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Short communication

Highly sensitive high-performance liquid chromatography–fluorimetric assay method for carboxypeptidase H activity

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Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC)–fluorimetric assay method has been developed for the determination of carboxypeptidase H activity based on the measurement of N-(5-dimethylaminonaphthalene-1-sulfonyl)glycine (dansyl-Gly) formed enzymatically from dansyl-Gly-L-Lys or dansyl-Gly-L-Arg. Dansyl-Gly is eluted faster than the substrates with an N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) buffer at pH 7.0 containing methanol, but eluted slower with an acidic buffer at pH 4.6. The new HPLC method separates the product and substrate in less than 5 min using an elution buffer at pH 7.0 containing 60% methanol. Using this method carboxypeptidase H activity has been detected in rat sciatic nerves. This HPLC method facilitates the assay of carboxypeptidase H activity in the enzyme samples from various tissues.

1. Introduction

Biologically active peptides have been proposed to be released by a post-translational processing pathway [1–4]. Initial cleavage at pairs of basic amino acid residues is regulated by an endopeptidase. Following endoproteolytic action, a carboxypeptidase is required to remove the basic amino acids from the C-terminus of the peptides [5–7]. Among many carboxypeptidases, carboxypeptidase H (CPH, EC 3.4.17.10) (previously referred to as "carboxypeptidase B-like enzyme" [8], "enkephalin convertase" [5], and "carboxypeptidase E" (CPE) [7,9]) is thought to be a putative prohormone processing enzyme [1,10,11].

In previous studies on the CPH assay, mainly three different methods have been reported: HPLC–UV detection using 3(2-furyl-acryloyl)-L-Ala-L-Lys (FA-Ala-Lys) or FA-Ala-Arg as a substrate [12], extraction–fluorimetry using dansyl-L-Phe-L-Ala-L-Arg [6,13] and extraction–radioisotopic assay using [^{3}H]benzoyl-L-Phe-L-Ala-L-Arg [8,14] and [^{125}I]acetyl-L-Tyr-L-Ala-L-Arg [15].

In the present study, we have developed a

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rapid and highly sensitive HPLC–fluorimetric CPH assay method. The method combines the advantages of the HPLC–UV method and extraction–fluorimetry which utilizes dansyl-Gly-Lys or dansyl-Gly-Arg as an enzyme substrate and detects highly fluorescent dansyl-Gly liberated from the substrate.

2. Experimental

2.1. Materials

Dansyl-Gly was purchased from Tokyo Kasei (Tokyo, Japan). Dansyl-Gly-Arg and dansyl-Gly-Lys were synthesized by a conventional liquid-phase method with N-hydroxysuccinimide and N,N′-dicyclohexyl carbodiimide [16]. Male Wistar rats weighing 200–300 g were obtained from the Sankyo laboratory (Tokyo, Japan) and housed with ad libitum access to chow and water. A 12:12 light–dark cycle was maintained over 10 days. The animals were killed by decapitation and the pituitary glands, dorsal root ganglia and sciatic nerves were immediately separated on ice. The sciatic nerves were divided into two groups.

2.2. Sample preparation

Each tissue was minced by scissors and homogenized by sonication in 100 μl of 25 m*M* sodium acetate buffer (pH 5.5) containing 100 m*M* NaCl. The homogenate from rat pituitary gland or from one group of the sciatic nerves was used for the enzyme activity measurement. The homogenate from the dorsal root ganglia or from the other group of the sciatic nerves was centrifuged at 100 000 *g* for 60 min and the precipitate was resuspended in 100 μl of the same buffer by sonication. Both soluble and precipitated fractions were used as the soluble and membrane-associated enzyme, respectively.

2.3. Determination of carboxypeptidase H enzyme activity

The enzyme activity was measured by the HPLC–fluorimetric method as described below. The reaction mixture (100 μl of total volume) in a 500-μl Eppendorf tube with cap consisted of 25 m*M* sodium acetate buffer (pH 5.5), 100 m*M* NaCl, 10 μ*M* dansyl-Gly-Lys (Arg), and the enzyme sample. The mixture was incubated at 37°C for 15 h. The reaction was terminated by heating for 5 min in a boiling-water bath. After centrifugation at 10 000 *g* for 5 min, 20 μl of the supernatant was analyzed by HPLC. The enzyme activity is determined by the calibration curve of standard dansyl-Gly on HPLC, and is expressed as pmol of dansyl-Gly formed per hour for each sample.

2.4. HPLC analysis

An HPLC system (JASCO) equipped with a C_{18} reversed-phase polymer column (BAS C_{18}, 120 × 4 mm I.D., 5 μm) (BAS, Tokyo, Japan) was used for the analysis. HPLC was carried out at a flow-rate of 0.8 ml/min with a mobile phase composed of 10 m*M* Hepes buffer (pH 7.0) containing 60% methanol. Peaks were monitored with a fluorimeter (821FP, JASCO) using excitation at 333 nm and emission at 533 nm.

2.5. Statistics

Data are represented as a mean ± S.E.M. and analyzed by a two-tailed unpaired Student's *t*-test.

3. Results

As CPH-like activity in the homogenate, soluble and membrane-associated samples decreased to about one tenth by freezing and thawing (data not shown), the enzyme assay was started one day after the homogenization of each sample. The enzyme reaction was almost linear for 15 h in the presence of a small amount of protein and half of the substrate in the reaction mixture. This phenomenon was also found in another processing enzyme, peptidyl-glycine α-amidating monooxygenase [16]. After 15 h, the enzyme reaction did not increase linearly with time.

The retention times of dansyl-Gly, dansyl-Gly-Lys and dansyl-Gly-Arg on HPLC–fluorimetry

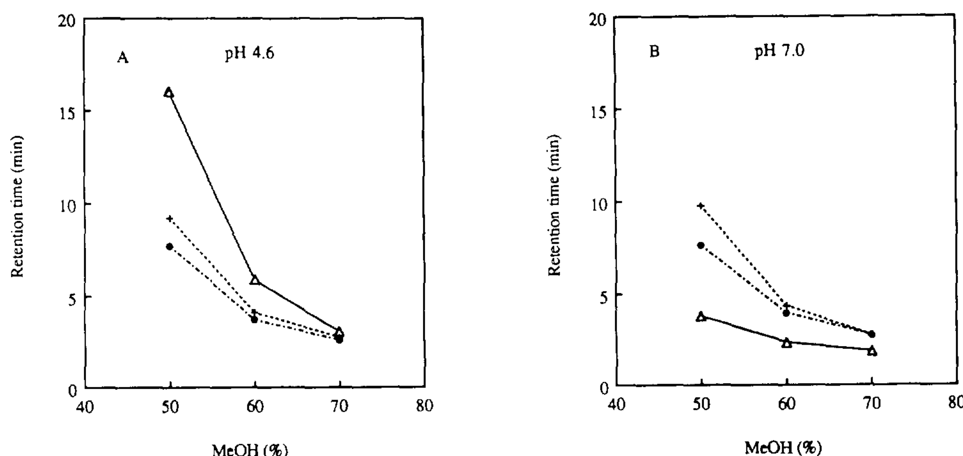

Fig. 1. Effects of different concentrations of methanol and different pH values on retention times of dansyl peptides in HPLC–fluorimetry. Detailed experimental conditions are described in Section 2. (A) Elution buffer was 10 m*M* sodium acetate (pH 4.6) plus different concentrations of methanol (50:70, v/v). (B) Elution buffer was 10 m*M* Hepes-Na (pH 7.0) + methanol (50:70, v/v). Each dansyl peptide was 10^{-6} *M*; (△) dansyl-Gly, (+) dansyl-Gly-Arg, (●) dansyl-Gly-Lys.

were examined as a function of pH and methanol content of elution buffer (Fig. 1). As shown in Fig. 1, the methanol concentration at pH 4.6 significantly affected the retention time of dansyl-Gly. Dansyl-Gly was eluted slowly with a lower methanol concentration (50% methanol: 17.0 min) and eluted rapidly with a higher methanol concentration (70% methanol: 3.0 min). At pH 7.0 (Fig. 1B), however, the retention time of dansyl-Gly was not much affected by the methanol content in the solvent. Comparatively, dansyl-Gly-Arg and dansyl-Gly-Lys were eluted with similar retention times at both pH values (Figs. 1A,B). From these data, 60% (v/v) methanol in 10 m*M* Hepes buffer (pH 7.0) was chosen as elution solvent.

The HPLC profiles of the enzyme reaction mixtures in which rat pituitary gland (0.1 μg protein/tube), rat sciatic nerves (1.9 μg/tube) or control (without the enzyme sample) was added, are shown in Figs. 2A–C, respectively. The elution pattern of standard dansyl-Gly (20 pmol) is also shown (Fig. 2D). The chromatograms in Figs. 2A,B feature two peaks arising from dansyl-Gly and dansyl-Gly-Lys. The product (dansyl-Gly, peak 1) by the CPH-like hydrolysis was eluted at 2.5 min and the substrate (dansyl-Gly-Lys, peak 2) was eluted at 4.0 min. These results suggested that the CPH-like activity in rat sciatic nerves was about 50 times lower than that in rat pituitary gland. The calibration curve was linear from 100 fmol to 10 nmol of dansyl-Gly (data not shown).

The CPH-like activity both in rat pituitary gland and in rat sciatic nerves also showed a linear relationship with the wet weight of each tissue used in one test tube (Fig. 3). For the pituitary gland, the CPH-like activity (2–26 pmol/h) was directly proportional to the tissue's wet weight used between 1.5 and 37 μg/tube, while for the sciatic nerves the enzyme activity (0.5–3.6 pmol/h) was also proportional to the tissue's wet weight used between 10 and 110 μg/tube. These results demonstrate that the CPH-like activity varies in proportion to the amount of CPH-like enzyme in the range of tissue weights used, and that the activity in the sciatic nerves is 20-fold less than that in the pituitary gland.

We investigated the CPH-like activity in the soluble and membrane-associated fractions of rat sciatic nerves and dorsal root ganglia (Fig. 4). As shown in Fig. 4, the CPH-like activity was detected in each fraction. For the sciatic nerves, the CPH-like activity was 6.5 pmol h^{-1} cm^{-1} in the soluble fraction and was 13 pmol h^{-1} cm^{-1} in the membrane-associated fraction. For the dorsal root ganglia, the activities were 3 and 12 pmol/h

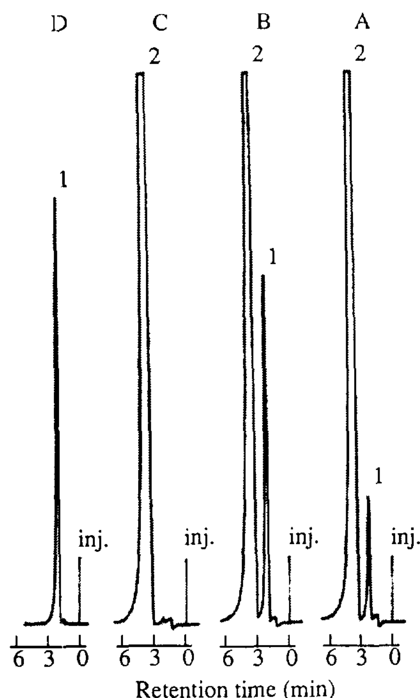

Fig. 2. HPLC–fluorescent elution patterns of carboxypeptidase H-like activity in the homogenates of rat pituitary gland and sciatic nerves. (A, B and C) Reaction mixture (100 μl of total volume) contained 25 m*M* sodium acetate buffer (pH 5.5), 100 m*M* NaCl, 10 μ*M* dansyl-Gly-Lys and enzymes (A: 1.9 μg protein of rat sciatic nerve homogenate; B: 0.1 μg protein of pituitary gland; C: without enzyme). The reaction was performed at 37°C for 15 h, and stopped by heating in a boiling-water bath for 5 min. After centrifugation at 10 000 *g* for 5 min, 20 μl of the supernatant was subjected to HPLC analysis [10 m*M* Hepes-Na (pH 7.0)–methanol (4:6), flow-rate 0.8 ml/min]. (D) 20 pmol of standard dansyl-Gly. 1: dansyl-Gly; 2: dansyl-Gly-Lys; inj.: injection point.

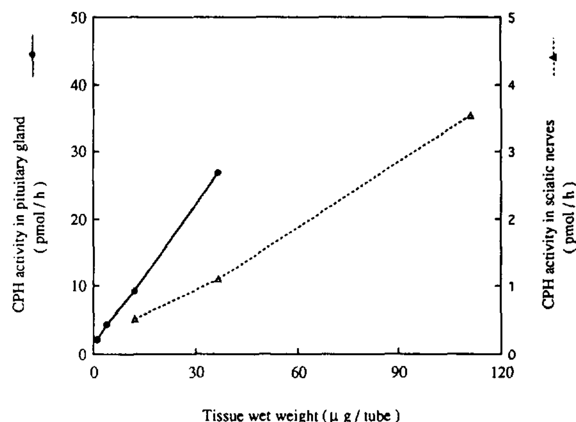

Fig. 3. Carboxypeptidase H-like activity at various concentrations of the enzymes in rat pituitary gland and sciatic nerves. Sample preparation and assay conditions are described in Section 2. The enzyme activity is expressed as pmol/h of dansyl-Gly formed enzymatically. (●) Rat pituitary gland, (△) sciatic nerves.

per gland in the soluble and membrane-associated fractions, respectively.

4. Discussion

Several assay methods for CPH and other carboxypeptidase B-like enzymes have been reported which are based on radiometric [8,14,15], fluorimetric [6,13] and UV absorbance [12] assays combined with either extraction technique or HPLC [12].

Although detection of as little as 0.2 pg of CPE can be achieved with the radiometric assay for CPE especially using ^{125}I-Ac-Tyr-Ala-Arg [15], the extraction procedure of transferring the enzymatic product to a non-aqueous phase may be somewhat tedious and the need of handling the radioactive compounds requires special facilities and care, which limits its utilization. The fluorimetric and UV absorbance assays are generally less sensitive than the radiometric assay [15]. However, the HPLC–UV absorbance assay method developed by Grimwood et al. [12] may be convenient for the measurement of carboxypeptidase activity. Their HPLC analysis by the detection of FA-Ala formed from FA-Ala-Arg as an enzyme substrate can be completed within 7 min per one run: the enzyme product and substrate are eluted at 6.1 min and 4.3 min, respectively, at a flow-rate of 2.0 ml/min.

In the present study, we have developed a rapid and sensitive HPLC–fluorimetric CPH assay method. Dansyl-Gly-Lys and dansyl-Gly-Arg were synthesized and used as a fluorescent substrate for CPH. After the enzyme reaction is stopped, highly fluorescent dansyl-Gly liberated

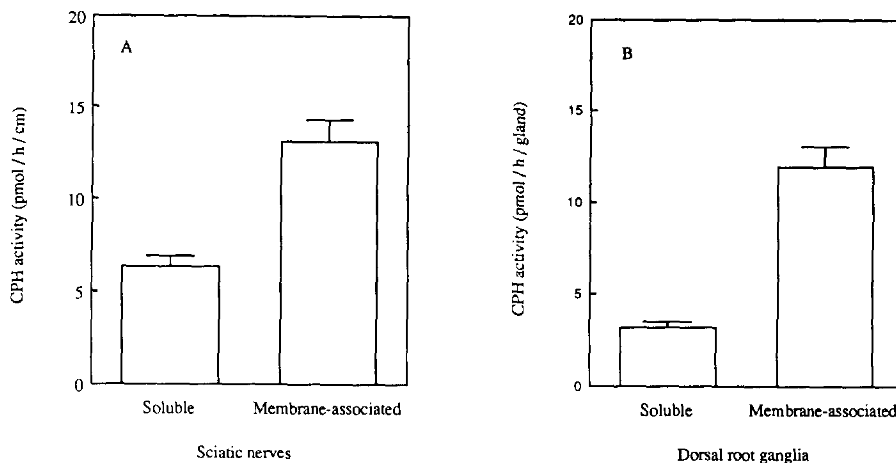

Fig. 4. Carboxypeptidase H-like activity in the soluble and membrane-associated fractions in rat sciatic nerves and dorsal root ganglia. Sample preparation for both fractions is described in Section 2. (A) Rat sciatic nerves. (B) Dorsal root ganglia. Data are shown as mean ± S.E.M. of 4 animals.

from the substrate is analyzed by means of HPLC. By examining the HPLC conditions, we have found that dansyl-Gly is eluted faster than dansyl-Gly-Lys or dansyl-Gly-Arg using a mobile phase of 60% methanol in 10 m*M* Hepes buffer (pH 7.0). Under these conditions dansyl-Gly and dansyl-Gly-Arg are eluted in 2.5 min and 4.0 min, respectively, with almost complete separation (Fig. 2), and as little as 30 fmol of dansyl-Gly can be detected. This enables us to quantitate dansyl-Gly in less than 5 min and to analyze CPH-like enzymes directly from a variety of samples.

Elution behaviour of the dansyl compounds in HPLC is largely dependent on the pH of the buffer used as one of the eluent components and on the methanol content (Fig. 1). Methanol shortens the retention time of the dansyl derivatives in HPLC as shown in Fig. 1. At pH 7.0, dansyl-Gly is eluted faster than dansyl-Gly-Lys and dansyl-Gly-Arg, whereas, at pH 4.6, dansyl-Gly is eluted slower than dansyl-Gly-Lys and dansyl-Gly-Arg. If the carboxyl group of dansyl-Gly is dissociated to a negatively charged carboxyl anion at pH 7.0 and is present as an uncharged acidic form at pH 4.6, this phenomenon could be explained in terms of the difference in hydrophobic adsorption ability between the anion form and the acidic form of the carboxyl group of dansyl-Gly and presumably the pK_a of the dansyl-Gly. In contrast, there may be no change in ionized forms of dansyl-Gly-Lys and dansyl-Gly-Arg between both pH values and then the retention times are similar at different pH values. Based on these results, we have chosen 60% methanol in 10 m*M* Hepes buffer (pH 7.0) as elution solvent in reversed-phase HPLC and studied CPH-like activity in various tissues.

Using our HPLC–fluorimetric CPH assay method, we have investigated the distribution of CPH-like activity in the soluble and membrane-associated fractions of rat sciatic nerves and dorsal root ganglia. As a result, it has been found that there is an active form of CPH not only in rat sciatic nerves but also in rat dorsal root ganglia (neuronal cell body), and in both fractions (Fig. 4). Hook et al. [11] have reported that in the hypothalamo-neurohypophysial system of rats, CPH in the neuronal perikarya of the supraoptic nucleus is almost inactive, while the enzyme in the nerve terminals of the posterior pituitary is active. From those results, together with their immunoblotting analysis, they have insisted on the axonal transport of CPH in the rat's hypothalamo-neurohypophysial system. Although we have observed CPH-like activity in

the dorsal root ganglia, the discrepant result from Hook et al. may be merely due to different experimental conditions such as different tissues.

In summary, we have developed a rapid and sensitive HPLC–fluorimetric assay method for CPH determination by detecting highly fluorescent dansyl-Gly from CPH action on dansyl-Gly-Arg. Using this method we found the CPH-like activity in rat sciatic nerves and its axonal transport. This method will also be applicable to other carboxypeptidases such as carboxypeptidase B.

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