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

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Human renal dipeptidase (RDPase) was purified from surgically removed kidneys of renal stone patients by affinity chromatography using its specific inhibitor, cilastatin, as the ligand. The partial purified RDPase of 6 mg exhibited specific activity of 99.4 unit/mg with 2,029 fold purification. It was composed of a slow moving major band (96%) and a fast moving minor band (4%). The minor band was not a contaminant as it showed a dipeptidase-specific activity. The kinetic parameters determined with glycyldehydrophenylalanine (Gdp) as synthetic substrate were Vmax, 322.6 μmol/min/mg and Km, 0.102 mM. This experiment provided biochemical evidences that surgically removed, nonfunctional kidneys in respect of glomerular filtration still retained high activity of renal dipeptidase.

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

INTRODUCTION

Renal dipeptidase (EC 3.4.13.11), also referred as dehydropeptidase I, is an integral membrane protein localized on the microvilli of renal proximal tubules (Welch and Campbell, 1980; Littlewood *et al.*, 1989). It acts upon a variety of dipeptides but not on esters, tripeptides, or proteins (Campbell, 1970). In 1982, Kim and Campbell reported that purified porcine RDPase hydrolyze novel β-lactam antibiotic, N-formimidoyl thienamycin (imipenem). Imipenem, an amide derivative of thienamycin produced by *Streptomyces cattleya*, was introduced in 1970s as a novel β-lactam antibiotic with wide range specificities and high efficacies toward various penicillin/cephalosporin-resistant microorganisms (Kahan *et al.*, 1976; Kahan *et al.*, 1983; Horadam *et al.*, 1980; Tally *et al.*, 1980; Toda *et al.*, 1980). However, its urinary recovery was very low when tested on animals (Kropp *et al.*, 1982) as it was hydrolyzed by mammalian β-lactamase, RDPase (Kim and Campbell, 1982; Matsuda *et al.*, 1985). Currently, imipenem is in the market with the product name of Primaxin in U.S.A. and Tienam in Korea. It is manufactured from Merck Sharp and Dohme, U.S.A. as 1 : 1 complex with cilastatin, the specific competitive inhibitor of RDPase.

It was reported that RDPase hydrolyze LTD_4 to LTE_4 (Campbell *et al.*, 1988). Leukotriene C_4, D_4 and E_4 are known as the major components of Slow Reacting Substances of Anaphylaxis (SRS-A) where LTD_4 is the most active bronchospasmogen (Lewis *et al.*, 1990). Physiological role of RDPase in relation to LTD_4 hydrolysis in the kidney is not clear, yet.

The kinds of substrates acted upon by RDPase indicate that it is an important enzyme to study. Renal dipeptidase has been isolated from kidneys of rat (Farrell *et al.*, 1987; Hirota *et al.*, 1987), porcine (Kim and Campbell, 1983; Hooper and Turner, 1989) and human (Campbell *et al.*, 1984; Mitsuhashi *et al.*, 1988).

Purification of RDPase from animals was most widely performed according to the method of Armstrong *et al.* (1974) employing techniques of n-butanol solubilization, isoelectric precipitation, ammonium sulfate fractionations and preparative polyacrylamide gel electrophoresis. Some researchers substituted n-butanol solubilization by enzyme digestion with phosphatidylinositol-specific phospholipase C (Hooper and Turner, 19 89; Littlewood *et al.*, 1989; Adachi *et al.*, 1990). Recently most of the researchers switched the lengthy steps of the traditional methods to affinity chromatographic technique employing cilastatin as the ligand. Cilastatin is a competitive inhibitor of RDPase (Kim and Campbell, 1982; Campbell *et al.*, 1984).

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Current studies were undertaken to examine if the surgically removed, nonfunctional kidneys of renal stone patients retain RDPase activity, and if so, to purify the human RDPase from this rare source material.

MATERIALS AND METHODS

Surgically removed kidney specimens of renal stone patients were obtained from Renal Clinic of Chonbuk National University, Korea, and kept frozen until use. Cilastatin was kindly provided by Mr.H. Kropp of Merck Sharp and Dohme, U.S.A. Glycyldehydrophenylalanine was synthesized by the method previously described (Campbell *et al.*, 1963). CNBr-activated sepharose 4B was purchased from Sigma. Suprasil cuvettes of 2 mm light path were purchased from Fisher Scientific, U.S.A. All manipulations were performed at 4℃ or in the ice bath, unless stated otherwise.

Purification of human renal dipeptidase

Human RDPase was purified according to the method of Campbell *et al.* (1984). Frozen kidneys were thawed at room temperature, the clinging fat on outside of tissue were removed and chopped into small pieces. It was homogenized with 10 vol (v/w) of 33 mM sodium phosphate buffer, pH 8.0 containing 5% glycerol (w/v), centrifuged for 10 min at 5,000×g and the supernatant was referred as 'homogenate'. The homogenate was solubilized with chilled n-butanol to a final concentration of 20% for 43 hours, fractionated with ammonium sulfate to give 0-50% saturation by adding 314 g/L. After overnight standing, it was centrifuged for 20 min at 23,000×g and more ammonium sulfate (172 g/L) was added to the supernatant to precipitate the 50-75% saturation fraction. After centrifugation, the pellet was resuspended in a small volume of 2 mM Tris-HCl, pH 7.4 followed by dialysis. Further purification was achieved by the affinity chromatography technique according to Kropp *et al.*(1982) using cilastatin as the ligand. One gram of CNBr-activated sepharose 4B was reacted with 5 ml of cilastatin solution (1 mg/ml) and packed into a 10 ml disposable column. Solid NaCl was added to the ammonium sulfate fraction (50-75%) to give a final concentration of 0.5 M and applied to the column followed by thorough washing. The cilastatin-bound enzyme was eluted with 10 ml of cilastatin solution (1 mg/ml) and OD280 nm peak fractions were dialyzed individually to remove cilastatin. Each fraction was assayed for RDPase with Gdp as substrate. The fractions of activity peak were pooled and concentrated with ultrafiltration unit using PM30 membrane and referred as 'affinity fraction'.

Analytical procedures

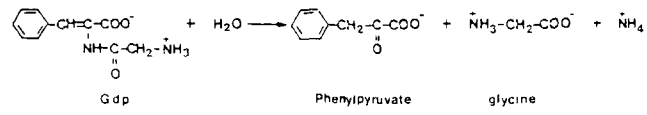

Fig. 1. Chemical reactions of Gdp hydrolysis.

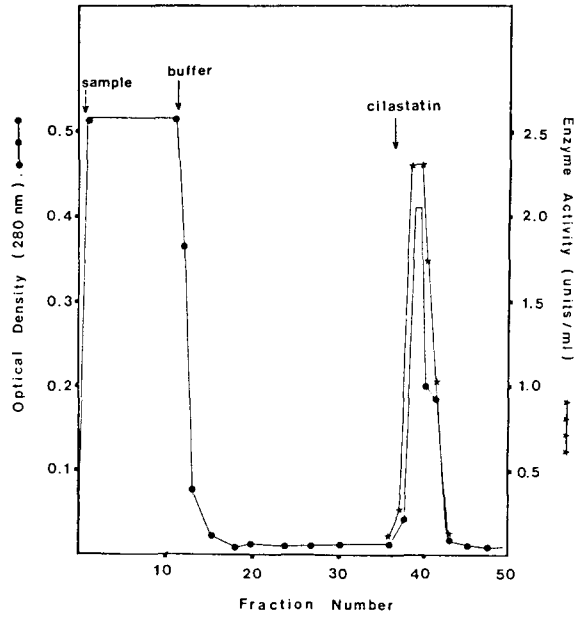

Fig. 2. Affinity chromatographic profile of human RDPase. The ammonium sulfate fraction (50-75% saturation) of human RDPase (10 mg/ml in 2 mM Tris-HCl, pH 7.4 with 0.5 M NaCl) was applied to the 1.5 ml bed volume of cilastatin-coupled affinity chromatographic column at the flow rate of 0.5 ml/min. It was washed with 0.1 M Tris-HCl pH 8 with 0.5 M NaCl until OD_{280} return to stable baseline. Ligand-bound RDPase was eluted with 10 mg of cilastatin dissolved in 10 ml of 0.1 M bicarbonate buffer, pH 8.3 with 0.5 M NaCl. Each fraction of the protein peak was dialyzed individually and assayed for RDPase activity with Gdp as described under MATERIALS AND METHODS.

Activity of RDPase was determined by the rate of Gdp hydrolysis as its chemical reaction shown Fig. 1 (Campbell, 1970). The enzyme activity in 2.5 ml total reaction volume was measured at 275 nm with 5×10^{-5} M Gdp as substrate by observing its fall as function of time (Rene and Campbell, 1969). The molar extinction coefficient of Gdp ($E_{1cm}^{275nm}=1.56\times10^4$) (Campbell *et al.*, 1961) was used for calculation of specific activity which was expressed as μmole of Gdp hydrolyzed/min/mg protein. The protein concentration was measured by the protein dye binding method of Bradford (1976) with BSA as the standard protein. Kinetic measurements of RDPase with Gdp as substrate in 0.5 ml total reaction volumes were performed as described above but using 2 mm light path cuvette.

Native polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Ornstein

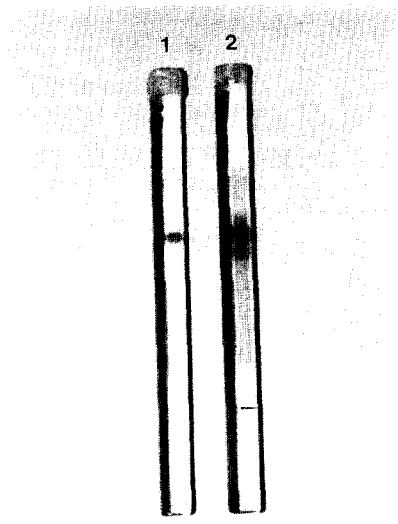

Fig. 3. Protein and Activity staining. The gels were made of 2.5% stacking and 7.5% separating tube gel and electrophoresis was carried out for 35 min with 4 mamp/gel. In gel 1 (protein staining), human RDPase (5 μg) was stained for protein with Coomassie Brilliant Blue R-250 followed by destaining with methanol-acetic acid-H_2O (40 : 7 : 53). In gel 2 (activity staining), the staining reaction was carried out in 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.

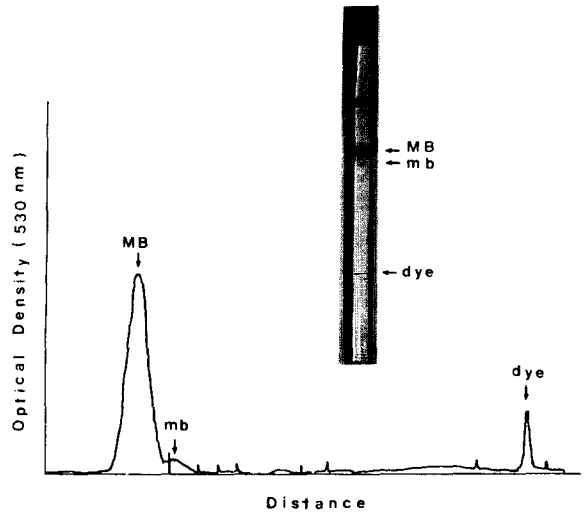

Fig. 4. Chromoscan of Coomassie stained native PAGE gel. The Coomassie stained native PAGE gel (gel 1 of Fig. 3.) was scanned at 530 nm with aperture of 0.3×5 mm. MB, major band; mb, minor band.

(1964). Dipeptidase activity of electrophoresed gel was detected using the method of Sugiura *et al.* (1977).

RESULTS AND DISCUSSION

A kidney was surgically removed from the patient when it was in irreversibly degenerated state and incapable of glomerular filtration. Five such kidneys were obtained and tested prior to purification. Only three kidneys were used for purification because almost no activity was found from two of them. Renal cortex had been used for RDPase purification (Kim and Campbell, 1982; Rene and Campbell, 1969) but whole kidneys were used in this experiment as they were so severely degenerated and clear distinction between cortex and medulla could not be made.

Sometimes RDPase was released from the membrane by phosphatidylinositol-specific phospholipase C (Hooper and Turner, 1989; Littlewood *et al.*, 1989; Adachi *et al.*, 1990) instead of n-butanol. This enzyme digestion took the advantage of RDPase being a glycosyl-phosphatidylinositol-anchored ectoenzyme. In this way, the inositol-containing RDPase was released from the membrane leaving the lipid portion. In contrast, n-butanol solubilization released the complete integral enzyme containing the lipid portion as well. The latter solubilization method was employed in this purification.

The major purification steps employed in this experiment were n-butanol solubilization, ammonium sulfate fractionations and affinity chromatography. The most critical and efficient step was affinity chromatography as shown in Fig. 2. Most of the contaminating proteins could be effectively removed from the column leaving RDPase on the matrix. Affinity chromatography was one of the best choice for protein purification for its specificity as demonstrated by the overlapping protein and the activity peaks. Another advantage of this method was that large volume of sample could be applied to such small column. The pooled fractions between #38 and #42 of Fig. 2 contained 6 mg of 2,029 fold purified RDPase with the specific activity of 99.4 unit/mg protein as summarized in Table 1. The specific activity of the starting homogenate was 0.05 unit/mg. The total activity recovery was 71%. Campbell *et al.* (1984) purified human RDPase from the kidney of a drowned victim [personal communication]. By the same technique, they achieved 1,991 fold purification and obtained affinity fraction with 65.7 unit/mg starting from 0.033 unit/mg of homogenate. Comparison of these purification results of two different source materials suggested that severely degenerated nonfunctional kidneys of renal patients can serve as an excellent source of RDPase. It also indicated that the kidney which failed to perform glomerular filtration does not necessarily mean all other renal functions also impaired. The physiological role of RDPase was suggested as the hydrolysis of L-dipeptides present in the glomerular filtrate, with the resultant free amino acids transported across the brush border microvilli by Na^+ gradient-dependent processes (Welch

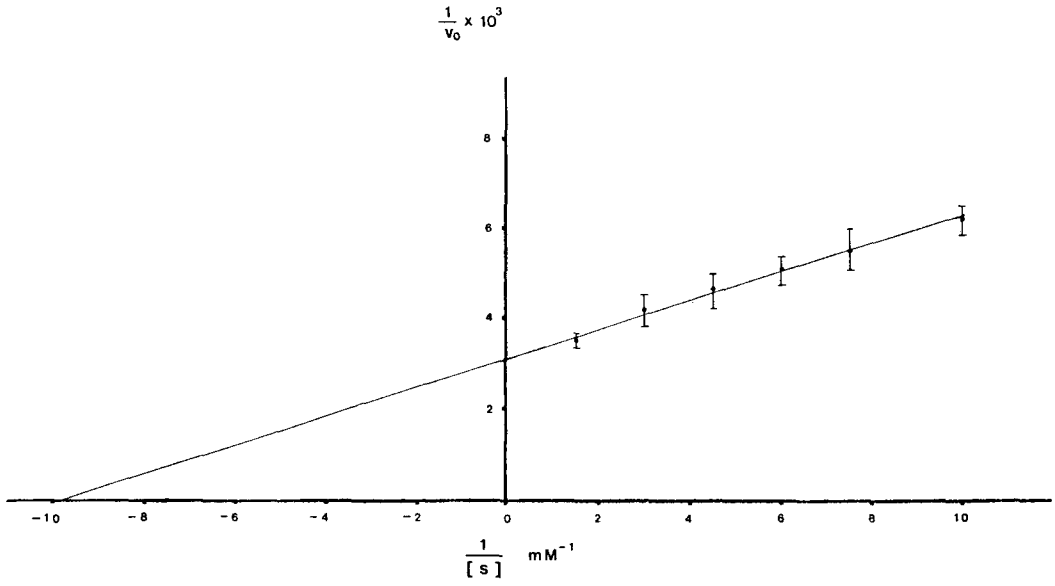

Fig. 5. Lineweaver-Burk plots of RDPase. Catalytic activity of RDPase was measured with Gdp as substrate using 2 mm light path cuvette at 37℃ as described under MATERIALS AND METHODS.

Table I. Purification of human renal dipeptidase

Fraction	Total protein (mg)	Total activity (unit)	Activity recovery (%)	Specific activity (*unit/mg)	Purification factor
Homogenate	17,061	833.45	100.0	0.05	1
Affinity	6	591.33	70.95	99.40	2,029

*unit is defined as μmol Gdp hydrolyzed per min.

and Campbell, 1980). Thus, RDPase may be indirectly involved in salvage of dipeptide amino acids that otherwise would be lost in the urine.

Electrophoresed gels of purified enzyme were subjected to staining for protein and dipeptidase (Fig. 3). Gel 1 of Coomassie staining exhibited two bands with Rfs of 0.26 (major band) and 0.33 (minor band). The minor band was not a contaminant but the RDPase itself as it was shown positive in gel 2 of dipeptidase-specific reaction. The minor band was calculated approximately 4% of the total protein from the chromoscan of the Coomassie stained gel (Fig. 4). The nature of the minor band can not be said more than what is described already at this stage.

The kinetic parameters determined from the double reciprocal plots (Fig. 5) with Gdp as substrate were Vmax, 322.6 μmol/min/mg and Km, 0.102 mM. Apparently, the minor band did not affect the shape of the plots maybe because its contribution in protein concentration have been negligible.

The results of this investigations demonstrated that some of the surgically removed kidney specimens from renal stone patients can be used as an excellent source material for the purification of human RDPase. Nothing can be said from this experiment about at what stage and how the two nonfunctional kidneys in respect of glomerular filtration lost its RDPase activity. However, it provided biochemical evidences that some of the nonfunctional kidneys still retained high activity of renal dipeptidase suggesting its normal physiological functions possible.

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