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High-performance liquid chromatographic determination of PZ-peptidase activity

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ABSTRACT

A rapid and sensitive assay method for the determination of PZ-peptidase activity is reported. This method is based on the monitoring of the absorption at 320 nm of 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu (PZ-Pro-Leu), enzymatically formed from the substrate 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide), after separation by high-performance liquid chromatography using a C_{18} reversed-phase column by isocratic elution. This method is sensitive enough to measure PZ-Pro-Leu at levels as low as 10 pmol, yields highly reproducible results and requires less than 5.5 min per sample for separation and determination. The optimum pH for PZ-peptidase activity was 7.5-8.0. The K_m and V_{max} values were 166.7 μM and 5.35 pmol/min . μg protein, respectively, with the use of enzyme extract obtained from mouse whole brain. The approximate molecular mass of this enzyme was estimated to be 64 000 by gel filtration. PZ-peptidase activity was strongly inhibited by Zn^{2+}, Cu^{2+} and p-chloromercuriphenylsulphonic acid. By using this method, PZ-peptidase activity could be readily detected in a single mouse pituitary gland. Among the tissues examined in various mouse brain regions, the highest specific activity of the enzyme was found in cerebral cortex. The sensitivity and selectivity of this method will aid in efforts to examine the physiological role of this peptidase.

INTRODUCTION

The collagenase of *Clostridium histolyticum* is commonly assayed with 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide), a synthetic peptide with an amino acid sequence based on the -Gly-Pro-Xaa- tripeptide repeating pattern of the helical region of collagen [1]. It is not hydrolysed by mammalian collagenase, but is cleaved by another enzyme, PZ-peptidase (EC 3.4.99.31), which is widely distributed in mammalian tissues [2].

The assay method using PZ-peptide as substrate has the advantage that the chromogenic product, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu (PZ-Pro-Leu), is not water soluble and can be assayed after extraction with an organic solvent. However, this method also has a few disadvantages. This conventional procedure is obviously non-specific as all PZ-peptides eventually produced by non-enzymatic hydrolysis may be extracted into the organic phase; moreover, the extraction step does not allow the optimum reproducibility to be obtained. A greater disadvantage is the low sensitivity. The direct

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analysis of the reaction mixture by a high resolution technique would alleviate the problems concerning selectivity and reproducibility.

In this paper, we describe an advantageous assay method for PZ-peptidase using high-performance liquid chromatography (HPLC) on a reversed-phase column to achieve a rapid and selective separation of substrate and product. Using this sensitive assay method, PZ-peptidase activity was discovered in mouse pituitary gland for the first time. Moreover, we describe some physico-chemical properties of PZ-peptidase in mouse brain in order to investigate the real physiological roles of this enzyme in the central nervous system (CNS).

EXPERIMENTAL

Materials

PZ-peptide and PZ-Pro-Leu were purchased from Fluka (Buchs, Switzerland). N-2,4-Dinitrophenyl-L-phenylalanine (DNP-Phe), cytochrome c, ribonuclease A, myoglobin, α-chymotrypsinogen A, ovalbumin, bovine serum albumin (BSA), BSA dimer, phenylmethylsulphonyl fluoride (PMSF), iodoacetic acid (IAA), N-ethylmaleimide (NEM), *p*-chloromercuriphenylsulphonic acid (PCMS) and pepstatin A were obtained from Sigma (St. Louis, MO, USA). Soybean trypsin inhibitor, bacitracin and 1,10-phenanthroline hydrochloride were obtained from Wako (Osaka, Japan). Sephacryl S-300 HR was purchased from Pharmacia (Uppsala, Sweden). Acetonitrile was of chromatographic grade (Cica-Merck). Other chemicals and solvents were of analytical-reagent grade.

Animals

Male ICR mice weighing 20-25 g were purchased from Charles River and housed on a 12-h light-dark cycle for at least 1 week before the beginning of all experiments. Food and water were available *ad Zibitum.* All operations were carried out at 0-4°C unless stated otherwise. Mice were killed by decapitation. After washing the whole brain with saline, it was cut into small pieces and homogenized in nine volumes of 0.32 *M* sucrose with a glass-PTFE homogenizer. The homogenate was centrifuged at 100 000 g for 80 min and the supernatant obtained was used as an enzyme source.

For the investigation on localization of the enzyme activity, various brain regions, *i.e.,* bulbus olfactorius, pons-medulla, cerebellum, hypothalamus, hippocampus, cerebral cortex, striatum, midbrain and pituitary gland, were dissected on ice. Tissues were homogenized in nineteen volumes of 0.32 *M* sucrose with a glass–PTFE homogenizer. The homogenates were used as an enzyme source.

Assay for PZ-peptidase 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. The reaction mixture contained 60 m*M* Tris–HCl buffer (pH 8.0), 6 m*M* $CaCl_2$, 240 m*M* NaCl, 0.24 m*M* PZ-peptide and enzyme plus water in a total reaction volume of 250 μl. Incubation was carried out at 37°C and the reaction was terminated by heating at 95°C for 5 min in boiling water. After addition of DNP-Phe as an internal standard, the reaction mixture was centrifuged and the clear supematant obtained was evaporated *in vacuo* using an AS160 Speed-Vac concentrator (Savant, Farmingdale, MA). The resulting residue was dissolved into acetonitrile-water (55:45, v/v) containing 0.1% of trifluoroacetic acid, and an aliquot of the mixture was subjected to HPLC analysis. For micro-assay, the incubation mixture was reduced to a total volume of 50 μl instead of 250 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 into picomoles from the peak height of DNP-Phe added as an internal standard. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of the substrate into the corresponding product in 1 min at 37°C.

Chromatographic conditions

Analysis of the product was performed using a Japan Spectroscopic HPLC system consisting of a Model 880-PU pump, 875-UV UV detector

(fixed at 320 nm), 860-CO column oven, 880-50 degasser, 880-02 gradient unit, 802-SC system controller and 807-IT integrator. The system was operated at room temperature at a flow-rate of 1.32 ml/min employing a Finepak SIL C18S (particle size 5 μm) reversed-phase column (150 x 4.6 mm I.D.) (Jasco) fitted with a LiChrosorb RP-18 guard gel (15 × 3.2 mm I.D.; particle size 5 μm) (Cica-Merck). The mobile phase was acetonitrile-water (55:45, v/v) containing 0.1% of trifluoroacetic acid.

Determination of molecular mass

All of the following procedures were carried out at 0-4°C. Mouse whole brain (3.7 g) was homogenized in nine volumes of 0.25 M Tris–HCl buffer (pH 8.0) with a glass-PTFE homogenizer. The homogenate was centrifuged at 100 000 *g* for 80 min and the resulting supernatant was concentrated to 5 ml by ultrafiltration (Amicon YM 10 membrane) and subjected to size-exclusion chromatography on a Sephacryl S-300 HR column (96.5 x 2.6 cm I.D.) equilibrated with 0.25 *M* Tris–HCl buffer (pH 8.0), and eluted with same buffer at a flow-rate of 0.5 ml/min. The UV absorbance of the eluate was monitored at 280 nm, and column fractions (2.5 ml per tube) were assayed for enzyme activity. The following proteins were used as molecular mass markers: BSA (dimer 130000, monomer 67 000), ovalbumin (43 000), α-chymotrypsinogen A (25 000), soybean trypsin inhibitor (20 100), myoglobin (18 800), ribonuclease A (13 700) and cytochrome c (12 500).

Effects of various metal ions and inhibitors on enzymatic activity

The active fractions eluted from the Sephacryl S-300 HR column were pooled and used as the source of enzyme for this experiment. Enzyme solution was preincubated in the absence or presence of various chemicals (0.1 and 1 m*M*) in 60 m*M* Tris–HCl buffer (pH 8.0) for 5 min at 37°C. Immediately thereafter, 0.24 m*M* PZ-peptide (final concentration) was added to the reaction mixture and incubated at 37°C for the desired periods. After stopping the reaction, the samples were treated as described above.

Protein determination

Protein concentration was measured by the Lowry method as modified by Hat-tree [3] using BSA as a standard protein.

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 10 to 2000 pmol. The calibration graph for DNP-Phe also showed good linearity from 50 to 2000 pmol. Fig. 1 shows the chromatographic patterns of the reaction mixture after incubation with 24.9 μg of protein prepared from mouse whole brain supematant for 30 min. The blank incubation (Fig. 1A) contained PZ-

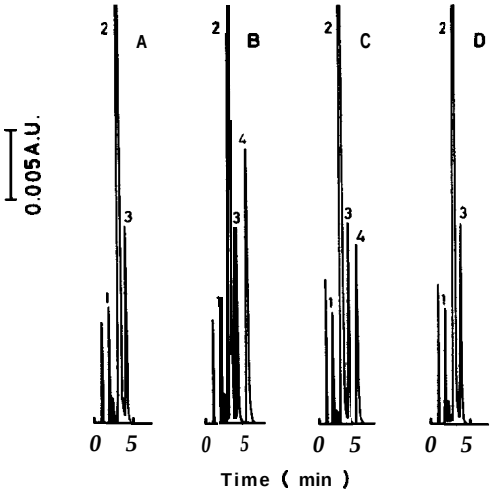

Fig. 1. HPLC elution patterns of PZ-peptidase activity determined using enzyme in mouse whole brain. Conditions as described under Experimental. Peaks: 1 = impurity contained in PZ-peptide; 2 = PZ-peptide; 3 = DNP-Phe; 4 = PZ-Pro-Leu. A 5500-pmol amount of DNP-Phe (internal standard) was added to each sample after incubation. (A) Blank incubation: PZ-peptide was incubated without enzyme at 37°C for 30 min. (B) Standard incubation: 5500 pmol of PZ-Pro-Leu were added to a sample tube before incubation as a standard sample. The two peak heights of DNP-Phe and PZ-Pro-Leu correspond 500 pmol. (C) Experimental incubation: PZ-peptide was incubated with 24.9 μg of protein in mouse whole brain at 37°C for 30 min. (D) Control incubation: a control tube without the enzyme was incubated, the same amount of active enzyme was added and the resulting tube was kept in an ice-bath before heating at 95°C for 5 min.

peptide and DNP-Phe, and the standard incubation (Fig. 1B) contained exogenous PZ-Pro-Leu in addition to PZ-peptide and DNP-Phe. The retention times for PZ-peptide, DNP-Phe and PZ-Pro-Leu were 3.0, 3.9 and 5.1 min, respectively (Fig. 1A and B). As shown in the blank incubation (Fig. 1A), an unknown peak, presumably originating from an impurity in the substrate, was found at 1.8 min near the peak of the solvent. The experimental incubation under the standard assay conditions (Fig. 1C) showed a significant amount of PZ-Pro-Leu at 5.1 min, whereas the control incubation (Fig. 1D) did not show any peak of PZ-Pro-Leu.

The enzyme reaction was found to be linear with time at 37°C for at least cu. 60 min (data not shown).

The pH dependence of enzyme activity was investigated in both 60 m*M* sodium phosphate buffer (pH 6.0-8.0) and 60 m*M* Tris–HCl buffer (pH 7.5-9.0). The catalytic activity of the enzyme was greatest at a pH of approximately 7.5-8.0, with very little activity below pH 6.0 and above pH 9.0 (Fig. 2).

PZ-peptidase activity was investigated as a function of the amount of enzyme extract obtained from mouse whole brain. Perfect linearity was observed for plots of the amount of PZ-Pro-Leu, from $7.84 \cdot 10^{-5}$ to $3.02 \cdot 10^{-4}$ units, formed enzymatically from PZ-peptide against those of enzyme (100 000 *g* supematant fraction). By using the micro-assay system, the enzyme activity was detectable as levels as low as $4.80 \cdot 10^{-6}$ units (Fig. 3).

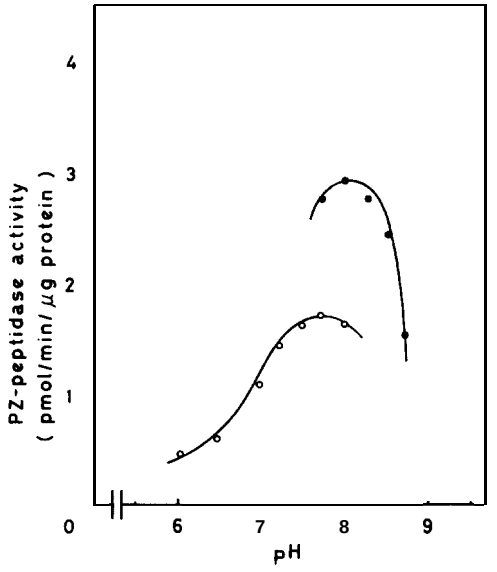

Fig. 2. Effect of pH on PZ-peptidase activity: (0) 60 m*M* sodium phosphate buffer (pH 6.0-8.0) and (●) 60 m*M* Tris–HCl buffer (pH 7.5–9.0) were used. Incubation was carried out at 37°C for 30 min.

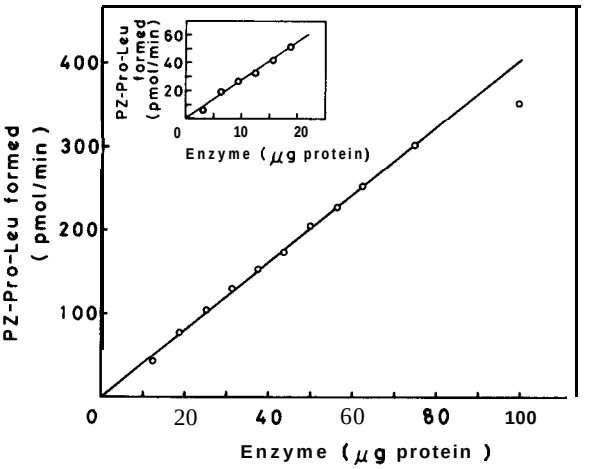

Fig. 3. Velocity of PZ-peptidase reaction determined at various concentrations of enzyme in mouse whole brain. The standard assay conditions were used and incubation was carried out at 37°C for 20 min. Inset: the enzyme activity was measured by means of a micro-assay system.

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-

TABLE I

DISTRIBUTION OF PZ-PEPTIDASE ACTIVITY IN MOUSE BRAIN HOMOGENATE

Values are means ±SEM for four experiments.

Brain region	Enzyme activity (pmol/min . mg protein)
Bulbus olfactorius	893 ± 61
Pons-medulla	477 ± 34
Cerebellum	591" 32
Hypothalamus	524 ± 33
Hippocampus	756 ± 16
Cerebral cortex	1276 ± 41
Striatum	586 ± 28
Midbrain	779 ± 35
Pituitary gland	1020 ± 46

peptidase. The Michaelis constant (K_m) and the maximum velocity (V_{max}) towards the PZ-peptide were calculated to be 166.7 ± 3.0 μM and 5.35 ± 0.28 pmol/min . μg protein, respectively.

We applied this standard assay method for the detection of PZ-peptidase activity in various mouse brain regions (Table I). It can be seen that the PZ-peptidase activity was distributed unevenly in the mouse CNS. Among the tissues examined, the highest specific activity of the enzyme was found in the cerebral cortex and the lowest in the pons-medulla. A high level of activity was also observed in the pituitary gland. Moderate levels of activity were seen in the bulbus olfactorius, hippocampus and midbrain.

Further, some enzymatic and physico-chemical properties of PZ-peptidase in mouse whole brain were studied by this standard assay method. An estimation of the molecular mass of the enzyme was made by the gel filtration method using a Sephacryl S-300 HR column. The void volume of the column was determined with Blue Dextran 2000. From a plot of log M_r *versus* K_{av} for protein standards, the molecular mass of the enzyme was estimated to be approximately 64000 (Fig. 4).

The effects of various metal ions and inhibitors

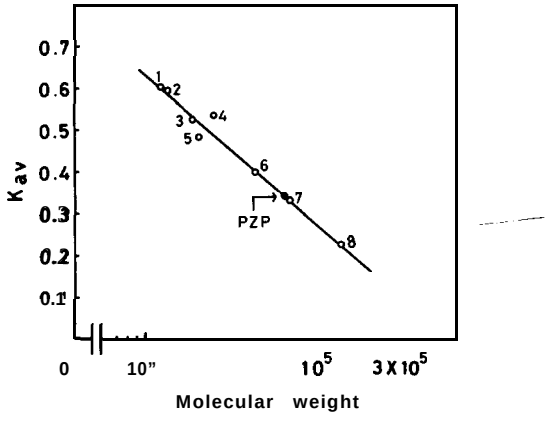

Fig. 4. Estimation of the molecular mass of PZ-peptidase on a Sephacryl S-300 HR column. The protein markers used were (1) cytochrome c, 12500; (2) ribonuclease A, 13700; (3) myoglobin, 18 800; (4) a-chymotrypsinogen A, 25 000; (5) soybean trypsin inhibitor, 20 100; (6) ovalbumin, 43 000; (7) BSA monomer, 67000; (8) BSA dimer, 130 000; and PZ-peptidase (PZP), molecular mass estimated to be 64 000. Experimental details as described under Experimental.

on PZ-peptidase activity in mouse whole brain were examined at a final concentration of 1 and 0.1 mM. As shown in Table II, metals such as Zn^{2+} and Cu^{2+} completely inhibited the enzyme activity at 1 mM, whereas Ca^{2+} and Mg^{2+} had no effect. On the other hand, Mn^{2+} enhanced the enzyme activity to 1.6-fold compared with the control level at 1 mM. EDTA, a chelating agent, had no effect on enzyme activity, but 1,10-phenanthroline was found to be inhibitory to the enzyme. PCMS, a typical thiol protease inhibitor, inhibited the enzyme activity completely at a final concentration of 0.1 mM. However, there was no inhibition by other thiol protease inhibitors such as IAA and NEM and PMSF (serine protease inhibitor). Further, the PZ-peptidase activity was not affected by pep-

TABLE II

EFFECTS OF METAL IONS AND INHIBITORS ON PZ-PEPTIDASE ACTIVITY IN MOUSE WHOLE BRAIN

Reagent	Final concentration (mM)	PZ-peptidase activity (% of control)
None	–	100
$MnCl_2$	1	164.3
	0.1	121.6
$CaCl_2$	1	100.2
	0.1	95.1
$MgCl_2$	1	99.3
	0.1	100.7
znso,	1	0
	0.1	7.5
$CuSO_4$	1	0
	0.1	31.7
EDTA	1	105.1
	0.1	106.5
1 ,lO-Phenanthroline	1	47.0
	0.1	88.6
p-Chloromercuriphenyl sulphonic acid	1	0
	0.1	0
Iodoacetic acid	1	117.3
	0.1	105.5
N-Ethylmaleimide	1	97.4
	0.1	94.7
Phenylmethylsulphonyl fluoride	1	67.3
	0.1	95.6
Pepstatin A	32 μg/ml	95.5
Soybean trypsin inhibitor	32 μg/mol	92.4
Bacitracin	80 μg/ml	89.1

statin A (acid protease inhibitor), soybean trypsin inhibitor and bacitracin.

DISCUSSION

As pointed out in other HPLC enzymatic assays, the direct analysis of the product of enzyme action, separated from the substrate and other interfering substances, offers several advantages over previous spectrophotometric methods.

The proposed sensitive assay method for PZ-peptidase activity using an HPLC–spectrophotometric detection system has a few advantages. First, it is very sensitive. The limit of the sensitivity was about 10 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. We especially emphasize that enzyme activity was able to be detected in a single mouse pituitary gland using this sensitive assay method. Typically, 15-20 assays could be performed with the extract from a single mouse pituitary. Second, the substrate and the product are separated completely in less than 5.5 min. The modified assay method for this enzyme activity by HPLC, which we reported previously [4], still had the disadvantage that it is time consuming for the complete separation of several peaks. However, the present assay method overcame this problem. Third, more accurate quantification of the product and better reproducibility were ensured by the use of an internal standard (DNP-Phe) and the elimination of an extraction step.

The physiological role of PZ-peptidase in animals has not been clarified except for the observation that PZ-peptidase plays a part in the late stages of the degradation of collagen in peripheral tissues [5], but there is no direct evidence for this. On the other hand, only one observation has been reported about findings of PZ-peptidase in CNS, in which PZ-peptidase activity continuously decreased with maturation in rat brain [6]. However, the physiological role of this enzyme in CNS is also still unclear. In order to search for it in CNS, some properties of PZ-peptidase in mouse brain were examined and compared with those in peripheral tissues.

The approximate molecular mass of PZ-peptidase from mouse brain is 64000 on gel filtration, and its optimum pH is cu. 7.5-8.0. The effects of various metal ions and inhibitors on the PZ-peptidase are also investigated (Table II). The PZ-peptidase in mouse brain is completely inhibited by metals such as Zn^{2+} and Cu^{2+} and by PCMS, but not by other metals (Ca^{2+} and Mg^{2+}), serine protease inhibitor, acid protease inhibitor and trypsin inhibitor, indicating that this enzyme is a metallo-endopeptidase with thiol dependence. These properties are very similar to those of the enzymes that have been isolated from peripheral tissues and fluids [5–14]. On the other hand, the PZ-peptidase activity in pituitary gland is higher than that in other brain regions (Table I). From these observations, we conclude that some 'of the enzymes reported as "PZ-peptidases" may function as peptidases hydrolysing collagen-related peptides, but others may be related to the processing of certain hormones. The real physiological role of PZ-peptidase in CNS remains a subject for further investigation. Recently, PZ-peptidase has been shown to be identical with soluble metallo-endopeptidase (EC 3.4.24.15) [15] and endo-oligopeptidase (EC 3.4.22.19) [16]. Because of this, there is considerable interest in the physiological role of PZ-peptidase.

In conclusion, the rapid and accurate assay method described in this paper may be a useful means for investigating the roles of PZ-peptidase *in vivo*.

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