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High-performance liquid chromatographic–colorimetric assay for glycine carboxypeptidase activity

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Abstract

A rapid and sensitive assay method for the determination of glycine carboxypeptidase activity has been reported. This method is based on the monitoring of the absorption at 460 nm of 4-dimethylaminoazobenzene-4′-sulfonyl-Gly-L-Phe, enzymatically formed from the substrate 4-dimethylaminoazobenzene-4′-sulfonyl-Gly-L-Phe-Gly, after separation by high-performance liquid chromatography (HPLC) using a TSK gel ODS-80TM reversed-phase column by isocratic elution. This method is sensitive enough to measure 4-dimethylaminoazobenzene-4′-sulfonyl-Gly-L-Phe at concentrations as low as 1 pmol and yield highly reproducible results and requires less than 7.5 min per sample for separation and quantitation. The pH optimum for glycine carboxypeptidase activity was 4.8 to 5.4. The K_m and V_{max} values were respectively 21.1 μmol and 3.73 pmol/μg/h with the use of enzyme extract obtained from bovine pituitary. Glycine carboxypeptidase activity was strongly inhibited by Ag^+, Cu^{2+} and *p*-chloromercuriphenylsulfonic acid. Among the organs examined in a mouse, the highest specific activity of the enzyme was found in testis. The sensitivity and selectivity of this method will aid in efforts to examine the physiological role of this peptidase.

Keywords: Glycine carboxypeptidase; Enzymes

1. Introduction

Most neuropeptides and peptide hormones are synthesized as large precursor proteins, and these precursors have been shown to undergo tissue- and region-specific processing [1–4]. Initial cleavage at pairs of basic amino acid residues is regulated by an endopeptidase such as furin or prohormone convertase 2 and 3 [5]. Following endoproteolytic action, a carboxypeptidase activity is required to remove the basic amino acids from the C-terminus of the peptides [6]. After the action of the carboxypeptidase, peptides with a C-terminal glycine residue are converted into the C-terminal amide by pep-

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tidylglycine α-amidating monooxygenase [7]. Conversely, the physiological process of inactivation of biologically active peptides by enzymes may be important in the regulation of the biological activity of their peptides. Several peptidases, known as ectoenzymes, are expressed on cell surfaces as integral membrane proteins with their catalytic sites directed towards the extracellular substrates [8]. Some of the ectopeptidases, including neutral endopeptidase-24.11 and aminopeptidase M, are believed to be physiologically important inactivators of neuropeptides in the central nervous system [9].

An estimated one-half of all biologically active peptides have an α-amide structure at their carboxyl termini. Among the remaining nonamidated-peptides, several peptides such as bovine adrenal medulla docosapeptide (BAM-22P) [10] and γ-melanocyte stimulating hormone (γ-MSH) [11] have a glycine residue at their carboxyl termini, and glycine carboxypeptidase (GCP) (EC 3.4.17.4) may be involved in the inactivation pathway of these peptides.

The most commonly used assay system for detecting and estimating GCP activity is based on the ability of enzyme preparation to convert the synthetic peptide Z-Gly-Leu to the corresponding Z-Gly and Leu. The degree of conversion to product is determined by derivatization of Leu formed enzymatically with ninhydrin reagent [12].

In this paper, we describe a new and sensitive assay method for GCP activity using 4-dimethylaminoazobenzene-4′-sulfonyl-Gly-L-Phe-Gly (dabsyl-Gly-Phe-Gly) as substrate by high-performance liquid chromatography (HPLC) on a reversed-phase column to achieve a rapid and selective separation of substrate and product. This system possesses several distinct advantages when compared with the above procedures and, therefore, is suitable for a routine assay of GCP activity. This assay may also be useful means for studying the roles of GCP in the catabolic pathway of biologically active peptides.

2. Experimental

2.1. Chemicals

4-Dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl-chloride), ascorbic acid, 1,10-phenanthroline monohydrate, bacitracin and soybean trypsin inhibitor were purchased from Wako (Tokyo, Japan). N-Ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, *p*-chloromercuriphenylsulfonic acid (PCMS), diisopropylfluorophosphate (DFP), iodoacetic acid (IAA), β-nicotinamide adenine dinucleotide (β-NAD), β-nicotinamide adenine dinucleotide, reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate (β-NADP), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), reduced glutathione and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Gly-L-Phe-Gly and Gly-L-Phe were from Bachem Feinchemikalien (Bubendorf, Switzerland). Gly-L-Phe-NH_2 was from Novabiochem (Läufelfingen, Switzerland). Acetonitrile was of chromatographic grade (Wako). Other chemicals and solvents were of analytical-reagent grade.

2.2. Preparation of enzyme source

Two different enzyme preparations were used.

(i) Bovine pituitaries were obtained from a local slaughter house. Extracts of bovine pituitaries were prepared and subjected to ammonium sulfate precipitation according to the procedure of Murthy et al. [13].

(ii) Male ICR mice weighting 20–25 g were purchased from Charles River (Japan) and housed on a 12 h light–dark cycle for at least 1 week before the beginning of experiments. Food and water were available ad libitum. All procedures were performed at 4°C unless indicated otherwise. Mice were killed by decapitation. After washing the mouse organs with saline, they were cut into small pieces and homogenized in nine volumes of 0.25 mol and/or 0.32 mol sucrose with a glass–PTFE homogenizer. The homogenates were used as an enzyme source for the investigation on localization of the enzyme activity.

2.3. Peptide synthesis

Dabsyl-Gly-Phe-Gly was synthesized by the method of Lin and Chang [14] with minor modifications. In brief, 180 μmol of Gly-L-Phe-Gly was dissolved

in 5 ml of 50 mmol sodium carbonate–sodium bicarbonate buffer. To this peptide solution, 45 μmol of dabsyl chloride in 5 ml of acetone was added. The tightly capped mixture was allowed to react at 70°C for 15 min in a water bath with constant shaking, and thereafter the acetone was evaporated under reduced pressure. 4-Dimethylaminoazobenzene-4′-sulfonyl-Gly-L-Phe (dabsyl-Gly-Phe) and 4-dimethylamino-azobenzene-4′-sulfonyl-Gly-L-Phe-NH_2 (dabsyl-Gly-Phe-NH_2) were also prepared in a similar manner using Gly-L-Phe and Gly-L-Phe-NH_2 as the starting materials, respectively. All synthetic dabsylated products were purified by reversed-phase HPLC on a TSK gel ODS-80TM column (300×7.8 mm I.D., Tosoh) before use [15]. Following purification, their structures were confirmed by nuclear magnetic resonance and infrared spectra.

2.4. Assay for glycine carboxypeptidase activity

The principle of the assay method for GCP activity is based on the colorimetric measurement at 460 nm of dabsyl-Gly-Phe formed enzymatically from the substrate, dabsyl-Gly-Phe-Gly, after separation by HPLC. The reaction mixture contained 50 mmol sodium acetate buffer (pH 5.2), 50 μmol dabsyl-Gly-Phe-Gly, 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 centrifugation, dabsyl-Gly-Phe-NH_2 was added to clear supernatant as the internal standard, and an aliquot of the mixture obtained was subjected to HPLC analysis. The peak area of dabsyl-Gly-Phe was measured and converted to pmol from the peak area of dabsyl-Gly-Phe-NH_2 added as an internal standard. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 pmol of substrate into the corresponding product in 1 min at 37°C.

2.5. Chromatographic conditions

Analysis of the product was performed using a Japan Spectroscopic Co. HPLC system consisting of a 880-PU pump, 875-UV variable-wavelength detector, 860-CO column oven, 880-50 degasser, 880-02 gradient unit, 802-SC system controller and 807-IT integrator. The system was operated at 35°C at a flow-rate of 0.75 ml/min employing a TSK gel ODS-80 TM (particle size, 5 μm) reversed-phase column (150×4.6 mm I.D.) fitted with a TSK guard gel ODS-80TM (15×3.2 mm I.D., particle size, 5 μm). The mobile phase consisted of 0.01 mol sodium acetate buffer (pH 3.80)–acetonitrile (45:55, v/v). The analytes were detected at 460 nm.

2.6. Protein determination

Protein concentration was measured by the Lowry method as modified by Hartree [16] using BSA as standard protein.

3. Results

This HPLC–colorimetric detection system for the measurement of dabsyl-Gly-Phe-Gly and dabsyl-Gly-Phe was found to be very sensitive. The calibration graph for dabsyl-Gly-Phe and dabsyl-Gly-Phe-NH_2 injected showed good linearity from 1 to 1000 pmol. Fig. 1 shows the chromatographic patterns of the reaction mixture after incubation with 10.7 μg of protein prepared from bovine pituitary for 5 h. The blank incubation (Fig. 1A) contained dabsyl-Gly-Phe-Gly and dabsyl-Gly-Phe-NH_2, and the standard incubation contained exogenous dabsyl-Gly-Phe in addition to dabsyl-Gly-Phe-Gly and dabsyl-Gly-Phe-NH_2 (Fig. 1B). The retention times for dabsyl-Gly-Phe-Gly, dabsyl-Gly-Phe, and dabsyl-Gly-Phe-NH_2 were 4.2, 5.2 and 6.8 min, respectively (Fig. 1A, B). The experimental incubation under the standard assay conditions (Fig. 1C) showed a significant amount of dabsyl-Gly-Phe at 5.2 min, whereas the control incubation did not show any peak of dabsyl-Gly-Phe (Fig. 1D).

The enzyme reaction was found to be linear with time at 37°C at least for about 6 h (data not shown).

The pH dependence of enzyme activity was investigated in 50 mmol sodium acetate buffer (pH 3.4–6.6). The catalytic activity of the enzyme was greatest at a pH of approximately 4.8–5.4, with very little activity below pH 4.2 and above pH 6.4 (Fig. 2).

GCP activity was investigated as a function of the amount of enzyme extract obtained from bovine

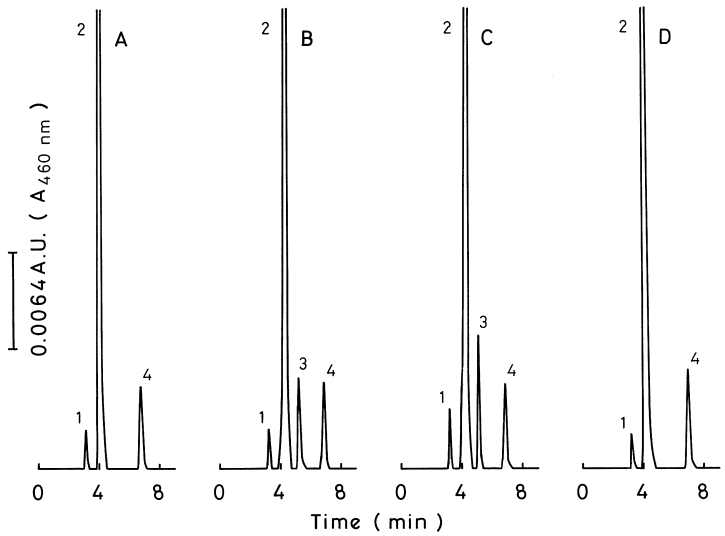

Fig. 1. HPLC elution patterns of GCP activity determined using enzyme in bovine pituitary. Conditions are described in Section 2.5. Peaks: 1=unknown compound; 2=dabsyl-Gly-Phe-Gly; 3=dabsyl-Gly-Phe; 4=dabsyl-Gly-Phe-NH_2. 300 pmol of dabsyl-Gly-Phe-NH_2 (internal standard) was added to each sample after incubation. (A) Blank incubation: dabsyl-Gly-Phe-Gly was incubated without enzyme at 37°C for 5 h. (B) Standard incubation: 300 pmol of dabsyl-Gly-Phe was added to a sample tube before incubation as a standard sample. The two peak areas of dabsyl-Gly-Phe and dabsyl-Gly-Phe-NH_2 correspond to 50 pmol. (C) Experimental incubation: dabsyl-Gly-Phe-Gly was incubated with bovine pituitary extract at 37°C for 5 h. (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.

pituitary. Perfect linearity was observed for plots of the amount of dabsyl-Gly-Phe, at least from 0.19 to 2.15 units, formed enzymatically from dabsyl-Gly-Phe-Gly against those of enzyme (data not shown).

Various reduced and oxidized flavin and pyridine nucleotides (FAD, FMN, β-NADPH, β-NADP, β-NADH, β-NAD), as well as ascorbate and reduced glutathione, were examined for their ability to stimu-

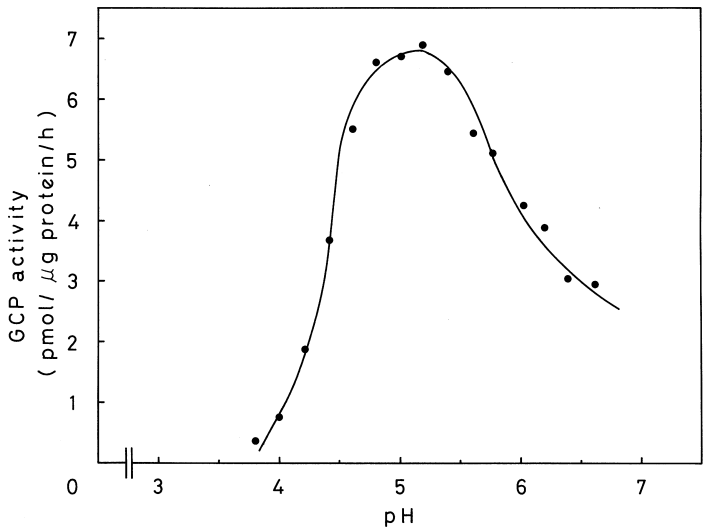

Fig. 2. Effects of pH on GCP activity in bovine pituitary: 50 mmol sodium acetate buffer (pH 3.4–6.6) was used. Incubation was carried out at 37°C for 5 h.

late the GCP activity. All of cofactors tested had no stimulating effect on the GCP activity (data not shown).

A Lineweaver–Burk plot was obtained from the effect of the concentration of dabsyl-Gly-Phe-Gly on the rate of formation of dabsyl-Gly-Phe by GCP. The Michaelis constant (K_m) and the maximum velocity (V_{max}) toward the dabsyl-Gly-Phe-Gly were calculated to be 21.1 μmol and 3.73 pmol/μg/h, respectively.

We applied this standard assay method for the determination of the effects of various metal ions and protease inhibitors on GCP activity in bovine pituitary extract. As shown in Table 1, metals such as Ag^{+} and Cu^{2+} completely inhibited the enzyme activity at 1 mmol, whereas Mn^{2+}, Ca^{2+} and Mg^{2+} had no effect. 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

Table 1
Effects of various metal ions and inhibitors on GCP activity in bovine pituitary

Reagent	Final concentration (m*M*)	GCP activity (% of control)
None		100
$MnCl_2$	1	104.2
	0.1	106.3
$CaCl_2$	1	105.5
	0.1	108.9
$MgCl_2$	1	103.4
	0.1	109.8
$ZnSO_4$	1	69.6
	0.1	97.6
$CuSO_4$	1	0
	0.1	61.0
$AgNO_3$	1	0
	0.1	0
EDTA	1	96.1
	0.1	105.0
1,10-Phenanthroline	1	74.7
	0.1	95.6
Diisopropylfluorophosphate	1	24.3
	0.1	32.3
Phenylmethylsulfonyl fluoride	1	21.4
	0.1	78.1
Iodoacetic acid	1	94.8
	0.1	110.5
N-Ethylmaleimide	1	100.9
	0.1	100.5
p-Chloromercuriphenyl-sulfonic acid	1	0
	0.1	0
Bacitracin	50 μg/ml	100.6
Soybean trypsin inhibitor	20 μg/ml	106.2
Pepstatin A	20 μg/ml	109.9

Table 2
Distribution of GCP activity in mouse organs

Organ	GCP activity (pmol/μg protein/h)
Spleen	0.249±0.013
Brain	0.068±0.002
Kidney	0.067±0.003
Liver	0.069±0.003
Lung	0.115±0.021
Testis	0.507±0.032

Note: values are means±S.E. for six experiments.

activity completely at a final concentration of 0.1 mmol. However, there was no inhibition by other thiol protease inhibitors such as IAA and NEM. Furthermore, GCP activity was not affected by bacitracin, soybean trypsin inhibitor and pepstatin A (acid protease inhibitor). GCP activity was partially affected by other reagents (Zn^{2+}, DFP, PMSF).

Finally, we examined the distribution of GCP activity in various mouse organs (Table 2). The enzyme activity was determined using the homogenates obtained from spleen, brain, kidney, liver, lung and testis of a six-week-old mouse. It was found that the GCP activity was distributed unevenly in mouse organs. Among the organs examined, the highest specific activity of the enzyme was found in the testis, and the lowest in the brain, kidney and liver. A moderate level of activity was observed in the spleen.

4. 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 of earlier methods [12].

Herein we reported a new assay method for GCP activity by the HPLC–colorimetric detection system using dabsyl-Gly-Phe-Gly as substrate. The carboxyl-terminal Phe-Gly was selected because it mimics that of γ-MSH, and bovine pituitary extract was used as a source of GCP activity because γ-MSH was mainly distributed in this tissue. The proposed sensitive assay method for GCP activity has a few advantages. First, it is very sensitive. The limit of the sensitivity was about 1 pmol of dabsyl-Gly-Phe formed enzymatically. Second, the substrate and the product are separated completely in less than 7.5 min. Third, more accurate quantitation of the product and better reproducibility were guaranteed in our method by the employment of an internal standard (dabsyl-Gly-Phe-NH_2). Finally, an important structural feature of the dabsylated substrate is that the dabsyl group prevents the ionization of the amino terminus of the peptides. Thus, the net charge of the substrate remains constant in the pH range of 5 to 9. Since the structural change may complicate the assessment of pH effects on enzyme activity, the use of the dabsylated substrate permits the unambiguous study of pH effects.

Since the physiological role of GCP in animals has not been investigated so far, we described a few physiological properties of GCP in bovine pituitary in order to search for it in the central nervous system. The effects of various chemical reagents and protease inhibitors on GCP activity are investigated (Table 1). The GCP activity in bovine pituitary is completely inhibited by metals such as Ag^{+} and Cu^{2+} and by typical thiol protease inhibitor (PCMS), but not by other metals (Mn^{2+}, Ca^{2+} and Mg^{2+}), EDTA, other thiol protease inhibitors (IAA and NEM), acid protease inhibitor and trypsin inhibitor, indicating that this enzyme is a metallo-carboxypeptidase with thiol dependence. The inhibition pattern on GCP activity described above is not similar to that of the enzymes that have been purified from brewer's yeast. The GCP activity in brewer's yeast was partially inhibited by EDTA and IAA, whereas DFP, a typical serine protease inhibitor, had no effect on enzyme activity [12]. The optimum pH of the GCP purified from brewer's yeast is slightly acidic (pH 6.0–6.2). In the present study, we showed that the enzyme in bovine pituitary has a different pH optimum (around 5.0). However, it is not clear whether the use of different substrates could be responsible for some of the differences observed.

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

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