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High-performance liquid chromatography of N-terminal tryptophan-containing peptides with precolumn fluorescence derivatization with glyoxal

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ABSTRACT

A precolumn fluorescence derivatization method combined with high-performance liquid chromatography is described for the sensitive and selective determination of N-terminal tryptophan-containing peptides. The peptides and tryptophan were converted into fluorescent derivatives with glyoxal in a moderately acidic medium (pH 4.5). The derivatives were separated on a reversed-phase column with isocratic elution with an aqueous mobile phase composed of acetonitrile, methanol and phosphate buffer (pH 6.0), and subsequently detected by fluorimetry. The derivatization technique provided the respective N-terminal tryptophan-containing oligopeptides with single fluorescent peaks in chromatography. The detection limits for the peptides were 55–382 fmol per 100-μl injection volume at a signal-to-noise ratio of 3. The method also allowed the facile detection of an N-terminal tryptophyl fragment in the enzyme reaction mixture of dynorphin A with trypin.

INTRODUCTION

High-performance liquid chromatography (HPLC) is advantageous for the separation of various peptides. Many detection techniques in HPLC have been proposed for the determination of peptides, including radioimmunoassay, mass spectrometric, ultraviolet (UV) absorption, electrochemical activity and fluorescence measurements.

Radioimmunoassay [1] as an off-line detection method in HPLC generally offers high sensitivity and selectivity for peptides, but it is difficult to obtain specific antibodies, especially for oligopeptides. Mass spectrometric detection [2] coupled with off-line HPLC needs expensive instrumentation and refined operation, although the method shows satisfactory structural specificity for peptides. UV detection [3] at wavelengths between 200 and 280 nm and electrochemical detection [4] are frequently influenced by either various UV-absorbing components or oxidizing components in biological samples and in the mobile phase, although these techniques are convenient for the detection of peptides without derivatization. Fluorescence detection utilizing fluorescamine [5] or *o*-phthalaldehyde [6] as fluorogenic reagents selective for primary amines shows insufficient selectivity for the target peptides.

On the other hand, fluorogenic reagents that can recognize an amino acid residue of peptides are available for the facile detection of particular

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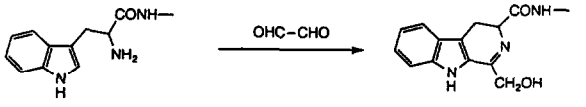
peptides in complex samples such as enzymatic digests [7]. In this respect, we have developed fluorescence derivatization methods in HPLC for the selective determination of arginine-containing peptides with benzoin reagent [8,9] and of N-terminal tyrosine-containing peptides with hydroxylamine–cobalt(II)–borate reagent [10–13].

We previously reported [14] that phenylglyoxal reacted selectively with tryptophan (Trp) in a strongly acidic medium to give three fluorescent compounds, of which the major product was 1-(1-hydroxybenzyl)-β-carboline. In a further study [15] on a survey of more suitable fluorogenic reagents for Trp and/or Trp-containing peptides, using glyoxal, methylglyoxal and four synthesized analogues of phenylglyoxal substituted with electron-donating group(s) as the reagent, we found that glyoxal could offer intense fluorescence for N-terminal Trp-containing peptides in a weakly acidic solution under relatively mild reaction conditions.

We therefore considered that the glyoxal reaction may be useful for converting N-terminal Trp-containing peptides into the corresponding fluorescent derivatives that can be separated by HPLC; the expected structure on the basis of the reaction product of Trp with phenylglyoxal is shown here. In this work, we studied the con-



N-terminal Trp-containing peptide possible fluorophore

ditions of precolumn fluorescence derivatization and HPLC separation that give single fluorescent peaks in chromatography. The efficiencies of the HPLC method were also evaluated with respect to the determination of the synthetic peptides and the simple detection of peptide fragments produced by enzymatic digestion of a heptadecapeptide, dynorphin A, with trypsin.

EXPERIMENTAL

Materials and solutions

The N-Terminal Trp-containing peptides listed in Table I (six species), Ala–Trp, Lys–Trp–Lys and dynorphin A were obtained from Sigma (St. Louis, MO, USA). Stock solutions of these synthetic peptides (1.0 μmol/ml each) were prepared in methyl Cellosolve, then diluted to 100 nmol/ml with water and stored at −80°C. The stock solutions were diluted with water to appropriate concentrations before use and used within 1 day. Glyoxal as an aqueous solution (21.9 *M*) from Sigma was diluted with water to obtain a 0.2 *M* solution, which was usable as a reagent solution for at least 1 month when stored at 4°C. Other chemicals were of the highest purity available.

Derivatization procedure

A portion (100 μl) of peptide solution was mixed with 100 μl each of 0.2 *M* succinate buffer (pH 4.5) and 0.2 *M* glyoxal solution. The mixture was heated at 100°C for 30 min. A portion (100 μl) of the final reactions mixture was used for HPLC.

Apparatus and HPLC conditions

The HPLC system consisted of a Tosoh (Tokyo, Japan) HLC-803D high-performance liquid chromatograph, a Rheodyne Model 7125 syringe-loading sample injector (100-μl loop) and a Hitachi F-1000 spectrofluorimeter fitted with a 12-μl flowcell. A reversed-phase column of TSKgel ODS-80T$_M$ (150 × 4.6 mm I.D., particle size 5 μm) (Tosoh) was used. The column temperature was ambient (25 ± 4°C). For the separation of the fluorescent derivatives of peptides on the column, isocratic elution with an aqueous mobile phase of acetonitrile–methanol–20 m*M* phosphate buffer (pH 6.0) (23:17:60, v/v/v) was carried out at a constant flow-rate of 0.8 ml/min. The fluorescence intensity of the column eluate was monitored at 465 nm (emission) with excitation at 275 nm.

Uncorrected fluorescence excitation and emission spectra of the eluates from the fluorescent peaks were measured with a Hitachi F-2000 spectrofluorimeter in 10 × 10 cm quartz cells; spectral band widths of 10 nm were used in both the excitation and emission monochromators.

RESULTS AND DISCUSSION

HPLC separation

The three N-terminal Trp-containing peptides, Trp–Leu, Trp–Gly–Gly and Trp–Met–Asp–Phe–NH_2, and Trp were converted into the corresponding fluorescent derivatives under the recommended reaction conditions.

When the derivatization mixture of the peptides and Trp was subjected to reversed-phase HPLC on an ODS column (TSKgel ODS-80T_M), single fluorescent peaks were observed for the respective compounds. Their peaks were mutually separated within 22 min by isocratic elution with the above mobile phase (Fig. 1).

The elution of the peaks for the peptides was delayed when the pH of the phosphate buffer in the mobile phase was <5.5, although no effect of pH on the retention time of the Trp peak was detected (Fig. 2). The peptide derivatives in the reaction mixture fluoresced most intensely at pH 6.0, although the derivative of Trp fluoresced intensely at pH >9.0 (Fig. 3). Therefore, the maximum peak heights for the respective peptides were observed at pH 6.0 and phosphate

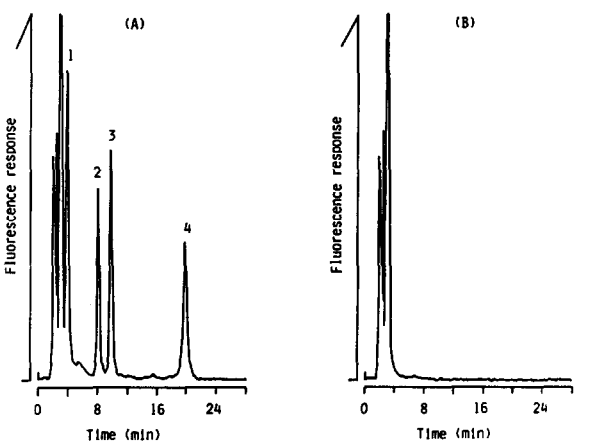


Fig. 1. Chromatograms of (A) the fluorescent derivatives of three N-terminal Trp-containing peptides and Trp and (B) the reagent blank. A portion (100 μl) of a mixture of the peptides and Trp (250 pmol/ml for Trp–Gly–Gly and 500 pmol/ml for the others) was treated under the recommended conditions for derivatization and HPLC. Peaks: 1 = Trp–Gly–Gly; 2 = Trp; 3 = Trp–Leu; 4 = Trp–Met–Asp–Phe–NH_2.

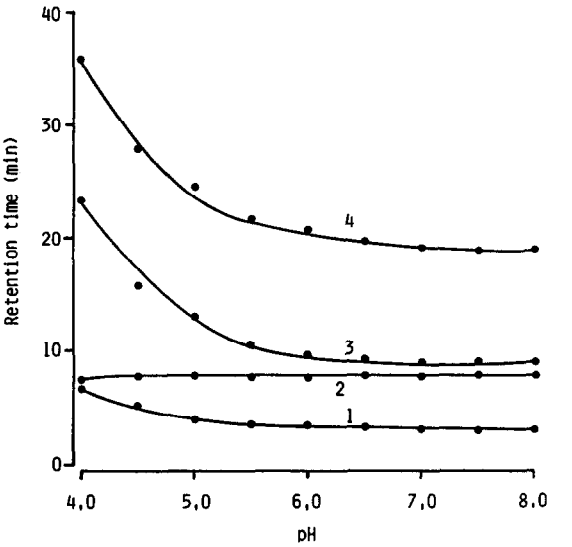


Fig. 2. Effect of pH of 20 m*M* phosphate buffer in the mobile phase on the separation. Portions (100 μl) of the mixture of the peptides and Trp were treated as in Fig. 1 except that buffers of various pH were used in the mobile phase. Numbers on curves as for peaks in Fig. 1.

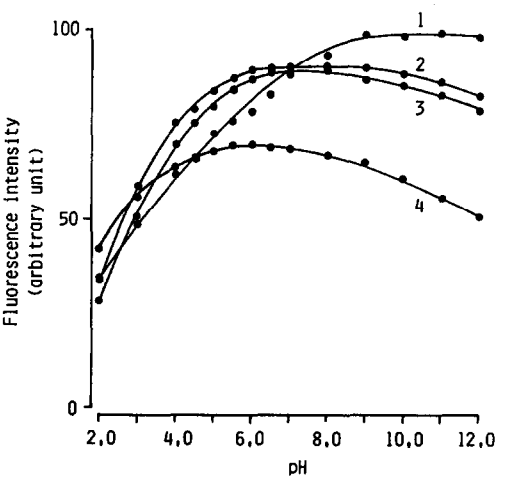


Fig. 3. Effect of pH of the reaction mixture on the fluorescence intensity after the derivatization. Portions (100 μl) of the final derivatization mixture (500 pmol of each compound per tube) were diluted with 1.0 ml of 40 m*M* Britton–Robinson buffer (pH 2–12) and the fluorescence intensities of the resulting solutions were measured manually with emission at 465 nm and excitation at 275 nm. 1 = Trp; 2 = Trp–Gly–Gly; 3 = Trp–Leu; 4 = Trp–Met–Asp–Phe–NH_2.

buffer of pH 6.0 was selected for use in the mobile phase.

The fluorescence excitation and emission maxima of the peptide derivatives in the column eluates were all around 275 and 465 nm, respectively, although the Trp peak showed the respective maxima at 286 and 455 nm. At the former wavelengths the peak height of Trp was approximately 72% of that at the latter wavelengths.

Derivatization and determination

The fluorescence derivatization of the three N-terminal Trp-containing peptides with glyoxal occurred most effectively with succinate buffer of pH 4.5. Buffer concentrations, >0.15 *M* resulted in constant peak heights for the peptides and Trp with; 0.2 *M* succinate buffer of pH 4.5 was adopted in the procedure.

Higher temperatures in the range 40–100°C allowed the derivatization of the peptides and Trp to proceed more rapidly, and at 100°C nearly maximum peak heights were achieved on heating for 30 min (Fig. 4). With heating at 140°C for 5 min, the peak heights were also maximum and almost the same as those obtained at 100°C for 30 min. In these instances, no by-product peaks due to the degradation of the peptides were found in the chromatograms.

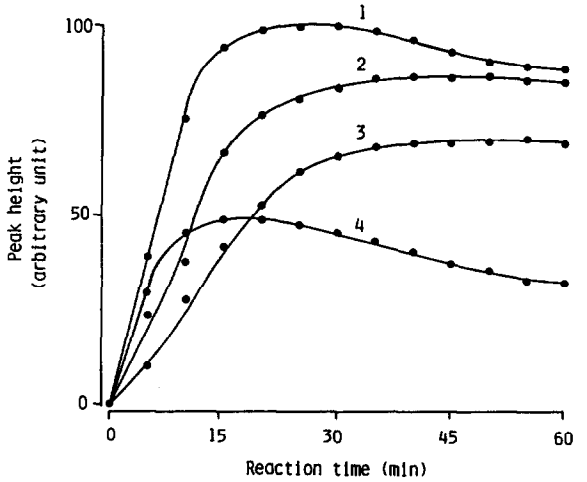


Fig. 4. Effect of reaction time at 100°C on the fluorescence derivatization. Portions (100 μl) of the mixture of the peptides and Trp were treated as in Fig. 1 except that the reaction mixtures were heated for various periods in the derivatization. Numbers on curves as on peaks in Fig. 1.

Maximum and constant peak heights from the peptides were attained at glyoxal concentrations >0.1*M*; 0.2*M* was adopted for the fluroescence derivatization.

The fluorescent derivatives of the peptides were fairly stable; the corresponding peak heights did not change when the derivatization mixture after the reaction was allowed to stand in an ice–water bath for 2 h and no peaks due to by-products were detected in the chromatograms.

Under the established conditions for the fluorescence derivatization, the peptides without an N-terminal Trp residue (Ala–Trp and Lys–Trp–Lys), Trp-related compounds (5-hydroxytryptophan, serotonin, melatonin, kynurenine and 3-hydroxykynurenine), amines (histamine, epinephrine, norepinephrine, spermidine, spermine), nineteen L-α-amino acids, amino sugars (galactosamine and N-acetylgalactosamine) and nucleic acid bases (adenine, guanine, thymine, cytosine and uracil) did not form any fluorescent derivatives.

Table I gives the detection limits and the retention times of various N-terminal Trp-containing oligopeptides, Trp, tryptamine and tryptophamide (Trp-NH_2) obtained by the proposed precolumn fluorescence derivatization HPLC method. These compounds gave single

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF N-TERMINAL TRP-CONTAINING PEPTIDES AND TRP-RELATED COMPOUNDS

Compound	Retention time (min)	Detection limit[a] (fmol)
Trp–Gly–Gly	3.7	55
Trp–Gly	4.0	97
Trp–Ala	4.8	79
Trp–NH_2	7.4	143
Trp	7.8	314
Tryptamine	7.8	311
Trp–Trp	8.0	4166
Trp–Leu	9.2	192
Trp–Met–Asp–Phe–NH_2	18.2	382

[a] Defined as the amount per 100-μl injection volume giving a signal-to-noise ratio of 3.

peaks in the chromatograms. The detection limits for the compounds other than Trp–Trp were in the range 55–382 fmol in a 100-μl injection volume at a signal-to-noise ratio of 3. The peak height due to the fluorescent derivative of Trp–Trp was unexpectedly low, being *ca.* 1% of that for Trp–Gly–Gly at an equimolar concentration (500 pmol/ml). This weak fluorescence may be caused by a low derivatization yield for Trp–Trp and/or internal molecular quenching of fluorescence due to the absorption of excitation energy with its C-terminal Trp. The Trp residue in peptide molecules exhibits its native fluorescence, the excitation and emission maxima of which are around 290 and 350 nm, respectively.

The native fluorescence of the Trp-containing peptides was not detected with the present detector, because they have different emission wavelengths to those of the peptide derivatives, although the native fluorescence intensities of the peptides at equimolar concentrations were *ca.* 20% of the intensities given by the glyoxal reaction when the optimum wavelengths for emission and excitation were used in each instance.

The calibration graphs for the peptides Trp–Gly–Gly, Trp–Leu and Trp–Met–Asp–Phe–NH_2 of the peak height *versus* concentration (0.25–500 pmol per derivatization mixture) were all linear with correlation coefficients of 0.998–0.999. In within-day assays, the relative standard deviations ($n = 10$) of the peak heights were 3.0, 1.3 and 4.2% for Trp–Gly–Gly, Trp–Leu and Trp–Met–Asp–Phe–NH_2, respectively, at the concentrations used for Fig. 1.

Fig. 5 shows the application of the present method to the detection of the tryptic digest of dynorphin A (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln; 5 nmol used for the enzyme reaction). In the sample, Trp–Asp–Asn–Gln and many other C-terminal Lys- or Arg-containing peptides are produced in the course of the enzyme reaction, as trypsin mediates the hydrolysis of peptide bonds at the carboxyl sides of lysyl and arginyl residues. In the chromatograms obtained with the present fluorescence detection and conventional UV detection, the fluorescent derivative

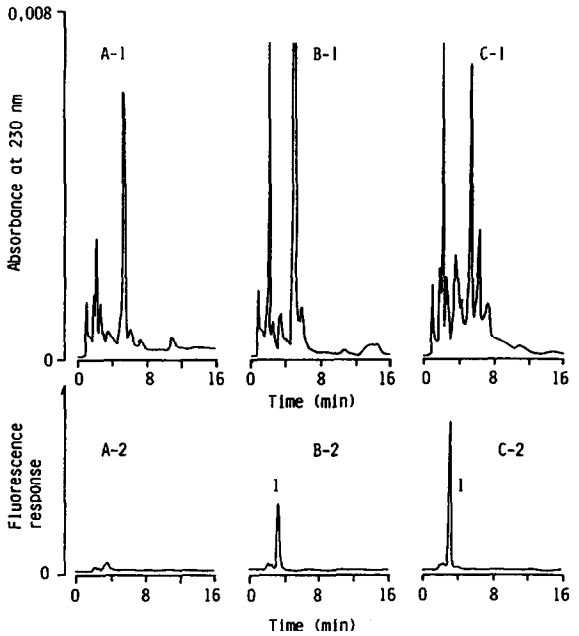


Fig. 5. Chromatograms of the peptide fragments produced by tryptic digestion of dynorphin A. A 50-μl portion of 100 nmol/ml dynorphin A was mixed with 100 μl of 50 m*M* sodium phosphate buffer (pH 8.0) and 50 μl of 1.0 mg (122 000 units)/ml trypsin and the mixture was incubated at 37°C for (A) 0, (B) 5 and (C) 30 min. A 20-μl portion of the enzyme reaction mixture was chromatographed for UV detection (A-1, B-1, C-1). Another 100-μl portion of the mixture was used for the fluorescence derivatization, and a 100-μl portion of the final reaction mixture was chromatographed for fluorescence detection (A-2, B-2, C-2). HPLC conditions: mobile phase, acetonitrile–methanol–20 m*M* phosphate buffer (pH 6.0)–water (14:14:60:12, v/v); other conditions as under Experimental. Peak 1 = fluorescent derivative of Trp–Asp–Asn–Gln.

due to the N-terminal Trp-containing fragment was readily detected with fluorescence detection. With UV detection, however, it was difficult to identify the fragment because many other peaks were detected.

CONCLUSION

This is the first method for the selective fluorescence derivatization HPLC of N-terminal Trp-containing peptides. It permits the sensitive determination of the peptides at the sub-picomole level. The sensitivity of the method is nearly three and two orders of magnitude higher

than those of the manual fluorimetric method [15] and conventional HPLC–UV detection method, respectively. The proposed method is probably useful for the simple identification of Trp-containing fragments of high-molecular-mass peptides in various enzymatic digests. The specificity of the method may also be an aid for the quantitative or qualitative determination of biogenic N-terminal Trp-containing peptides such as delta-sleep-inducing peptides (Trp–Ala–Gly–Gly–Asp–Ser–Gly–Glu) [16], a metabolic fragment peptide of cholecystokinin (Trp–Met–Asp–Phe–NH_2) [17] or unknown bioactive peptides in the complex matrices of mammalian tissues and fluids. Studies on these aspects are in progress.

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