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# HIGHLY SENSITIVE ASSAY FOR PZ-PEPTIDASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A rapid and highly sensitive assay method for measuring PZ-peptidase activity in newborn rat brain is described. The method is based on monitoring the absorption at 320 nm of PZ-Pro-Leu enzymatically formed from the substrate, PZ-L-Pro-L-Leu-Gly-L-Pro-D-Arg, after separation by high-performance liquid chromatography using a reversed-phase column. This method is sensitive enough to measure PZ-Pro-Leu at concentrations as low as 5 pmol, and is able to make the column ready for the next injection within 10 min after the preceding injection. By using this method, PZ-peptidase activity was discovered in clonal osteoblastic cells derived from newborn mouse calvaria.

## INTRODUCTION

Collagenase from *Clostridium histolyticum* cleaves the α-chains of collagen at the X–Gly bond in sequences of the general form, Pro-X-Gly-Pro, where X is any amino acid[1]. This observation led to the development of synthetic substrates for collagenase such as benzyloxycarbonyl-Gly-L-Pro-L-Leu-Gly-L-Pro-L-Ala[2] and 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg[3]. The latter substrate is commonly abbreviated to "PZ-peptide".

PZ-peptide has been widely used in searching for animal proteases with collagenase-like specificity[4–14]. This substrate has the advantage that the chromogenic product, PZ-Pro-Leu, is not water soluble and can be assayed after extraction with an organic solvent. However, this method has a few disadvantages also. As the substrate has the same absorption spectrum as the product, PZ-Pro-Leu, its assay needs to be preceded by separation from the remaining substrate. The extraction with an organic solvent is time consuming and laborious. A more disadvantageous point is the low sensitivity.

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On the other hand, Kojima *et al.*[15] have reported a highly sensitive fluorescence assay method for collagenase-like peptidase (CL-peptidase) activity using (succinyl-Gly-L-Pro-L-Leu-Gly-L-Pro)-4-methylcoumaryl-7-amide as substrate instead of PZ-peptide. However, this substrate for CL-peptidase is also hydrolysed by another enzyme. Therefore, we had to develop a highly sensitive assay method for PZ-peptidase to avoid the above confusion.

Many enzyme assays have been performed using high-performance liquid chromatography (HPLC). For example, Marceau *et al.*[16] reported a sensitive assay method of carboxypeptidase N in human plasma using hippuryl-L-lysine as the substrate. In this method the product, hippuric acid, was determined by HPLC.

In this paper, we describe an advantageous assay method for PZ-peptidase using HPLC on a reversed-phase column to achieve a rapid and selective separation of substrate and product.

## EXPERIMENTAL

### *Materials*

4-Phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide) and 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu (PZ-Pro-Leu) were purchased from Fluka (Buchs, Switzerland). Bovine serum albumin (BSA) was obtained from Wako (Tokyo, Japan). Potassium methanesulphonate and N,N′-di-(2,4-dinitrophenyl)-L-lysine (di-DNP-Lys) were obtained from Tokyo Kasei (Tokyo, Japan). Acetonitrile was of chromatographic grade (Wako). (Succinyl-Gly-L-Pro-L-Leu-Gly-L-Pro)-4-methylcoumaryl-7-amide (Suc-GPLGP-MCA) and 7-amino-4-methylcoumarin (AMC) were purchased from the Protein Research Foundation (Osaka, Japan). Other chemicals and solvents were of analytical-reagent grade.

Newborn rats were killed by decapitation. After washing the brain with saline, it was cut into small pieces and homogenized in 5 volumes of 5 m*M* Tris–HCl buffer (pH 7.5) with a glass–PTFE homogenizer. The homogenate was centrifuged at 100 000 *g* for 60 min and the supernatant was used as enzyme source.

### *Assay of enzyme activity*

The principle of the assay method for PZ-peptidase activity is based on the spectrophotometric measurement at 320 nm of PZ-Pro-Leu liberated enzymatically from the substrate, PZ-peptide, after separation by HPLC (Fig. 1).

The incubation mixture contained (in a final concentration) 50 m*M* Tris–HCl buffer (pH 7.5), 0.2 m*M* PZ-peptide, 5 m*M* $CaCl_2$ and enzyme plus water in a total volume of 300 μl. The blank and standard tubes contained water and 6 nmol of PZ-Pro-Leu, instead of enzyme, respectively. Incubation was carried out at 37°C, and the reaction was terminated by heating at 95°C for 3 min. After addition of 53 μl of acetonitrile containing 3.53–17.7 nmol of di-DNP-Lys as the internal standard, the reaction mixture was centrifuged at 15 600 *g* for 10 min and an aliquot of the clear supernatant was injected into the HPLC column. For micro-assay, the incubation mixture was reduced to a total volume of 60 μl instead of 300 μl, and the volumes of other reagents added were also reduced to one-fifth. The peak height of PZ-Pro-Leu was measured and converted to pmol from the peak height of di-DNP-Lys added as an internal standard. One unit of enzyme activity is expressed as that amount which catalyses the hydrolysis of 1 μmol of the substrate per minute.

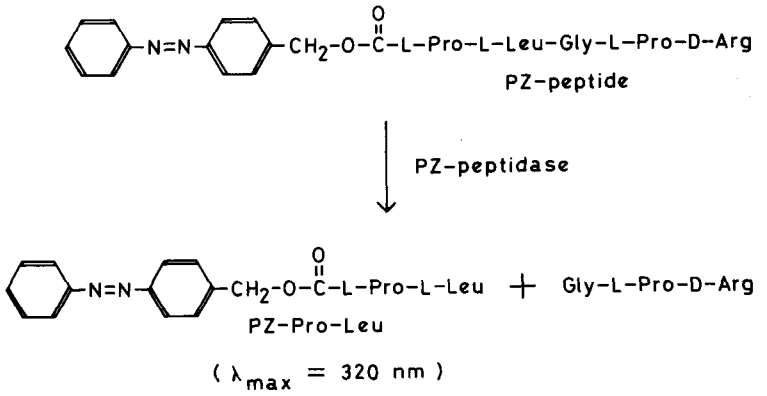


Fig. 1. Principle of the spectrophotometric assay of PZ-peptidase with PZ-peptide as the substrate.

*Chromatographic conditions*

Analysis of the product was performed on a chromatographic system consisting of an NSP-800-DX pump (Nihon Seimitsu Kagaku, Japan), an NS-310A variable-wavelength absorption detector (Nihon Seimitsu Kagaku) fixed at 320 nm and a Hitachi gel No. 3056 (particle size 5 $\mu$m) reversed-phase column (150 × 4.6 mm I.D.) (Hitachi, Japan). The system was operated at room temperature at a flow-rate of 0.86 ml/min. The mobile phase consisted of 0.01 *M* sodium acetate buffer containing 0.1% of potassium methanesulphonate (pH 5.30)–acetonitrile (63:37, v/v).

*Other methods*

The protein concentration was determined according to the method of Lowry *et al.*[17] with BSA as the standard using a Hitachi 220A spectrophotometer. CL-peptidase activity was measured according to the method of Kojima *et al.*[15] using Suc-GPLGP-MCA as substrate. The principle of the assay is based on the fluorimetric measurement of AMC liberated from the reaction product, Gly-L-Pro-MCA, by the second enzyme reaction with dipeptidyl-peptidase IV.

## RESULTS

This HPLC–spectrophotometric detection system for the measurement of PZ-peptide and PZ-Pro-Leu was found to be very sensitive. The calibration graph for PZ-Pro-Leu injected showed good linearity from 5 to 900 pmol. The calibration graph for di-DNP-Lys also showed good linearity from 25 to 1000 pmol. Fig. 2 shows the chromatographic patterns of the reaction mixture after incubation with 15 $\mu$l of newborn rat brain supernatant for 20 min. The blank incubation (Fig. 2A) contained PZ-peptide and di-DNP-Lys and the standard incubation contained exogenous PZ-Pro-Leu in addition to the PZ-peptide and di-DNP-Lys (Fig. 2B). The retention times for di-DNP-Lys, PZ-Pro-Leu and PZ-peptide were 3.4, 4.3 and 6.6 min, respectively (Fig. 2A and B). As shown in the blank incubation (Fig. 2A), an unknown peak, presumably originating from an impurity in the substrate, was found at 2.8 min near the peak of the solvent. The experimental incubation under the standard assay con-

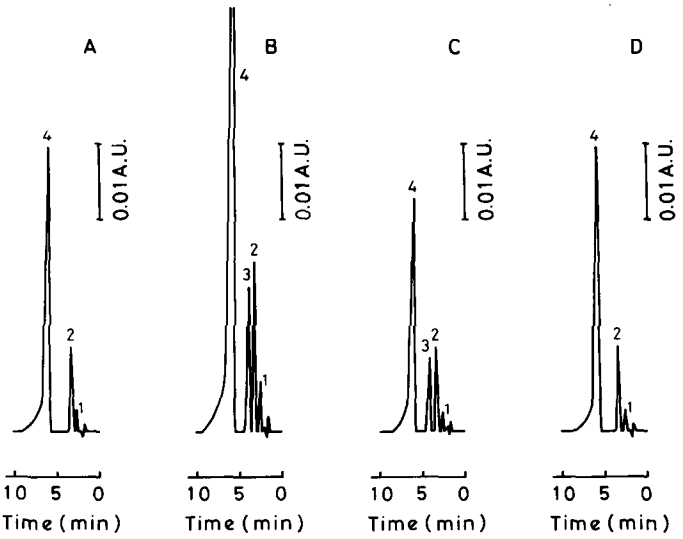


Fig. 2. HPLC elution patterns of PZ-peptidase activity determined using enzyme in newborn rat brain. Conditions were as described under Experimental. Peaks: 1, impurity contained in PZ-peptide; 2, di-DNP-Lys; 3, PZ-Pro-Leu; 4, PZ-peptide. 17.65 nmol of di-DNP-Lys (internal standard) were added to each sample after incubation. (A) Blank incubation; PZ-peptide was incubated without enzyme. (B) Standard incubation; 6 nmol of PZ-Pro-Leu were added to a sample tube before incubation as a standard sample. The peak heights of PZ-Pro-Leu and di-DNP-Lys correspond to 340 pmol and 1 nmol, respectively. The injection volume for the standard incubation was twice those of the other incubation tubes (A, C and D). (C) Experimental incubation; PZ-peptide was incubated with 142.5 $\mu$g of protein in newborn rat brain at 37°C for 20 min. (D) Control incubation; a control tube without the enzyme was incubated, then the same amount of active enzyme was added and the resulting tube was kept in an ice-bath until heated at 95°C for 3 min.

ditions (Fig. 2C) showed a significant amount of PZ-Pro-Leu at 4.3 min, whereas the control incubation did not show any peak of PZ-Pro-Leu (Fig. 2D).

The enzyme reaction was found to be linearly related to time at 37°C for about 30 min (Fig. 3). When the enzyme reaction was carried out at 25°C, a linear relationship was obtained for about 60 min (data not shown).

In Fig. 4, PZ-peptidase activity is shown as a function of the amount of enzyme extract obtained from newborn rat brain. Perfect linearity was observed for plots of the amount of PZ-Pro-Leu, from $1.95 \cdot 10^{-5}$ to $2.18 \cdot 10^{-4}$ units, formed enzymatically from PZ-peptide against those of enzyme (100 000 *g* supernatant fraction). By using the micro-assay system, the enzyme activity was detectable at levels as low as $2.73 \cdot 10^{-6}$ units.

A Lineweaver–Burk plot was obtained from the effect of the concentration of PZ-peptide on the rate of formation of PZ-Pro-Leu by PZ-peptidase. The Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) towards the PZ-peptide were calculated to be 248 $\pm$ 4.5 $\mu M$ and 10.47 $\pm$ 0.56 nmol/min/ml, respectively.

A comparison between the present HPLC–spectrophotometric assay method and a widely used extraction method[3] for the activity of PZ-peptidase in mouse tissues is shown in Table I. The enzyme activity was determined in the supernatant fractions (10 000 *g*) obtained from the homogenates of brain, kidney, liver and spleen

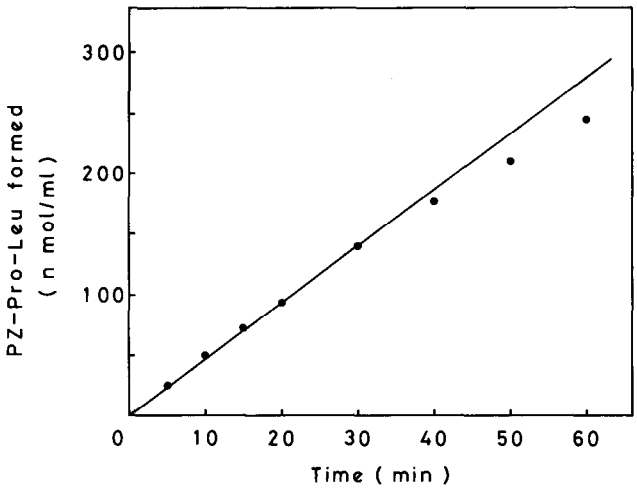


Fig. 3. Time-dependent changes of PZ-Pro-Leu enzymatically formed from the substrate. The standard assay conditions are described under Experimental. Incubation was carried out at 37°C for the indicated periods.

of a mouse 9 weeks old. The enzyme activities measured by the two methods were in close agreement. It can be seen from the Table I that the highest specific activity of the enzyme was found in brain and the lowest in liver. On the other hand, PZ-peptidase activity was discovered for the first time in cloned osteoblastic cells (MC3T3-El) by our method, and the activity was about 0.26 mU/mg protein.

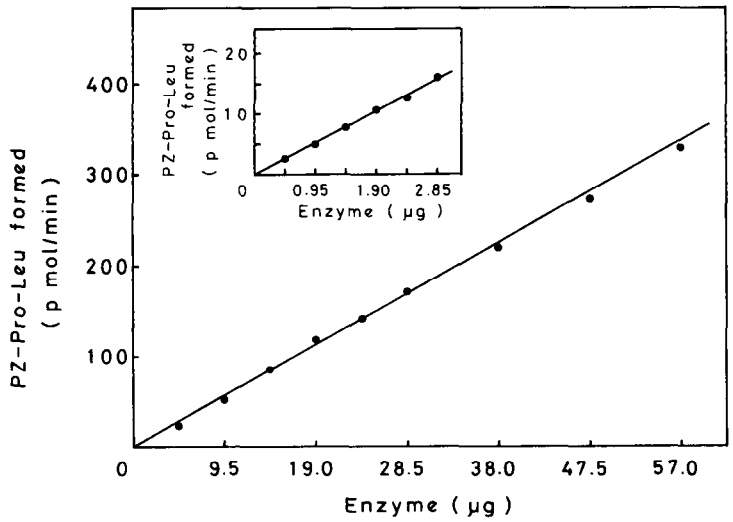


Fig. 4. Velocity of PZ-peptidase reaction determined at various concentrations of enzyme in newborn rat brain. The standard assay conditions were used and incubation was carried out at 37°C for 20 min. Insert: the enzyme activity was measured by means of a micro-assay system.

TABLE I

COMPARISON BETWEEN THE HPLC–SPECTROPHOTOMETRIC ASSAY METHOD AND A WIDELY USED EXTRACTION METHOD FOR ASSAY OF THE ACTIVITY OF PZ-PEPTIDASE IN MOUSE TISSUES

| *Tissue* | *PZ-peptidase activity (mU/mg protein)** | |
|---|---|---|
| | *HPLC method* | *Extraction method*[3] |
| Brain | 2.27 ± 0.30 | 1.60 ± 0.42 |
| Kidney | 1.49 ± 0.16 | 1.25 ± 0.27 |
| Liver | 0.86 ± 0.05 | 0.83 ± 0.11 |
| Spleen | 0.96 ± 0.13 | 0.85 ± 0.13 |
| Cloned osteoblastic cells (MC3T3-El)** | 0.26 ± 0.04 | Below detection limit |

* Each value is the mean ± S.D. obtained from five separate experiments.

** Clone MC3T3-El cells were isolated from line MC3T3-E cells derived from newborn C57BL/N6 mouse calvaria[18].

TABLE II

CL-PEPTIDASE AND PZ-PEPTIDASE ACTIVITIES IN 10 000 *g* SUPERNATANT FRACTIONS OF MOUSE TISSUES

Activities were measured at pH 7.5 in 50 m*M* Tris–HCl buffer.

| *Tissue* | *Enzyme activity (mU/mg protein)* | | |
|---|---|---|---|
| | *CL-peptidase* | *PZ-peptidase (HPLC method)* | $\frac{[CL\text{-}peptidase]}{[PZ\text{-}peptidase]}$ *ratio* |
| Brain | 8.84 | 2.27 | 3.89 |
| Kidney | 4.17 | 1.49 | 2.80 |
| Liver | 5.20 | 0.86 | 6.05 |
| Spleen | 8.32 | 0.96 | 8.67 |

The CL-peptidase and PZ-peptidase activities in mouse tissues are shown in Table II. It can be seen that the CL-peptidase activities in brain and spleen were the same and that in kidney was about half of this level. As shown in Table II, the ratio between the CL-peptidase and PZ-peptidase was variable in mouse tissues.

## DISCUSSION

The proposed highly sensitive assay method for PZ-peptidase activity using an HPLC–spectrophotometric detection system has several advantages. First, it is very sensitive. The limit of the sensitivity was about 5 pmol of PZ-Pro-Leu formed enzymatically. On the other hand, the sensitivity of the widely used extraction assay method is only 5 nmol, because the absorbance at 320 nm in controls is very high compared with the present HPLC–spectrophotometric detection system. Second, the substrate and the product are separated completely in less than 10 min.

Collagen is a major component of the bone matrix and it is very important to determine the regulatory mechanisms of collagen metabolism in bone tissue. The enzymes for both the synthesis and degradation of collagen regulate its total amount. Kodama *et al.*[18] established a clonal osteoblastic cell line, MC3T3-El, from newborn mouse calvaria. This cell has the capacity to produce collagen and differentiate into osteoblast-like cells, forming calcified tissue *in vitro* similar to their *in vivo* counterparts. Hence this cell is very useful for studying the mechanism of collagen metabolism in addition to osteoblastic differentiation. On the other hand, PZ-peptidase is widely distributed in many animal tissues. The real physiological role of PZ-peptidase in animals has not yet been made clear, but it has been observed that collagen degradation is closely correlated with the PZ-peptidase activity in post partum uterus, tumour tissue and in developing chick embryo skin[19–21]. From these observations, PZ-peptidase is thought to be an indicator of collagen catabolism. However, PZ-peptidase activity in MC3T3-El cells was too low to be detected using the widely used extraction assay method. It should be noted, therefore, that the newly developed HPLC–spectrophotometric detection system was able to detect PZ-peptidase activity in MC3T3-El for the first time (Table I).

The comparison between CL-peptidase and PZ-peptidase activities in mouse tissues determined using Suc-GPLGP-MCA and PZ-peptide, respectively, as substrates is shown in Table II. However, as can be seen, the ratio of the rates of hydrolysis of the two substrates were variable in mouse tissues, suggesting that the CL-peptidase assayed with Suc-GPLGP-MCA may be different from PZ-peptidase. Both the CL-peptidase and PZ-peptidase hydrolysed the Leu–Gly bond of each substrate, but Suc-GPLGP-MCA is also a good substrate for post-proline cleaving enzyme[22], because post-proline cleaving enzyme is a proline-specific endopeptidase[23]. Therefore, the use of Suc-GPLGP-MCA as the substrate in place of PZ-peptide gave a confused result. Perhaps this confusion may be solved by our newly developed highly sensitive assay method for PZ-peptidase using PZ-peptide as the substrate.

In conclusion, this HPLC assay method may be useful for investigating the roles of PZ-peptidase *in vivo*.

## REFERENCES

1 A. Nordwig, *Adv. Enzymol.*, 34 (1971) 155.
2 Y. Nagai, S. Sakakibara, H. Noda and S. Akabori, *Biochim. Biophys. Acta*, 37 (1960) 567.
3 E. Wünsch and H. G. Heidrich, *Hoppe-Seyler's Z. Physiol. Chem.*, 333 (1963) 149.
4 G. Gries, H. Buresch and L. Strauch, *Experientia*, 26 (1970) 31.
5 S. Aswanikumar and A. N. Radhakrishnan, *Biochim. Biophys. Acta*, 276 (1972) 241.
6 H. G. Heidrich, O. Kronschnabl and K. Hannig, *Hoppe-Seyler's Z. Physiol. Chem.*, 354 (1973) 1399.
7 E. Koren and S. Milkovic, *J. Reprod. Fertil.*, 32 (1973) 349.
8 S. Aswanikumar and A. N. Radhakrishnan, *Biochim. Biophys. Acta*, 304 (1973) 210.
9 M. Hino, G. Nakano, M. Harada and T. Nagatsu, *Arch. Oral Biol.*, 20 (1975) 19.
10 S. Aswanikumar and A. N. Radhakrishnan, *Biochim. Biophys. Acta*, 384 (1975) 194.
11 M. Hino and T. Nagatsu, *Biochim. Biophys. Acta*, 429 (1976) 555.
12 T. I. Morales and J. F. Woessner, *J. Biol. Chem.*, 252 (1977) 4855.
13 A. Ito, K. Naganeo, Y. Mori, S. Hirakawa and M. Hayashi, *Clin. Chim. Acta*, 78 (1977) 267.
14 M. Nagelschmidt, T. Unger and H. Struck, *Biochim. Biophys. Acta*, 571 (1979) 105.
15 K. Kojima, H. Kinoshita, T. Kato, T. Nagatsu, K. Takada and S. Sakakibara, *Anal. Biochem.*, 100 (1979) 43.

16 F. Marceau, A. Drumheller, M. Gendreau, A. Lussier and S. St. Pierre, *J. Chromatogr.*, 266 (1983) 173.
17 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
18 H. Kodama, Y. Amagai, H. Sudo, S. Kasai and S. Yamamoto, *Jpn. J. Oral Biol.*, 23 (1981) 899.
19 L. Strauch, *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, Academic press, New York, 1970, p. 1675.
20 E. Keiditsch and L. Strauch, *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, Academic Press, New York, 1970, p. 1617.
21 J. F. Woessner, *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, Academic Press, New York, 1970, p. 1663.
22 T. Kato, in preparation.
23 M. Koida and R. Walter, *J. Biol. Chem.*, 251 (1976) 7593.