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High-performance liquid chromatographic determination of proteins by post-column fluorescence derivatization with thiamine reagent

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ABSTRACT

A post-column derivatization method for the high-performance liquid chromatography of peptides and proteins giving a fluorescence intensity proportional to the number of peptide bonds is described. Peptide bonds were chlorinated with hypochlorite and the N-chlorite formed was allowed to react with thiamine to give fluorescent thiochrome. This method was applied the determination of membrane-forming proteins of microorganisms.

INTRODUCTION

The determination of proteins and peptides by high-performance liquid chromatography (HPLC) has been extensively investigated in recent years [1–7]. In most of the HPLC methods, the proteins and peptides were usually detected spectrophotometrically because most proteins and peptides absorb at 210 or 280 nm. Although the absorbance at 210 nm reflected the actual content of proteins because it is based on the absorption by the peptide bonds in the protein molecule, it is seriously affected by various UV-absorbing substances in biological samples. On the other hand, the absorbance at 280 nm is subject to interferences by contaminants, but is not proportional to the actual content of proteins because it reflects the amount of aromatic amino acids.

There have also been reports of post-column fluorescence derivatization methods with *o*-phthalaldehyde [8,9] or fluorescamine [9,10] for peptides of low molecular weight, with alkaline ninhydrin [11] or benzoin [12] for arginine-containing peptides and with hydroxylamine–cobalt (II) [13] or a phenol-sensitive electrode [14,15] for tyrosine-containing peptides. However, these methods were also based on the reaction of terminal amino groups or particular side-chains and did not reflect the actual amount of proteins.

Kinoshita *et al.* [16] reported a fluorimetric method in which the peptide bonds of protein were chlorinated and allowed to react with thiamine to give thiochrome.

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We have recently applied this principle to the flow-injection analysis (FIA) of proteins in which the fluorescence intensity was proportional to the number of peptide bonds [17].

In this study, we have devised an HPLC method based on this principle that facilitates the detection of various proteins and peptides at the same level of sensitivity.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA), thyroglobulin, γ-globulin, myoglobin, lysozyme, ovalbumin, cytochrome *c*, α-chymotrypsin, α-chymotrypsinogen A, trypsinogen, ribonuclease and jack bean meal were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium hypochlorite solution (Antiformin), sodium nitrite, thiamine hydrochloride, Brij-35, anhydrous sodium sulphate, sodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate and other chemicals were of analytical-reagent grade from Wako (Osaka, Japan).

Deionized, distilled water was used throughout.

Mobile phase and derivatization reagents

The mobile phase for HPLC was prepared by adding 0.1 *M* sodium sulphate to 0.1 *M* phosphate buffer (pH 7.5). The solution was filtered through a 0.45-μm microfilter (Fuji Photo Film, Tokyo, Japan) and degassed prior to use.

Hypochlorite reagent was prepared by diluting commercial sodium hypochlorite solution (Antiformin) with 0.05 *M* phosphate buffer (pH 7.5), adjusting the pH to 7.5 and the final concentration of available chlorine to 0.8% with 0.05 *M* sodium hydrogenphosphate solution and 0.05 *M* sodium dihydrogenphosphate solution and adding 0.1% Brij-35.

Thiamine reagent was prepared by dissolving 8.0 g of sodium nitrite and 40 mg of thiamine hydrochloride in *ca.* 100 ml of 0.05 *M* phosphate buffer (pH 7.5), adjusting the pH of the solution to 7.5 using 0.05 *M* sodium hydrogenphosphate solution and 0.05 *M* sodium dihydrogenphosphate solution and diluting the resulting mixture to 200 ml with 0.05 *M* phosphate buffer (pH 7.5). The final concentrations of sodium nitrite and thiamine hydrochloride were 4 and 0.02% (w/v), respectively. This reagent was stable for at least 24 h at room temperature and for 1 week in a refrigerator.

The above reagent solutions were filtered to remove suspended material before use.

Chromatographic system

Fig. 1 shows a schematic diagram of the HPLC system. Chromatographic separations were carried out on a 30 cm × 7.5 mm I.D. TSKgel-G3000SW column (Tosoh, Tokyo, Japan), operated at ambient temperature. The mobile phase and fluorescence reagents were delivered using LC-6A high-pressure semi-micro solvent-delivery systems (Shimadzu, Kyoto, Japan). Samples were injected using a KMT-60A HPLC autosampler (Kyowa-Seimitu, Tokyo, Japan) equipped with a 20-μl loop.

The column eluate was first passed through an SPD-6A UV detector (Shimad-

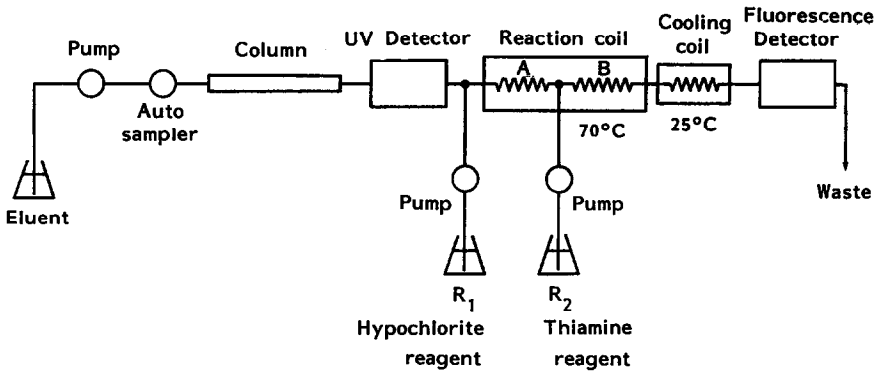

Fig. 1. Schematic diagram of the separation and post-column fluorescence derivatization of proteins.

zu) equipped with a 20-μl flow cell set at 210 or 280 nm, and then it was introduced into a fluorescence reactor system. The hypochlorite reagent was delivered to the eluate stream at a tee-mixer at a flow-rate of 0.2 ml/min, and then the stream passed through PTFE reaction coil A (3 m × 0.5 mm I.D.) immersed in a thermostated water-bath (Thermo-minder, Type Ace-80; Taiyo Service Centre, Tokyo, Japan) maintained at 70°C. After the chlorination reaction, the thiamine reagent was delivered to the reaction steam at a flow-rate of 0.2 ml/min. Then the mixture was passed through PTFE reaction coil B (5 m × 0.5 mm I.D.) immersed in a water-bath at 70°C.

The effluent from coil B was passed through a PTFE cooling coil (1 m × 0.5 mm I.D.). The fluorescence generated was monitored with an RF-535 spectrofluorimeter (Shimadzu) equipped with a xenon lamp and a 20-μl flow cell, with excitation and emission wavelengths of 370 and 440 nm, respectively.

Preparation of microbiological samples

The ammonium sulphate fraction of *Escherichia coli* cell debris was prepared as follows. Cultured bacteria cells were suspended in 1/15 *M* phosphate buffer (pH 6.5) and disrupted ultrasonically, and cell debris was removed by centrifugation and fractionated with 30 and 70% ammonium sulphate solution [18]. The suspension of precipitated crude protein in 70% ammonium sulphate was dissolved in 0.05 *M* phosphate buffer (pH 7.5) and the solution obtained was used as a sample.

RESULTS AND DISCUSSION

Optimization of the post-column reaction conditions

Proteins were separated in the gel-filtration mode in which phosphate buffer (pH 7.5) was used as the mobile phase. The components of the mobile phase were the same as those of the carrier solution in our previous FIA method for proteins [17].

The post-column reaction conditions were examined by varying the concentrations of the reagents. Fig. 2 shows that the fluorescence intensity increases with increase in the concentration of available chlorine and reaches a plateau at 0.8%. It also demonstrates that the fluorescence intensity increases with increase in the concentration of sodium nitrite and the maximum fluorescence intensity was observed at 4%.

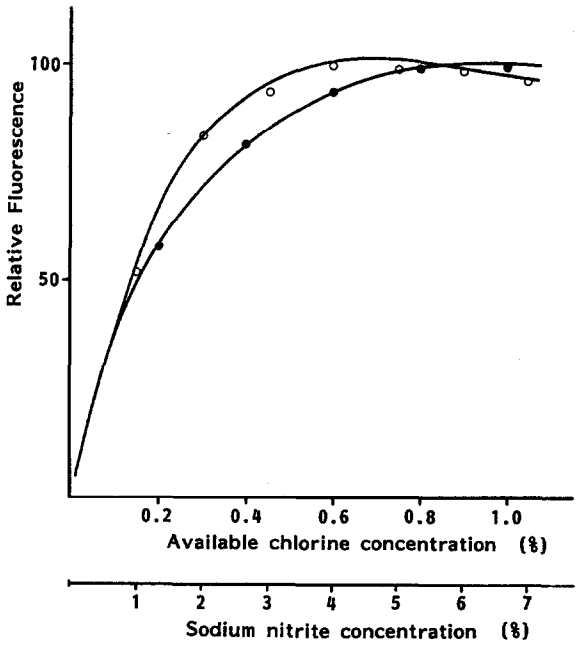

Fig. 2. Effect of the concentrations of available chlorine (●) and nitrite (○) on the fluorescence development of BSA (2 μg per injection). HPLC and post-column reaction conditions as in text, except for reagent concentrations.

The concentration of thiamine in the reagent was adjusted to 0.02% (w/v), where the maximum fluorescence was observed.

System performance

Fig. 3 shows the HPLC profile of the standard proteins which were simultaneously detected utilizing the absorbance at 210 or 280 nm and fluorescence intensity.

The fluorescence intensity hardly fluctuated among the different proteins, whereas the absorbance at 210 nm gave a higher peak for thyroglobulin than for other proteins. Further, considerable differences in the absorbance at 280 nm were observed among the proteins. For example, the peak height of lysozyme was *ca.* five times larger than that of BSA. The peak-height ratio of cytochrome *c* to ovalbumin measured at 210 nm was 1.1 and that measured at 280 nm was 3.2, but that measured by the present method was almost unity.

Fig. 4 shows the calibration graphs for α-chymotrypsin, BSA, trypsinogen, myoglobin and thryroglobulin. Linear relationships between the peak responses and the amounts of the proteins were observed in the range 20 ng–2 μg per injection (20 μl). The limit of detection by the present method for BSA was 10 ng per injection at a signal-to-noise ratio of 2.0; this value was equal to that of the absorbance at 210 nm. The relative standard deviation was 1.9% ($n = 10$) for 500 ng in a 20-μl injection.

Fig. 5 shows the relationships between the number of peptide bonds and the peak responses in the present method and the UV 280-nm method. The fluorescence responses in the present method were found to be proportional to the number of

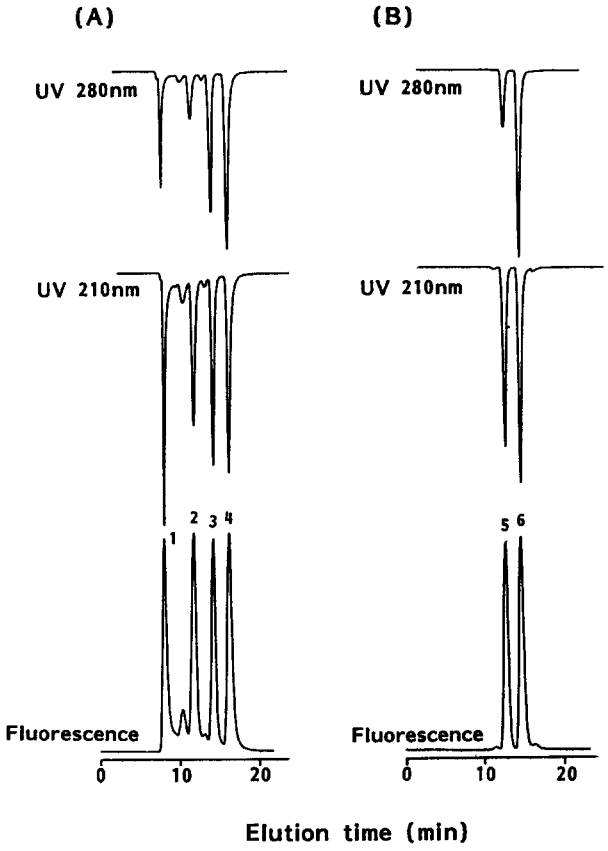

Fig. 3. Chromatograms of a mixture of standard proteins (2 μg each), obtained by dual UV detection at 210 or 280 nm and by the present method. (A) Thyroglobulin, BSA, myoglobin and lysozyme; (B) ovalbumin and cytochrome *c*. Column, TSKgel-G3000SW (30 cm × 7.5 mm I.D.); mobile phase, 0.1 *M* phosphate buffer (pH 7.5) containing 0.1 *M* sodium sulphate; flow-rate, 0.8 ml/min. Peaks: 1 = thyroglobulin; 2 = BSA; 3 = myoglobin; 4 = lysozyme; 5 = ovalbumin; 6 = cytochrome *c*.

peptide bonds, whereas the absorbances at 280 nm deviated substantially from a linear relationship. These results suggest that the present detection method is advantageous for mixtures of proteins having different amino acid compositions.

Fig. 6 shows the chromatograms of jack bean meal and the ammonium sulphate fraction of *E. coli* cell debris. The chromatograms obtained by the present method are similar to those given by the method using the absorption at 210 nm. The large response at a retention time of 26 min shown in Fig. 6A is due to the ammonium sulphate used for the fractionation. However, the peak has a very different retention time to those of the peptides and proteins.

Ionic surfactants, such as sodium dodecyl sulphate (SDS) and Triton X-100, which interfere in protein determinations based on UV absorption at 210 nm, did not affect the present method. Therefore, the present method is also useful for the study

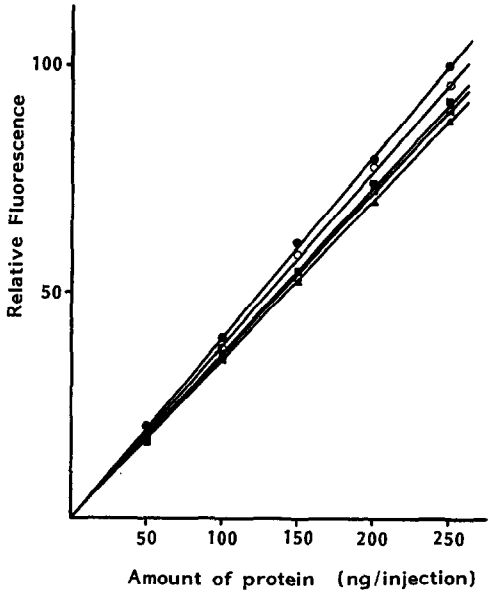

Fig. 4. Calibration graphs for (●) α-chymotrypsin, (○) trypsinogen, (■) myoglobin, (□) BSA and (▲) thyroglobulin obtained by the present method.

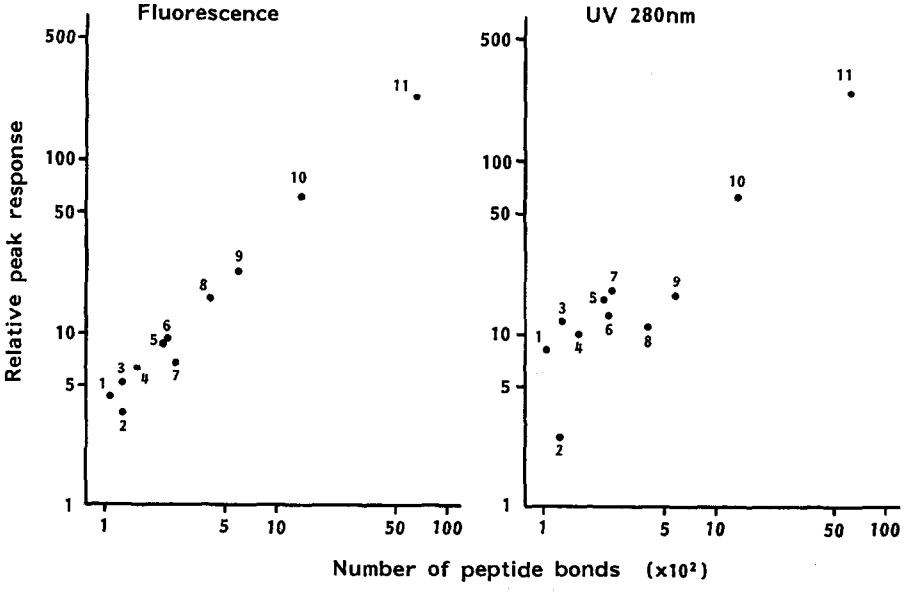

Fig. 5. Relationships between number of peptide bonds and peak responses obtained by the UV 280-nm method and the present method. A 20-μl volume of a sample containing 2 μg of each protein was injected. 1 = Cytochrome *c*; 2 = ribonuclease; 3 = lysozyme; 4 = myoglobin; 5 = α-chymotrypsin; 6 = trypsinogen; 7 = α-chymotrypsinogen A; 8 = ovalbumin; 9 = BSA; 10 = γ-globulin; 11 = thyroglobulin.

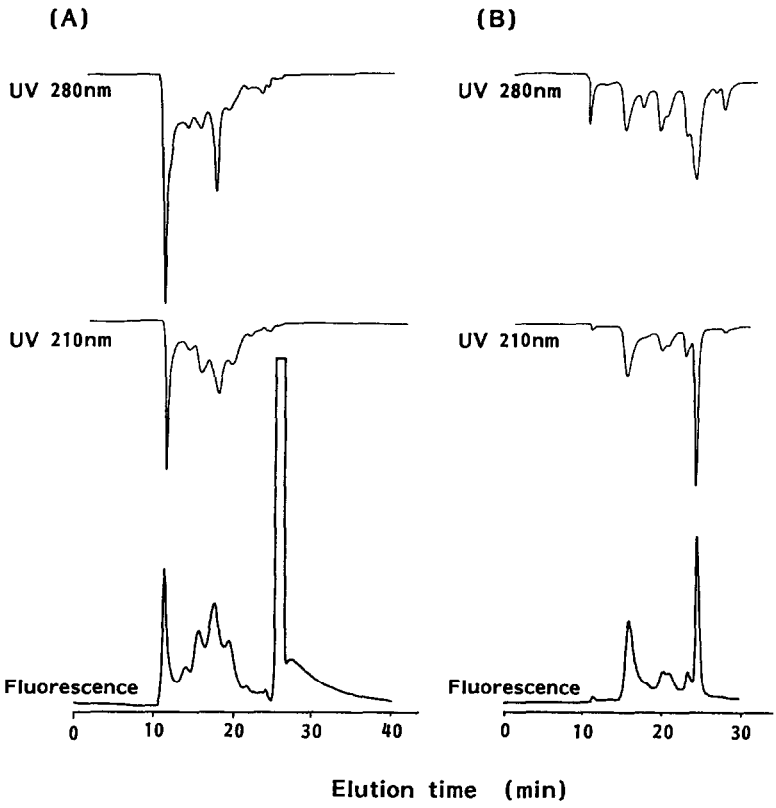

Fig. 6. Chromatograms of the ammonium sulphate fraction of (A) *E. coli* cell debris and (B) jack bean meal obtained by dual detection with the UV 210- or 280-nm method and the present method. Column, TSKgel-G3000SW (30 cm × 7.5 mm I.D.); mobile phase, 0.1 *M* phosphate buffer (pH 7.5) containing 0.1 *M* sodium sulphate; flow-rate, 0.8 ml/min.

of membrane-forming proteins which are usually analysed after eluation with such ionic surfactants.

Detection conditions in HPLC other than gel filtration mode, such as the ion-exchange and reversed-phase modes, are currently under investigation.

REFERENCES

1 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
2 T. Kadoya, T. Ogawa, H. Kuwahara and T. Okuyama, *J. Liq. Chromatogr.*, 11 (1988) 2951.
3 J. S. Swan, M. Azadpur, A. J. Bharucha and M. A. Krafczyk, *J. Liq. Chromatogr.*, 11 (1988) 3385.
4 B. S. Welinder, H. H. Sorensen and B. Hansen, *J. Chromatogr.*, 462 (1989) 255.
5 K. Fukano, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 166 (1978) 47.
6 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519.
7 N. Hirata, M. Kasai, Y. Yanagihara and K. Noguchi, *J. Chromatogr.*, 434 (1988) 71.
8 S. Terabe, A. Tsuchiya and T. Ando, *Bunseki Kagaku*, 33 (1984) 361.
9 T. D. Schlabach, *J. Chromatogr.*, 266 (1983) 427.
10 R. W. Frei, L. Michel and W. Santi, *J. Chromatogr.*, 126 (1976) 665.
11 Y. Hiraga, K. Shirono, S. Ohishi, S. Sakakibara and T. Kinoshita, *Bunseki Kagaku*, 33 (1984) E279.

12 M. Ohno, M. Kai and Y. Ohkura, *J. Chromatogr.*, 392 (1987) 309.
13 M. Ohno, M. Kai and Y. Ohkura, *J. Chromatogr.*, 421 (1987) 245.
14 S. Mousa and D. Couri, *J. Chromatogr.*, 267 (1983) 191.
15 M. W. White, *J. Chromatogr.*, 262 (1983) 420.
16 T. Kinoshita, J. Murayama, K. Murayama and A. Tsuji, *Chem. Pharm. Bull.*, 28 (1980) 641.
17 T. Yokoyama, N. Nakamura and T. Kinoshita, *Anal. Biochem.*, 184 (1990) 184.
18 Y. Kato, K. Komiya, Y. Sawada, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 305.