A Low Picomole Fluorometric Detection System for Amino Acid Analysis

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An amino acid detection system was developed for low picomole analysis of amino acids including proline, based on OPA-post-labeling with a non-switching NaClO flow system. A computer-controlled HPLC equipped with a three-solvent gradient mixer was assembled with the detection system to analyze 17 kinds of amino acids in 85 min with a stable base line. The optimum conditions of the NaClO and OPA solutions were determined as 0.002%–0.22 ml/min and 0.16%–0.26 ml/min, respectively. The use of NaClO caused only approximately 30% decrease in fluorescence response of the usual amino acids, except for Pro and Cys, the latter even being enhanced by about 10 times. The detection limit for Pro and Cys was 500 and 1000 fmol, respectively, and that of the usual amino acids was 100 fmol. The calibration curves of all amino acids showed good linearity in a range between 5 and 500 pmol. This system was used as a detector for enantiomeric analysis of glutamic acid and cysteic acid with a reversed phase HPLC during stereochemical studies on the natural peptide derivative, oxyecdystin.

Improvements to the sensitivity and convenience of automatic amino acid analyzers have always been sought by those who deal with biologically important natural peptides which are often obtained in only trace quantities. The introduction of the fluorogenic reagents, fluorescamine and *o*-phthalaldehyde (OPA),¹⁾ has stimulated research in this field, since the sensitivity of detection of amino acids using these reagents is up to 100 times higher than the usual ninhydrin method; the fluorescence method can detect the amino acids in a pmol range. However, the method suffers from the serious drawback that secondary amino acids such as proline cannot be detected.

A combination of fluorescamine and *N*-chlorosuccinimide (NCS) seemed to solve the problem; NCS oxidizes Pro to 4-aminobutanal, which is subsequently detected with fluorescamine.²⁾ Fluorescamine, however, has several technical difficulties due to its instability with moisture, anhydrous acetone being used for its storage solution. Mixing the or-

ganic solvent and the buffer solution used for amino acid separation often causes bubble formation in the line. On the other hand, NCS is unsuitable for OPA-detection because it interferes with the reaction. Other oxidants, sodium hypochlorite (NaClO)³⁾ and chloramine-T,⁴⁾ were also known to convert Pro into OPA-sensitive derivatives, and have been utilized for automatic amino acid analysis using the switching flow method, where the oxidant solution is connected to the line only when Pro is to be detected.

Böhlen *et al.* have adopted a switching flow system using NaClO as the oxidant, because considerable destruction of amino acids was found when the oxidant was continuously added to all of the amino acids.⁵⁾ The switching method suffered from several problems such as base line fluctuation and operation difficulty.

Ishida *et al.* reported a non-switching flow method using NaClO as the oxidant.⁶⁾ They applied a stepwise gradient system to elute the amino acids and stated that the calibra-

tion curve for each amino acid is linear from a few pmol level to a few tens of nmol level, but they did not show their calibration curve nor actual analytical data at the pmol level. Here we describe the non-switching flow method using a linear gradient elution system that can analyze 5~500 pmol amino acids including Pro, which offers practical and routine analysis using NaClO and OPA.

MATERIALS

Most of the chemicals used were the highest grade commercial products and were used without further purifi-

cation. Sodium citrate, citric acid, 2-mercaptoethanol and Brij-35 (20% w/v) were purchased from Pierce Chem. Corp. Sodium hypochlorite solution (chlorine concentration 10%), potassium hydroxide, sodium hydroxide and boric acid were from Nakarai Chemicals, Ltd. *o*-Phthalaldehyde (OPA) was from E. Merck. The amino acid calibration mixture was from Midorijuji. Proline, aspartic acid and serine were from Ajinomoto Corp. Ethanol was distilled twice from ninhydrin. Water was distilled before use twice from a quartz flask after being passed through a column packed with charcoal and an ion exchange resin.

METHODS

1) *Flow system.* The total flow system is illustrated schematically in Fig. 1. A microcomputer controlled HPLC (JASCO, Tri-Rotar-III) equipped with a three-

FIG. 1. Schematic Representation of the Amino Acid Analyzer.

TABLE I. COMPOSITION OF BUFFERS

Separation buffer	A	B	C
Na-Citrate·2H ₂ O	7.74 g ^a	88.2 g	0
Citric acid	20.0 g ^a	0	0
Na-Perchlorate	17.0 g	42.1 g	0
NaOH	0	0	8.0 g
EtOH	70 ml	0	0
H ₂ O	930 ml	1 liter	1 liter
Reagent buffer		Reagent solution	
Boric acid	25.0 g	OPA solution (0.16%)	
KOH	23.0 g	OPA	1.6 g in 10 ml EtOH
Brij-35	1.0 g	HS~OH	4.0 ml
H ₂ O	1 liter	Reagent buffer	1 liter
		NaClO solution (0.002%)	
		NaClO	0.02 g (0.2 ml, commercial solution)
		Reagent buffer	1 liter

^a Taken from ref. 7.

solvent gradient mixer and a heating oven was used as the basic instrument. It was equipped with an ammonia-filter column (JASCO AECpak, 4.6×125 mm) and a separation column (JASCO AAPak $5 \mu\text{m}$, 4.6×125 mm). An additional reciprocal pump (JASCO, SP-024-2) with two plunger units, each of which worked separately, was used to introduce the NaClO and OPA reagents into the eluate through dumper column ($44 \sim 88 \mu\text{m}$ glass beads, 2.0×250 mm). A short reaction coil A (0.3×1000 mm) was used for NaClO oxidation at 60°C for 6.8 sec. Reaction coil B (0.3×2000 mm) was followed by a bed reactor packed with glass beads ($44 \sim 88 \mu\text{m}$, 4.6×34 mm) to complete the reaction with OPA in 21 sec with minimum peak broadening. A fluorometric detector (JASCO, FP-115 filter type) was used with excitation at 365 nm and emission at 440 nm, and data-processor (Shimadzu Co., Chromatopak C-R2A) was employed to compensate the off-base line during the gradient elution.

2) *Operation of the system.* Table I shows the composition of the buffers for separating the amino acids, solvent A being the starting buffer and solvent B the final

buffer. Both buffers contained NaClO_4 instead of NaCl that was used previously⁷⁾ to protect the stainless steel line. Solvent C was used to regenerate the column and a common borate buffer was used for two reagent solutions. All buffers were conveniently prepared by simply weighing the reagents accurately without pH adjustment. Two reagent solutions were kept in the dark at 4°C throughout the analysis. The OPA solution was stable for a week, whereas the NaClO solution had to be renewed after 15 hr. The amino acids were eluted at a flow rate of 0.4 ml/min according to the following program: (i) linear gradient from 100% A to 85% A + 15% B for 25 min, (ii) linear gradient from 85% A + 15% B to 15% A + 85% B for 25 min, (iii) 15% A + 85% B constant for 15 min, (iv) 100% C for 10 min to wash the separation column, and (v) 100% A for 45 min to make the column ready for the next analysis. The separation column, the dumper columns, the reaction coils and the bed reactor were kept at 60°C in an oven for efficient separation of the amino acids and acceleration of the reactions. This operation gave good separation of the usual 17 amino acids and ammonia within 85 min. A one-cycle analysis requires 120 min.

FIG. 2. The Influence of Total Flow Rate of an 0.08% OPA Solution and Reagent Buffer on the Fluorescence Response of the Serine Peak (100 pmol).

	0.08% OPA solution	Reagent buffer
○-----○	varied	0
●-----●	0.32 ml/min	varied
●-----●	0.26 ml/min	varied
●-----●	pH of the effluent	

RESULTS

The fluorescence intensity of the amino acids analyzed in the non-switching flow system was strongly influenced by the flow rate and concentration of the NaClO solution and the effect of these factors upon the fluorescence response of Asp and Ser was investigated. Asp and Ser gave similar results and only the Ser case is shown in Fig. 2.

First, the optimum total flow rate of the two reagents was determined with a constant concentration of 0.08% OPA at various flow rates of the OPA solution ranging from 0.26 to 1.10 ml/min without adding NaClO. The resultant fluorescence response of Ser is shown in Fig. 2, which indicates the highest response of the amino acids at a flow rate of 0.48 ml/min. The buffer was then introduced in a variety of flow rates, while the flow rate of the OPA solution was kept constant at 0.26 and 0.32 ml/min. The results are summarized in Fig. 2, suggesting that the total flow rate had the dominant effect on the response of amino acids, while the flow rate of the OPA solution had very little effect. In other words, sufficient OPA was supplied even at the flow rate of 0.26 ml/min, the optimum total flow rate being 0.48 ml/min in each case. Above a flow rate of 0.48 ml/min, the peak heights decreased due to the dilution effect, since the peak areas were constant. Flow rates less than 0.48 ml/min caused an improper pH level to complete the fluorophore formation. Similar results were obtained by using 0.16% OPA at a flow rate of 0.26 ml/min.

Secondly, the optimum concentration of NaClO was determined from the sensitivity of Pro, Ser and Asp. The total flow rate of the OPA and NaClO solutions was maintained at 0.48 ml/min and the flow rate of the 0.16% OPA solution was kept at 0.26 ml/min. In Fig. 3, the fluorescence responses of Pro and Ser are plotted against the concentration of NaClO. A plateau was reached at 0.002% NaClO and higher concentrations offered very little enhancement of fluorescence, while the fluorescence response of other amino acids

FIG. 3. Effects of the Amount of NaClO on the Fluorescence Responses of Ser (100 pmol) and Pro (200 pmol).

Conditions: 0.16% OPA, 0.26 ml/min; total flow rate, 0.48 ml/min.

gradually decreased with an increased concentration of NaClO. Thus, the optimum concentration of NaClO for the analysis of all amino acids was determined to be 0.002%.

Standard amino acids containing 10 pmol each were analyzed under this condition and the results are shown in Fig. 4. In Table II, the relative intensity of each amino acid under this condition is compared with that analyzed without the addition of NaClO. With NaClO, most of the amino acids were detected at about 70% the intensity of those without NaClO, except for Cys which gave ten-times more intense fluorescence by the introduction of NaClO. A linear correlation was obtained between fluorescence intensity and the concentration of amino acids in the range of 5 to 500 pmol. Figure 5 shows some examples. The minimum detection limits for Pro, Cys and other amino acids were 500 fmol, 1000 fmol and 100 fmol, respectively.

DISCUSSION

The major difficulty to be overcome in the

FIG. 4. Chromatogram of a Standard Amino Acid Calibration Mixture.

Amount of amino acids: 10 pmol each. Conditions: JASCO AAapak (4.6 × 125 mm); buffer A, 0.2 N Na-citrate; buffer B, 1.2 N Na-citrate; buffer C, 0.2 N NaOH (see Table I); buffer flow rate, 0.4 ml/min. Gradient profile: 0~25 min (100%A to 85%A + 15%B), 25~50 min (85%A + 15%B to 15%A + 85%B), 50~65 min (15%A + 85%B, constant), 65~75 min (100%C, constant), 75~120 min (100%A, constant). OPA solution: 0.26 ml/min. NaClO solution: 0.22 ml/min. Temperature of the column and reaction system: 60°C.

TABLE II. EFFECTS OF NaClO ON THE FLUORESCENCE RESPONSE OF AMINO ACIDS

Amino acids	Relative response with/without NaClO	Amino acids	Relative response with/without NaClO
Asp	0.66	Met	0.76
Thr	1.04	Ile	0.92
Ser	0.77	Leu	0.74
Glu	0.69	Tyr	0.65
Pro	—	Phe	0.67
Gly	1.01	Lys	0.69
Ala	0.78	His	0.86
Cys	10	Arg	0.95
Val	0.73	(NH ₃)	0.59)

Amount of amino acids: 100 pmol each.

combination of OPA-NaClO reagents for fluorometric amino acid analysis was how to enhance the detection sensitivity of Pro without affecting the detection sensitivity of the other amino acids in the non-switching method. Ishida *et al.*⁶⁾ reported a non-switch-

ing stepwise-gradient flow system for the automatic analysis of amino acids including Pro at the pmol level, but no analytical charts were presented at such a level. Our studies on optimization of the sensitivities have yielded successful results for routine analysis of each amino acid including Pro and Cys using a non-switching linear gradient flow system in the range of 5 to 500 pmol. Figure 4 shows the analysis of 10 pmol of each amino acid. The linearity in the detection of all standard amino acids in our case was satisfactory for amounts less than 500 pmol (Fig. 5), although Böhlen *et al.* stated that in a non-switching flow system significant destruction of the amino acids was observed and, therefore, quantitative measurement was impossible.⁵⁾

The use of a microcomputer-controlled HPLC with a three solvent gradient mixer and a column oven made is possible to construct a highly sensitive amino acid analyzer. At a high sensitivity, the conventional stepwise gradient

FIG. 5. Standard Curves for the Determination of Four Amino Acids.

Other amino acids gave similar results, although they are not shown in this figure.

elution of amino acids is sometimes accompanied by troublesome ghost peaks, but a linear gradient elution solves this problem by broadening the ghost peaks into the base line level. Additionally, the linear gradient elution system needs only two separation buffers instead of three. These buffers and the common reagent buffer afforded sufficiently reproducible data without pH adjustment simply by mixing the reagents in accurate amounts. A gradual alteration of the base line during the gradient elution was compensated by the data-processor, by which a routine analysis of amino acids at lower than 10 pmol became possible.

Application

The application of this highly sensitive detection system is not restricted to the usual amino acid analysis. It is also effective for detection in other separation methods such as reversed phase chromatography and the gel filtration of primary amines and peptides. An example of enantiomeric analysis of subnano-

mol amino acids was reported by Hare and co-workers⁸⁾ using an ODS column with a mixture of acetonitrile and aqueous buffer at pH 5.5, with Cu^{++} and *N,N*-di-*n*-propyl-L-alanine (DPA) as the chiral eluent. We applied our OPA detection system for this enantiomeric amino acid analysis during the course of a study on the stereochemistry of oxycadystin, a heptapeptide composed of glutamic acids (3), cysteic acids (3) and glycine (1).⁹⁾ The optical isomers of Glu and cysteic acid were separated by a Develosil ODS column ($5\mu\text{m}$, $4.6 \times 250\text{mm}$) using only the chiral buffer [8mM DPA and 4mM $\text{Cu}(\text{OAc})_2$] without acetonitrile. To save analytical time was employed such a flow rate program as 0.2ml/min in 0~30 min and then 0.4 ml/min in 30~55 min. We employed 0.2M potassium borate as the reagent buffer which was sufficient to adjust the pH of the eluate in this case. NaClO was eliminated from the reagent buffer since no proline was found in the peptide. A typical chromatogram for enantiomeric separation of glutamic acid and cysteic acid at 100 pmol is

FIG. 6. Chromatogram of D,L-CysSO₃H, D,L-Glu and Gly.

Amount of amino acids: 100 pmol each. Conditions: Develosil ODS-5 (5 μ m, 4.6 $\times$ 250 mm); eluate, 4 mM Cu(OAc)₂ and 8 mM *N,N*-di-*n*-propyl-L-alanine (pH 5.5); flow rate, 0.2 ml/min (0 ~ 30 min) then 0.4 ml/min (30 ~ 55 min). OPA solution 0.16% OPA in 0.2 M K-borate buffer (0.48 ml/min). Reagent buffer: 0.2 M K-borate buffer (0.48 ml/min).

shown in Fig. 6. Using this system revealed that oxycadystin comprised three L-glutamic acids, two L-cysteic acids and one D-cysteic acid as well as glycine. The OPA-post-labeling detection system is applicable not only to the above example but also to "flow injection analysis" of amino acids or peptides, a study of which is now in progress.

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