 µg of pcDNA3.1/HisA vector (control cells). After incubation for 5 h,

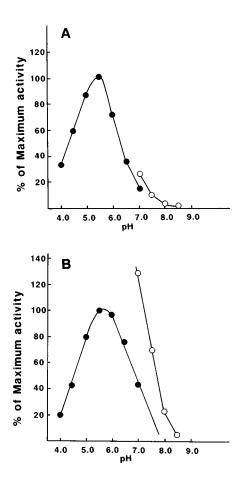


Figure 2 pH profile of purified rat kidney DPP II

(A) Lys-Ala-NA-cleaving activity. (B) Gly-Pro-NA-cleaving activity. ●, 170 mM cacodylate buffer; ○, 50 mM Hepes buffer. Results are mean values of three determinations.

l ml of DMEM containing 20 % fetal calf serum was added, and the cells were incubated for a further 48 h. The cells were collected, washed with PBS (1.06 mM KH₂PO₄/2.97 mM Na₂HPO₄/0.9 % NaCl, pH 7.4), and resuspended in 1 ml of PBS. The suspension was then sonicated with three 10 s bursts on ice and subsequently subjected to three cycles of freezing (in liquid nitrogen) and thawing (at 37 °C). The crude lysate was assayed for protein contents and Lys-Ala-MCA cleaving activity. For the latter, 0.02 µmol of each of *o*-phenanthroline and bestatin were preincubated at 37 °C for 10 min with the cell lysate. The reaction was initiated by the addition of 0.02 µmol of Lys-Ala-MCA. The recombinant protein was partially purified from the lysate according to the same purification procedures used for the rat kidney DPP II.

RESULTS

Purification and properties of the purified enzyme

The purification was 2255-fold, and the yield was 14.8 % with respect to the total activity of the $(NH_4)_2SO_4$ fraction (Table 1). SDS/PAGE and Western-blot analysis of the purified enzyme are shown in Figure 1. The DPP II activity during purification (steps 1–3 as listed in Table 1) was assayed in the presence of 0.02 µmol of bestatin to inhibit the aminopeptidase activity. The rat kidney enzyme (1 mg/ml in 10 mM sodium phosphate buffer,

pH 7.6, containing 0.15 M NaCl and 2 mM mercaptoethanol) was stable at -20 °C for 3 months without loss of activity, but after 1 year the activity had dropped to 70% of the original activity.

Mono-amino-acid-NA and Cbz-Gly-Pro-NA were not hydrolysed at all, even if 100 times more purified enzyme and reaction times longer than those used for the assay with Lys-Ala-NA as the substrate were employed. K_m values for Lys-Ala-MCA were the same as those of pig seminal plasma and human kidney enzymes [20,30]. Di-isopropyl fluorophosphate (1 mM) and 50 mM Tris caused 88 and 65 % inhibition, respectively, of the control enzyme activity. On the other hand, 1 mM *o*-phenanthroline, 1 mM E-64 [(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-

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	G CTG L TCC S CCT P GGC G	P GTC V AAAT N TTC F GAC D	L TAC Y GGCO A S CTT L	P AAC ACG R ACG R D AAAA K	A TCC S GCC A GCA A E GCA A	N TCT S TGG W CTC L GCC A	P GGC D CGC R AGC S	V ATG M TAC Y CAG Q N	K GAA E CAG Q CAA Q CAA I	V P A CCCC P A A TAC Y I	G TGT C TGT C TGC C TTGC F	C TTT F T CCTC L S	E GAC D XGAG E XGAT D XGAT N	R ATC I ATC I ACA T GGT G	L TAC Y AAT TGG W GAC D	L CAG Q CTG GGC G G CTA L	S ATG M ACC T T STG V GAC D	E TAC Y TTT F TGG W CCC P	G CAA Q GAC D CCT P TGG W	Q AGC S AGC R CGG R CGG A	R TGT C AAC N SCCA P G G	I GCT A AAC D GAC D GGT G	M GAC D CTC V TCC G G	G P ACA T CTG L CTG L ATC	L ACT T GAC D CAG Q CAG	R GGGC G XATG M XACC T XCGC R	A TTGI C TTTC F CAGO S CAAO	L G G CCT P TTC F	A T T CAAAA E T T GAAAA E T T G G G G G G G G G G G G G G G G G	21 108 31 11' 34 126 3' 139 40
	G CTG L TCC S CCT P CCT P CCCT C C C C C C C C C C C	P GTC V AAAT N TTC F GAC D	L TAC Y GCCC A TCT S CCTT L	P AAC N AGG R CAGG R D CAAA K	A TCC S GCC A GCA A GCA A GCA A GCA	N TCT S TGG W CTC L CTC L CTC A	P GGC G G G G G G G G C G C G C G C G C	V ATG M TAC Y CAG Q N XAAC N	K GAA E Q Q Q Q ATC I	V ACCCC P AGCCC A ATAC Y ZATT I CATT	G TIGI C TIGI C TIGI C TIGI C TIGI C F	C TTT F ACG T C C C C C C C C C C C	E GAC D GAG E CAAC N CAAC	R ATC I ATC I ATC I ACA T G G T G CTA	L TAC Y AAT TGG W GAC D GAC	L CAG Q CTG G G G CTA L CTC	S ATG M ACC T C C T C C C C C C C C C C C C C C	E TAC Y TTT F TGG W CCCC P GCA	G CAA Q GAC D CCT P TGG W TCT	Q AGC S AGC S CGG R CGG R CGG A	R TGT C XAAC N GCCA P GCCA G G TTCA	I GCT A AAC D GAC D GGT G GAA	M GAC D CTC V TCC W CCC G G G G G G	G P ACA T CCTG L ATC I	L ACT T GAC Q CAG Q CAG Q CAG	R GGC G XATG M XACC T XCGC R TTCT	A TIGI C TIC F C AGC S C AAC	L GGC G CCT P TTC F CCTG L CCTG	A T T CAAAA E TTGGG W SAGTA	20 108 31 117 34 120 37 120 37 120 37 120 37 120 37 120 37 120 37 120 37 120 37 120 120 120 120 120 120 120 120
	G CTG L TCC S CCCT P CCCT G C CCCT S	P GTC V AAAI N TTC F GAC D AATC I	L TAC Y GCC A S CTT L S CTT L I I	P AAC N AGG R CAT D XAAA K SGCT A	A TCC S GCC A GCA A E GCA A C GCC A C C A C C A C C A C C C C	N TCT S TGG W L CTC L GCC A CTC T	P GGC G G G G G G G G G C G C G C G C G	V ATG M TAC Y CAG Q N XAAC N CAA	K GAA E Q Q CAA Q Q CAA I I GGGG	V P GGCC A A TAC Y L ATTAC Y GGGA	G TGT C TGT C TGC C TGC F GCA	C TTTT F T CCTC L CTCC S CAT	E GAC D GAG E KGAT D XAAC N XAAC N	R ATC I ATC I ATC I T CACA T G G CTA	L TAC Y AAT TGG W GAC D GAC D	L CAG Q CTG G G G CTA L CTC L	S ATG M ACCC T CGTG CGTG C V GACC D CGTG C C CGTG C C C CGTG C C C C C C C	E TAC Y TTT F TGG W CCCC P GCA	G CAA Q GAC D CCT P TGG W TCT S	Q AGC S CGG R CGG R CGG A X AAC N	R TTGT C XAAC N S CCA P G G G TTCA S	I GCT A AAC D GAC D GAC GAA E	M GAC D CTGC W CGGG G G G G G C D	G P P CCCC P CCCC L CCCC P	L ACT T GAC D CAG Q CAG Q CCG P	R GGC G XATG M XACO T XCGC R XCGC R T TCT S	A TIGI C TIC F CAGO S CAAC S CAAC	L GGCC P TTC F CCTG L SGTG V	A T T CACTG T CACAGE W CACAGE E	21 100 3: 111 3: 120 3 112 3 4 133 4 4 4 4 4 4
	G CTG L TCC S CCCT P G G CCCT G TCA S	P GTC V AAT TTC F GAC D ATC I	L TAC Y CCCA A CTCI S CCTI L CATI I	P AAAC N AGG R CAAGG D CAAAA K SGCT A	A TCC S GCC A GCA A E GCA A C GCC V C C C C C C C C C C C C C C C C	N TCT S TGG W CTC L CTC L GCC A C T	P GGC G G G G G G G G C G C G C G C G C	V ATG M TAC Y CAG Q CAG N CAA N CAA		V P GGCCC A A TACCC A C A TACCC P C A C A C C C C C C C C C C C C C	G TGT C TGT C TGC C TGC F F GCA	C TTT F ACG T CTC L CTC S CAL	E GAC D GAG E CAAC N CAAC H	R ATC I ATC I ATC I CACA T GGT G GCTA	L TAC Y AAT TGG W GAC D GAC	L CAG Q CTG G G G CTA L CTC L AGA	S ATG M ACC T CTG GAC D CTA	E TAC Y TTT F TGG W CCCC P GCA A	G CAA Q GAC D CCT P TGG W TCT S CAA	Q AGC S AGC S CGG R CGG R A CGG A N	R TGT C XAAC N SCCA P G G G G TCA S	I GCT A AAC D GAC D GAC C C AA C GAC C C AA C C C AA C C C C	M GAC D CTG V TGG G G G G G G G G G G G G G G G	G P ACA T CTG L ATC I CCCG P	L ACT T GAC D CAG Q CAG Q CAG Q CAG Q TGG	R GGGC G ATG M ACC T CGC R S CCCI	A TIGI C TIC F F CAGC S CAAC S CAAC V V CGC	L GGCC P CCTG F CCTG L CCTG V CCTG	A TACTG T NGAAA E TTGGG W SAGTA SAGTA E TAAGT	28 106 31 117 34 126 37 126 37 40 135 40 144 43 155
	G CTG L TCC S CCCT P CCCT G G CCCT S CCCT R	P GTC V AAAI F GAC D AAC K	L TAC Y GCC A S CTCI S CTCI L CTCI L	P CAACC N CAGG R CAGGAT D CAAAA K CAAAA K CGAA E	A TCC S GCC A GCA E GCA A GCA A GCC V GCT A	N TCT S TTGG W CTC L GCCC A CTC T TACCC T	P GGC D CGC R CGC R CGC S CACC S CACC S CACC L	V ATG M TAC Y CAG Q CAAC N X CAAC N X CAAC N X CAATT I	K E CAG Q Q CAT C I CAT C I C CAG R	V P GGCCC A A ATAC Y I CATT I CATT I CATT C A C A C A C C A C C C C C C C C	G TIGI C TIGI C TIGC F F G G A TIGC	C TTT F ACC T C C C C C C C C C C C C C C C C C	E GAC D GAG E CAAC N N CAAC H AGCA A	R ATC I ATC I ATC C A C C T C C T C C T C C T C C T A C C T C C T C C C A C C C C	L TAC Y AAT TGG W GAC D GAC D GAC	L CAG Q CTG G G G CTA L CTC L AGA	S ATG M ACC T CTG GAC D CTA AGA L	E TAC Y TTT F TGG W CCCC P GCA A AAG K	G CAA Q GAC D CCT P TGG W TCT S CAA Q	Q AGC S AGC R CGG R A CGG A N N CCA	R TTGT C XAAC N GCCAA P GCCAA G G TTCA S AGCA A	I GCT A AAC D GGCT G GAC E GAC E	M GAC D GTG V TGG G G G G G C D G G C A	G P ACA T CTG L CTG L SATC P CCCC P	L ACT T GAC D CAG Q CAG Q CAG Q CAG Q CAG Q TGG	R GGC G ATG M ACC T T C GCC C C C C C C C C C C C C C C	A TGI C TTC F C AGC S S AAC V C C C C S S C C C TTC C C TTC C F C S S C S C S C S C S C S C S C S	L G G CCT F CCTG L CCTG C CCTG V CCTG V CCTG V CCTG	A T T CACTG T CACAGE W CACAGE E	21 33 111 34 112 3 3 112 3 3 4 4 4 5 155 4

Figure 3 Nucleotide and deduced amino acid sequences of rat kidney DPP II

Nucleotides and amino acid residues are numbered on the right. The amino acid sequence is numbered with respect to the N-terminal Leu of the mature protein. The asterisk indicates the termination codon. The potential signal peptide cleavage site is shown by the open arrow. The sequences of the three tryptic peptides obtained by automated Edman degradation are underlined. Six potential N-linked glycosylation sites are double-underlined. The positions of the two PCR primers are shown with two dashed arrows above the nucleotide residues. The polyadenylation sequence near the 3' end is also underlined.

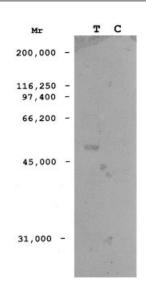
PDPII RDPII HQPP HPCP	1 -36 1 1	ASPPEPGFREV MGLHPCSPVDHGVPSWVLVLLLTLGLCSLQATADSVLDPDFREN MGSAPWAPVLLLALGLRGLQAGARRAPDPGFQER MGRRALLLLLSFLAPWATIALRPALRALGSLHLPTNPTSLPAVAKNYSV * - * * * * *	11 8 34 50
PDPII RDPII HQPP HPCP	9 35	-YFEQLLDHFNFERFGKKTFRQRFLVSDKFW -YFEQYMDHFNFESFSNKTFGQRFLVSDKFWKMGEGPIFFYTGNEGDIWS -FFQQRLDHFNFERFGNKTFPQRFLVSDRFWVRGEGPTFFYTGNEGDVWA LYFQQKVDHFGFNTVKTFNQRYLVADKYWKKNGGSILFYTGNEGDIIW .*.* .***.* *** **.**.* * *******	41 57 83 98
RDPII HQPP HPCP	84	LANNSGFIVELAAQQEALLVFAEHRYYGKSLPFGVQSTQRG-YTQLLTVE FANNSGFVAELAAERGALLVFAEHRYYGKSLPFGAQSTQRG-HTELLTVE FCNNTGFMWDVAEELKAMLVFAEHRYYGESLPFGDNSFKDSRHLNFLTSE .**.***. *.************************	132
RDPII HQPP HPCP	133	GXSXGG QALADFAVLLQALRHNL-GVQDAPTIAFGGSYGGMLSAYMRMKYPHLVAG QALADFAELLRALRRDL-GAQDAPAIAFGGSYGGMLSAYLRMKYPHLVAG QALADFAELIKHLKRTIPGAENQPVIAIGGSYGGMLAAWFRMKYPHMVVG ******* * * * * * ********* ********	181
RDPII HQPP HPCP	182	ALAASAPVIAVAGLGNPDQFFRDVTADFYGQSPKCAQAVRDAFQQIKDLF ALAASAPVLAVAGLGDSNQFFRDVTADFEGQSPKCTQGVREAFRQIKDLF ALAASAPIWQFEDLVPCGVFMKIVTTDFRKSGPHCSESIHRSWDAINRLS *******. * * * * * * * * * * * * * * *	231
RDPII HQPP HPCP	232	LQG-AYDTISQNFGTCQSLSSPKDLTQLFGFARNAFTVLAMMDYPYPTNF LQG-AYDTVRWEFGTCQPLSDEKDLTQLFMFARNAFTVLAMMDYPYPTDF NTGSGLQWLTGALHLCSPLTS-QDIQHLKDWISETWVNLAMVDYPYASNF * * * * * * * * * * * * *	280
RDPII HQPP HPCP	281	LGPLPANPVKVGCERLLSEGQRIMGLRALAGLVYNSSGMEPCFDIYQ LGPLPANPVKVGCDRLLSEAQRITGLRALAGLVYNASGSEHCYDIYR LQPLPAWPIKVVCQYLKNPNVSDSLLLQNIFQALNVYNYSGQVKCLNISE * **** * * * * * * * * * * * * * * *	327
RDPII HQPP HPCP	328	MYQSCADPTGCGTGSNARAWDYQACTEINLTFDSNNVTDMFPEIPFSDEL LYHSCADPTGCGTGPDARAWDYQACTEINLTFASNNVTDMFPDLPFTDEL TATSSLGTLGWSYQACTEVVMPFCTNGVDDMFEPHSWNLKE *. * * ****** * * * * ***	377
RDPII HQPP HPCP	378	RQQYCLDTWGVWPRPDWLQTSFWGGDLKAASNIIFSN GDLD PWAGGGIQR RQRYCLDTWGVWPRPDWLLTSFWGGDLRAASNIIFSN GNLD PWAGGGIRR LSDDCFQQWGVRPRPSWITTMYGGKNISSHTNIVFSN GELD PWSGGGVTK * *** *** *. * * *	427
RDPII HQPP HPCP	428	H NLSTSIIAVTIQGGAHHLDLRASNSEDPPSVVEVRKLEATLIREWVAAAR NLSASVIAVTIQGGAHHLDLRASHPEDPASVVEARKLEATIIGEWVKAAR DITDTLVAVTISEGAHHLDLRTKNALDPMSVLLARSLEVRHMKNWIRDFY	477
RDPII HQPP HPCP	478	LKQPAEAQWPGPK 464 REQ-QPALRGGPRLSL 492 DSAGKQH 495	

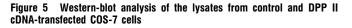
Figure 4 Alignment of the deduced amino acid sequence of rat DPP II with those sequences of porcine DPP II, human QPP and human PCP

Identical residues are shown with asterisks. Dashes are gaps introduced to maximize alignment. Consensus sequences (GXSXGG and GXXD) and potential active residue (H) of serine proteases are indicated above these amino acid residues. PDPII, porcine DPP II; RDPII, rat DPP II; HQPP, human QPP; HPCP, human PCP.

Leu-agmatin] or 0.1 mM diprotin A (DPP IV inhibitor) did not inhibit it significantly, as was reported previously [9].

The optimum pH of rat kidney DPP II in 170 mM cacodylate buffer with Lys-Ala-NA used as the substrate was pH 5.5, the same as found when universal buffer (0.0286 M citric acid/KH₂PO₄/H₃BO₃/barbital buffer, pH 3.0–10.0) was used, as described previously [9]. The activity of the enzyme at pH 7.0 in 50 mM Hepes buffer was only 25.6 % (31.9 nmol/min per mg of protein) of that observed at pH 5.5 in cacodylate buffer (124.4 nmol/min per mg of protein, Figure 2A). However, the activity with Gly-Pro-NA as the substrate became 129 % (12.75 versus 9.88 nmol/min per mg of protein, Figure 2B).





C, control lysate; T, DPP II cDNA-transfected lysate (20 µl each).

Isolation and sequencing of cDNA encoding rat DPP II

Three peptides from the trypsin digest of the purified enzyme were successfully isolated and sequenced. The amino acid sequences of the three peptides and the retention times (in min) of each peptide on each column (Pepkat, Shim-pack, respectively) were as follows: peptide 1, YYGK (7.3, 13.3); peptide 2, DLTQLFGFAR (8.1, 15.0); and peptide 3, GGAHHLDLR (9.5, 16.6). The peptide sequences were used as probes to search for translation products in the rat EST database. Several EST clones were identified, and the largest clone (AI716264) contained 435 bases of sequence, including a polyadenylation signal and poly(A)⁺ tail, and encoded peptide 3, GGAHHLDLR. A rat kidney cDNA was amplified by PCR using two primers based on the nucleotide sequence of the EST clone (AI716264), for screening the rat kidney cDNA library. Four positive clones were isolated from the library. Three of these clones had the same molecular mass as the insert cDNA, and the 5' regions of these insert cDNAs also had the same nucleotide sequence, indicating that the three clones were identical. The cDNA was 1710 bp long with an open reading frame of 1500 bp encoding a protein of 500 amino acid residues (Figure 3). A potential initiation codon (ATG) was found beginning at nucleotide 30, and the nucleotide sequence surrounding this codon (GGCATGG) obeyed Kozak's rule [31]. In the 3' non-coding region, a polyadenylation signal and $poly(A)^+$ tail were found, so the cDNA contained the complete open reading frame.

The N-terminal 10 amino acids derived from proteinsequencing analysis of the purified enzyme matched perfectly residues 37–46 of the deduced sequence, and the peptide sequences analysed also matched the three portions of the molecule, indicating that the cDNA encoded the entire mature protein (Figure 3). The mature protein had 464 amino acid residues with a theoretical molecular mass of 51400 Da. On the other hand the molecular mass of DPP II purified from rat kidney was estimated to be 60000 ± 2800 Da by SDS/PAGE. The predicted primary structure of the protein contained six potential N-glycosylation sites distributed throughout the molecule (Figure 3), which might account for the difference between

Table 2 Kinetic parameters of the purified enzyme

In the calculation of $k_{\rm cat}$ the apparent molecular mass of the purified enzyme was taken as 120000 Da.

Substrate	Concentration (mM)	<i>K</i> _m (mM)	$k_{\rm cat} ({\rm s}^{-1})$	$k_{\rm cat}/K_{\rm m} \ ({\rm mM}^{-1} \cdot {\rm s}^{-1})$
Lys-Ala-NA Gly-Pro-NA Lys-Ala-MCA	0.1—1 0.05—0.4 0.025—0.4	$\begin{array}{c} 0.763 \pm 0.068 \\ 0.192 \pm 0.05 \\ 0.26 \pm 0.02 \end{array}$	656 46.7 54.8	859 243 211
Gly-Pro-MCA	0.00625-0.1	0.072 <u>+</u> 0.002	73.6	1022

the theoretical molecular mass and that estimated by SDS/ PAGE, as in the case of prolyl carboxypeptidase (PCP) [22]. The mature protein is thought to contain carbohydrate.

The 36-amino acid peptide upstream from the N-terminal Leu of the mature protein most probably contains a signal peptide and a propeptide. An Internet-based analysis employing the signal-prediction server SignalP V1.1 (http://genome.cbs.dtu.dk/services/SignlP/index.html/) predicted this stretch to represent a typical N-terminal signal sequence: a positively charged N-terminal region (Met⁻³⁶-Trp⁻²¹), a central hydrophobic region (Val⁻²⁰-Leu⁻¹¹) and a polar C-terminal region (Cys⁻¹⁰-Ala⁻⁴) [32,33]. The presence of Ala at the -4 and -6 positions is consistent with a signal-peptidase-cleavage site at Ala⁻⁴-Asp⁻³ [34] and the remaining three residues are thought to comprise a propeptide (Asp⁻³-Val⁻¹). However, experiments to determine whether DPP II is initially synthesized as an inactive proenzyme have not yet been performed.

A search of the National Biomedical Research Foundation-The Protein Information Resource (NBRF-PIR) protein sequence database with the primary structure of rat DPP II showed that it had highly significant similarity to human N-terminal proline-specific dipeptidase (QPP, 78 % identity [24]) and human lysosomal PCP (38 % identity). Furthermore, the deduced amino acid sequence of DPP II contained the consensus sequences GXSXGG, for the active-site serine residue, and GXXD, for the aspartic acid residue of serine-type proteases, such as are found in prolyl oligopeptidases [23], PCP and QPP (Figure 4).

Functional expression of DPP II in COS-7 cells

To determine whether the cDNA encoded an active DPP II, we transfected COS-7 cells with the cDNA cloned into the pcDNA3.1/HisA expression vector. The protein content of the cell lysate was $674.5 \pm 88 \ \mu \text{g/ml}$ for the control cells (n = 6) and $723.7 \pm 107 \,\mu\text{g/ml}$ for the transfected cells (n = 4), and the corresponding Lys-Ala-MCA-cleaving activity was 211 ± 24 and 9985.2±1400 pmol/min per ml. Thus transfected COS-7 cells showed 50 times higher Lys-Ala-MCA-hydrolysing activity than the control COS-7 cells. Figure 5 shows the existence of expressed DPP II in transfected-cell lysate. The activity with Gly-Pro-MCA as the substrate was about 120 times higher than that of the control cells (results not shown). The partial purified recombinant enzyme showed the same properties as the native enzyme with respect to gel filtration, immunoblotting and pH profile (results not shown). The $K_{\rm m}$ values for Lys-Ala-MCA and Gly-Pro-MCA were 0.26 and 0.07 mM, respectively (Table 2).

DISCUSSION

The molecular mass of rat DPP II was about 60000 Da by SDS/PAGE and 120000 Da by gel filtration (results not shown). These findings suggest that the rat DPP II is composed of two

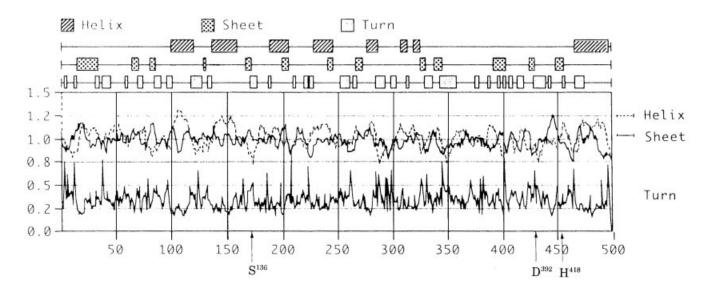


Figure 6 Schematic representation of secondary-structure predictions for rat DPP II

Calculations were made by using the Chou and Fasman algorithm with Macintosh sequence-analysis software. The positions of the potential active amino acid residues (S¹³⁶, D³⁹² and H⁴¹⁸) are indicated by arrows.

identical subunits. The results were the same for the molecular masses of bovine pituitary and guinea pig testis enzymes [35,36]. The predicted primary structure of the protein contained six potential N-glycosylation sites (Figure 3), which might account for the difference between the theoretical molecular mass and that estimated by SDS/PAGE, as in the case of PCP [22]. The mature protein is thought to contain about 14 % carbohydrate. From our study, we can conclude that DPP II belongs to the family S 28 of the clan SC, as was predicted previously by Rawlings and Barrett [21].

Recently, an enzyme (QPP) related to the cell death of quiescent lymphocytes was isolated and cloned [24]. It had the same substrate specificity as DPP II and IV [24,37]. QPP also shared the same properties of DPP II with respect to molecular mass, lysosomal localization and serine peptidase classification, and was assigned to family S 28 of clan SC [23]. Underwood et al. [24] showed that QPP and DPP II were different with respect to their pH profiles. The optimum pH of rat kidney DPP II in 170 mM cacodylate buffer with Lys-Ala-NA as the substrate was pH 5.5, the same as in universal buffer described previously [9]. The activity of the enzyme at pH 7.0 in 50 mM Hepes buffer was only 25.6% of that observed at pH 5.5 in cacodylate buffer (Figure 2A). However, the pH profile of purified DPP II using Gly-Pro-NA as the substrate, as shown in Figure 2(B), showed the same behaviour as that of QPP. This profile was dependent on the buffers used and on the nature of the substrates' P1 and P1' sites, because the pH profiles tended to be similar with Lys-Ala-MCA as the substrate (cacodylate buffer, pH 5.5, 100 % versus Hepes buffer, pH 7.0, 45 %) and with Gly-Pro-MCA as the substrate (cacodylate buffer, pH 5.5, 100 % versus Hepes buffer, pH 7.0, 113%).

The amino acid homology between rat kidney DPP II and QPP showed 78 % identity over 500 residues (Figure 4). Moreover, the N-terminal amino acid sequence of human placental DPP II and human QPP were consistent with 32 out of 33 residues by a search of the NBRF-PIR protein sequence database (accession no. S77568). Therefore, it is conceivable that QPP is human DPP II. The active site of serine proteases classified as clan SC has the Ser-Asp-His triad, and it shows an α/β hydrolase fold [23]. The protein secondary structure of DPP II predicted by the Chou and Fasman algorithm used by Macintosh Vector sequence analysis software is shown in Figure 6. Ser¹³⁶ in the GXSXGG motif and His⁴¹⁸ are in the α/β hydrolase fold, but Asp³⁹² in the GXXD motif is not. Especially, active-site Ser¹³⁶ exists between the fifth β -sheet and the third α -helix region from the N-terminus, as shown in Figure 6. Its position is consistent with the results from X-ray crystallographic analysis on proline aminopeptidase [38].

It is probable that DPP II causes apoptosis in quiescent lymphocytes [37]. Moreover, the activity of this enzyme significantly increased in the gingival crevicular fluid of periodontal patients (M. Ohno, N. Ota, K. Fukasawa and K. M. Fukasawa, unpublished work). However, it remains to be explained why DPP II in various human carcinoma cells was found in a 6–24fold higher concentration than that in normal human fibroblasts [39]. Because many cytokines have N-terminal Xaa-Pro or Xaa-Ala sequences [40], identification of endogenous substrates in each cell type remains to be accomplished for DPP II, as reported for DPP I [41].

We dedicate this paper to Dr Minoru Harada and the late Dr George G. Glenner, who encouraged K.F. and K.M.F. in this interesting research field. We are grateful to Tomoko Takei for secretarial assistance.

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