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PURIFICATION AND PROPERTIES OF HUMAN KIDNEY DIPEPTIDASES *

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Summary

Two dipeptidases from human kidney were purified with L-Leu-L-Leu as a substrate, and polyacrylamide gel (pH 9.4) electrophoresis showed that the peptidase obtained were homogeneous. On the basis of their electrophoretic mobilities the peptidases were classified as peptidases F (fast) and S (slow). The isoelectric point of both enzymes was estimated to be p*I* 4.7. The molecular weights of peptidase F and S by Sephadex G-200 gel filtration were estimated to be 135 000 and 200 000, respectively. From the result of SDS polyacrylamide gel electrophoresis, it was found that peptidase F consisted of two subunits with equal molecular weights of 66 000 and that peptidase S consisted of two subunits with molecular weights of 94 000 and 115 000. Both enzymes were stained with periodic acid-Schiff reagent in polyacrylamide gels, indicating that the enzymes were glycoproteins. Thermal stabilities of the two peptidases were very different. On heat treatment, peptidase F was stable up to 60°C but peptidase S became unstable above 40°C. Substrate specificities of the two peptidases were similar, and the enzymes hydrolyzed dipeptides such as L-Ala-L-Ala, L-Met-L-Met, Gly-L-Leu, L-Leu-Gly, L-Phe-L-Tyr, Gly-Gly and L-Leu-L-Leu to a significant extent.

Introduction

In recent years, a number of reports have dealt with peptidases in human [1,2] and mammalian tissues [3—5]. Campbell et al. [6] reported purification and some properties of dipeptidase from the hog kidney. René [7,8] demonstrated that this peptidase was similar and possibly identical to renal dipeptidase of the pig and human kidney tissue and that the renal dipeptidase was

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found in normal human urine but not in the serum.

In previous report we have demonstrated that the activity of the peptidase using L-Leu-L-Leu as a substrate was markedly elevated in the serum of patients with liver diseases [9]. Furthermore the purification and properties of the peptidase from human liver has also been described [10]. We have already reported that the dipeptidase in human kidney can be distinguished from that in human liver by means of polyacrylamide gel electrophoresis [11]. The dipeptidase was also found in human urine of patients with renal disease. From these results, the enzyme in human urine seems to have leaked from the kidney tissue.

In this paper, we describe the purification and the properties of dipeptidases from human kidney.

Materials and Methods

DEAE-cellulose and CM-cellulose were obtained from Brown Co. (U.S.A.). The following peptides were used in the study: L-Leu-L-Leu, purchased from Fluka AG (Switzerland); L-Phe-L-Tyr and L-Leu-Gly-Gly from Mils-Yeda Ltd. (U.S.A.); Gly-Gly, Gly-L-Leu, Gly-L-Ser, Gly-L-His, L-Leu-Gly, L-Leu-NH_2, L-Ala-L-Ala, L-Met-L-Met, L-Phe-L-Pro, L-Pro-L-Phe. L-His-L-Leu, Gly-Gly-Gly, L-Leu-L-Leu-L-Leu, L-Ala-Gly-Gly, benzoyl-Gly-Gly (Bz-Gly-Gly), benzoyl-L-Leu-Gly (Bz-L-Leu-Gly), α-*N*-benzoyl-L-Arg, benzyloxycarbonyl-Gly-Gly (CBz-Gly-Gly), Hippuryl-L-Phe, L-Leu-β-naphthylamide (L-Leu-β-Nap), L-Cys-di-β-naphthylamide (L-Cis-di-β-Nap) from Sigma Chemical Co. (U.S.A.). Peroxidase (Sigma Type II, 195 units/mg) was obtained from Sigma Chemical Co. (U.S.A.).

L-Amino acid oxidase was purified from venom of *Agkistrodon caliginosus* by the method described previously [9].

The crude enzyme of dipeptidase was prepared as follows. Frozen human kidney (100 g) was homogenized with 300 ml of water and 100 ml of *n*-butanol (kidney/water/*n*-butanol, 1 : 3 : 1) and centrifuged at 10 000 × *g* for 10 min. The supernatant was treated with 60% acetone at 0°C to precipitate the protein. Distilled water was added to the precipitate, and the soluble fraction was used as the crude enzyme.

Peptidase activity using L-Leu-L-Leu as a substrate was measured in same manner as described previously [9]. The incubation mixture contained 4 μmol of L-Leu-L-Leu in 1 ml of 50 mM Tris · HCl buffer (pH 8.0), 20 μl of L-amino acid oxidase (20 units/ml), 2 ml of color reagent in which 8 mg of 4-aminoantipyrine, 20 μl of *N*,*N*-dimethylaniline and 300 units of peroxidase were dissolved in 100 ml of 50 mM Tris · HCl buffer (pH 8.0), and 0.1 ml of the enzyme solution. The reaction was carried out at 37°C for 20 min and stopped by addition of 1 ml of 0.1 M acetic acid. Absorbance was measured at 550 nm. A unit of dipeptidase activity is defined as 1 μmol of L-Leu-L-Leu hydrolyzed per min at 37°C.

The substrate specificity of the peptidase was assayed according to the ninhydrin-peptidase method of Campbell et al. [6]. Protease activity was determined by the method of Matsubara et al. [12].

Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

Disc electrophoresis in polyacrylamide gel (7.5% gel, pH 9.4) was carried out according to the procedure of Davis [14]. After electrophoresis, the gel was added to 12.5% trichloroacetic acid to fix the protein and then stained with 1% Amido Black 10B or periodic acid-Schiff reagent [15]. The staining of enzyme activity was also performed using the formazan system described previously [10].

SDS disc electrophoresis in polyacrylamide gel (5%) was performed by the method of Weber and Osborn [16].

Results

Purification of dipeptidases from human kidney

The crude enzyme was fractionated with ammonium sulfate. The precipitate between 40—60% saturation of $(NH_4)_2SO_4$ was collected and dialyzed against 15 l of 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$ for 20 h at 4°C.

The enzyme was applied to a DEAE-cellulose column (3 × 20 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$. After washing the column with 500 ml of the same buffer, the peptidase was eluted with a linear concentration gradient from 0 to 0.3 M NaCl in 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$. Fig. 1 shows that the peptidase activity is in one peak.

The active fraction was concentrated through a membrane filter (Diafilter MC-2 Type, Membrane: G-10T, Nihonshinkugijutsu Co. Ltd., Japan) and the concentrated fraction was adjusted to 10 ml by 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$ and 0.1 M NaCl. The preparation was applied to a Sephadex G-150 column (3.5 × 95 cm) equilibrated with the above buffer. In

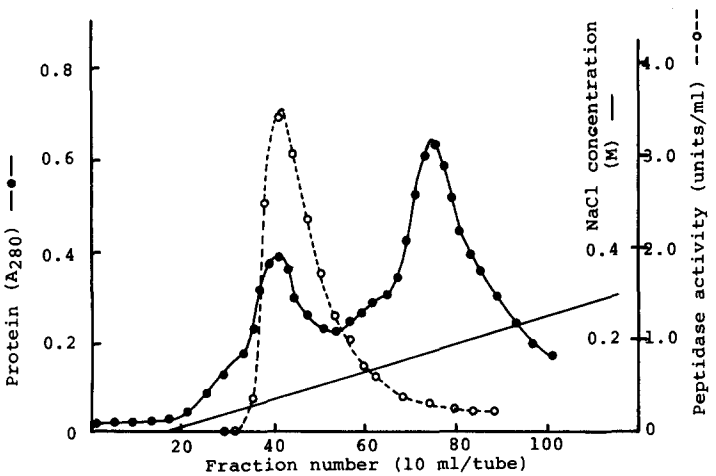

Fig. 1. Purification of dipeptidase from human kidney by DEAE-cellulose chromatography. The crude enzyme was applied to a DEAE-cellulose column (3.0 × 20 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$. —•—, protein (A_{280}); ---o---, peptidase activity; ———, NaCl concentration.

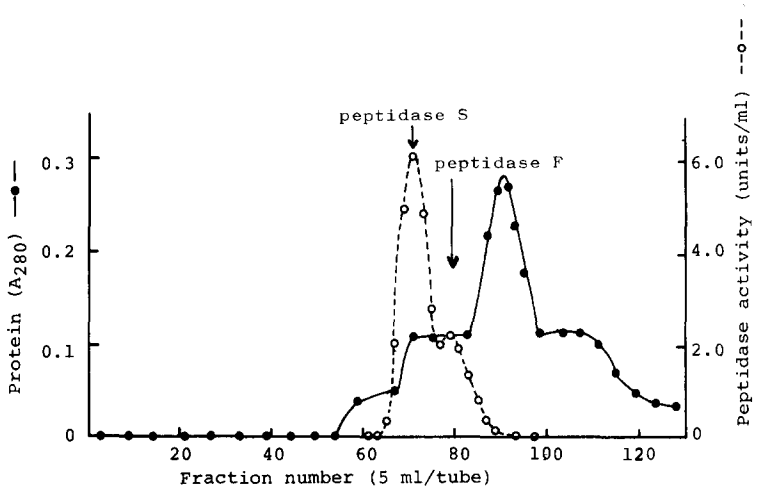

Fig. 2. Purification of dipeptidases from huamn kidney by Sephadex G-150 gel filtration. DEAE-cellulose fraction was applied to a Sephadex G-150 column (3.5 × 95 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$ and 0.1 M NaCl. —●—, protein (A_{280}); - - -o- - -, peptidase activity.

Fig. 2 the peptidase activity was observed at two different fraction numbers. The early active fraction eluted from a Sephadex G-150 column was hereafter referred to as peptidase S, and the late active fraction as peptidase F. These active fractions were collected separately, and the buffer was exchanged with 10 mM acetate buffer (pH 6.0) containing 10^{-5} M $MnCl_2$.

Each peptidase fraction was passed through a CM-cellulose column (2 × 15 cm) equilibrated with 10 mM acetate buffer (pH 6.0) containing 10^{-5} M $MnCl_2$,

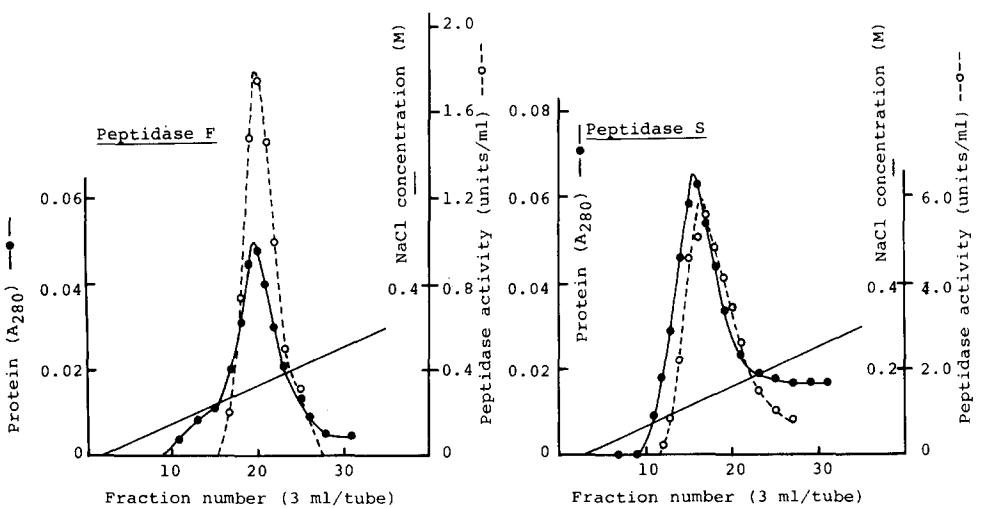

Fig. 3. Purification of peptidase F and S from human kidney by DEAE-Sephadex A-50 chromatography. CM-cellulose fraction was applied to a DEAE-Sephadex A-50 column (0.9 × 8 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$. —●—, protein (A_{280}); - - -o- - -, peptidase activity; ———, NaCl concentration.

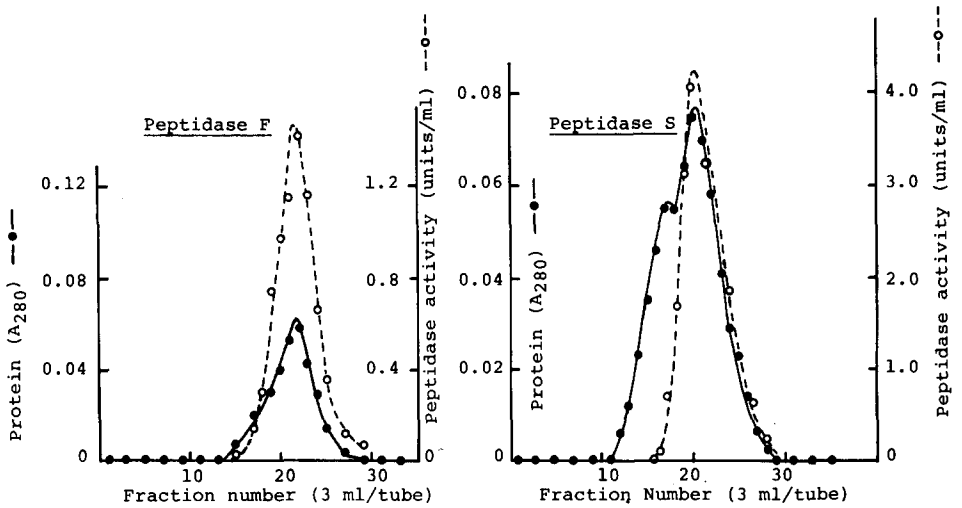

Fig. 4. Purification of peptidase F and S from human kidney by Sephadex G-150 rechromatography. DEAE-Sephadex A-50 fraction was applied to a Sephadex G-150 column (2 × 40 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$ and 0.1 M NaCl. —•—, protein (A_{280}); ---o---, peptidase activity.

and the effluent was collected and the buffer was exchanged with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$.

Each active fraction was applied to a DEAE-Sephadex A-50 column (0.9 × 8 cm) equilibrated with 10 mM Tris · HCl (pH 7.5) containing 10^{-5} M $MnCl_2$. After the column was washed with 20 ml of the same buffer, the peptidase was eluted with a linear concentration gradient from 0 to 0.3 M NaCl in 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$. The elution pattern is shown in Fig. 3.

Each fraction purified by DEAE-Sephadex A-50 column chromatography was applied to a Sephadex G-150 column (2 × 40 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.5) containing 10^{-5} M $MnCl_2$ and 0.1 M NaCl. The elution pattern is shown in Fig. 4.

Each active fraction was collected and used as a purified peptidase preparation. The above-mentioned purifying procedure is summarized in Table I. The purity of final preparations was about 100-fold with a recovery of 2.4% for peptidase F and about 110-fold with a recovery of 8.5% for peptidase S.

To prove homogeneity of the purified enzymes, the preparations were subjected to disc electrophoresis (7.5% polyacrylamide gel, pH 9.4). Each enzyme gave a single band of protein, carbohydrate and activity in the gels as shown in Fig. 5. Since both enzymes were stained with periodic acid-Schiff reagent in polyacrylamide gels, the enzymes are considered to be glycoprotein.

Optimum pH and pH stability

The optimum of the two dipeptidases was assayed in the following procedure. 0.1 ml of the enzyme solution was incubated with 4 μmol of L-Leu-L-Leu dissolved in 1 ml of 10 mM buffer (acetate buffer, pH 4.0—6.0; phosphate buff-

TABLE I

PURIFICATION OF PEPTIDASE FROM HUMAN KIDNEY

Step of purification	Activity (units) *	Protein ($A_{280\ nm}$)	Specific activity **	Yield of activity (%)
Crude enzyme	793	1550	0.512	100
Precipitation with $(NH_4)_2SO_4$ (40—60% satn.)	412	368	1.12	52.0
DEAE-cellulose	321	43.4	7.40	40.5
Sephadex G-150				
Peptidase F	69.3	4.15	16.7	8.74
Peptidase S	187	4.25	49.9	23.5
Peptidase F				
CM-cellulose	52.2	1.74	30.0	6.58
DEAE-Sephadex A-50	25.2	0.642	39.2	3.18
Sephadex G-150 rechromatography	18.6	0.375	49.6	2.35
Peptidase S				
CM-cellulose	93.8	2.11	44.5	11.8
DEAE-Sephadex A-50	75.2	1.50	50.1	9.48
Sephadex G-150 rechromatography	67.2	1.16	57.9	8.47

* L-Leu-L-Leu hydrolyzed μmol per min.

** L-Leu-L-Leu hydrolyzed μmol per min per 280 nm absorbance unit.

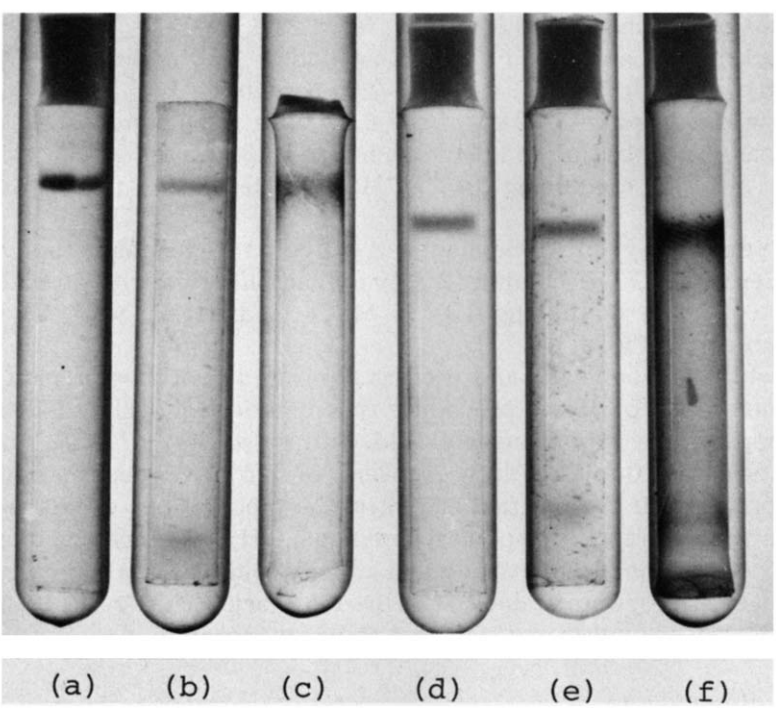

Fig. 5. Disc electrophoresis of peptidase F and S from human kidney. Conditions: pH 9.4, 3 mA/tube, 90 min, 4°C. Gels (a) and (d) were stained with Amido Black 10B, gels (b) and (e) with periodic acid-Schiff reagent, and gels (c) and (f) for activity with L-Leu-L-Leu as a substrate.

er, pH 6.0—7.5; Tris · HCl buffer, pH 7.5—9.0; carbonate buffer, pH 9.0—11.0; phosphate/NaOH buffer, pH 11.0—12.0) at 37°C for 15 min. The incubation was stopped by heating the reaction mixture for 5 min in a boiling water bath. The released L-leucine was determined by the standard method. The optimum pH values of peptidase F and S were found to be pH 9.0 and pH 9.0—11.0, respectively. Peptidase S showed a wider range of the optimum pH. Examination of pH stabilities of the two dipeptidases was performed as follows. Each enzyme was incubated at various pH's (using the same buffer mentioned above) at 37°C for 30 min, and then the remaining activity was determined. Both enzymes were stable in a pH range of pH 8.0—11.0, but extremely unstable below pH 6.0.

Thermal stability and optimum temperature

The two dipeptidase were treated in 10 mM Tris · HCl buffer (pH 8.0) for 30 min at various temperatures, and the remaining activity was determined. Peptidase F was stable up to 60°C, but the activity of peptidase S decreased above 40°C. From this result, it was found that peptidase F was more stable than peptidase S to heat treatment. Examination of optimum temperature of the two dipetidases was carried out as follows. 0.1 ml of the enzyme solution was incubated with 4 μmol of L-Leu-L-Leu dissolved in 1 ml of 50 mM Tris · HCl buffer (pH 8.0) at the temperature indicated (30—80°C). The incubation was stopped by heating for 5 min in a boiling water bath. The released L-leucine was determined by the standard method. The optimum temperatures of peptidase F and S were found to be 70°C and 40—45°C, respectively.

Molecular weight and subunits

The molecular weights of petidases F and S were estimated to be 135 000 and 200 000, respectively, by Sephadex G-200 gel filtration.

The molecular weights of the subunits of the two enzymes were determined by SDS polyacrylamide gel electrophoresis. The molecular weights of the subunits were estimated to be 94 000 and 115 000 for peptidase S, and 66 000 for peptidase F. It was found that each enzyme was a dimer consisting of two subunits.

K_m value

The K_m values of peptidase F and S were determined by the standard assay using various concentrations of L-Leu-L-Leu and estimated to be 0.056 and 0.086 mM, respectively.

Isoelectric point

The isoelectric points of both peptidases were the same in their values (p*I* 4.7) determined by isoelectric focusing with 1% ampholyte (pH 3.0—6.0).

Effect of metal ions and reagents on peptidase F and S

The effects of metal ions and reagents on both enzymes were similar, as shown in Table II. The two enzymes were activated by 10^{-3} M Cd^{2+} and inhibited by 10^{-3} M Co^{2+}. Other metal ions tested did not affect the activities of the enzymes. Chelators such as *o*-phenanthroline, EDTA and cysteine, oxidising

TABLE II

EFFECT OF VARIOUS METAL IONS AND REAGENTS ON ACTIVITY OF HUMAN KIDNEY PEPTIDASES

The enzyme was incubated with the metal ions and reagents in 10 mM Tris · HCl buffer (pH 8.0) at 37°C for 30 min. 1 mM metal ions and reagents were used in the enzyme system. After the incubation mixture had been diluted 1000-fold with 10 mM Tris · HCl buffer (pH 8.0), the remaining activity was assayed. F, peptidase F; S, peptidase S.

Metal ion	Remaining activity (%)		Reagent	Remaining activity (%)	
	F	S		F	S
None	100	100			
NaCl	87	92	*o*-Phenanthroline	32	25
$CaCl_2$	85	95	EDTA	76	76
$MgCl_2$	86	99	Diisopropyl fluorophosphate	103	100
$ZnCl_2$	94	83	Monoiodoacetate	102	102
$CdCl_2$	139	121	*p*-Chloromercuribenzoate	128	121
$CuCl_2$	69	78	*N*-Bromosuccinimide	29	19
$NiCl_2$	83	87	Iodine	25	19
$MnCl_2$	83	108	Cysteine	23	15
$CoCl_2$	55	62	2-Mercaptoethanol	101	76
$FeCl_2$	76	77			
$HgCl_2$	80	72			

agents such as *N*-bromosuccinimide and iodine significantly inhibited the activities of both enzymes.

Specificities of peptidase F and S

Specificity for substrate was investigated and the result is shown in Table III. The specificities of the two enzymes for peptide substrate gave similar results. The two enzymes hydrolyzed dipeptides such as L-Ala-L-Ala, L-Met-L-Met, Gly-L-Leu, L-Leu-Gly, L-Phe-L-Tyr, Gly-Gly and L-Leu-L-Leu to a great extent, but the two peptidases did not hydrolyze tripetides except that pep-

TABLE III

SUBSTRATE SPECIFICITIES OF PEPTIDASE F AND S FROM HUMAN KIDNEY

The values were calculated as percentage of L-Leu-L-Leu hydrolysis. F, peptidase F; S, peptidase S.

Substrate	Relative activity (%)		Substrate	Relative activity (%)	
	F	S		F	S
L-Leu-L-Leu	100	100	L-Met-L-Met	311	223
L-Leu-Gly	134	132	L-Phe-L-Tyr	142	123
L-Leu-NH_2	0	0	L-His-L-Leu	20	0
L-Leu-L-Leu-L-Leu	0	58	L-Phe-L-Pro	0	0
L-Leu-Gly-Gly	0	0	L-Pro-L-Phe	0	21
Gly-L-Leu	271	153	Bz-Gly-Gly	0	0
Gly-Gly	123	136	Bz-L-Leu-Gly	0	0
Gly-L-Ser	86	50	α-*N*-Benzoyl-L-Arg	0	0
Gly-L-His	79	51	Hippuryl-L-Phe	0	0
Gly-Gly-Gly	0	0	CBz-Gly-Gly	0	0
L-Ala-L-Ala	448	332	Casein	0	0
L-Ala-Gly-Gly	0	0			

tidase S hydrolyzed L-Leu-L-Leu-L-Leu to a medium extent. From these results, the two enzymes are considered to be dipeptidase; no trace for aminopeptidase, carboxypeptidase or endopeptidase is found.

Discussion

The two dipeptidases which hydrolyze L-Leu-L-Leu are present exclusively in the cytosol fraction from human kidney. Purification of the enzymes in the cytosol fraction was performed successfully when the kidney was treated with *n*-butanol. It seemed that the treatment with *n*-butanol was necessary for separating the enzyme protein from the lipid material and for solubilization of the enzymes.

Kim et al. [17,18] reported the purification and some properties of two peptidases from the rat intestinal brush border membrane. These authors found that the sugar contents of the enzymes were different and that the amino acid compositions and the molecular weights of both enzymes were very similar. Moreover, the enzymes were immmunologically identical. By contrast, the present study showed that the isoelectric points of the two peptidases from human kidney were similar and that the molecular weights of the enzyme determined by Sephadex G-200 gel filtration and SDS disc electrophoresis were very different. The two peptidases from human kidney, therefore, may be different enzyme proteins.

Kim et al. [19] reported that the peptide hydrolases in the cytosol fraction from small intestinal mucosa of rat and man showed a marked difference in the thermal stability with L-Ala-Gly and L-Ala-Gly-Gly as substrates. It was interesting to note that there was a significant difference in the thermal stabilities between the two dipeptidases from human kidney: peptidase F was stable up to 60°C and peptidase S became unstable above 40°C.

The peptidases purified from human kidney were stained with periodic acid-Schiff reagent in polyacrylamide gel, but the peptidase from human liver [10] was not. These observations indicate that the two peptidases from human kidney are glycoprotein and that the kidney peptidases are different for enzyme protein from the liver peptidase.

A number of peptidases are known to be metalloenzymes containing zinc [20,21]. Since peptidase F and S from human kidney were activated by Cd^{2+} and inhibited by chelators such as *o*-phenanthroline, EDTA and cysteine, it is possible that the enzymes are metalloenzymes.

Although the two peptidases from human kidney effectively hydrolyzed dipeptides such as L-Ala-L-Ala, L-Met-L-Met, Gly-L-Leu, L-Leu-Gly, L-Phe-L-Tyr, Gly-Gly and L-Leu-L-Leu, these enzymes did not hydrolyze tripeptides except that peptidase S hydrolyzed L-Leu-L-Leu-L-Leu. The absence of the peptidase activity against CBz-peptide or Bz-peptides indicates that a free amino group is required for the enzymic catalysis. Peptidase F and S did not hydrolyze L-Leu-NH_2 or L-Leu-β-naphthylamide which was used as substrate of leucine aminopeptidase. From these observations it is suggested that the carboxyl group of the C-terminal amino acid of peptides has to be in a close proximity to the required amino group for the hydrolysis to occur. These results led us to a conclusion that the two enzymes were dipeptidases which possessed a consid-

erable range of substrate specificity. As far as the substrate specificity is concerned, the two dipeptidases purified by us were very different from the microsomal dipeptidase which was partially purified from the human kidney by René [7].

In order to clarify the relationship between the peptidases in human urine and kidney, further enzymological and immunological studies are in progress.

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