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# ASSAY FOR ENKEPHALIN-DEGRADING PEPTIDASES IN RAT BRAIN TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE POST-COLUMN FLUORESCENCE DETECTION

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## SUMMARY

The activities of enkephalin-degrading peptidases such as enkephalinases A and B in rat brain tissues were simultaneously assayed by a high-performance liquid chromatographic method with fluorimetric detection with an automatic reaction system. Tyrosine and tyrosine-containing peptides produced enzymatically from the substrate, methionine-enkephalin, were separated by gradient elution on a reversed-phase column (TSK gel ODS-120T), and then converted into fluorescent derivatives for detection by reaction with hydroxylamine, cobalt(II) and borate reagents. The method permits the simple and sensitive detection of N-terminal tyrosine-containing fragments of the enkephalin peptide. The limits of detection are 5–20 pmol per assay tube for the N-terminal tyrosine-containing fragments. The enzyme activities in the regionally separated tissues were 54–191 pmol/min·mg protein for enkephalinase A and 79–153 pmol/min·mg protein for enkephalinase B, which were calculated from the formation of Tyr–Gly–Gly and Tyr–Gly, respectively, during the enzyme reaction.

## INTRODUCTION

The endogenous methionine-enkephalin (ME; Tyr–Gly–Gly–Phe–Met) and leucine-enkephalin (LE; Tyr–Gly–Gly–Phe–Leu) have opiate-like activity in the nervous system [1]. Recently, these opioid peptides have been found to act in vivo as neurotransmitters via interaction with opiate receptors [2]. Therefore, it is important to determine whether specific enzymes are responsible for the physiological inactivation of the released peptides.

Several peptidases for the degradation of enkephalins have been demonstrated to be present in brain tissues; they are aminopeptidase (EC 3.4.11.2) [3], dipeptidyl carboxypeptidase (EC 3.4.24.11), known as enkephalinase (Ease) A [4],

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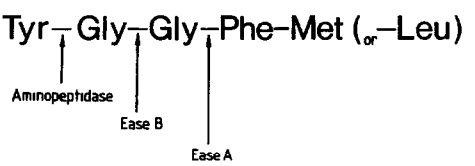


Fig. 1. Enzymatic hydrolysis of enkephalins by brain homogenates.

and dipeptidyl aminopeptidase (EC 3.4.14.4), known as Ease B [5]. These enzymes cleave at different peptide bonds in the enkephalin molecule (Fig. 1).

Of the enzymes, aminopeptidase does not have a specific function for the enkephalin degradation in vivo [6] because of its localization in brain tissues and its affinity for enkephalins, although this enzyme generally reveals a high activity in brain homogenates. However, Ease A has a significant role in the physiological inactivation of enkephalins in vivo [4,7] because enkephalins perfused into the cerebroventriculus of a rat brain are mainly degraded at the Gly–Phe bond, and further because the distribution of Ease A in cellular fractions is parallel to that of enkephalin receptor. Ease B is not sufficiently characterized.

For the assay of the above enzymes in tissue samples, radiochemical methods [8–11] coupled with high-performance liquid chromatography (HPLC) or thin-layer chromatography have usually been employed, using tritiated enkephalins as the substrate, in which the radioactivities of the degradation products, Tyr, Tyr–Gly and Tyr–Gly–Gly, are measured after the chromatographic separation of the products.

We previously developed a simple method [12] for the determination of N-terminal tyrosine-containing peptides involving ME and LE by reversed-phase HPLC with on-line post-column fluorescence detection utilizing a novel reaction with hydroxylamine, cobalt(II) and borate reagents [13]. The post-column derivatization system yields an intense fluorescence with maximum wavelengths for excitation and emission near 335 and 435 nm, respectively, for N-terminal tyrosine-containing peptides. Therefore, the peptide fragments of enkephalins that retain a tyrosyl residue at the N-terminus of the peptide can be detected selectively at the picomole level on-column using fluorescence detection. Free Tyr is also detectable with this detection system. However, the fluorescence of Tyr is approximately one fifth that of the N-terminal tyrosine-containing fragments.

This paper describes a practical application of the method to the simple and sensitive assay of the activities of enkephalin-degrading enzymes, particularly of Eases A and B in crude samples of rat brains. The striatum region of the brains as the enzyme preparation and ME as the substrate were employed to establish the assay procedure.

## EXPERIMENTAL

### *Chemicals and solutions*

The following synthetic peptides were purchased from the Peptide Institute (Osaka, Japan) or Sigma (St. Louis, MO, U.S.A.): Tyr–Gly, Tyr–Gly–Gly, Tyr–Gly–Gly–Phe, ME and LE. Captopril was a product of Sankyo (Tokyo, Japan)

and bestatin was obtained from Nihon Kayaku (Tokyo, Japan). Water was deionized and distilled before use. Other chemicals were of analytical-reagent grade. Solutions of peptides and reagents were prepared in water, unless indicated otherwise.

*Mobile phase for HPLC.* Eluents A and B were acetonitrile–0.3 *M* sodium phosphate buffer (pH 2.3)–water (1:20:79 and 3:1:1, v/v/v, respectively). The eluents were degassed before use.

*Reagent solutions for post-column derivatization.* Hydroxylamine (20 m*M*)–cobalt(II) (50 $\mu M$) solution was prepared by mixing equal volumes of 40 m*M* hydroxylamine hydrochloride and 0.1 m*M* cobalt(II) chloride hexahydrate. The mixture was usable for at least one month when stored in a refrigerator. Borate solution (0.3 *M*) was prepared by dissolving 9.27 g (0.15 mol) of boric acid in ca. 400 ml of water, adjusting the pH to 11.4 with 0.3 *M* sodium hydroxide solution and diluting to 500 ml with water. The reagent solutions were degassed thoroughly before use.

*Sample preparation*

Male Sprague–Dawley rats (220–240 g) were anaesthetized with diethyl ether and killed by dehaematization. The brain was removed quickly and separated into striatum, cortex, pituitary, hypothalamus, hippocampus and amygdala regions. The tissues were stored at −80°C until used.

A portion (ca. 100 mg) of each tissue was homogenized with 2 ml of water and the homogenate was centrifuged at 800 *g* for 10 min. The precipitate was mixed vigorously with 5 ml of 1 mg/ml Triton X-100 solution. The mixture was allowed to stand for 1 h, then centrifuged at 2000 *g* for 25 min. The above procedure was carried out at 0–4°C. A portion of the supernatant was used as a sample for the enzyme reaction. The protein concentration of the enzyme sample was measured by the method of Lowry et al. [14] with bovine serum albumin as the standard protein.

*Enzyme reaction*

A 30-$\mu$l portion of the enzyme sample was quickly mixed with 50 $\mu$l of 0.5 *M* tris(hydroxymethyl)aminomethane (Tris)–hydrochloric acid buffer (pH 7.4), 50 $\mu$l of 1 m*M* bestatin as an inhibitor of aminopeptidase, 20 $\mu$l of 40 m*M* captopril as an inhibitor of angiotensin-converting enzyme (ACE) and 50 $\mu$l of 100 $\mu M$ ME as the substrate. The mixture was incubated at 37°C for 30 min. The reaction was terminated by heating at 100°C for 2 min. The mixture was then centrifuged at 2000 *g* for 25 min. A 50-$\mu$l portion of the supernatant was used for HPLC. For the enzyme blank, the same procedure was carried out but without the substrate.

*Chromatography and apparatus*

Fig. 2 shows a schematic diagram of the HPLC system for the determination of products in the enzyme reaction mixture. PTFE tubing (0.5 mm I.D.) was used to connect the column outlet and the detection system.

Enkephalins and their N-terminal tyrosine-containing fragments in a 50-$\mu$l

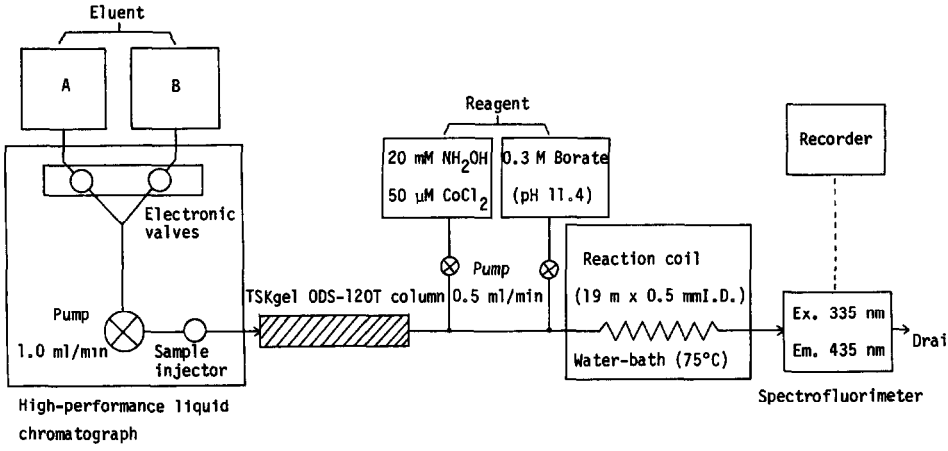


Fig. 2. Schematic diagram of on-line post-column fluorescence derivatization HPLC for the determination of N-terminal tyrosine-containing peptides.

portion of the enzyme reaction mixture were separated by gradient elution of acetonitrile in the mobile phase (eluents A and B) on a reversed-phase column (200 mm×4 mm I.D.) packed with TSK gel ODS-120T (particle size 5 μm) (Tosoh, Tokyo, Japan). The acetonitrile gradient in the mobile phase is indicated on the right-hand axis of Fig. 3. The mobile phase was pumped at a flow-rate of 1.0 ml/min by a Hitachi 638-30 high-pressure pump fitted with a programmed electronic controller for the electronic valves placed prior to the pump inlet for various gradient elutions. The column temperature was ambient (24±4°C).

The column eluate was conducted to the fluorescence reactor system. In the system, the hydroxylamine–cobalt(II) solution and the borate solution were added to the eluate stream by a Hitachi 633 reagent-delivery pump at a flow-rate of 0.5 ml/min. The mixture was passed through a PTFE reaction coil (19 m×0.5 mm I.D.) immersed in a water-bath at 75±1°C.

The fluorescence in the last eluate was monitored at 435 nm (emission) and 335 nm (excitation) with a Hitachi 650-10LC spectrofluorimeter equipped with an 18-μl flow cell.

## RESULTS AND DISCUSSION

### *Determination of enkephalin fragments*

Fig. 3 shows the chromatograms obtained by the HPLC method with on-line fluorescence detection for the enzyme reaction mixture of a striatum sample. The degradation products (i.e., Tyr, Tyr–Gly and Tyr–Gly–Gly) and the intact substrate ME could be successfully separated and determined fluorimetrically. The retention times of these compounds were 11.6 min for Tyr, 15.5 min for Tyr–Gly–Gly, 17.5 min for Tyr–Gly and 23.0 min for ME. The HPLC method could also separate other related peptides such as Tyr–Gly–Gly–Phe and LE, the retention times of which were 20.9 and 24.2 min, respectively, under the same conditions as in Fig. 3.

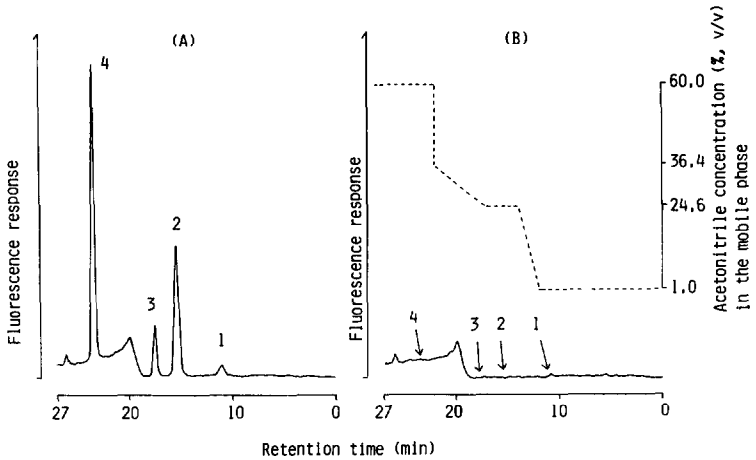


Fig. 3. Chromatograms obtained with fluorescence detection after the enzymatic reaction of (A) ME and (B) water for the blank. The conditions for the enzyme reaction and HPLC are described under Experimental. Peaks (with amounts, pmol per assay tube, in parentheses): 1 = Tyr (225); 2 = Tyr-Gly-Gly (605); 3 = Tyr-Gly (305); 4 = ME (3835). The arrows in chromatogram B indicate the retention times of the corresponding peptides and Tyr.

As previously described [13], the fluorescence detection system does not detect peptides that have no tyrosyl residue at their N-terminal position in the molecules, because these peptides are not converted into the fluorescent derivatives by the reaction with hydroxylamine, cobalt(II) and borate reagents. Therefore, the separation of other fragments (e.g., Gly–Gly–Phe–Met, Gly–Phe–Met, Phe–Met, Gly–Gly–Phe, Gly–Gly and Gly–Phe) was unnecessary. Detailed conditions for the separation and fluorescence detection were reported previously [12].

Calibration graphs (each a single plot) for the fragments, which were constructed by adding the synthetic compounds to the incubation mixture after the enzymes had been inactivated, were all linear up to at least 5 nmol per assay tube (200 $\mu$l). The correlation coefficients ($r$) for the straight lines were 0.997–0.999. The limits of detection based on a signal-to-noise ratio of 2 were 80 pmol for Tyr, 20 pmol for Tyr–Gly, 20 pmol for Tyr–Gly–Gly and 24 pmol for Tyr–Gly–Gly–Phe per assay tube, which correspond to amounts of 5–20 pmol in a 50-$\mu$l injection volume. The relative standard deviations of the peak heights for the fragments of the enkephalin peptides (2 nmol each per assay tube) were 1.8–3.4% ($n=10$). The enzyme sample used for the assay contained $106 \pm 9$ $\mu$g of protein (ca. 0.6 mg of the striatum tissue) per assay tube. The endogenous enkephalins and the related fragments were not detected by the present method, as shown in Fig. 3B.

*Assay of Eases A and B*

Eases A and B are membrane-bound enzymes [4,5]. Hence Triton X-100 was used to solubilize these enzymes from the cell membrane of brain tissue, as described elsewhere [10]. For the assay of the enzyme activities in the crude preparation, the enzyme reaction was carried out in the presence of 200 $\mu M$ bestatin and 5 m$M$ captopril in the reaction mixture. Bestatin strongly inhibits aminopeptidase B and leucine aminopeptidase [15]. In the absence of bestatin, only

Tyr was produced from the substrate ME because the rate of the catalytic reaction of the aminopeptidase is significantly higher than those of Eases A and B [6] (Fig. 4A). ACE has an Ease A-like activity [16]. However, the sample enzyme activity was inhibited efficiently by adding captopril to the incubation mixture (Fig. 4B). Captopril is a specific inhibitor of ACE [18].

LE and the related peptides were individually examined as the substrate instead of ME for the enzyme assay, and the resulting products and their production amounts are shown in Table I. Under the recommended conditions for the enzyme reaction, LE in addition to ME was degraded to Tyr, Tyr–Gly and Tyr–Gly–Gly and not degraded to Tyr–Gly–Gly–Phe. On the other hand, Tyr–Gly–Gly and Tyr–Gly did not give their degradation fragments. The results demon-

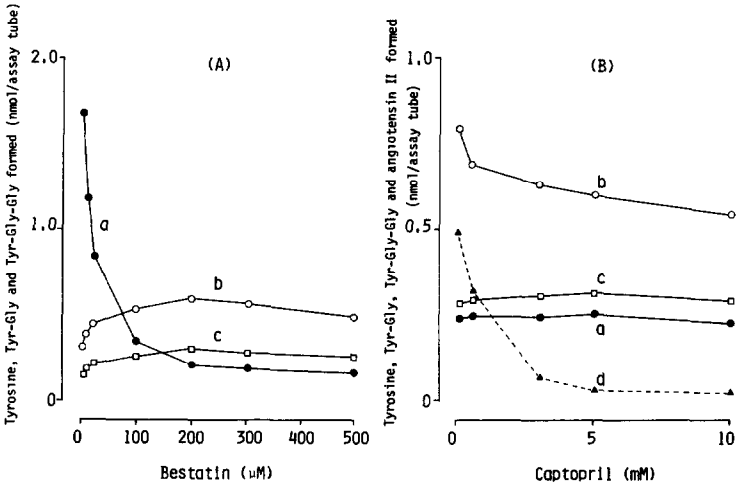


Fig. 4. Effects of the concentrations of (A) bestatin and (B) captopril on the formation of Tyr, Tyr-Gly and Tyr-Gly-Gly from ME and angiotensin II from angiotensin I. Curves: (a) Tyr; (b) Tyr-Gly-Gly; (c) Tyr-Gly; (d) angiotensin II. The activity of ACE in the enzyme preparation was assayed by the method of Sakamoto et al. [17] using the substrate (200 $\mu M$ in the incubation mixture) of angiotensin I.

TABLE I

DEGRADATION OF ENKEPHALINS AND RELATED PEPTIDES BY ENZYMES IN BRAIN HOMOGENATE

N.D. = not detected.

| Substrate (2.5 nmol per assay tube) | Product (pmol per assay tube) | | | |
|---|---|---|---|---|
| | Tyr | Tyr-Gly | Tyr-Gly-Gly | Tyr-Gly-Gly-Phe |
| ME | 255 | 305 | 605 | N.D. |
| LE | 320 | 345 | 290 | N.D. |
| Tyr-Gly-Gly-Phe | 140 | 80 | N.D. | – |
| Tyr-Gly-Gly | N.D. | N.D. | – | N.D. |
| Tyr-Gly | N.D. | – | N.D. | N.D. |

strate that the formation of both Tyr–Gly–Gly and Tyr–Gly from enkephalins is obviously due to the catalysis of Eases A and B, respectively, and also that these products are not further hydrolysed during the incubation.

Fig. 5 shows the time course at various incubation temperatures for the enzymatic production of Tyr–Gly–Gly and Tyr–Gly. At 37°C, the production rates were both higher than those at 25, 50 and 60°C. At 60°C, neither peptide was produced. The data indicate that Eases A and B are both thermally unstable. Hence, the enzyme reaction was readily terminated by boiling at 100°C for 2 min.

In other methods [9–11] for the assay of Ease A, Tris–hydrochloric acid buffer (pH 7.4) has generally been used for the enzyme reaction, although the enzyme used was purified either completely or partially. However, the pH profile of the buffer did not show the optimum pH at 7.4 in the present assay procedure using the crude enzyme preparation. The formation of Tyr–Gly–Gly was increased at a low pH in the range 5–8. The increment for their peptide production in the acidic medium composed of Tris and hydrochloric acid may be due to the activation of ACE, as ACE is activated with chloride ion [19]. In fact, at pH 5.8 the activity of ACE in the same sample as that for Fig. 4B, which was calculated from the formation of angiotensin II from angiotensin I [17], was greatly increased at the 0.7 nmol level of angiotensin II even in the presence of the inhibitor captopril. Phosphate buffer could not be employed instead of the buffer used, however, because Ease A was strongly inhibited by phosphate ion [20]. On the other hand, the formation of Tyr–Gly was maximal at pH 6.4.

In the present method, Tris–hydrochloric acid buffer (pH 7.4) was tentatively employed in order to compare the enzyme activity obtained by the present method with those given by other methods [9–11].

The amounts of Tyr–Gly–Gly and Tyr–Gly formed enzymatically were proportional to the amounts of the sample protein up to at least 140 $\mu$g per assay tube. The Michaelis constants ($K_m$ values) were 94 $\mu M$ for Ease A and 48 $\mu M$ for Ease B.

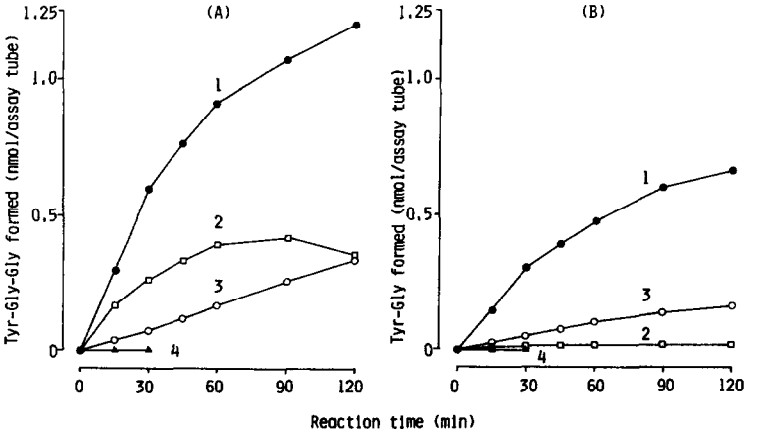


Fig. 5. Effects of incubation time and temperature on the formation of (A) Tyr-Gly-Gly and (B) Tyr-Gly. Curves: 1, 37°C; 2, 50°C; 3, 25°C; 4, 60°C.

TABLE II

ACTIVITIES OF ENKEPHALINASES A AND B IN VARIOUS TISSUES OF RAT BRAIN

| Tissue | Activity (pmol/min·mg protein) | |
|---|---|---|
| | Ease A | Ease B |
| Striatum | 191 | 95 |
| Hippocampus | 81 | 153 |
| Amygdala | 80 | 111 |
| Pituitary | 75 | 78 |
| Cortex | 74 | 157 |
| Hypothalamus | 54 | 79 |

The specific activities of Eases A and B in several regions of rat brains were measured (Table II). The Ease A activity was highest in the striatum region, as found by other workers [8,9]. In contrast, the Ease B activity was not as high in the striatum region and relatively high in the cortex and hippocampus regions.

This study has indicated that the HPLC method with on-line fluorescence derivatization using hydroxylamine, cobalt(II) and borate reagents should be useful for the simultaneous determination of N-terminal tyrosine-containing fragments produced from enkephalins by the enzyme reaction. Both the selectivity and sensitivity are sufficient to allow the assay of the enkephalin-degrading enzymes in crude samples of brain tissues.

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