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ON-LINE POST-COLUMN FLUORESCENCE DETECTION FOR N-TERMINAL TYROSINE-CONTAINING PEPTIDES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A detection system based on on-line post-column fluorescence derivatization is described for the determination of N-terminal tyrosine-containing peptides by reversed-phase high-performance liquid chromatography. The peptides are automatically converted into fluorescent derivatives by reaction with hydroxylamine, cobalt(II) and borate after peptide separation on a reversed-phase column (TSKgel ODS-120T) followed by passage through an ultraviolet absorbance detector. The reaction system permits the fluorescence detection at 435 nm (emission) with excitation at 335 nm for N-terminal tyrosine-containing synthetic peptides in as little as picomole amounts. The facile fluorescence detection of N-terminal tyrosine-containing fragments produced from methionine-enkephalin by enzymatic degradation using a rat brain homogenate was achieved by comparison with the ultraviolet absorption detection at 215 nm.

INTRODUCTION

Enkephalins (EKs), methionine–enkephalin (MEK; Tyr – Gly – Gly – Phe – Met) and leucine–enkephalin (LEK; Tyr – Gly – Gly – Phe – Leu), which have a tyrosyl residue at an N-terminus in their molecules, are bioactive pentapeptides involved in the repression of pain sensation in mammalian brain [1]. Recently, studies on the enzymatic metabolism of EKs have been undertaken [2–4].

The determination of EKs is usually carried out by radioimmunoassay (RIA). Although RIA methods [5,6] offer sufficient sensitivity for the individual peptides, there is still difficulty in preparing a specific antibody especially for the oligopeptides. High-performance liquid chromatography (HPLC) is often used for the isolation of the peptides from biological samples. Mass spectrometric detection coupled with off-line HPLC can be used to determine EKs in tissue

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extracts [7] because of its high sensitivity and structural specificity for the target peptides. However, the method is technically difficult for the simultaneous determination of biogenic peptides. HPLC detection is generally based on ultraviolet (UV) absorption between 200 and 230 nm [8] or the electrochemical activity of the tyrosyl residues in the peptide molecules [9]. However, the latter detection methods for peptides are disturbed by either various UV-absorbing components or oxidizing and reducing components in biological samples and/or in the mobile phase.

A novel fluorescence reaction using hydroxylamine, cobalt(II) and borate was previously reported by our laboratory; it produces highly fluorescent compounds for N-terminal tyrosine-containing synthetic peptides under mild conditions [10], although the chemical structure of the fluorescent products remains unknown. The manual procedure for the reaction does not permit any fluorescence development from synthetic peptides with no tyrosyl residue at the N-terminus such as Gly–Tyr and Phe–Arg–Gly. In the procedure, however, tyrosine and tyramine fluoresce slightly; their intensities are less than 5% of that given by an equimolar concentration of MEK.

In a further study, we have developed a reversed-phase HPLC method for N-terminal tyrosine-containing peptides including EKs coupled with on-line post-column fluorescence detection by means of that fluorescence reaction. As an example of the biological application of the HPLC system, the determination of N-terminal tyrosine-containing fragments produced from MEK by enzymatic degradation with rat brain homogenate prepared from the cerebral cortex region is also described.

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–Tyr, Gly–Tyr, Phe – Gly, Tyr – Gly – Gly, Tyr–Gly–Gly–Phe, MEK, LEK, vasopressin (Cys – Tyr – Phe – Gln – Asn – Cys – Pro – Arg – Gly – NH_2), kallidin (Lys – Arg – Pro – Pro – Gly – Phe – Ser – Pro – Phe – Arg) and angiotensin II (Asp – Arg – Val – Tyr – Ile – His – Pro – Phe). Water was deionized and distilled before use. Other chemicals were of analytical reagent grade.

Mobile phase for HPLC. Eluents A and B were mixtures of acetonitrile, 0.3 *M* sodium phosphate buffer (pH 2.3) and 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 μM) solution was prepared by mixing equal volumes of 40 m*M* hydroxylammonium chloride 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 of boric acid in ca. 400 ml of water, adjusting the pH to 11.0 with 0.3 *M* sodium hydroxide solution and diluting with water to 500 ml. The reagent solutions were thoroughly degassed before use.

Chromatography

Fig. 1 shows a schematic diagram of the HPLC system constructed for the analysis of N-terminal tyrosine-containing peptides. PTFE tubing (0.5 mm I.D.) (Gasukuro Kogyo, Tokyo, Japan) was used. Volumes of sample solution in the range 50–100 μl were injected. Peptides in the sample were separated on a reversed-phase column (TSKgel ODS-120T, 200×4 mm I.D., particle size 5 μm) (Toyo Soda, Tokyo, Japan) with gradient elution of acetonitrile in eluents A and B. The acetonitrile concentration in the mobile phase is indicated in Fig. 2. The mobile phase was pumped at a flow-rate of 1.0 ml/min. A Hitachi 638-30 high-performance liquid chromatograph fitted with a programming electronic controller was used. The column temperature was ambient (24±4°C).

The column eluate was first passed through a Hitachi 638-41 UV detector set at 215 nm and equipped with a flow cell (13 μl), in order to detect the intact peptides, then it was conducted to a 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 of the reagent solutions and the column eluate 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 from each N-terminal tyrosine-containing peptide in the last eluate was monitored at 435 nm (emission) with excitation at 335 nm. The spectral bandwidths for both wavelengths were 10 nm with a Hitachi 650-10LC spectrofluorimeter equipped with a flow cell (18 μl).

Preparation of rat brain homogenate

Male Wistar rats (220–240 g) were anaesthetized with diethyl ether and killed by dehaematization. The cerebral cortex region in the brain was quickly removed and stored at −80°C until used. A portion (50 mg) of the tissue was homogenized at 0–4°C with 10 ml of 40 m*M* tris(hydroxymethyl)aminomethane (Tris)–hydrochloride buffer (pH 7.4) containing 1% Triton X-100 as described previously [11]. The homogenate was centrifuged at 2000 *g* for 25 min. The supernatant was then used for the enzymatic degradation of MEK. The protein concentration in the supernatant was measured by the method of Lowry et al. [12] with bovine serum albumin as the standard protein.

Enzymatic degradation of MEK with rat brain homogenate

A portion (50 μl) of the supernatant was mixed with 100 μl of 100 nmol/ml MEK and 50 μl of water or 0.1 m*M* bestatin as an inhibitor of aminopeptidase [13]. The mixture was incubated at 37°C for 30 min and the reaction was terminated by heating at 100°C for 1 min. The mixture was centrifuged at 2000 *g* for 10 min. A 50-μl portion of the supernatant was injected into the HPLC system. Peak heights were used for the quantitative evaluation of the chromatograms.

RESULTS AND DISCUSSION

Post-column fluorescence derivatization

In the manual method [10], N-terminal tyrosine-containing synthetic peptides gave intense fluorescence when heated at 100°C for 1–5 min with hydrox-

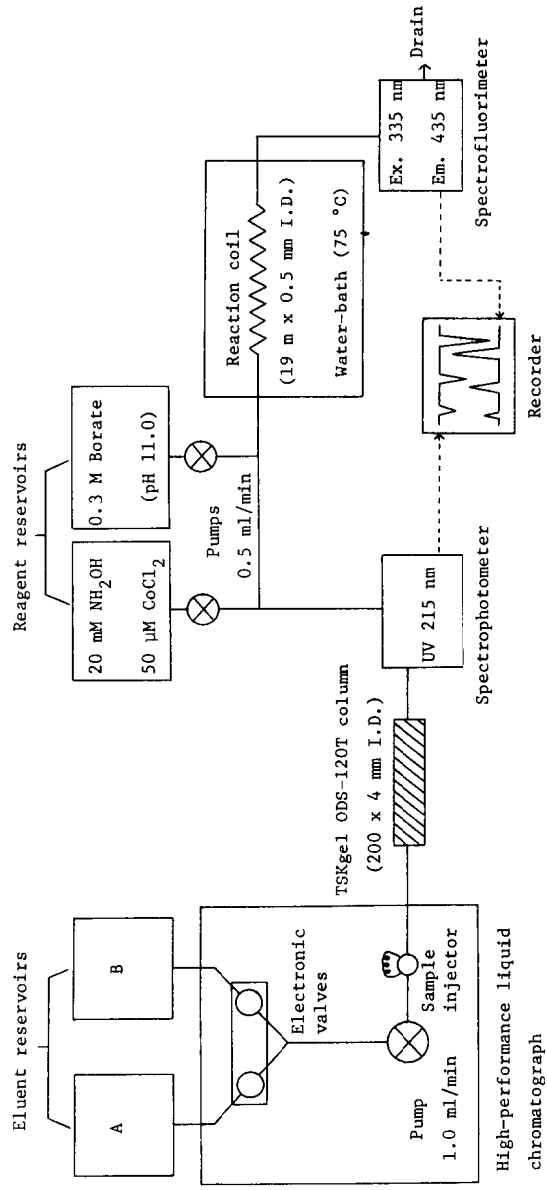

Fig. 1. Schematic diagram of post-column fluorescence derivatization for the HPLC determination of N-terminal tyrosine-containing peptides.

ylamine, cobalt(II) ion and borate in a weakly alkaline solution (pH 9 ± 0.5). When this reaction is applied to the reactor in the present HPLC system (Fig. 1), aqueous solutions of hydroxylamine, cobalt(II) and borate should be mixed continuously with the column eluate (pH 2.3). The reaction conditions of the post-column fluorescence derivatization were studied by separating six N-terminal tyrosine-containing synthetic peptides (Tyr–Gly, Tyr–Tyr, Tyr–Gly–Gly, Tyr–Gly–Gly–Phe, MEK and LEK).

Hydroxylammonium chloride (20 m*M*) and cobalt(II) chloride (50 μM) in the reagent solution (pH 4.0) provided the maximum heights of the fluorescent peaks produced from the tested peptides. The chloride ion in the solution was not essential to the fluorescence reaction and acetate and/or oxalate ions could be used instead. Maximum and almost maximum peak heights were attained at pH 11.0 of the borate solution; a concentration of 0.3 *M* gave the maximum peak height. The borate solution could not be mixed with the hydroxylamine–cobalt(II) solution before introduction into the reaction coil because of the instability of hydroxylamine in the alkaline borate solution. The mixture of the column eluate, the hydroxylamine–cobalt(II) solution and the borate solution in the reaction coil resulted in a pH range of 8.7–9.2.

The peak heights of the tested peptides, except Tyr–Tyr, increased with increase in the reaction temperature in the range 40–80 °C; that of Tyr–Tyr reached a maximum and constant value at 75–80 °C. At 80 °C or higher, an irregular baseline was often observed on the chromatogram because of the formation of air bubbles in the reaction coil; 75 °C was recommended for the reactor system.

The length of the reaction coil (0.5 mm I.D.) affected the fluorescence intensities from the tested peptides. Maximum peak heights for the peptides were achieved with a 19-m coil. With a longer coil the peaks were broadened, but the peak areas remained almost constant; the peak widths observed with a 30-m coil were approximately 1.5 times greater than those with the selected 19-m coil. The reaction time through the coil was ca. 1.8 min as calculated from the coil volume and the flow-rates of the mobile phase and the reagent solutions. The actual time of passage through the reactor system from the outlet of the UV detector to the fluorescence detector was 2.4 min. This time corresponds to the lag time for fluorescence detection.

The fluorescence excitation and emission maxima of the tested peptides were around 335 and 435 nm, respectively. No significant shift of the maximum wavelengths of the fluorescences was observed when the volume ratio of acetonitrile in the mobile phase was altered during the gradient elution, although some fluorescent substances give shifted fluorescence spectra, depending on the polarity of the solvent [14].

Determination of N-terminal tyrosine-containing peptides

Oligopeptides with molecular masses between 500 and 3000 have been separated well on a reversed-phase column by elution with a mobile phase composed of acetonitrile and acidic buffers (pH 2–4) [15]. Thus, the separation of the peptides tested here (molecular masses 200–1200) was carried out under similar conditions to those of the conventional HPLC.

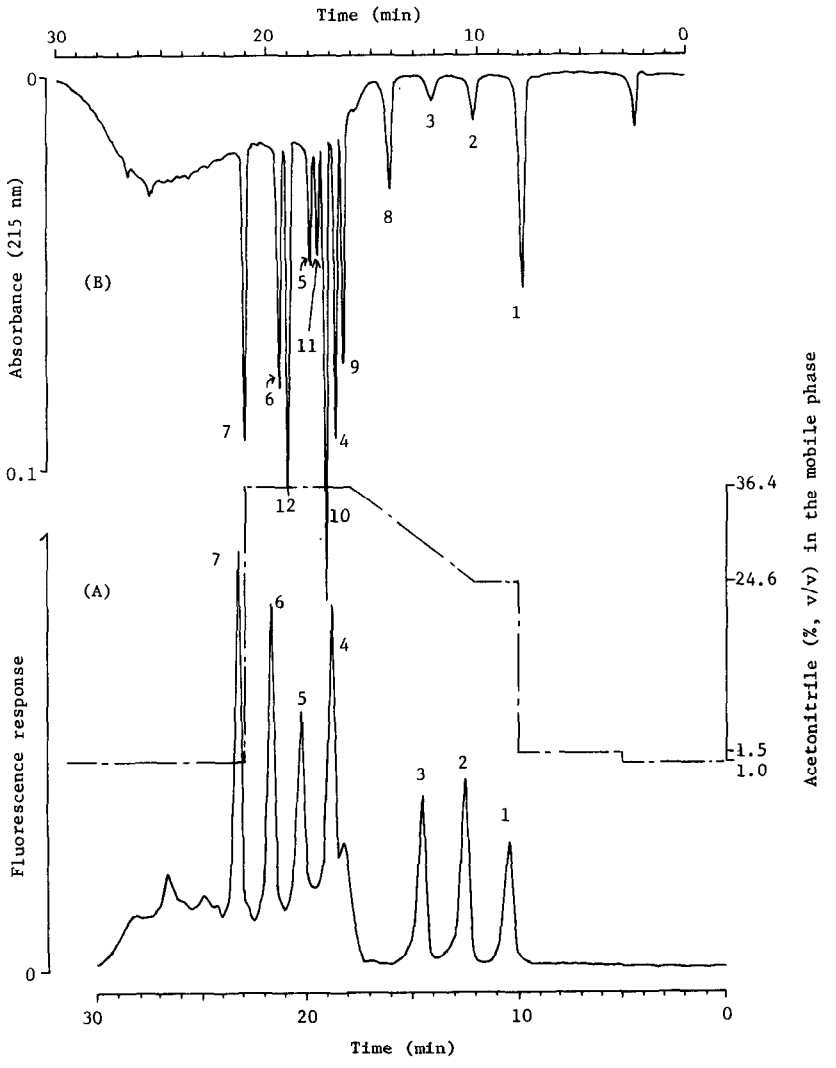

Fig. 2. Reversed-phase chromatograms obtained with (A) fluorescence and (B) UV detection of synthetic peptides and tyrosine. Peaks (nmol per 100-μl injection volume): 1, tyrosine (2.0); 2, Tyr-Gly-Gly (0.5); 3, Tyr-Gly (0.5); 4, Tyr-Tyr (0.5); 5, Tyr-Gly-Gly-Phe (0.5); 6, MEK (0.5); 7, LEK (0.5); 8, Gly-Tyr (1.0); 9, Phe-Gly (0.5); 10, vasopressin (0.5); 11, kallidin (0.5); 12, angiotensin II (0.5). For HPLC conditions, see text.

Fig. 2 shows chromatograms obtained with the fluorescence and UV detectors after reversed-phase HPLC separation of a standard mixture of six N-terminal tyrosine-containing peptides (Tyr–Gly, Tyr–Tyr, Tyr–Gly–Gly, Tyr–Gly–Gly–Phe, MEK and LEK), three other tyrosine-containing peptides (Gly–Tyr, angiotensin II and vasopressin) and two tyrosine-free peptides (Phe–Gly and kallidin) and tyrosine. With the fluorescence detection, the six N-terminal tyrosine-containing peptides and tyrosine were selectively detected from the other peptides with no tyrosyl residue at the N-terminal position in the

sequence (Fig. 2A). In contrast, UV detection at 215 nm, which has been widely used for peptide detection, permitted the detection of 0.1–1.0 nmol amounts of the peptides tested (Fig. 2B). In addition, other synthetic biological compounds (2 nmol each) such as amino acids (phenylalanine and tryptophan), organic acids (propionic acid, phenylpyruvic acid and sorbic acid) and steroids (oestriol and oestrone-3-sulphate) were also detectable with UV detection [16]. However, these substances were not detected with the present fluorescence method. Tyrosine, phenylalanine and tryptophan, in a peptide molecule or free, exhibit weak native fluorescence at an emission wavelength of 270–350 nm due to the intrinsic fluorescence of the benzene ring. However, their fluorescence did not interfere with the present fluorescence detection of the N-terminal tyrosine-containing peptides because they have different fluorescence wavelengths.

The calibration graphs (each a single plot) of peak heights versus amounts injected for the six N-terminal tyrosine-containing synthetic peptides (100–500 pmol) and tyrosine (0.4–2.0 nmol) with both detection methods were linear (Fig. 3). The correlation coefficients (r) for the straight lines were 0.995–0.999 for the fluorescence detection and 0.997–0.999 for the UV detection. The detection limits for the peptides and tyrosine (pmol per 100-μl injection volume) in the fluorescence and UV detections were 20 and 16 (tyrosine), 5 and 15 (Tyr–Gly–Gly), 5 and 20 (Tyr–Gly), 4 and 13 (Tyr–Tyr), 6 and 14 (Tyr–Gly–Gly–Phe) and 5 and 10 (MEK and LEK), respectively, at a signal-to-noise ratio of 2. The results indicate that the fluorescence detection for the tested N-terminal tyrosine-containing peptides is slightly more sensitive than the UV detection. However, the fluorescence detection of tyrosine was less sensitive than the UV detection, as the present post-column reactor system, similarly to the previous manual method [10], did not give an intense fluorescence for tyrosine. The relative standard deviations of the peak heights for the N-terminal tyrosine-containing peptides (0.5 nmol each) and tyrosine (2.0 nmol) were 1.8–6.0% with fluorescence detection and 1.5–5.6% with UV detection ($n=10$ in each instance).

Determination of enzymatically degraded MEK fragments

The following peptidases for the degradation of EKs were demonstrated to be present in mammalian brain tissues [17]. They are aminopeptidase, which degrades EKs at the Tyr–Gly bond [18,19], dipeptidyl aminopeptidase (e.g., enkephalinase B [20]), which splits EKs at the Gly–Gly bond, and dipeptidyl carboxypeptidase (e.g., enkephalinase A [21] and angiotensin converting enzyme [22]), which degrades EKs at the Gly–Phe bond. Therefore, tyrosine and two N-terminal tyrosine-containing peptide fragments (i.e., Tyr–Gly and Tyr–Gly–Gly) other than Tyr–Gly–Gly–Phe can be produced by the enzymatic degradation of MEK with the peptidases in a brain homogenate.

Fig. 4 shows the chromatograms obtained by the present HPLC method with fluorescence and UV detectors for the enzyme reaction mixture after treatment with the degradation procedure in the presence of bestatin. With fluorescence detection (Fig. 4A), three fragment peaks of tyrosine (peak 1), Tyr–Gly–Gly (peak 2) and Tyr–Gly (peak 3) were apparently observed, while five fragment peaks were observed with UV detection (Fig. 4B). However, the latter method

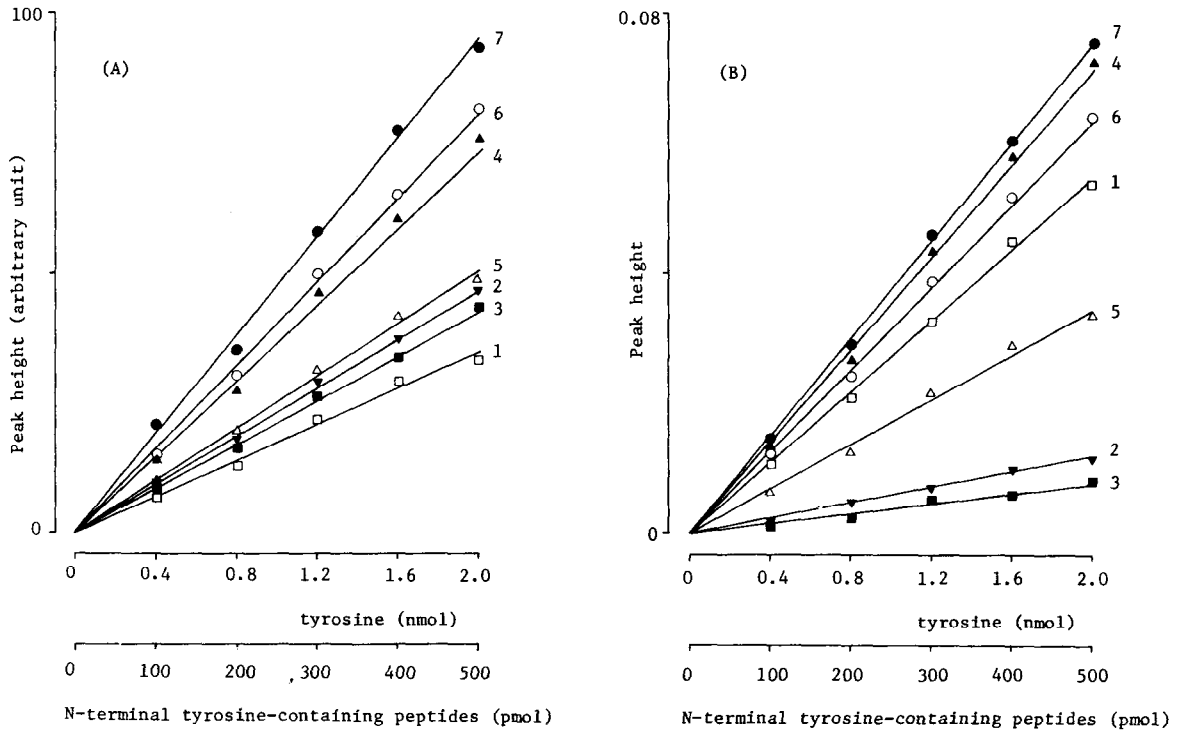

Fig. 3. Calibration graphs obtained with (A) fluorescence and (B) UV detection for six N-terminal tyrosine-containing synthetic peptides and tyrosine. Graphs: 1, tyrosine; 2, Tyr-Gly-Gly; 3, Tyr-Gly; 4, Tyr-Tyr; 5, Tyr-Gly-Gly-Phe; 6, MEK; 7, LEK.

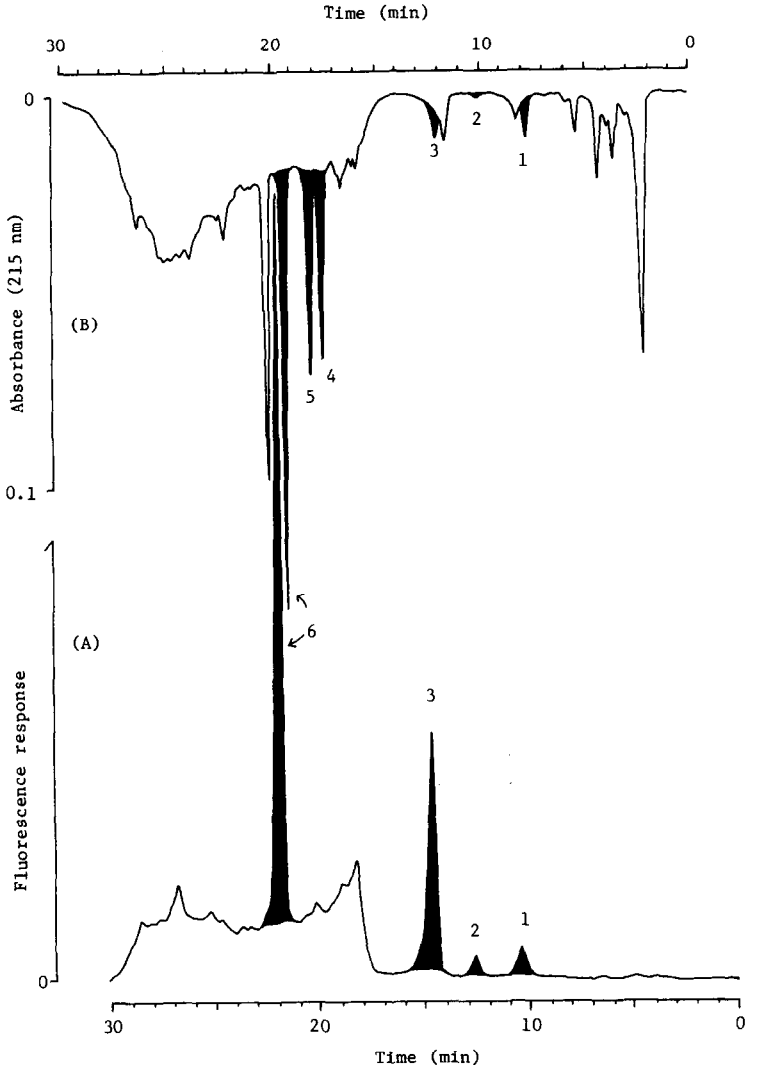

Fig. 4. Chromatograms obtained with (A) fluorescence and (B) UV detection for the enzymatic degradation of MEK in the presence of bestatin with rat brain homogenate. Sample size: 50 μl. Peaks: 1, tyrosine; 2, Tyr-Gly-Gly; 3, Tyr-Gly; 4 and 5, unidentified fragments produced from MEK; 6, MEK. Peaks other than shaded peaks were also observed when the same degradation procedure without addition of MEK was carried out. HPLC conditions as in Fig. 2.

detected several UV-absorbing compounds in the enzyme reaction mixture, which interfered with the determination of the fragment peaks 1 and 3. In addition, the chromatogram obtained with the UV detection became more complex when the sample was repeatedly analysed, as various UV-absorbing substances retained on the column were detected after being carried over. Therefore, the quantitative determination based on the fluorescent peaks of the fragments produced and the undegraded MEK was performed by the standard additions method; each syn-

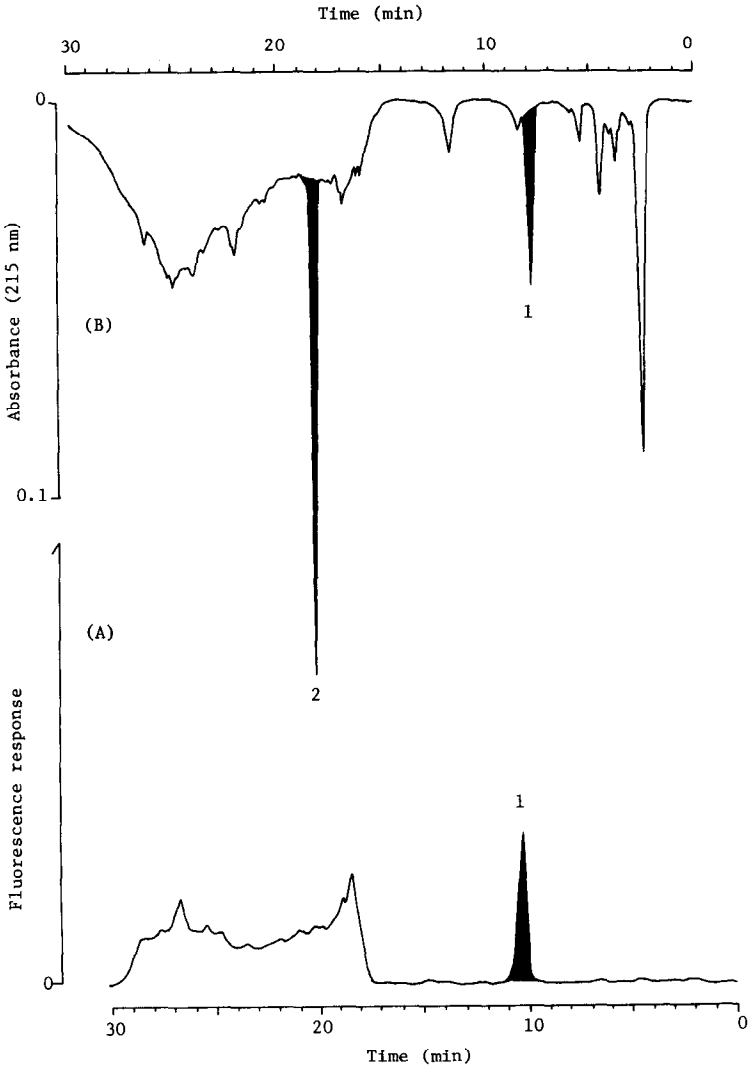

Fig. 5. Chromatograms obtained with (A) fluorescence and (B) UV detection for the enzymatic degradation of MEK in the absence of bestatin with the same homogenate as in Fig. 4. Sample size: 50 μl. Peaks: 1, tyrosine; 2, unidentified fragment produced from MEK. Peaks other than shaded peaks were also observed when the same degradation procedure without addition of MEK was carried out. HPLC conditions as in Fig. 2.

thetic peptide was added to the enzyme reaction mixture in the absence of MEK as the substrate after the enzyme reaction was terminated.

The amounts of the tyrosine-containing fragments and that of the undegraded MEK were 0.53 nmol for tyrosine, 0.68 nmol for Tyr–Gly, 0.05 nmol for Tyr–Gly–Gly and 1.19 nmol for MEK in 50 μl of the enzyme reaction mixture when 2.5 nmol of MEK were subjected to the enzyme degradation. Hence the total amount (1.26 nmol) of the products corresponds to 96% of the amount (1.31 nmol) of the degraded MEK. The 4% loss in the production of the fragments may

be caused by the assay error within the standard deviation of the present method; otherwise, some metabolism other than the peptidase degradation is also conceivable, for example, an enzymatic modification of the tyrosyl residue of MEK.

The enzyme reaction was carried out in the presence of bestatin, which inhibits aminopeptidase B and leucine aminopeptidase [23]. In its absence (Fig. 5), MEK was completely degraded and two UV-absorbing peaks were alternatively observed in the chromatogram (Fig. 5B). One (peak 1) of the fragment peaks was detected with fluorescence detection (Fig. 5A), and thus the peak is due to tyrosine produced from MEK by the enzyme reaction. The amount of tyrosine produced was 2.48 nmol, as determined with fluorescence detection. This value is very close to that of the degraded MEK. Thus, another UV-absorbing fragment (peak 2) may be Gly–Gly–Phe–Met, although the retention time of the synthetic peptide was not examined under the present HPLC conditions.

The tissue preparation used for the above degradation studies contained 94 ± 8 μg of protein (approximately 0.25 mg of the tissue) in 50 μl of the enzyme reaction mixture. We examined whether endogeneous EKs and related peptides (e.g., Tyr–Gly, Tyr–Gly–Gly and Tyr–Gly–Gly–Phe) interfered with the detection of the fragments produced from MEK and also whether those peptides were newly produced from EK precursors [24] in the tissue preparation by the enzyme reaction. When the tissue preparation in the absence of MEK was treated in the same way as in the experiments shown in Figs. 4 and 5, the chromatograms obtained for the control samples were compatible with the chromatograms of Figs. 4 and 5, but from which the peaks for the fragments produced and the undegraded MEK peak, respectively, were excluded. The same was also true when the control sample was treated with no incubation. The results demonstrate that the endogenous EKs and the related peptides present in the tissue preparation were not sufficiently detected by the present HPLC method and that the corresponding peptides were not produced from EK precursors under the present conditions of the enzyme reaction.

The present HPLC system, based on on-line post-column fluorescence detection using hydroxylamine, cobalt(II) and borate reagents, is useful for the sensitive determination of N-terminal tyrosine-containing peptides such as EKs and their fragment peptides.

The results obtained for the enzymatic degradation of MEK using the rat brain homogenate suggest that the production of fragments of Tyr–Gly–Gly and Tyr–Gly was caused by the catalysis of enkephalinase A (involving angiotensin-converting enzyme) and enkephalinase B, respectively, in the tissue preparation. Hence the activities of both enkephalinase A and B may be simultaneously assayed using the present HPLC system. This study is currently in progress.

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