[Chem. Pharm. Bull. 34(3)1176—1183(1986)]

Differential Determination of Human Liver and Pancreas Dipeptidase Activities by Using Specific Antibody-Conjugated Paper Disks

YOSHIMASA ITO,*,[a] MASAHIRO IWAKI,[a] TARO OGISO,[a] KAZUYUKI HIRANO,[b] MAMORU SUGIURA,[b] SHUNJI SAWAKI,[c] and KATSUHITO SUGIURA[d]

Faculty of Pharmaceutical Sciences, Kinki University,[a] 4–1, Kowakae 3-chome, Higashi-Osaka 577, Japan, Gifu Pharmaceutical University,[b] 6–1, Mitahora-higashi 5-chome, Gifu 502, Japan, First Department of Internal Medicine, Aichi Medical University,[c] Nagakute-chou, Aichi-gun, Aichi 480–11, Japan, and Fujita Gakuen Health University Hospital,[d] Kutsukake-chou, Toyoake 470–11, Japan

(Received August 8, 1985)

A new method was developed for the determination of liver and pancreas dipeptidase activities in serum by using specific antibody-conjugated paper disks. The immunoreaction between the antibody-conjugated paper disks and the dipeptidases reached a plateau within 7 h at 4 °C, and no dipeptidase activity was detected in the reaction medium thereafter. The calibration curves obtained by the proposed method passed through the origin and were linear in the ranges of 0—200 ng of liver dipeptidase and 0—37.5 ng of pancreas dipeptidase. The bound enzymes from human liver and pancreas had higher K_m values and lower V_{max} values toward L-Leu–L-Leu than those of the corresponding free enzymes. However, with L-Ala–L-Ala as a substrate, the K_m and V_{max} values of the pancreas dipeptidase bound on the paper disk and the free enzyme were almost the same. The recoveries of added liver and pancreas dipeptidases from serum were more than 96%. The proposed method showed a good precision. The normal value of liver dipeptidase activity in serum was 1.62 ± 0.40 I.U./l of serum (mean $\pm$ S.D.). The activities were elevated in acute hepatitis (18.7 ± 18.2 I.U./l, $p < 0.001$ *vs.* normal) and liver cancer (12.3 ± 12.9 I.U./l, $p < 0.001$). The normal value of pancreas dipeptidase activity in serum was 0.74 ± 0.99 I.U./l of serum. The activities were remarkably elevated in pancreatic cancer (5.22 ± 2.68 I.U./l, $p < 0.001$) and acute pancreatitis (4.82 ± 2.84 I.U./l, $p < 0.001$). These results suggest that this method may be useful in the clinical diagnosis of hepatic and pancreatic diseases.

Keywords——differential determination of dipeptidase; dipeptidase; immunoassay; hepatic disease; pancreatic disease; antibody-conjugated paper disk

A number of serum enzymes have been utilized for clinical diagnosis, especially enzymes that originate from tissues affected by diseases. There have been several reports on the multiple forms of some enzymes originating from various tissues.[1–3] Therefore, it is sometimes difficult to assess the significance of the levels of enzyme activity in serum. However, immunological methods are advantageous for measuring the serum concentrations of specific antigens.[4,5]

In previous reports we demonstrated that the activity of the dipeptidase toward L-Leu–L-Leu as a substrate was elevated markedly in the sera of patients with hepatic and pancreatic diseases.[6] Subsequently, we purified the dipeptidases from human liver and pancreas, and clarified some of their enzymological and immunological properties.[7,8] The results suggested that the dipeptidases from human liver and pancreas were distinguishable immunologically.

In this paper, we describe a convenient method of dipeptidase measurement using specific antibody-conjugated paper disks, and we present the results of assay of serum dipeptidase

activities in patients with various diseases by this method.

Experimental

Materials——One-year-old New Zealand white rabbits were used for immunization. Complete Freund's adjuvant was purchased from Difco Co. (U.S.A.). L-Leu–L-Leu was purchased from Fluka AG Co. (Switzerland), and L-Ala–L-Ala from Sigma Chemical Co. (U.S.A.). Filter paper (No. 51A) for the preparation of the paper disks was purchased from Toyo Roshi Co., Ltd. (Japan). Peroxidase (Sigma type II, 195 units/mg) was obtained from Sigma Chemical Co. (U.S.A.). L-Amino acid oxidase was purified from the venom of *Agkistrodon caliginosus* by the method described previously.[9] Human liver and pancreas dipeptidases were purified by the procedures described previously.[7,8] The preparations were proven to be homogeneous on disc and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The specific activities of liver and pancreas enzymes against L-Leu–L-Leu were 45.2 and 63.2 units/mg, respectively. That of the pancreas enzyme against L-Ala–L-Ala was 267 units/mg. These enzyme preparations were used as antigens for immunization and as standards for the proposed method.

Preparation of Antibodies to Human Liver and Pancreas Dipeptidases——Antibody to each dipeptidase was obtained by immunization of a rabbit with 1 mg of enzyme protein for the first injection followed by three further injections of 0.5 mg at two- or three-week intervals. The enzyme was emulsified with complete Freund's adjuvant and administered subcutaneously. The crude immunoglobulin G (IgG) fraction was precipitated with 33% saturated ammonium sulfate, followed by passage through a column of DE-52.[10] The preparation obtained by the above procedures was further purified by proteinA- Sepharose column chromatography. Each antibody (500 mg) was applied to a protein A-Sepharose column (1.5 × 5.0 cm) equilibrated with 20 mM potassium phosphate buffer (pH 8.0). The column was washed with 50 ml of the same buffer and eluted with 1 M acetic acid. The purified IgG's were used as antibodies to liver and pancreas dipeptidases.

Preparation of Antibody-Conjugated Paper Disk——Antibodies to liver and pancreas dipeptidases were conjugated to paper disks according to the method of Ceska and Lundkrist.[11] Paper disks with a diameter of 6 mm were punched out from the above-mentioned filter paper. The paper disks were soaked with distilled water. They were then mixed with 80 ml of CNBr solution (2.7 g of CNBr in 80 ml of distilled water). The pH was brought to 10.5 with 1 M NaOH for 30 min. The liquid was then aspirated and the disks were washed with 100 ml of 5 mM $NaHCO_3$. This washing procedure was repeated 12 times. These disks were then washed twice with 100 ml of distilled water. A solution of 10 mg of antibody in 100 ml of 0.1 M $NaHCO_3$ was mixed with 2 g of the CNBr-activated paper disks, with stirring at 4 °C for 3 h. The disks were then washed with 200 ml of 0.1 M $NaHCO_3$. The whole washing procedure was performed at room temperature. The remaining reactive groups on the paper disks were blocked with 40 ml of 50 mM ethanolamine in 0.1 M $NaHCO_3$ for 3 h at room temperature. The disks were again washed twice with 100 ml of 0.5 M $NaHCO_3$ and thereafter with 0.1 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl. This latter washing procedure was repeated once more with an incubation period of 30 min. The disks were then washed twice with 100 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl, 0.3% bovine serum albumin, 0.6% Triton X-405 and 0.05% sodium azide (buffer P).

Standard Immunoassay——A piece of antibody-conjugated paper disk was incubated in a glass test tube (12 × 75 mm) with 50 μl of serum and 100 μl of buffer P. The reaction mixture was aspirated and discarded after incubation with shaking overnight at 4 °C. Then each disk was washed twice with 2.0 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl. For blank analysis, the antibody-conjugated paper disk was replaced with a normal rabbit IgG-conjugated one. Then the dipeptidase activities on the paper disks were determined by the methods described below.

Assay for Dipeptidase Activity toward L-Leu–L-Leu as a Substrate[9]: The incubation mixture contained 0.25 ml of 50 mM Tris–HCl buffer (pH 8.0) together with 4 μmol of L-Leu–L-Leu, 0.5 ml of color reagent containing 8 mg of 4-aminoantipyrine, 300 units of peroxidase and 20 μl of *N*,*N*-dimethylaniline in 100 ml of 50 mM Tris–HCl buffer (pH 8.0), 5 μl of L-amino acid oxidase (20 units/ml), and one paper disk as mentioned above. After incubation for 30 min at 37 °C, the reaction was stopped by addition of 0.25 ml of 0.1 M acetic acid. The absorbance was measured at 550 nm by the use of a Shimadzu CL-720 micro-flow spectrophotometer. The dipeptidase activity was presented as I.U./l of serum.

Assay for Dipeptidase Activity toward L-Ala–L-Ala as a Substrate[12]: The incubation mixture contained 0.1 ml of 50 mM sodium carbonate buffer (pH 9.0) together with 4.5 μmol of L-Ala–L-Ala, 0.2 ml of color reagent, which contained 20 mg of β-NAD^+, 0.5 mg of resazurin sodium salt, 6 units of diaphorase and 40 units of L-alanine dehydrogenase in the same buffer, and one paper disk as mentioned above. After incubation for 15 min at 37 °C, the reaction was stopped by addition of 2.5 ml of 50 mM NaOH. The fluorescence was measured at 568 nm for excitation and 589 nm for emission. The dipeptidase activity was presented as I.U./l of serum.

Other Determinations——Alanine and aspartate aminotransferase were determined by the method of Karmen,[13] leucine aminopeptidase by the method of Szasz,[14] and amylase by the method of Caraway.[15]

Results

Time Course of Immunoreaction on Specific Antibody-Conjugated Paper Disk

The time required for incubation of the antibody-conjugated paper disk with purified dipeptidases at 4 °C was established. The immunoreaction essentially reached a plateau within 7 h, shown in Fig. 1. No enzyme activity was detected in the reaction medium after the immunoreaction had reached the plateau. We decided that the standard incubation time for immunoreaction should be overnight or 8 h at 4 °C.

Kinetic Parameters of Dipeptidases Bound on Antibody-Conjugated Paper Disk

Figure 2 shows the Lineweaver–Burk plots for the dipeptidases bound on the paper disks and for the free enzymes using L-Leu–L-Leu and L-Ala–L-Ala as peptide substrates. Toward L-

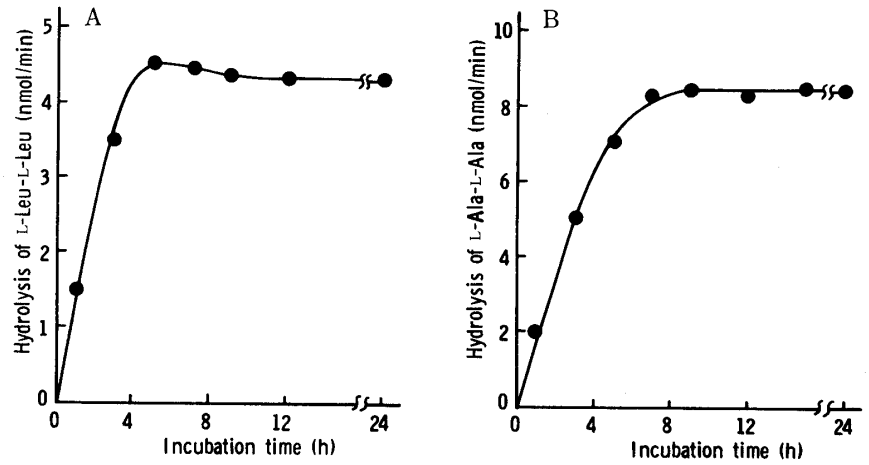

Fig. 1. Effect of Incubation Time on Immunoreaction

Human liver (160 ng, A) or pancreas dipeptidase (37.5 ng, B) was incubated at 4 °C for the indicated times with the corresponding antibody-conjugated paper disk. The other procedures were performed as described in Experimental.

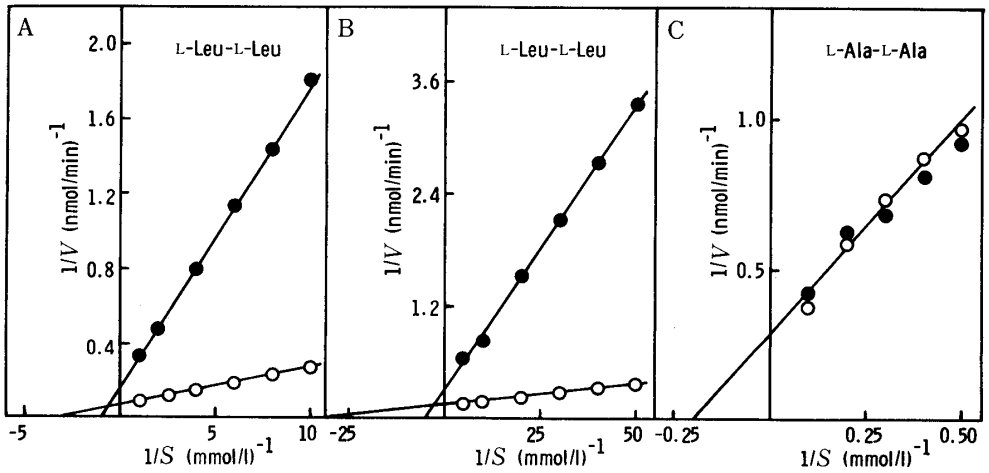

Fig. 2. Lineweaver–Burk Plots of Activities of Dipeptidases Bound on Antibody-Conjugated Paper Disks and Free Enzymes toward L-Leu–L-Leu and L-Ala–L-Ala as Substrates

A: K_m values of free (○) and bound liver enzymes (●) toward L-Leu–L-Leu were calculated to be 0.293 and 1.00 mM, respectively. B: K_m values of free (○) and bound pancreas enzymes (●) toward L-Leu–L-Leu were 0.033 and 0.21 mM, respectively. C: K_m values of both free (○) and bound pancreas enzymes (●) toward L-Ala–L-Ala were 5.18 mM.

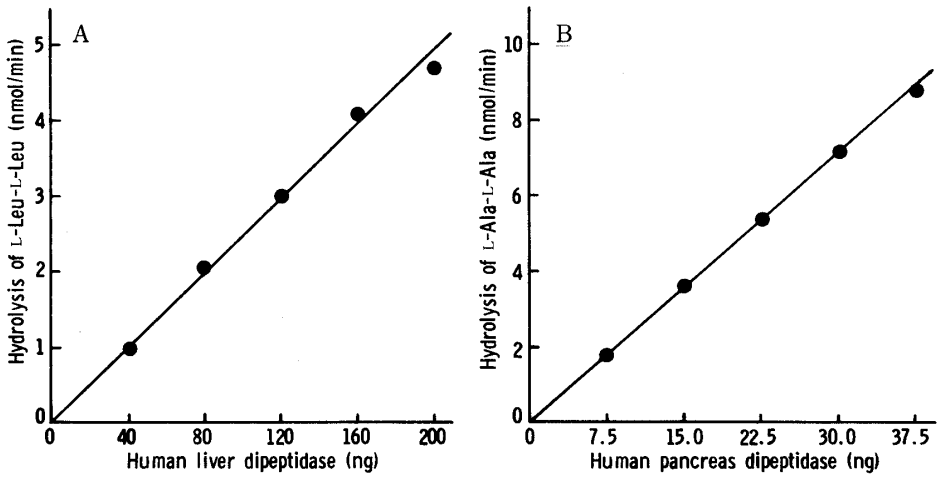

Fig. 3. Calibration Curves of Human Liver and Pancreas Dipeptidases Obtained by the Proposed Method

A, liver dipeptidase; B, pancreas dipeptidase.

Leu–L-Leu, the bound enzymes from human liver and pancreas had higher K_m values and lower V_{max} values than those of the corresponding free enzymes. However, toward L-Ala–L-Ala, the K_m and V_{max} values of the pancreas dipeptidase bound on the paper disk were almost the same as those of the free enzyme. We decided that L-Ala–L-Ala should be used for the assay of the pancreas dipeptidase activity and L-Leu–L-Leu for that of liver dipeptidase activity, because the liver enzyme can not hydrolyze L-Ala–L-Ala.[7]

Calibration Curve of Dipeptidases from Human Liver and Pancreas

Figure 3 shows the relationship between the protein contents of the two dipeptidases bound on the antibody-conjugated paper disks and the catalysis rates. The plots of the hydrolytic activity toward L-Leu–L-Leu *vs.* the amount of the liver dipeptidase (0—200 ng) were regressed to a straight line passing through the origin. The calibration curve of the pancreatic enzyme was also drawn, and was linear in the range of 0—37.5 ng, passing through the origin. The paper disks conjugated with antibodies to liver and pancreas dipeptidases could maximally bind about 500 ng of liver enzyme and about 200 ng of pancreas enzyme per one paper disk, and these enzyme activities could be determined successfully by the standard method described in Experimental except that the incubation time was reduced to one-half to one tenth.

Recovery and Precision of the Proposed Method

Dipeptidases purified from human liver and pancreas were added to a series of 10 sera chosen to cover a wide range of dipeptidase activities. The recoveries are given in Table I. The recoveries calculated from the calibration curves shown in Fig. 3 were 96—101% for liver dipeptidase, though those with respect to the free enzyme activity added were about 40%. The average recoveries of pancreas dipeptidase were 96—105%.

The precision of the assay was studied within a day and between days using sera samples. The within-day coefficients of variation (C.V.) were 0.85—3.8% for liver dipeptidase ($n=10$) and 2.2—5.1% for pancreas dipeptidase ($n=10$). The between-days precision (every day for 10 d) showed a C.V. of 2.1—5.7% for liver enzyme and 2.0—7.7% for pancreas enzyme.

Liver and Pancreas Dipeptidase Activities in Sera of Normal Subjects and Patients with Various Diseases

Figure 4 shows liver dipeptidase activities in sera of normal subjects and patients with

TABLE I. Analytical Recovery of Dipeptidase Activity Added to Serum

Enzyme	No. of serum	Dipeptidase activity (mI.U.)		Recovery (%)	
		Added	Recovered	A	B
Liver dipeptidase	10	2.0	0.77±0.03	38.5±1.5	96.3± 3.8
	10	5.0	1.96±0.11	39.2±2.2	98.0± 5.5
	10	10.0	4.02±0.41	40.2±4.1	101.0±10.3
Pancreas dipeptidase	10	0.5	0.48±0.01	96.0±2.0	98.0± 2.0
	10	2.0	2.03±0.10	102.0±5.0	105.0± 5.2
	10	5.0	4.97±0.17	99.4±3.4	100.0± 3.4

Recovery A was calculated as percentages of the free enzyme activity added and recovery B was calculated by using the calibration curves shown in Fig. 3.

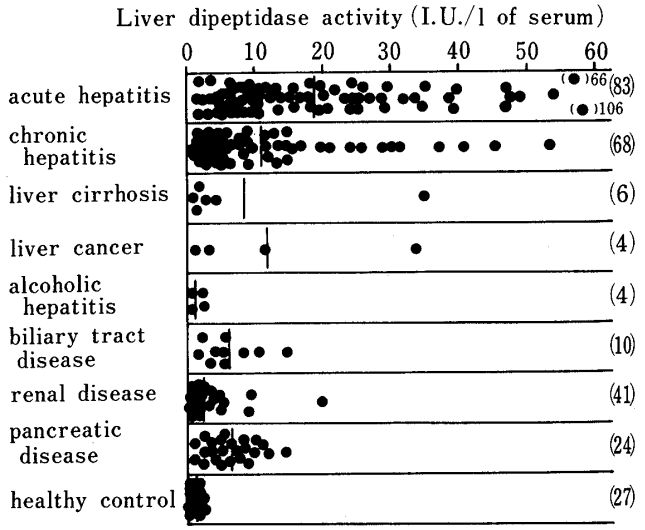

Fig. 4. Liver Dipeptidase Activities in Sera of Normal Subjects and Patients with Various Diseases as Determined by the Proposed Method

Bars indicate the mean value of serum dipeptidase activity. Figures in parentheses show the number of patients.

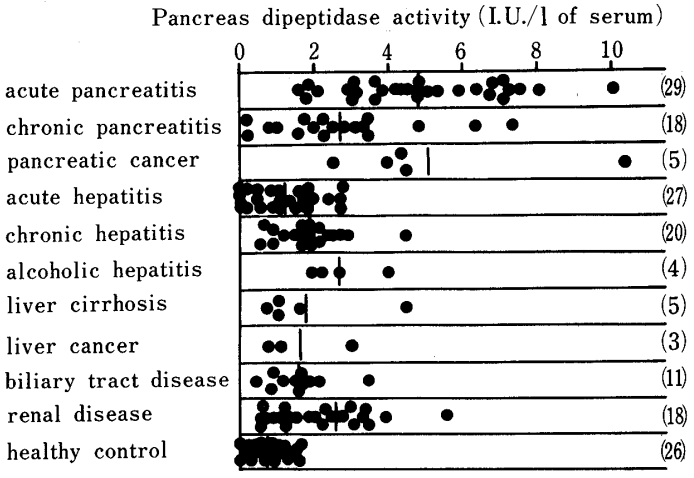

Fig. 5. Pancreas Dipeptidase Activities in Sera of Normal Subjects and Patients with Various Diseases as Determined by the Proposed Method

Bars indicate the mean value of serum dipeptidase activity. Figures in parentheses show the number of patients.

various diseases, determined by using the antibody-conjugated paper disks. The normal value was 1.62±0.40 I.U./l of serum (mean±S.D.). The serum dipeptidase activity was elevated in acute hepatitis (18.7±18.2 I.U./l, $p<0.001$ *vs.* normal), chronic hepatitis (11.0±12.0 I.U./l, $p<0.001$) and liver cancer (12.3±12.9 I.U./l, $p<0.001$). However, no elevation of the activity was observed in sera of patients with pancreatic diseases.

Figure 5 shows pancreas dipeptidase activities in sera from patients with various diseases.

TABLE II. Correlation between Dipeptidase Activity and Various Enzyme Activities in Sera of Patients with Liver and Pancreatic Diseases

Relation	Correlation coefficient (r)	
	Liver DPase[a] (51)	Pancreas DPase[a] (50)
ALT[b]	0.795	−0.175
AST[c]	0.766	0.078
ChE[d]	0.071	0.154
LAP[e]	0.742	0.112
AML[f]	−0.020	−0.329

The activities of liver and pancreas dipeptidases were determined by the proposed method using antibody-conjugated paper disks. Figures in parentheses show the number of serum samples. *a*) dipeptidase, *b*) alanine aminotransferase, *c*) aspartate aminotransferase, *d*) cholinesterase, *e*) leucine aminopeptidase, *f*) amylase.

The normal value was 0.74 ± 0.99 I.U./l of serum. The activities were remarkably elevated in sera of patients with pancreatic diseases, especially pancreatic cancer (5.22 ± 2.68 I.U./l, $p < 0.001$ *vs.* normal), and acute pancreatitis (4.82 ± 2.84 I.U./ l, $p < 0.001$).

Table II shows the relationships between dipeptidase activities using the antibody-conjugated paper disks and various enzyme activities in sera of patients with liver and pancreatic diseases. The liver dipeptidase activity was significant correlated with leucine aminopeptidase, alanine aminotransferase and aspartate aminotransferase activities. The pancreas dipeptidase activity showed no correlation with any of the serum enzyme activities assayed here.

Discussion

This report deals with the possibility of using an immobilized antibody to differentiate between antigenically different enzymes with similar substrate specificity. We succeeded in developing of a new enzyme assay system for serum dipeptidases by using of the specific antibody-conjugated paper disks. Many investigators have reported that aminopeptidases have hydrolytic activities toward some dipeptides.[16-19] Therefore, it is considered that dipeptide substrates such as L-Leu–L-Leu and L-Ala–L-Ala may be hydrolyzed not only by dipeptidase but also by aminopeptidase in serum. Consequently the total dipeptidase activity in serum may be presented as the sum of the dipeptide hydrolytic activities of both serum dipeptidase and aminopeptidase. However, when the serum dipeptidase activity is determined by the proposed method, it corresponds to the true liver or pancreas dipeptidase activity. Thus, the measurement of the tissue-specific dipeptidase by the proposed method may be more useful for evaluating the disease state of patients.

We tested various supporting materials for immobilizing the antibody. A polystyrene ball[20] or silicon rod,[21] which physically adsorbs the antibodies, binds then only at a low level. However, materials, such as paper disk[11] and agarose gel[22], which can bind the antibodies by covalent bonds have a higher binding capacity than the above supporting materials. The paper disk is efficient from a cost point of view, and can immobilize the antibodies easily.

As liver dipeptidase has a high substrate specificity for L-Leu–L-Leu and its activities in sera of normal subjects were higher than those of pancreas enzymes, this substrate could be used for the measurement of liver dipeptidase activity by use of the antibody-conjugated paper disk. With L-Leu–L-Leu as a substrate, the enzyme activity bound on the paper disk was reduced to about 40% of that of the corresponding free enzyme. It was suggested that the reduction of the activity may be due to conformational changes of the active site of the

enzyme and/or interference with the binding between the enzyme and L-Leu–L-Leu by the formation of the antigen–antibody complex. However, with L-Ala–L-Ala, which showed a high K_m value (5.2 mM) for pancreas dipeptidase as compared with L-Leu–L-Leu (0.033 mM for the free enzyme, 0.21 mM for the immobilized enzyme), the V_{max} and K_m values for the immobilized and free pancreas enzymes were almost the same. These results suggested that at high concentrations (more than twice the K_m value) of the peptide substrate, the catalytic activity and binding of dipeptidase may be unaffected by the formation of the enzyme-antibody complex.

As dipeptidase activity was not detected in the supernatant after incubation of the dipeptidase and the paper disk at 4 °C for 8 h, the enzyme was apparently bound almost 100% on the paper disk. McDonald *et al.*[23] similarly reported that the antibody immobilized on the pore glass had a high capacity for propandiol dehydrogenase from *Netuseria gonorrhoease* cells, and the recovery was 70—100%.

Ballard *et al.*[24] reported that the addition of 0.6% Triton X-405 to the reaction mixture gave a good immunoprecipitate between rat liver phosphoenolpyruvate carboxykinase and its antibody. In fact, adding Triton X-405 to the reaction mixture of the proposed immunoassay method resulted in good recovery of the enzyme activity and good precision of the assay. The addition of the detergent may remove interference with the immunoreaction owing to nonspecific bindings of some substances in the serum samples to the paper disks conjugated with antibodies.

By using anti-liver dipeptidase antibody-conjugated paper disk, the serum dipeptidase activity was found to be markedly elevated in patients with hepatic diseases such as acute hepatitis and liver cancer. On the other hand, by using the anti-pancreas dipeptidase antibody-conjugated paper disks, the serum enzyme activity was found to be elevated in patients with pancreatic diseases such as acute pancreatitis, chronic pancreatitis and pancreatic cancer, but not in patients with the above hepatic diseases. The activity also rose in patients with renal diseases. This elevation of the activity may be explained in terms of the failure of glomerular filtration and/or the metabolism of the enzyme in the kidney.

We are currently investigating the correlation between the serum dipeptidase activity and the concentration of the enzyme protein in various diseases.

References

1) P. G. W. Plagemann, K. F. Gregory, and F. Wroblewski, *J. Biol. Chem.*, **235**, 2282 (1960).
2) C. L. Markert (ed.), "Isozymes," Vol. 1, Academic Press, New York, 1975.
3) K. Hiwada, M. Terao, K. Nishimura, and T. Kokubu, *Clin. Chim. Acta*, **76**, 267 (1977).
4) S. A. Berson and R. S. Yallow, *J. Clin. Invest.*, **38**, 1996 (1959).
5) R. S. Shneider, P. Lindquist, E. T. Wong, K. E. Rubenstein, and E. F. Ullman, *Clin. Chem.*, **19**, 821 (1973).
6) M. Sugiura, Y. Ito, K. Hirano, S. Sawaki, M. Morikawa, and M. Tsuboi, *Clin. Chim. Acta*, **102**, 161 (1980).
7) M. Sugiura, Y. Ito, K. Hirano, and S. Sawaki, *Biochim. Biophys. Acta*, **481**, 578 (1977).
8) Y. Ito, M. Sugiura, and S. Sawaki, *J. Biochem.* (Tokyo), **94**, 871 (1983).
9) M. Sugiura, Y. Ito, K. Hirano, M. Sasaki, and S. Sawaki, *Clin. Chim. Acta*, **78**, 381 (1977).
10) C. A. Herbert, P. L. Pelham, and B. Pittman, *Appl. Microbiol.*, **25**, 26 (1973).
11) M. Ceska and U. Lundkrist, *Immunochem.*, **9**, 1021 (1972).
12) Y. Ito, Y. Watanabe, K. Hirano, M. Sugiura, S. Sawaki, and T. Ogiso, *J. Biochem.* (Tokyo), **96**, 1 (1984).
13) A. Karmen, *J. Clin. Invest.*, **34**, 131 (1955).
14) G. Szasz, *Am. J. Clin. Pathol.*, **47**, 607 (1967).
15) W. T. Caraway, *Am. J. Clin. Pathol.*, **32**, 97 (1959).
16) C. W. Garner and F. J. Behal, *Biochemistry*, **14**, 3208 (1975).
17) Y. S. Kim, E. J. Brophy, and J. A. Nicholson, *J. Biol. Chem.*, **251**, 3206 (1976).
18) E. M. Danielson, O. Noren, H. Sjostrom, J. Ingram, and A. J. Kenney, *Biochem. J.*, **189**, 591 (1980).
19) E. Kozak and S. S. Tate, *J. Biol. Chem.*, **257**, 6322 (1982).
20) E. Ishikawa, Y. Hamaguchi, and M. Imagawa, "Enzyme Immunoassay," ed. by E. Ishikawa, T. Kawai, and K.

Miyai, Igaku-Shoin, Tokyo, 1981, pp. 119—122.
21) K. Kato, H. Fukui, Y. Hamaguchi, and E. Ishikawa, *J. Immunol.*, **116**, 1554 (1976).
22) M. J. O'Sullivan, E. Gnemmi, D. Morris, G. Chieregatti, A. D. Simmonds, M. Simmons, J. W. Briges, and V. Marks, *Anal. Biochem.*, **100**, 100 (1979).
23) H. C. McDonald, H. H. Weetall, P. Simon, M. M. Takeguchi, and G. Odstrchel, *J. Solid-Phase Biochem.*, **3**, 151 (1978).
24) F. J. Ballard, M. F. Hopgood, L. Reshef, S. Tilghman, and R. W. Hanson, *Biochem. J.*, **144**, 199 (1974).