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Human Renal Dipeptidase from Kidneys of Renal Stone Patients: Partial Characterization

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Physico-chemical characterization of human renal dipeptidase was carried out. It was a glycoprotein with a subunit MW of approximately 47,700 dalton. The pH optimum was at 8 and its stable conformation was retained between pH 5 and 12. The kinetic parameters determined with imipenem, a novel β-lactam antibiotic, were Vmax, 5.21 μmol/min/mg; Km, 4.35 mM; and Ki with cilastatin, 0.25 μM. Cilastatin demonstrated reversible competitive inhibition.

Key words: Renal dipeptidase (RDPase), Imipenem, Cilastatin, Glycyldehydrophenylalanine (Gdp)

INTRODUCTION

Renal dipeptidase (EC 3.4.13.11; RDPase) was discovered from kidney in 1932 but it took 50 years to be recognized as it is today. It drew special attention since early 1980s because it hydrolyzed a novel β-lactam antibiotic, N-formimidoylthienamycin (imipenem; Kim and Campbell, 1982), and Leukotriene D_4 (Kozak and Tate, 1982).

Imipenem was introduced in 1970s as a semi-synthetic derivative of thienamycin (Kahan *et al.*, 1976) which was isolated from *reptomyces cattleya*, named from its characteristic orchid-pigmentation of sporulated aerial mycelium. Imipenem is a novel β-lactam antibiotic with wide spectrum specificities and high efficacies toward various penicillin/cephalosporin-resistant microorganisms (Alford, 1983; Bartmann and Tarbuc, 1982). However, it was hydrolyzed by RDPase in the kidney and its expected antibiotic potency was greatly reduced in experimental animals. It became clear that RDPase has to be controlled specifically and reversibly during imipenem administration (Kahan *et al.*, 1983). Imipenem is available for clinical use as 1 : 1 complex with cilastatin, named Primaxin.

Renal dipeptidase has been isolated from kidneys of rat (Farrell *et al.*, 1987; Hirota *et al.*, 1987), hog (Kim and Campbell, 1983; Hooper and Turner, 1989) and human (Campbell *et al.*, 1984; Mitsuhashi *et al.*, 1988). The source of human kidneys were not explained in those papers.

Current studies were undertaken to characterize the physicochemical properties of human RDPase including catalytic activity on imipenem hydrolysis and inhibition by cilastatin with the enzyme purified from the surgically removed kidneys of renal stone patients.

MATERIALS AND METHODS

Human RDPase was purified from surgically removed kidneys of renal stone patients as described in previous report (Park *et al.*, 1993). Imipenem and cilastatin were kindly provided by Mr. H. Kropp (Merck Sharp and Dohme), U.S.A. Gdp, a synthetic substrate of RDPase, was prepared by the method of Campbell *et al.* (1963). Quartz cuvettes of 10 mm and 1 mm light path were employed in enzyme assays using Gdp and imipenem, respectively. Kit of prestained MW markers, Sephacryl S-300 and reagents for stainings were purchased from Sigma Chemical Company, U.S.A.

All manipulations were performed at 4℃ or in the ice bath, unless stated otherwise.

Enzyme Assay

Hydrolysis of Gdp was carried out as described by

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Park *et al.* (1993). Activities were calculated from the molar extinction coefficient of Gdp ($E_{1\,cm}^{275\,nm} = 1.56 \times 10^4$: Campbell *et al.*, 1961) and expressed as μmol Gdp hydrolyzed/min/mg protein.

Polyacrylamide Gel Electrophoresis (PAGE) and Stainings

Native-PAGE gels were prepared according to the method of Ornstein (1964). The gel was made of 2.5% stacking and 7.5% separating tube gel and electrophoresis was carried out for 35 min with 4 mamp per gel. Individual electrophoresed tube gel was subjected to staining as follows. Protein staining of gel 1 was carried out overnight by keeping the gel in staining solution made of 0.1% Coomassie Brilliant Blue R-250 dissolved in methanol-acetic acid-water (40 : 7 : 53). Background of the gel was destained in the same solution without the dye. Activity staining reaction of gel 2 was performed by the method of Sugiura *et al.* (1977). The staining solution was composed of 5 mg p-iodonitrotetrazolium violet, 1 mg phenazine methosulfate, 1 mM Gdp and L-amino acid oxidase dissolved in 10 ml of 2 mM Tris-HCl, pH 7.6. Gel 3 was subjected to Periodic Acid-Schiff test for glycoprotein by the method of Kapitany and Zebrowski (1973). Electrophoresed gel was fixed for 10 min in 12.5% trichloroacetic acid and the subsequent reactions were carried out in the dark at 4℃. The gel was reacted with 1% periodic acid for 10 min followed by thorough washing in 15% acetic acid. It was reacted with Schiff reagent for 2 hours and the background stain was removed by washing the gel in 7% acetic acid with several changes. Glycoprotein appears as red band on the gel of clear background. The Schiff reagent was prepared as follows. Basic fuchsin (2.5 g) was dissolved in 500 ml distilled water. Hydrochloric acid (1 N, 50 ml), sodium meta bisulfite (4.25 g) and activated charcoal (10 g, washed with 1 N HCl) were added to the dye solution and the mixture was stirred overnight in the dark at 4℃. The charcoal was removed by centrifugation (13,200×g, 15 min) followd by passing through glass wool to remove the residual charcoal.

Determination of Native Molecular Weight

Molecular weight of native RDPase was determined by gel permeation chromatography using Sephacryl S-300. Fractions of 1 ml were collected with approximately 10 ml/hour flow rate. Apoferritin, alcohol dehydrogenase, BSA and cytochrome C were employed as MW markers beside Blue Dextran 2000. Cytochrome C peak was detected by measuring optical density at 380 nm, Blue Dextran 2000 at 465 nm and other proteins at 280 nm. Human RDPase peak was located by enzyme assay with Gdp.

Determination of Subunit Molecular Weight

The subunit MW was determined by the SDS-PAGE technique of Laemmli (1970) with 8% separating and 2.5% stacking gel. The prestained MW markers were pyruvate kinase, fumarase, fructose-6-phosphate kinase, lactate dehydrogenase and triose phosphate isomerase. Bromophenol blue was used as a tracking dye.

pH Profiles

Appoximate Universal Buffer (Perrin and Dempsey, 1974) was employed to determine the effect of pH on enzyme activity and stability at various pH ranging from 3 to 13. For enzyme activity plot (Curve A), human RDPase was assayed for 2.5 min with Gdp as substrate at various pH to determine the optimal pH. For enzyme stability plot (Curve B), RDPase was preincubated at various pH for two times the assay reaction time (5 min), then assayed at the optimal pH determined from the activity plot.

Imipenem Hydrolysis

Imipenem was dissolved in 50 mM MOPS buffer, pH 7.1. Human RDPase was assayed with imipenem ranging from 1.67 to 2.86 mM in the presence and absence of 0.15 μM cilastatin. Hydrolysis of imipenem was measured by the fall of optical density at 299 nm using 1 mm light path cuvettes. The enzyme acti-

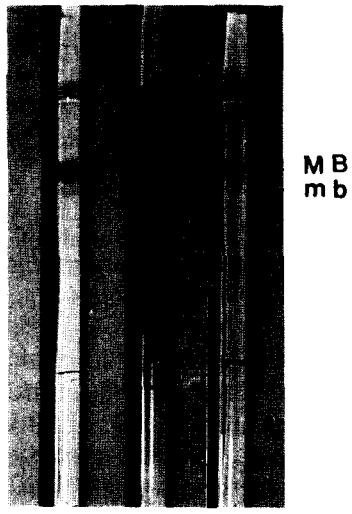

Fig. 1. Protein, Activity and PAS staining. The gels were made of 2.5% stacking and 7.5% separating tube gel. Gel 1 (5 μg protein) for Coomassie staining, gel 2 (0.6 μg protein) for activity staining, and gel 3 (7 μg protein) for PAS test were subjected to electrophoresis for 35 min with 4 mamp/gel. Each staining procedure was described under materials and methods.
MB, major band; mb, minor band

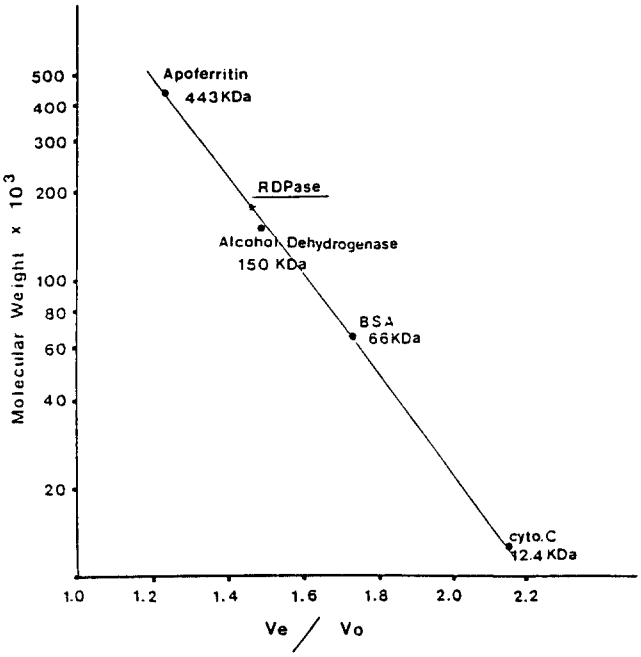

Fig. 2. Determination of native MW. Sephacryl S-300 was packed in 16×350 mm column and proteins were eluted with 2 mM Tris-HCl, pH 7.6. Fractions of 1 ml were collected and protein peaks of individual markers were identified as described under MATERIALS AND METHODS. Ve was elution volume of individual proteins. Vo was void volume required to elute Blue Dextran 2000.

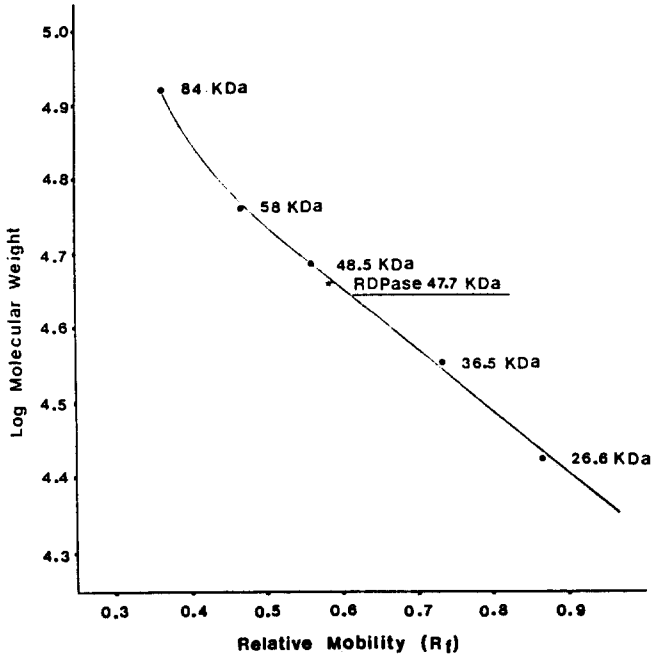

Fig. 3. Determination of subunit MW. SDS-Polyacrylamide gel was made of 8% separating and 2.5% stacking gel. Prestained MW markers of 3 μg each and human RDPase of 4 μg were loaded on each lane. The MW markers were fructose-6-phosphate kinase (84 kda), pyruvate kinase (58 kda), fumarase (48.5 kda), lactate dehydrogenase (36.5 kda), and triose phosphate isomerase (26.6 kda).

vity was expressed as μmol imipenem hydrolyzed/min/mg protein adopting $E\%_{1\,cm}^{299\,nm}=305$ for imipenem (Kropp *et al.*, 1980). Kinetic parameters were determined by standard graphical method of Lineweaver-Burk plots.

RESULTS AND DISCUSSION

In previous report (Park *et al.*, 1993), it was demonstrated that RDPase of renal patients was still active although the kidney failed to perform glomerular filtration. Some of the surgically removed kidneys of renal stone patients did not show any measurable RDPase activity and those kidneys were not used for enzyme purification. Physiological role of RDPase was proposed as the hydrolysis of L-dipeptides present in the glomerular filtrate, with the resultant free amino acids transported across the microvilli by Na^+ gradient-dependent processes (Welch and Campbell, 1980).

Electrophoresed gels of purified enzyme were subjected to staining for protein, dipeptidase and PAS test in Fig. 1. They exhibited a slow moving major band and a fast moving minor band. The minor band of gel 1 was approximately 4% of the total protein and was not a contaminant but the RDPase itself because it was shown positive in gel 2 of dipeptidase-specific reaction (Park *et al.*, 1993). Human RDPase is known to be anchored to membrane via phosphatidylinositol (Hooper and Turner, 1989; Littlewood *et al.*, 1989; Adachi *et al.*, 1990a). RDPase in this report was released from membrane with n-butanol instead of phosphatidylinositol-specific phospholipase C. The major band may be the entire RDPase (the membrane-bound form) while the minor band correspond to the soluble form without the lipid portion. This interpretation is partly confirmed by gel 3 where both bands clearly showed the red color of PAS-positive reaction indicating that they are glycoproteins. In most cases, the protein parts bearing carbohydrate are oriented exterior of the membrane. The composition or sequence of carbohydrate on RDPase has not been examined, yet.

In Fig. 2, the MW of native RDPase was estimated as 185,000-190,000 dalton. In Fig. 3, the subunit MW determined by SDS-PAGE was approximately 47,700 dalton. Campbell *et al.* (1984) suggested human RDPase as a tetrameric enzyme of 220,000 dalton with the subunit MW of 59,000 dalton. Adachi *et al.* (1990 b) calculated the unglycosylated human RDPase as 43,800 dalton from cDNA sequence.

The pH profile of activity shown in curve A of Fig. 4 exhibited the highest catalytic activity at pH 8. The

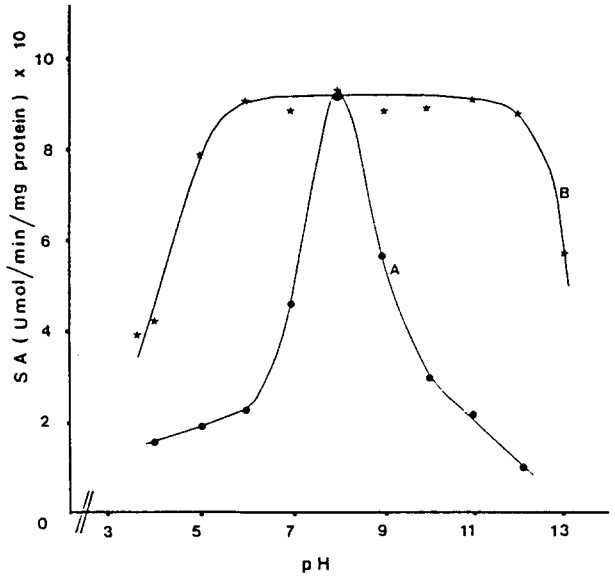

Fig. 4. Effect of pH on enzyme activity and stability. Enzyme activity plot (Curve A); Catalytic activity of human RDPase (8.75 ng) was measured for 2.5 min in 1 ml of 5.2×10^{-5} M Gdp as substrate at various pH. Enzyme stability plot (Curve B); Human RDPase was preincubated at various pH for 5 min, then assayed at pH 8. Each point represents the average of 4 measurements.

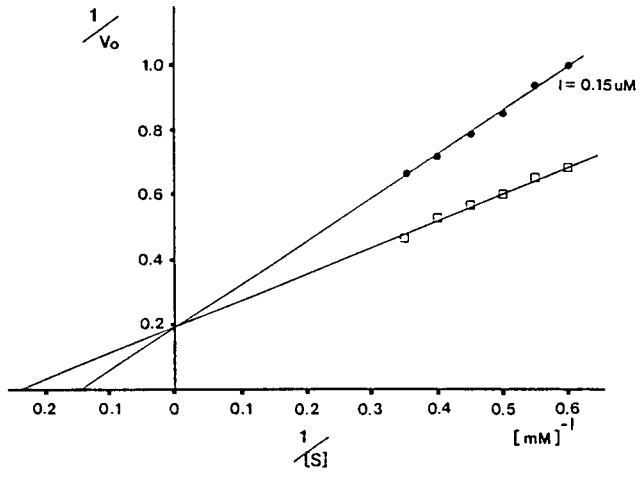

Fig. 5. Lineweaver-Burk plots of RDPase. The reaction mixture of 250 µl contained imipenem ranging from 1.67 to 2.86 mM in the absence or presence of 0.15 µM cilastatin and 0.44 µg of purified human RDPase. It was measured by the fall of OD299 nm, at 37℃ using 1 mm light path cuvettes. The enzyme activity was expressed as µmol imipenem hydrolyzed/min/mg protein.

stability profile in curve B demonstrated irreversible loss of activity below pH 5 and above pH 12. The result indicated that the stable native conformation of RDPase can be maintained when the enzyme kept between pH 5 and 12.

The kinetic parameters with imipenem as substrate were determined from Fig. 5. Vmax was 5.21 µmol/min/mg; Km, 4.35 mM; and the inhibition constant (Ki) for cilastatin, 0.25 nM. Cilastatin demonstrated reversible, competitive inhibition type. The kinetic values reported by Campbell *et al.* (1984) were Vmax, 44.5 µmol/min/mg; Km, 10.9 mM; and Ki for cilastatin, 0.73 µM. Values of Km and Ki in both reports may be considered in the similar range but Vmax exhibited a magnitude lower in RDPase from renal patients than drowned victim in Campbell et al. (1984; personal communication). Although Vmax is very low, RDPase has great potential to inactivate imipenem in renal stone patients during treatment of bacterial infection with this antibiotic. Some of the very severely degenerated kidney homogenate demonstrated no detectable RDPase activity, the amount of imipenem administration must be reduced to these patients to avoid possible toxicity due to imipenem overdose. Doses of imipenem higher than 100 mg per kg rabbit induced acute tubular necrosis (Kahn *et al.*, 1983). It also brought up the question about cilastatin which has been always coadministrated with imipenem to inhibit RDPase. Imipenem must be available without cilastatin instead of manufacturing imipenem and cilastatin complex (Primaxin) for those renal patients who has no or very low RDPase activity.

The results of this and previous investigations provided biochemical evidences that some of the nonfunctional kidneys from renal stone patients still have active RDPase indicating the need of its control as much as the individuals with healthy kidney during imipenem administration. The inhibition constant of cilastatin and its reversible, competitive inhibition type indicated effective clinical use of Primaxin in those renal patients. The need of cilastatin-free imipenem must be considered for some renal patients with no detectable RDPase activity.

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REFERENCES CITED

Adachi, H., Katayama, T., Inuzuka, C., Oikawa, S., Tsujimoto, M. and Nakazato, H., Identification of membrane anchoring site of human renal dipeptidase and construction and expression of a cDNA for its secretory form. *J. Biol. Chem.*, 265, 15341-15345 (1990a).

Adachi, H., Tawaragi, Y., Inuzuka, C., Kubota, I., Tsujimoto, M., Nishihara, I. and Nakazato, H., Primary structure of human microsomal dipeptidase deduced

from molecular cloning. *J. Biol. Chem.*, 265, 3992-3995 (1990b).

Alford, R. H., Comparison of ceftazidime and N-formimidoyl thienamycin against gentamicin-susceptible and resistant Pseudomonas aeruginosa. *J. Antimicrob. Chemother.* 11, 599-601 (1983).

Bartmann K. and Tarbuc, R., N-formimidoyl thienamycin-in vitro activity against bacteria resistant to beta-lactam antibiotics or gentamicin. *Infection,* 10, 361-370 (1982).

Campbell, B. J., Forrester, L. J., Zahler, W. L. and Burks, M., β-lactamase activity of purified and partially characterized human renal dipeptidase. *J. Biol. Chem.*, 259, 14586-14590 (1984).

Campbell, B. J., Lin, Y. C. and Bird, M. E., Renal aminopeptidase and copper-activated peptide hydrolysis. *J. Biol. Chem.*, 238, 3632-3640 (1963).

Campbell, B. J., Yudkin, W. H. and Klotz, I. M., Spectorphotometric properties of an intermediate in the enzymic hydrolysis of glycyldehydrophenylalanine. *Archiv. Biochem. Biophys.*, 92, 257-263 (1961).

Farrell, C. A., Allegetto, N. J. and Hitchcock, M. J. M., Cilastatinsensitive dehydrogenase I enzymes from three sources all catalyze carbapenem hydrolysis and conversion of leukotriene D_4 to leukotriene E_4. *Archiv. Biochem. Biophys.*, 256, 253-259 (1987).

Hirota, T., Nishikawa, Y., Tanaka, M., Fukuda, K., Igarashi, T. and Kitagawa, H., Localization of dehydropeptidase-I, an enzyme processing glutathione, in the rat kidney. *J. Biochem.*, 102, 547-550 (1987).

Hooper, N. M. and Turner, A. J., Ectoenzymes of the kidney microvillar membrane. *Biochem. J.*, 261, 811-818 (1989).

Kahan, F. M., Kropp, H., Sundelof, J. G. and Birnbaum, J., Thienamycin: development of imipenem-cilastatin. *J. Antimicrob. Chemother.*, 12, suppl. D: 1-35 (1983).

Kahan, J. S., Kahan, F. M., Goegelman, R., Currie, S. A., Jackson, M., Stapley, E. O., Miller, T. W., Miller, A. K., Hendlin, D., Mochales, S., Hernandez, S. and Woodruff, H. B., Thienamycin, A new β-lactam antibiotic. I. Discovery and isolation, *16th Interscience Conference on Antimicrob. Agent. Chemother., Chicago, Ill.,* Abstr. 227 (1976).

Kapitany, R. A. and Zebrowski, E. J., A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal. Biochem.*, 56, 361-369 (1973).

Kim, H. S. and Campbell, B. J., β-lactamase activity of renal dipeptidase against N-formimidoyl thienamycin. *Biochem. Biophys. Res. Comm.*, 108, 1638-1642 (1982).

Kim, H. S. and Campbell, B. J., Association of renal dipeptidase with the Triton-insoluble fraction of kidney microvilli. *J. Memb. Biol.*, 75, 115-122 (1983).

Kozak, E. M. and Tate, S. S., Glutathione-degrading enzymes of microvillus membranes. *J. Biol. Chem.*, 257, 6322-6327 (1982).

Kropp, H., Sundelof, J. G., Kahan, J. S., Kahan, F. M. and Birnbaum, J., MK0787 (N-formimidoyl thienamycin): Evaluation of in vitro and in vivo activities. *Antimicrob. Agent. Chemother.*, 17, 993-1000 (1980).

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (London), 227, 680-685 (1970).

Littlewood, G. M., Hooper, N. M. and Turner, A. J., Ectoenzymes of the kidney microvillar membrane. *Biochem. J.*, 257, 361-367 (1989).

Mitsuhashi, S., Fuse, A., Mikami, H., Saino, Y. and Inoue, M., Purification and characterization of human renal dehydropeptidase I. *Antimicrob. Agent. Chemother.*, 32, 587-588 (1988).

Ornstein, L., Disc electrophoresis-I Background and theory. *Annal. New York Acad. Sci.*, 121, 321-349 (1964).

Park, H. S., Kim, D. H., Kwark, H. S. E., Park, S. K., Kang, S. K., Chung, B. H. and Yoo, G. S., Human renal dipeptidase from kidneys of renal stone patients: Partial purification, *Archiv. Pharm. Res.*, 16(4), 295-299 (1993).

Perrin, D. D. and Dempsey, B., Approximate universal buffer; in Perrin, D. D. and Dempsey, B. (eds), *Buffer for pH and metal ion control,* Chapman and Hall, London, 1974, p. 156.

Sugiura, M., Yoshimasa, I. and Kazuyuki, H., Detection of dipeptidase and tripeptidase activities on polyacrylamide gel and cellulose acetate gel by the reduction of tetrazolium salts. *Anal. Biochem.*, 81, 481-484 (1977).

Welch, C. L. and Campbell, B. J., Uptake of glycine from L-alanylglycine into renal brush border vesicles. *J. Memb. Biol.*, 54, 39-50 (1980).