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Solid-phase derivatization of amino acids and peptides in high-performance liquid chromatography

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ABSTRACT

This paper presents solid-phase derivatization of amino acids and peptides on a hydrophobic polymeric reagent. Using cationic surfactants as a basic pH, the negatively charged amino acids and peptides are ion-paired and derivatized by a 9-fluoreneacetyl tagged polymeric reagent. Using an off-line derivatization approach, the effects of the ion-pair reagent, buffer pH, reaction temperature, etc., on the derivatization are evaluated. Derivatization of peptides of enzymatically digested cytochrome c is included to show the feasibility of solid-phase derivatization to peptide mapping.

INTRODUCTION

Amino acids and peptides are important biological compounds. Amino acid analyzers are mostly based on ion-exchange separation and postcolumn derivatization and detection [1]. Reversed-phase liquid chromatography for these species on C_{18}-silica columns is becoming more popular, offering shorter analysis times and higher sensitivity. Such approaches are increasingly replacing the traditional methods. Depending on the HPLC method used, unmodified amino acids and peptides are detected by UV at a short wavelength or derivatized with UV/fluorescence (FL)/electrochemical detectable tags. Direct UV detection is simple and straightforward, but less sensitive and selective. Thus, most of the analytical methods for amino acids and peptides are based on the reaction of amino groups by precolumn or postcolumn FL derivatization, such as with ninhydrin, *o*-phthalaldehyde, flurescamine, 9-fluorenylmethoxycarbony1 chloride (9-FMOC), phenylisothiocyanate , dansyl and dabsyl chloride, as major derivatization reagents for sensitive detection [2,3]. 9-Fluoreneacetyl chloride (9-FA-Cl), was recently introduced as a fluorescent reagent for the derivatization of primary and secondary amines [4]. The 9-FA-Cl reagent has the same reactivity as the 9-FMOC reagent, but it shows improved product stability and less interferences by its hydrolytic byproduct, mainly 9-FA acid.

Hydration of ionic analytes and hydrolysis of reactive derivatization reagents in basic buffer solution are the main obstacles for derivatization in aqueous solutions, particularly at low analyte concentrations. Surfactants have been well known to form an aqueous micellar solution, which has a hydrophobic microenvironment and performs an ion-pair extraction function with ionic compounds [5]. Phase transfer catalysis

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(PTC) was developed on the principle that the hydrophobic ion-pairing reagent transfers ionized analytes from an aqueous phase into an organic phase, where the analytes are derivatized with a fast reaction rate [6]. Using a micellar phase transfer catalysis (MPTC), efficient solution derivatizations have been developed for less nucleophilic ionic analytes, such as carboxylic acids [7–10].

Many solid-phase derivatization reagents have been developed for tagging hydrophobic nucleophiles [1 1–13]. However, charged amino acids can not easily get into the nonpolar polymeric reagent to be derivatized [14]. Derivatization efficiencies of amino acids were greatly reduced, as the free amino acids and peptides are soluble only in an aqueous phase. Thus, highly reactive solid-phase reagents, such as the polymeric hydroxybenzotriazole with 9-FMOC or 3,5-dinitrobenzoyl tags, were used to enhance the derivatization rate and efficiency for ionic nucleophiles. These solid-phase reagents have a limited stability in aqueous solutions, due to the competing hydrolysis process, especially at elevated temperatures and pH. Actually, reproducible and efficient solid-phase derivatization has never been successful for aqueous amino acids or peptides.

A phase transfer catalysis derivatization is developed in this paper for the efficient, solid-phase reactions of amino acids and peptides (Fig. 1). By incorporation of a cationic surfactant, negatively charged amino acids and peptides form ion-pairs which are extracted into the hydrophobic resin. Such ion-pair formation improves the mass transfer of ionic amino acids and peptides into the porous solid-phase reagent. These ion-paired, neutral complexes with their free amino groups react with the activated derivatization reagents to form an amide derivative bearing the detection tag. Conditions for solid-phase, 9-FA derivatization of amino acids were studied, including reaction time, temperature, buffer composition, and so forth. An example of the overall approach is provided using an enzymatic peptide digest of a cytochrome c sample, which was then derivatized and analyzed.

EXPERIMENTAL

Materials

Styrene-divinylbenzene copolymer (12 % cross-linked, 60 Å templated, 10–20 μm) was obtained from Supelco (Bellefonte, PA, USA). Amino acids, cytochrome c (horse heart), chymotrypsinogen A, trypsin-TPCK and trifluoroacetic acid were all obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN) solvent was generously donated by EM Science (Gibbstown, NJ, USA). Cationic surfactants were purchased from Aldrich (Milwaukee, WI, USA).

Instruments

Gradient separations of 9-FA tagged amino acid and peptide derivatives were performed on an automated Gilson HPLC system (Gilson

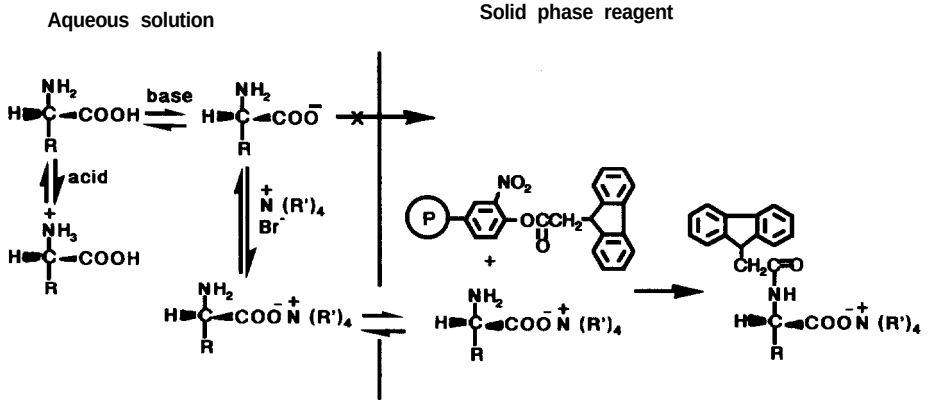

Fig. 1. Mechanism of phase transfer catalysis solid-phase derivatization for amino acids.

Medical Electronics, Middleton, WI, USA). This system consisted of a Gilson 232 auto-sampler, two Gilson 203 HPLC pumps, a Gilson 121 fluorescence detector with excitation at 254 nm and emission from 305-395 nm, a Gilson 811B dynamic mixer (1.5 ml), a Gilson 621 DataMaster, and an AST Premium 286 computer (AST Research, Irvine, CA, USA). The separation column was a YMC AP-303 300 Å ODS column (250 mm x 4.6 mm I.D.) from YMC (Morris Plains, NJ, USA). Chromatographic conditions were: mobile phase A: 0.05% (v/v) TFA in water; mobile phase B: 0.05% (v/v) TFA in ACN. The gradient program was: 0.00 min %B = 30.0; 16.00 min %B = 70.0; 22.00 min %B = 70.0; 22.50 min %B = 30.0.

Preparation of 9-FA tagged solid-phase reagent

The synthesis of the polymeric 9-FA derivatization reagent was performed as described in the literature [13,15]. Loading determinations of tag, by hydrolysis in a basic solution, showed 0.65 ± 0.05 mmol/g (n = 2) of tag content [4,11].

Synthesis of 9-FA amino acid standards

The authentic, standard derivatives of four amino acids were prepared by following a modified literature procedure, using 9-fluoreneacetyl chloride [16]. A 7.6 mmol sample of the amino acid was dissolved in 20 ml of 20% sodium carbonate. This solution was cooled to 0°C and 7.6 mmol of 9-fluoreneacetyl chloride (in 10 ml dioxane solution) was added dropwise, under stirring for 30 min. The solution was left at room temperature and stirred for 2 hours. The solution mixture was poured into 100 ml of distilled water. A 37% aqueous HCl solution was added to pH 2. The precipitate was collected and recrystallized from methanol. Mass spectrometric (MS) characterizations of these standards showed the expected structures. For example, the ammonia chemical ionization (Cl) mass spectrum of the 9-FA derivative of phenylalanine showed m/z values: 388.1; 386.1; 342.1; 300.2; 291.1; 170.1; 165.1; 121.1; 106.1; and 41.1 (M+ NH, = 388.1). Other mass spectra of similar 9-FA amino acid derivatives were obtained (not reported).

Acid hydrolysis of proteins

A l.O-mg sample of lyophilized protein, such as cytochrome c, was dissolved in 2 ml of 6 *M* HCl and heated under 90°C for 12 h in a sealed vial. The hydrolysate was dried under vacuum and reconstituted in 2.0 ml distilled water. This hydrolysate solution was directly used in the solid-phase derivatizations.

Trypsin digestion of cytochrome c

A solution of 2 mg/ml cytochrome c (from horse heart) was prepared in 100 m*M*, pH 8 ammonium bicarbonate buffer. Trypsin-TPCK was dissolved in the same buffer at a concentration of 0.1 mg/ml. To 0.5 ml of cytochrome c solution, 0.5 ml of trypsin solution was added. Digestion was kept at 37°C for 24 h, and then terminated by heating the digested solution at 100°C for 5 min [17]. The digested solution was directly derivatized with a solid-phase reagent.

Off-line derivatization

Off-line derivatizations were performed with 25 μl of analyte solution, 25 μl of sodium borate buffer solution, 25 μl of varying concentrations of a cationic surfactant and 12.5 μl ACN. Derivatization was performed in a water bath in a disposable pipet packed with 10 mg of 9-FA tagged reagent. After derivatization, the solid-phase reagent was washed with 1.0 ml of 70% ACN-water, 20 μl of which was injected into the HPLC.

RESULTS AND DISCUSSION

The cationic surfactants act as phase transfer catalysis agents by ion-pairing with a carboxyl group of the amino acids or peptides. The carboxyl group of amino acids is a weak acid, and needs a quaternary amine surfactant in order to form stable, hydrophobic, ion-pair complexes. Thus, type, size, and concentration of ion-pairing surfactant were important to the final ion-pair formation and to the derivatization efficiency.

Effect of ion-pair surfactant

To investigate the influence of ion-pair surfactant, off-line derivatizations of four amino acids

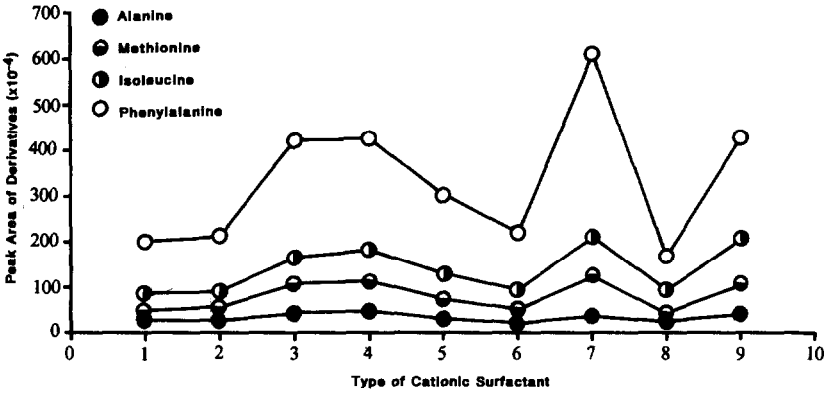

Fig. 2. Effect of surfactant type in solid-phase derivatizations. Off-line derivatization of *ca.* 10 mg 9-FA tagged reagent with 12.5 μl ACN, 25 μl 1.0 m*M* amino acid mixture, 25 μl of 20 m*M* surfactant and 25 μl of $Na_2B_4O_7$ buffer. After 10 mm reaction at 75°C, the polymeric reagent was washed with 1.0 ml 70% ACN and 20 μl of the washings was injected. 1 = Tetramethylammonium chloride; 2 = tetraethylammonium hydrogen sulfate; 3 = tetra-n-propylammonium hydrogen sulfate; 4 = tetra-*n*-butylammonium bromide; 5 = tetrabutylammonium hydrogen sulfate; 6 = tetrabutylammonium dihydrogen phosphate; 7 = hexadecyl(cetyl)trimethylammonium bromide (CTAB); 8 = trimethylphenylammonium bromide; 9 = benzyltriphenylphosphonium chloride.

with nine cationic surfactants were studied (Fig. 2). These nine surfactants had different ion-pairing tendencies, hydrophobicities, steric hindrances, and thus different phase transfer abilities. Of the nine surfactants tested, hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide, CTAB) provided the highest derivatization yield. Thus, this ion-pair reagent was chosen as the best surfactant for amino acid and peptide derivations. It is of course possible that for other peptides, different ion-pair reagents may prove more optimal.

The optimum concentration of an ion-pair surfactant is dependent on its ion-pair formation tendency and hydrophobicity in a derivatization buffer. Strong ion-pairing ability and hydrophobicity of the cationic surfactant both enhance the accessibility of amino acids to the immobilized reagent. Solid-phase derivatization efficiencies for amino acids were very low (cu. 15% for phenylalanine) without any cationic surfactant catalyst in the derivatization solution. With the CTAB phase transfer surfactant, an 85% derivatization efficiency was obtained for phenylalanine. Investigation of surfactant concentration showed that there was no apparent increase in the derivatization yield when the CTAB concentration was larger than 20 m*M*. Thus, 20 m*M* CTAB was used to perform these solid-phase derivatizations.

Derivatization temperature and time

Temperature optimization was performed by holding the reaction time at 10 min. The derivatization yields of amino acids increased as the reaction temperature increased (Fig. 3).

A temperature of 70°C was selected as the optimum derivatization temperature, although higher derivatization yields were obtained at 85°C. A high derivatization temperature tends to increase hydrolysis of the solid-phase reagent, rendering interference peaks from the hydrolysis by-products. The boiling point of ACN is 82°C, which limits the temperature of the derivatization. Thus, a final temperature of 70°C was a compromise between the desired and undesired effects. The effect of reaction time on the amino acid derivatization was investigated at 70°C (Fig. 4). Although an increase in derivatization yield was possible beyond 10 min, 70°C for 10 min were selected as the derivatization conditions in order to get good reproducibility and a short derivatization procedure.

Buffer pH of derivatization solvent

In order to derivatize amino acids, buffer solutions with a basic pH are needed to provide enough nucleophilicity to the amino group. Fig. 5 shows the pH effect on the derivatization yield of amino acids. At pH 9.1, the amino group is in the free base form and the nucleophilic car-

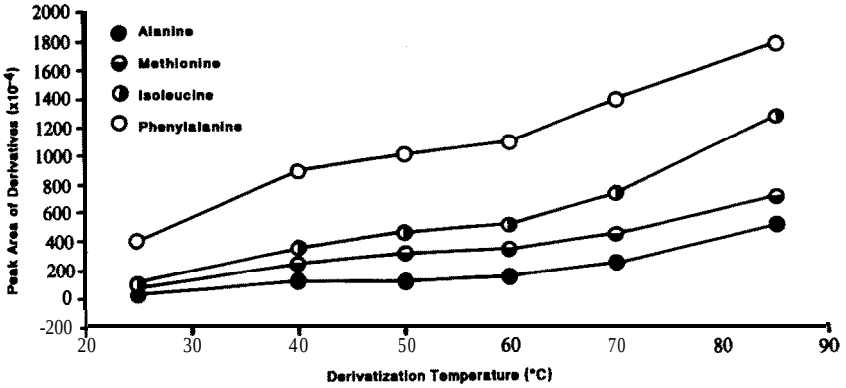

Fig. 3. Effect of temperature on off-line derivatization using CTAB; other conditions as in Fig. 2. Sample: amino acid mixture (1.0 m*M* in water).

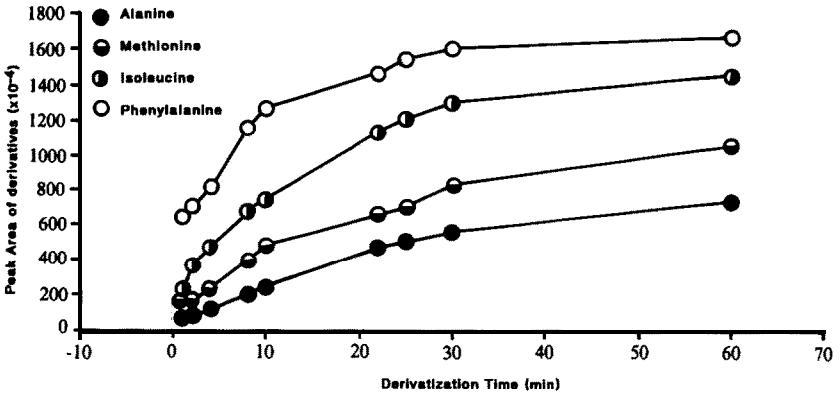

Fig. 4. Effect of time on off-line derivatization. Derivatization temperature 70°C; other conditions as in Fig. 2.

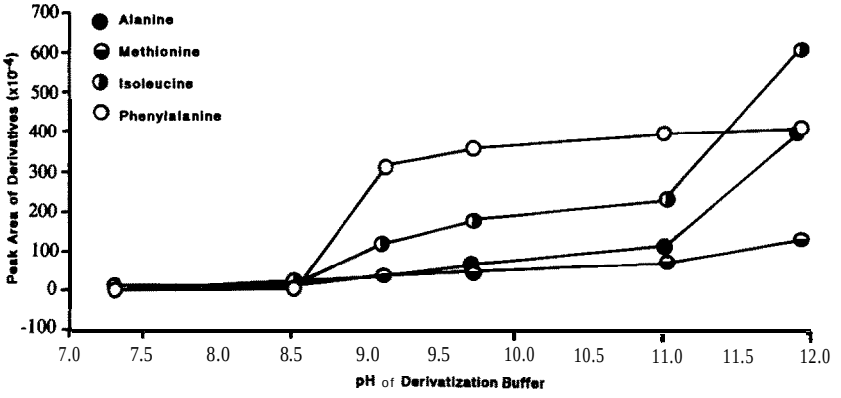

Fig. 5. Effect of pH on the derivatization yield of amino acids. Off-line derivatizations of 1.0 m*M* amino acids prepared in aqueous solution. Conditions as in Fig. 2, derivatization at 70°C for 10.0 min.

boxylate is negatively charged and ion-paired to the quaternary ammonium surfactant. Phase transfer now occurs with ease, and the nucleophilic amino group is derivatized by the hydrophobic polymeric reagent. At pH > 11, decomposition of the activated reagent was fast,

and the 9-FA acid hydrolysis by-product interfered in the chromatographic separation. Thus, pH 9.1 (sodium borate buffer) was selected as a compromise, to get reasonable percent derivatization and minimal reagent hydrolysis.

The ionic strength of the derivatization buffer suppresses the ion-pair dissociation, and affects the percent derivatization of amino acids by increasing the concentration of analyte in the polymeric support. The sodium borate concentration of the derivatization buffer needs to be maintained at levels high enough to ensure its basic buffer capacity and deprotonation of the amines. Derivatization yields of amino acids increased with sodium borate concentration, and a saturated sodium borate solution (0.23 *M* at room temperature) was the best for amino acid derivatizations.

Analyte hydrophobicity

Solid-phase derivatization is directly dependent on the actual concentration of each ion-paired amino acid in the polymeric reagent. This concentration is determined by the formation constant and the partition coefficient of the ion-paired, amino acid-quaternary ammonium complexes. Hydrophobic amino acids have stronger ion-pair formation constants, and with larger partition coefficients, they can be more efficiently extracted by the hydrophobic polymeric reagent. The less hydrophobic amino acids were expected to be less extractable. Consequently, they would be less derivatized by the polymeric reagent, as indeed was observed (Fig. 6).

Percent derivatization efficiencies of alanine, methionine, isoleucine and phenylalanine were 11 (8), 26 (6), 58 (5) and 85 (9), respectively, following their increased hydrophobicity. The numbers in parentheses are relative standard deviations (R. S.D.) of derivatization efficiency (n = 3) for each amino acid. Such big differences in derivatization efficiencies make the method somewhat less attractive for quantitative determinations of amino acid mixtures. However, from an applications point of view, this method is still practical, as long as the reaction conditions can ensure reproducibility of the method. The 9-FA tagged amino acid derivatives in the derivatizing buffer solution were stable over a two-week storage at ambient temperature, without any significant changes in peak intensities or retention times. Good stability of the final amino acid derivatives allowed for off-line derivatizations and provided reproducible results.

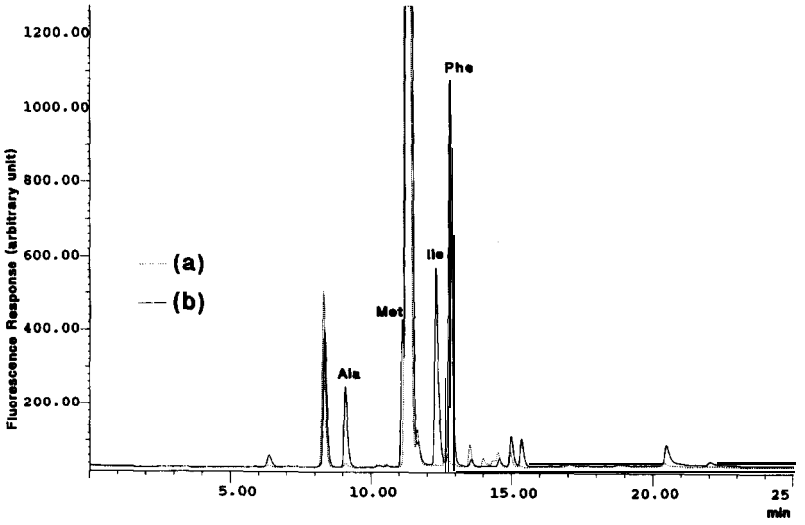

Fig. 6. Chromatogram of solid-phase derivatixation of amino acid mixture. Separation column: YMC AP-303 300 Å ODS column, 250 mm × 4.6 mm I.D.; detection: FL, excitation wavelength 254 nm, emission wavelength 305–395 nm, 0.001 relative fluorescence units. Gradient separation in 25 min at flow-rate 1.5 ml/min. Mobile phase A: 0.05% TFA-water, B: 0.05% TFA-ACN in gradient: 0.00 min %B = 30.0; 16.00 min, %B = 70.0; 22.00 min, %B = 70.0; 22.50 min, %B = 30.0. (a) Blank; (b) 1.0 m*M* amino acid mixture.

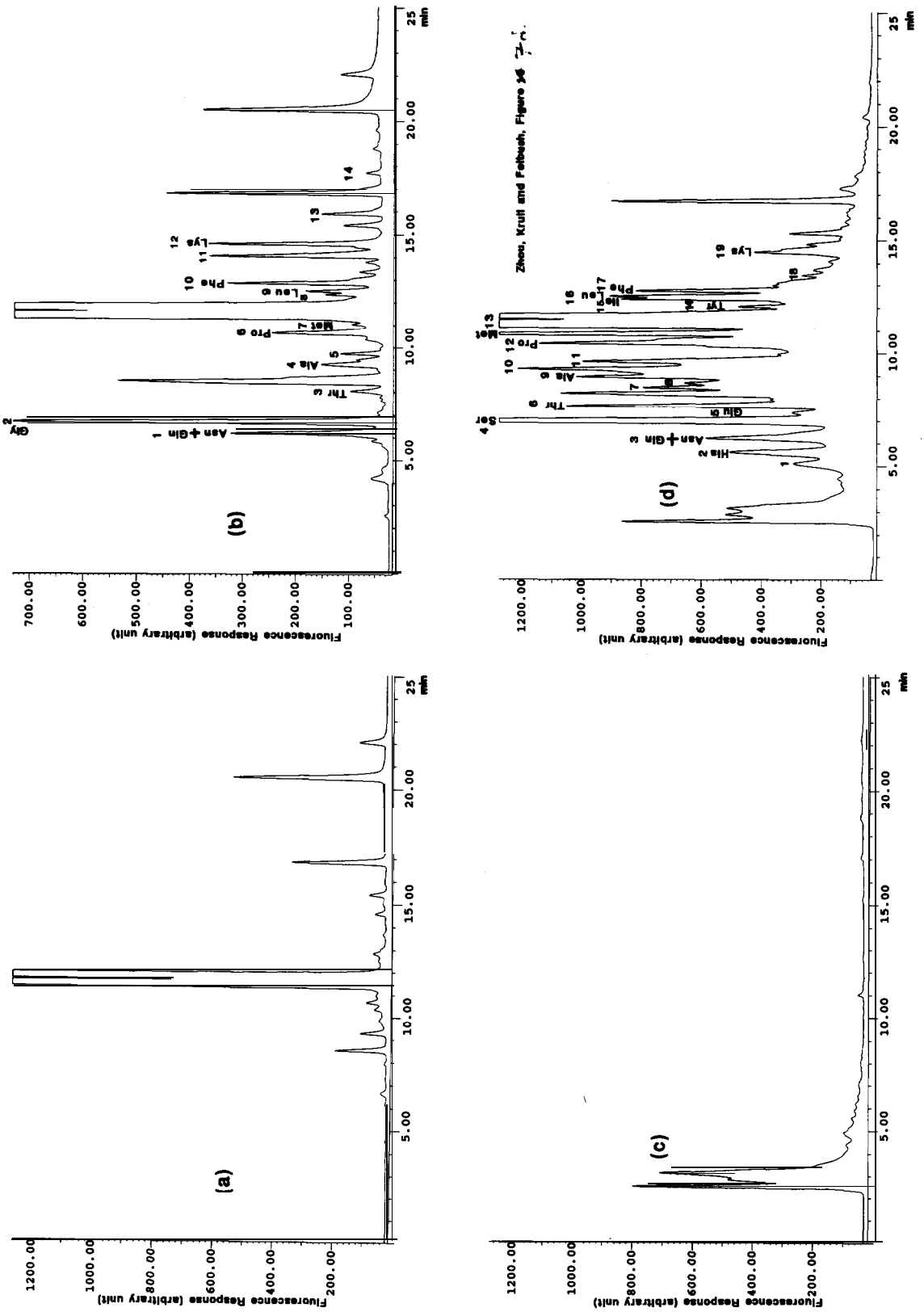

Fig. 7. Solid-phase derivatizations of protein hydrolysate. (a) Blank test of solid-phase reagent with water; (b) solid-phase derivatization of cytochrome *c* hydrolysate; (c) chymotrypsinogen A hydrolysate without derivatization; (d) solid-phase derivatization of chymotrypsinogen A hydrolysate. Separation and derivatization conditions as in Fig. 6.

Derivatization of amino acids from a protein hydrolysate

Fig. 7a is a blank test of the solid-phase reagent by off-line derivatization. The main peak at 11.3 min was confirmed to be 9-fluoreneacetic acid by a retention time comparison with an authentic standard under the same separation conditions. A chromatogram of the cytochrome c hydrolysate without derivatization showed no fluorescent response at the detection wavelengths. Fig. 7b shows a gradient separation of the solid-phase derivatized cytochrome c hydrolysate. There were 13 major derivative peaks. The resolutions of the 9-FA tagged amino acid derivatives were not fully optimized, some of the derivatives were not fully optimized, and thus some of the derivatives were not fully separated with these specific gradient conditions. However, these qualitative results demonstrated that the amino acids were derivatized by the solid-phase reagent. Because the derivatization percentage of amino acids with this method is analyte dependent, ratios of peak intensity in the chromatogram of the derivatized protein hydrolysate do not represent a quantitative ratio of the amino acid composition. Standard calibration plots would be needed in order to derive such more quantitative data.

Fig. 7c is a chromatogram of the underivatized protein hydrolysate from chymotrypsinogen A. There were few components which had fluorescence responses. Fig. 7d is a chromatogram of the derivatized chymotrypsinogen A hydrolysate. Sixteen major derivatization products were obtained with FL detection. Comparing Fig. 7b and d, obvious differences can be seen in the intensities and positions of derivative peaks. These different chromatograms qualitatively show the differences of the two proteins in their amino acid compositions.

Derivatization of enzymatically digested cytochrome c

An enzymatically digested cytochrome c was chosen as a sample of a peptide mixture. There were 14 major peptide peaks separated from digested horse cytochrome c with gradient reversed-phase separation and UV detection at 214 nm. Separation of the solid-phase derivatized digestion buffer (trypsin dissolved in ammonium bicarbonate buffer) showed the 9-FA acid from the hydrolysis of the solid-phase reagent and the derivatization product of ammonia, while the digested cytochrome c showed no fluorescent response without derivatization. Fig. 8 shows the derivatized peptides from a cytochrome c digestion with solid-phase derivatization, which has 15 major derivative peaks.

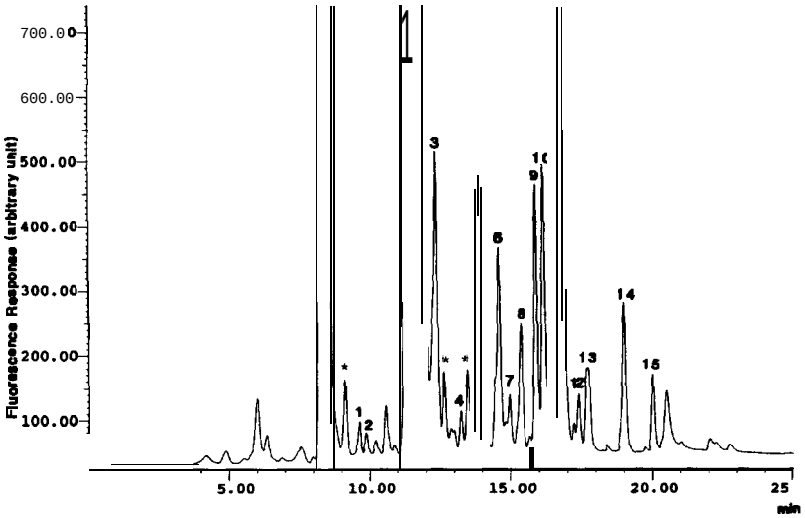

Fig. 8. Solid-phase derivatization of trypsin digested cytochrome c. Separation conditions as in Fig. 6. Peaks with * were from the derivatization of blank digestion solution (trypsin with buffer).

CONCLUSIONS

The authors have developed a reaction-detection technique using solid-phase, ion-pairing derivatization of amino acids and peptides. The high sensitivity of fluorescent derivations and the hydrophobic extraction function of polymeric reagents made this approach very promising for ionic nucleophiles. Due to the interference by 9-fluoreneacetic acid produced by hydrolysis of the derivatization reagent, an on-line derivatization of amino acids with this 9-FA tagged solid-phase reagent was unsuccessful. Immobilization of other derivatization tags, which do not introduce detection interference by hydrolysis products, are being investigated in our research group for on-line derivatizations.

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