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Automated precolumn derivatization of amino acids with *ortho*-phthalaldehyde using a hollow-fibre membrane reactor

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SUMMARY

An automated precolumn derivatization procedure was developed for the determination of amino acids, involving the reaction of amino acids with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol using a cation-exchange hollow-fibre membrane reactor, which is immersed in the reagent solution, and a trap column. Column switching is used to transfer OPA–amino acid derivatives from the trap column to an analytical C_{18} column, and the derivatives are subjected to gradient elution and fluorimetric detection. The detection limit is 0.3–2 pmol for each amino acid. For 20 pmol of amino acids, the precision is 0.9–5.0% (relative standard deviation, $n = 5$). The determination of amino acids in a protein hydrolysate sample is described.

INTRODUCTION

In recent years, automated procedures for pre- and postcolumn derivatization have been developed for the high-performance liquid chromatographic (HPLC) determination of various compounds. For the trace determination of amino acids, precolumn derivatization with dansyl chloride[1], 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole[2], *o*-phthalaldehyde (OPA)[3,4] and 9-fluorenylmethyl chloroformate[5] and phenyl isothiocyanate[6] has been employed.

Although the precolumn derivatization method with OPA is sometimes applied to amino acids, the precision of the method is dependent on the control of the reaction time. Therefore, various automated procedures have been developed, such as those using a peristalic pump[7,8], a reciprocating pump[9], or pneumatic pressure[10] to mix the sample and reagent, those using an automatic injector and a glass bead column (the former for injecting the sample and reagent and the latter for performing the derivatization reaction)[11,12] and those using a modified automatic injector[13,14]. Recently, an automatic injector which can automate precolumn derivatization has become commercially available. The problem with most such automated procedures is that they require the delivery, injection or dispensing of two or three solutions (sample and reagent solutions or these two plus the derivatized solutions). Errors in any of

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these procedures would have a detrimental effect on the precision and accuracy of the assay.

In a previous paper[15], we reported an HPLC method for the determination of amino acids using cation-exchange and anion-exchange hollow-fibre membrane reactors (HFMRs) immersed in hypochlorite, and OPA and 2-mercaptoethanol (2-ME) reagents, which are introduced into the main flow-stream by a concentration differential. This paper deals with automated procedures for the precolumn derivatization of amino acids with OPA and 2-ME using a cation-exchange HFMR. The advantage of the proposed method is that only one injection of the sample solution is needed because of the continuous supply of a constant amount of the reagent through the HFMR. This method was successfully applied to the determination of amino acids in a protein hydrolysate sample.

EXPERIMENTAL

Reagents and materials

Amino acids (L-form) were purchased from the Protein Research Foundation (Minoh, Osaka, Japan). OPA, 2-ME, and bovine serum albumin (BSA) were obtained from Nacalai Tesque (Kyoto, Japan). Acetonitrile of HPLC grade and other chemicals of analytical-reagent grade were obtained from Wako (Osaka, Japan). A cation-exchange HFMR (AFS-2) was obtained from Dionex (Sunnyvale, CA, U.S.A.).

Water prepared with a NANOpure II unit (Barnstead, Boston, MA, U.S.A.) was used for the preparation of the eluent and the sample solution. The OPA–2-ME reagent solution was prepared by dissolving 300 mg of OPA in 3 ml of ethanol and, after addition of 120 μl of 2-ME, diluting the solution to 20 ml with 20 m*M* borate buffer solution (pH 9.3).

Chromatography

The experimental setup is illustrated in Fig. 1. The pumps were a Trirotar-V pump (pump A) (Japan Spectroscopic, Tokyo, Japan) equipped with a gradient

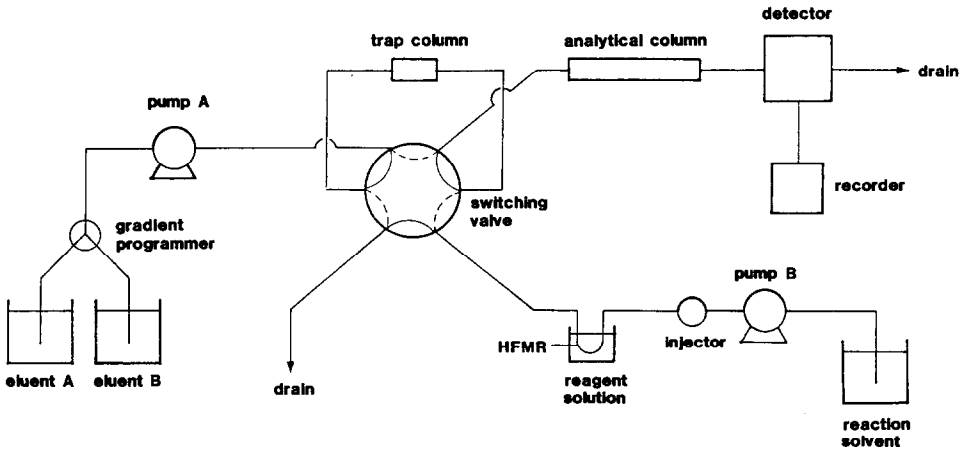

Fig. 1. Experimental setup. HFMR = hollow-fibre membrane reactor. Dashed lines, flow lines for the precolumn derivatization; solid lines, flow lines for separation and detection of the OPA–amino acid derivatives.

programmer (GP-A40; Japan Spectroscopic) and a degasser (KT-31, Showa Denko, Tokyo, Japan) for delivering the eluent and a Bip-I pump (pump B) (Japan Spectroscopic) for delivering the reaction solvent. The injector and automatic sampler were a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 100-μl loop or a Sil-9A Auto Injector (Shimadzu, Kyoto, Japan), respectively. The columns were a 250 × 4.6 mm I.D. column packed with Develosil ODS-5 (5-μm particle size) (Nomura Chemicals, Seto, Aichi, Japan), a guard column (30 × 4.6 mm I.D.) packed with the same materials and a trap column (10 × 4.6 mm I.D.) packed with Capcell Pak C_{18} (10–25-μm particle size) (Shiseido, Tokyo, Japan), which is a silicone-coated silica gel modified with octadecyl groups and has excellent stability against alkaline mobile phases such as at pH 9–10[16]. Other equipment consisted of a cation-exchange HFMR, 15 cm in length, a PT-8000 six-port switching valve (Tosoh, Tokyo, Japan), an F1000 spectrofluorimeter detector (Hitachi, Tokyo, Japan) equipped with a 12-μl flow-through cell and a C-R3A recorder–integrator (Shimadzu).

The eluents used were as follows: eluent A, 5 m*M* sodium dihydrogenphosphate–5 m*M* disodium hydrogenphosphate–acetonitrile (4:4:1, v/v/v); eluent B, 5 m*M* sodium dihydrogenphosphate–5 m*M* disodium hydrogenphosphate–acetonitrile (1:1:2, v/v/v); linear gradient, 0–70 min, 80% eluent B. The flow-rate was maintained at 0.8 ml/min. The reaction solvent, with the flow-rate maintained at 0.1 ml/min, was 20 m*M* borate buffer solution (pH 9.3).

The HFMR inserted between the injector and the trap column was immersed in 20 ml of OPA and 2-ME reagent. The HFMR was inserted in a PTFE tube (*ca.* 2 cm × 0.8 mm I.D.) and was attached to the connecting fittings (Japan Spectroscopic), as reported previously[17]. Precolumn reaction and separation were carried out at ambient temperature. Detection was performed with excitation at 340 nm and emission at 450 nm.

Automated precolumn derivatization procedure

Automated precolumn derivatization of amino acids with OPA was accomplished by employing the HFMR, a trap column and a six-port switching valve. Amino acid samples were introduced via the injector or the automatic sampler for the semi-automated or fully automated procedure, respectively. The samples reacted both in the HFMR and in the trap column. The OPA and 2-ME were delivered through the passive HFMR immersed in the reagent solution, by a concentration differential. The six-port valve was switched 2 min after injection of the sample. The OPA–amino acid derivatives were transferred to the analytical column in the back-flush mode and separated by gradient elution.

Preparation of protein hydrolysate sample

The protein sample (*ca.* 1 mg) was dissolved in 1 ml of 6 *M* hydrochloric acid and transferred to a hydrolysis tube. The tube was flushed with nitrogen, sealed and left in an oven at 110°C for 24 h. The hydrolysate sample was neutralized with 6 *M* sodium hydroxide solution and diluted 50-fold with 5 m*M* sodium dihydrogenphosphate–5 m*M* disodium hydrogenphosphate–acetonitrile (4:4:1, v/v/v). A 20-μl aliquot of the sample was injected onto the system described above.

RESULTS AND DISCUSSION

Precolumn reaction conditions for HFMR

In a previous paper[15], we reported the successful postcolumn derivatization of primary and secondary amino acids with OPA and 2-ME and with hypochlorite using cation- and anion-exchange HFMRs immersed in the reagent solution. The negatively charged membrane matrices permitted transport of both the uncharged and positively charged spcies due to the concentration gradient[15]. In this study, we tried to automate the precolumn derivatization of amino acids with OPA and 2-ME by using cation-exchange HFMR and a trap column in a precedure that included column switching.

The precolumn reaction conditions for Asp, Asn, Thr, Arg and Ala as typical amino acids were examined with respect to the various factors affecting the precolumn reaction: pH of the reaction solution, length of HFMR, reaction time and concentrations of OPA and 2-ME. The pH of the reaction solution used for the precolumn derivatization of amino acids with OPA was 9–10[7–14]. Therefore, we selected 20 m*M* borate buffer (pH 9.3) as the reaction solvent. The length of the HFMR, which is an important factor for introducing the reagent into the sample flow stream, was varied from 7.5 to 30 cm. Under the assumption that the derivatization reaction occurred completely, the leakage of Arg (basic amino acid) from the negatively charged membrane matrices was about 40, 65, 80% at HFMR lengths of 7.5, 15 and 30 cm, respectively (leakage calculated by comparison with standard loop injection of the corresponding OPA–amino acid derivatives). No leakage of other amino acids, except Asp (about 30% at a length of 15 cm), was observed in spite of the length of the HFMR. Therefore, 15 cm was chosen as the length of the HFMR. Since the leakage of ammonia from the negatively charged membrane matrices occurs to a greater extent than that of Arg, the present precolumn derivatization method is almost free from the influence of any ammonia which may contaminate the sample solutions.

The instability of OPA–amino acid derivatives is known to affect the precision and accuracy of precolumn derivatization methods[9,10]. The factor that must be controlled most precisely is the reaction time. In our precolumn reaction system, the factors affecting the reaction time were the flow-rate of the reaction solvent and the column switching time.

Fig. 2A and B show the effects of the flow-rate of the reaction solvent and the column switching time, respectively, on the fluorescence intensity of the OPA–amino acid derivatives. At 0.2 ml/min (column switching time 2 min), the peak of Asp was not observed owing to elution of the OPA–Asp derivative from the trap column. The fluorescence intensity of Arg increased with increase in flow-rate, because Arg leakage decreased with decrease in residence time. Therefore, the flow-rate of the reaction solvent was fixed at 0.1 ml/min.

The column switching time was varied from 1 to 5 min. Asp was eluted from the trap column at a column switching time of later than 4 min. The fluorescence intensity of the other amino acid derivatives was almost constant at column switching times longer than 2 min. Therefore, the column switching time was selected as 2 min.

Fig. 3A and B illustrate the effects of the concentrations of OPA and 2-ME on the fluorescence intensity. The fluorescence intensities of Arg and Ala were almost unaffected by the concentrations of OPA and 2-ME, whereas those of the other amino

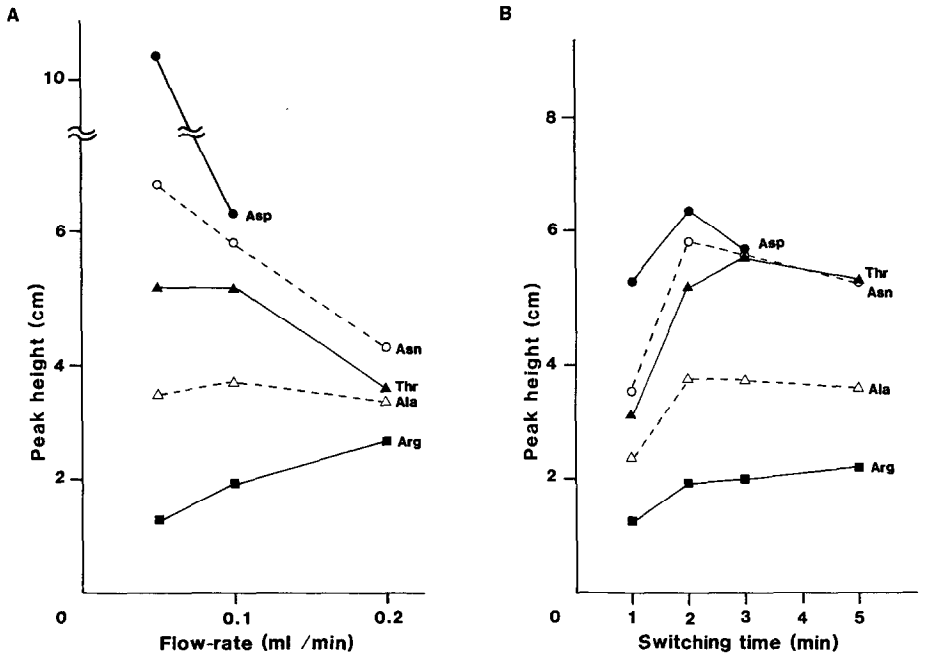

Fig. 2. Effects of (A) flow-rate of the reaction solvent and (B) column switching time on the fluorescence intensity of the OPA–amino acid derivatives. Amounts of 100 pmol of each amino acid were injected and the peak heights were measured. The concentrations of OPA and 2-ME were 15 mg/ml and 0.6%, respectively. (A) Switching time fixed at 2 min and (B) flow-rate of reaction solvent fixed at 0.1 ml/min.

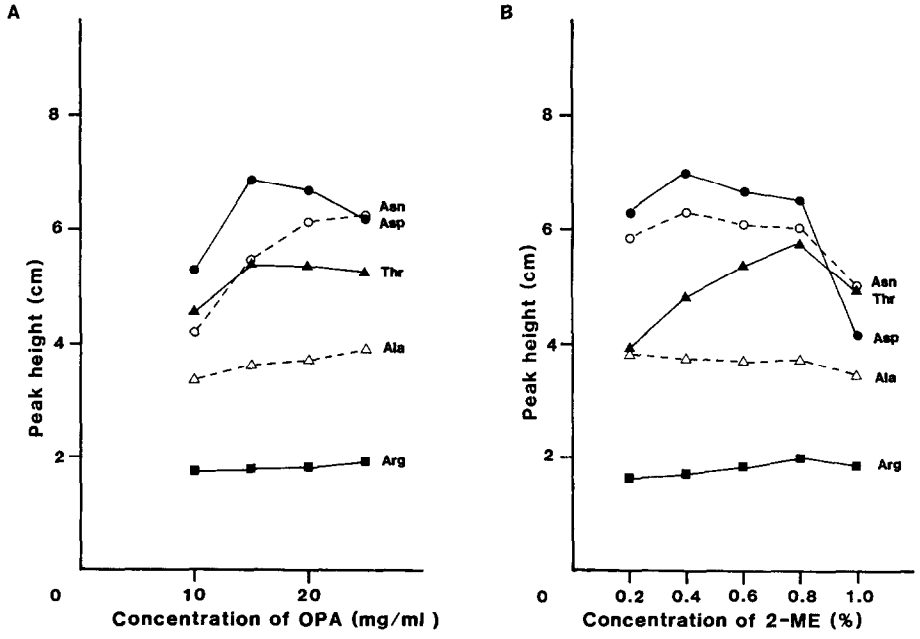

Fig. 3. Effects of concentrations of (A) OPA and (B) 2-ME on the fluorescence intensity of the OPA–amino acid derivatives. Amounts of 100 pmol of each amino acid were injected and the peak heights were measured. Flow-rate of reaction solvent, 0.1 ml/min; switching time, 2 min. (A) Concentration of 2-ME fixed at 0.6% and (B) concentration of OPA fixed at 15 mg/ml.

acids were considerably affected. The optimum concentrations of OPA and 2-ME were determined to be 15 mg/ml and 0.6%, respectively.

The precolumn reaction conditions described under Experimental were used for the assay of amino acids. The chromatogram of a standard mixture of amino acids, obtained under the optimum precolumn reaction conditions, is shown in Fig. 4.

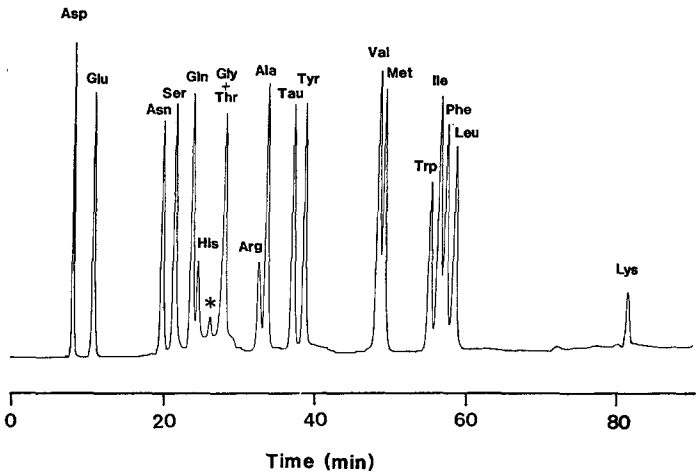

Fig. 4. Chromatogram of a standard mixture of amino acids. Amount injected, 100 pmol of each except for 50 pmol for Gly and Thr and 200 pmol for Lys. The asterisk indicates a system peak.

Reproducibility, linearity and detection limits

Table I lists the relative standard deviations (R.S.D.) ($n = 5$) for the measured peak heights of standard amino acids when 20 and 100 pmol were injected. The reproducibility was good for the amino acids studied. The measured peak heights were randomly scattered around a mean value, and there were no trends such as a constant decrease in peak height with time. This shows that at the reaction stage the optimum concentrations of OPA and 2-ME are maintained in spite of their continuous depletion. Although the OPA–2-ME reagent could still be used after a 24-h run, it was freshly prepared every day.

The calibration graph constructed from peak height *versus* absolute amount of each amino acid was linear over the range 2–500 pmol with a correaltion coefficient of 0.999 or above, and passed through the origin. The detection limits for amino acids with the proposed method are below 0.3–2 pmol and are determined by the purity of the reagent rather than by the signal-to-noise ratio, as reported previously[8].

Application to determination of protein amino acids

On the basis of the above findings, we applied the method to the determination of protein amino acids. Fig. 5 shows a chromatogram of a BSA hydrolysate sample. As the asparagine and glutamine residues were converted to aspartic acid and glutamic acids, respectively, during acid hydrolysis, they did not appear on the chromatogram. The composition of the amino acids agreed very well with that reported previously[15,18], as shown in Table II.

TABLE I

REPRODUCIBILITY OF AMINO ACID ASSAY

Relative standard deviations (R.S.D.) of five analyses. Amounts of Gly and Thr injected, 10 or 50 pmol of each.

Amino acid	*R.S.D. (%)*	
	20 pmol	*100 pmol*
Asp	5.0	4.0
Glu	2.9	2.9
Asn	2.1	2.6
Ser	4.7	2.3
His	1.6	3.1
Gly + Thr	4.0	3.3
Arg	4.6	3.6
Ala	2.0	1.8
Tau	1.1	0.6
Tyr	1.3	0.8
Met	0.9	1.0
Val	2.1	1.3
Trp	1.6	0.6
Ile	0.9	0.8
Phe	1.4	0.9
Leu	1.6	2.1
Lys	4.5	3.5

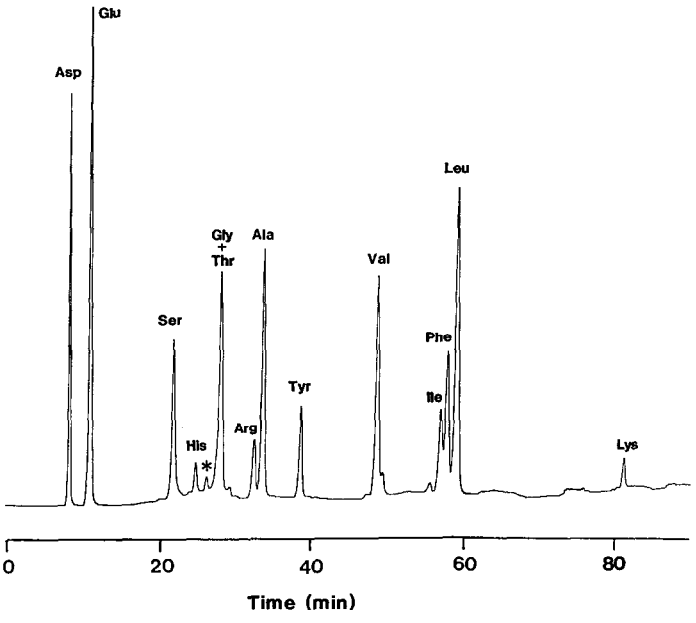

Fig. 5. Chromatogram of a BSA hydrolysate sample. The asterisk indicates a system peak.

TABLE II

AMINO ACID COMPOSITION OF BOVINE SERUM ALBUMIN

All values normalized to alanine = 1.00.

Amino acid	*Found*	*Known*
Asp + Asn	1.24	1.20
Glu + Gln	1.77	1.73
Ser	0.55	0.61
His	0.35	0.34
Arg	0.73	0.51
Ala	1.00	1.00
Tyr	0.37	0.37
Val	0.78	0.69
Ile	0.32	0.23
Phe	0.58	0.54
Leu	1.42	1.27
Lys	1.20	1.28

We are now investigating the application of this automated precolumn derivatization method to assays of biological substances and drugs in biological fluids followed by automated pretreatments such as deproteinization, extraction, and/or concentration.

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