Journal of Chromatography, 352 (1986) 169–177
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 711

o-PHTHALALDEHYDE–N-ACETYL-L-CYSTEINE AS A CHIRAL DERIVATIZATION REAGENT FOR LIQUID CHROMATOGRAPHIC OPTICAL RESOLUTION OF AMINO ACID ENANTIOMERS AND ITS APPLICATION TO CONVENTIONAL AMINO ACID ANALYSIS

NORIYUKI NIMURA* and TOSHIO KINOSHITA
School of Pharmaceutical Sciences, Kitasato University, 9-1 Shirokane-5, Minato-ku, Tokyo, 108 (Japan)

SUMMARY

A useful fluorescence derivatization reagent, *o*-phthalaldehyde–N-acetyl-L-cysteine, has been developed for the optical resolution of enantiomeric amino acids and for conventional amino acid analysis. Amino acids rapidly reacted with *o*-phthalaldehyde in the presence of *N*-acetyl-L-cysteine to give intensely fluorescent products, which were diastereoisomers. When this reaction was used in the precolumn derivatization of amino acid enantiomers, their diastereomeric derivatives were efficiently resolved on a reversed-phase column. Simultaneous analysis of common protein amino acid enantiomers was achieved by gradient elution within 70 min. In addition, the reagent was applied to the post-column derivatization of protein amino acids, combined with hypochlorite oxidation for the detection of imino acids, such as proline. Seventeen protein amino acids were sufficiently separated in 17 min by ion-pair chromatography with sodium dodecyl sulphate as a counter-ion, and they were determined at the same level of sensitivity by the above post-column fluorimetric detection system. Applications of both the precolumn and the post-column derivatization methods with the new reagent to hydrolysed protein samples are also described.

INTRODUCTION

o-Phthalaldehyde (OPTA)[1] has been widely used for the fluorometric analysis of amino acids[2–6] and biologically active amines[7–10]. OPTA readily reacts with primary amines in alkaline medium in the presence of thiol compounds, such as 2-mercaptoethanol or ethanethiol, to give highly fluorescent N-alkyl-2-alkylthio substituted isoindole derivatives. The reaction with OPTA–2-mercaptoethanol or OPTA–ethanethiol has been used for both precolumn[4–6] and post-column[2,3] derivatization in high-performance liquid chromatography (HPLC).

Numerous reagents[11–16] for chiral derivatization have been developed for the liquid chromatographic (LC) resolution of amino acid enantiomers. However, few reagents have enabled simultaneous separation and optical resolution of all common protein amino acids[17].

0021-9673/86/$03.50

In the course of our study on the simultaneous separation and resolution of amino acid enantiomers, we have attempted[18] the chiral derivatization by means of the OPTA reaction, combined with an optically active thiol compound, instead of 2-mercaptoethanol or ethanethiol, and have found that N-acetyl-L-cysteine (AcCys) is a suitable agent[18]. In addition, the OPTA–AcCys reagent was applicable to the post-column derivatization of amino acids in combination with sodium hypochlorite oxidation, which has been developed for the determination of imino acids, such as proline[19].

In the present study, OPTA–AcCys reagent was applied to the precolumn derivatization in the reversed-phase LC simultaneous analysis of protein amino acid enantiomers, and to the post-column derivatization for conventional protein amino acid analysis by reversed-phase ion-pair chromatography.

EXPERIMENTAL

Reagents and materials

OPTA and AcCys were obtained from Nakarai (Kyoto, Japan). Amino acids, organic solvents, and other reagents were purchased from Wako (Osaka, Japan) and Tokyo Chemical (Tokyo, Japan). Pure water for the preparation of all aqueous solutions was prepared by using a Milli-Q II system (Nihon Millipore, Tokyo, Japan). The reversed-phase packings, Develosil ODS (particle size, 5 μm; Nomura Chemical, Seto-shi, Japan) and Hypersil ODS (particle size, 3 μm; Shandon, Runcorn, U.K.), were packed into 200 × 6 mm I.D. and 50 × 6 mm I.D. stainless-steel tubes, respectively, in our laboratory by the conventional slurry-packing technique. The derivatization reagent for the precolumn or post-column derivatization, OPTA–AcCys, was prepared as follows. For the precolumn method, 8 mg of OPTA and 10 mg of AcCys were dissolved in 1 ml of methanol. When this reagent solution was stored in the refrigerator, the reagent was stable for at least a week. For the post-column method, 1.6 g of OPTA and 2.0 g of AcCys were dissolved in 10 ml of methanol, and the resulting mixture was diluted with 1 l of 0.2 *M* borate buffer (pH 9.2). Sodium hypochlorite was prepared by diluting 0.2 ml of commercial Antiformin® (10% aq. sodium hypochlorite solution) with 1 l of 0.2 *M* borate buffer (pH 9.2). Sodium dodecyl sulphate (SDS) solution as a mobile phase for the post-column method was prepared by dissolving 11.53 g (40 mmol) of SDS and 1 ml of 85% orthophosphoric acid in 1 l of the pure water.

Chromatographic systems

The Shimadzu LC-4A HPLC system (Kyoto, Japan) was used for the precolumn derivatization method. This system included a microprocessor-controlled dual plunger pump, a SIL-1A universal valve-injector, a CTO-2AS column oven, an FLD-1 fluorescence detector, provided with a low-pressure mercury lamp, which emits strong 360-nm light, and with an EM-4 filter to detect visible light beyond 405 nm, emitted from the fluorescent compounds in a 12-μl flow-cell, and a Chromatopack C-R2AX data processor/recorder. The column (Develosil ODS-5, 200 × 6 mm I.D.) was operated at 25°C and eluted by the mixture of 50 m*M* sodium acetate and methanol, the proportions of which were controlled by a microprocessor gradient programme. The mobile phase was delivered at a constant flow-rate of 1.0 ml/min.

For the post-column method, the mobile phase, consisting of 40 m*M* SDS–acetonitrile mixtures according to the gradient programme, was delivered at a constant flow-rate of 1.5 ml/min. Samples were injected with an autosample injector (Kyowa Seimitsu, Tokyo, Japan) in this system. Both the sodium hypochlorite reagent and the OPTA-AcCys reagent were delivered at a constant flow-rate of 0.7 ml/min by means of a double-plunger pump (Sanuki, Tokyo, Japan). The column eluate was first mixed with sodium hypochlorite in a T-piece, and then with the OPTA–AcCys reagent in another T-piece. The first mixture and the final mixture were allowed to flow successively through 60-cm long and 6-m long PTFE-tubing reaction coils (0.5 mm I.D.). The column (Hypersil ODS-3, 50 × 6 mm I.D.) and reaction coils were in a column oven at 50°C.

Precolumn derivatization procedure

To 10 μl of sample solution (amino acids dissolved in 0.1% hydrochloric acid) were progressively added 30 μl of 0.1 *M* sodium borate and 20 μl of OPTA–AcCys reagent. The resulting mixture was allowed to stand for 2 min at room temperature and then an aliquot of the solution was directly injected into the chromatographic system.

Protein hydrolysis

The protein sample (*ca.* 100 μg) was dissolved in 50 μl of 6 *M* hydrochloric acid in a Reacti-Vial®, and the vial was allowed to stand in a oven at 150°C for 2 h. After hydrolysis, the vial was cooled, and then one drop of the resulting hydrolysate was collected with a glass pipette into each of the sample cups of the autosampler of the HPLC system. The hydrolysates were evaporated to dryness in a desiccator over potassium hydroxide pellets. Then the residue in one sample cup was redissolved in 200 μl of 0.1 *M* hydrochloric acid solution for the precolumn method, and that in the other sample cup was redissolved in 100 μl of the mobile phase (40 m*M* SDS).

RESULTS AND DISCUSSION

We have previously developed a chiral derivatization reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), for the ressolution of amino acids[11,20] and catecholamines[21]. GITC is applicable to the resolution of various β-aminoalcohols[22–24], such as β-adrenergic antagonists, and to the simultaneous analysis of common protein amino acid enantiomers[17], but is difficult to obtain commercially. Therefore it was desirable to develop a more readily available reagent.

Roth[1] has developed a OPTA–thiol reagent for the fluorometric determination of primary amines, and Benson and Hare[2] have optimized the reaction conditions for the post-column derivatization of amino acid analysis. Since Simmons and Johnson[25] elucidated the reaction mechanism and structure of the reaction products, many attempts have been made to apply this reagent to the precolumn derivatization[4–9] of amines and amino acids. We planned to combine OPTA with an optical active thiol compounds to obtain diastereomeric isoindole derivatives from amino acid enantiomers and to separate them by reversed-phase HPLC. N-Acetyl-L-cysteine, which is readily available, was found to be an effective chiral derivatization agent[18]. Amino acids rapidly reacted with OPTA in the presence of AcCys to give

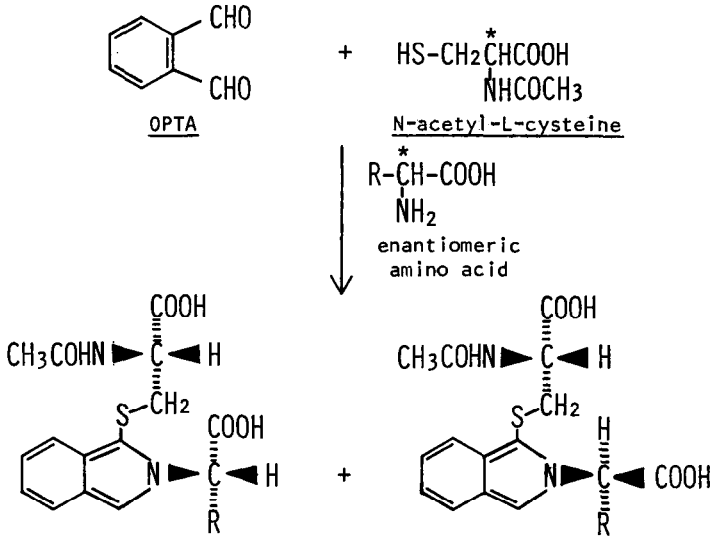

Fig. 1. Mode of reaction of OPTA–AcCys with amino acid enantiomer.

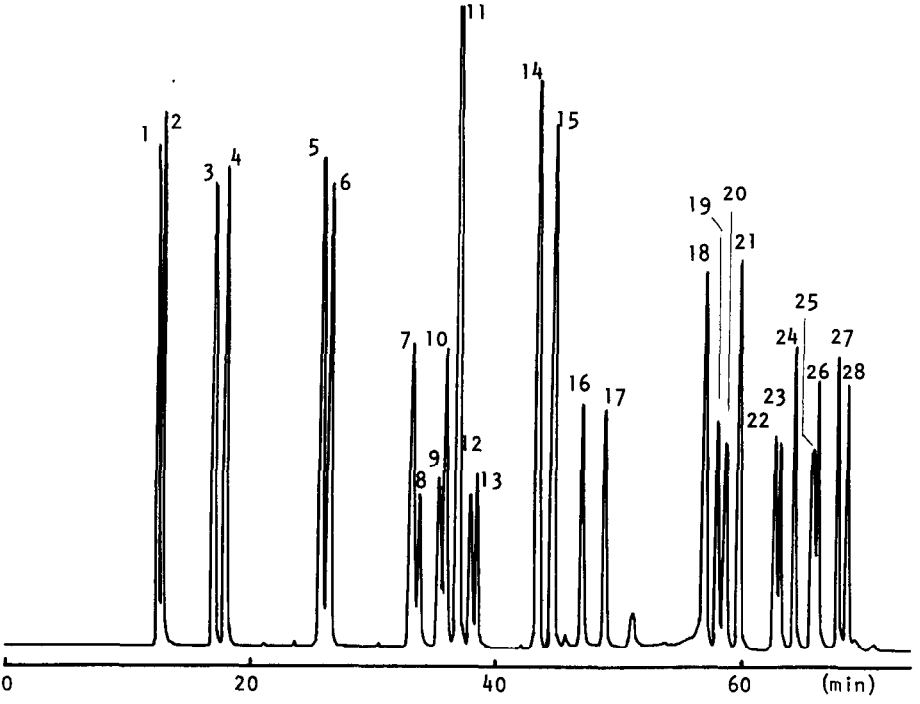

Fig. 2. Chromatogram of OPTA–AcCys derivatives of standard protein amino acid enantiomers. Column, Develosil ODS (particle size 5 μm, 200 × 6 mm I.D.) at 25°C; mobile phase, (A) 50 m*M* sodium acetate, (B) methanol; gradient, 0–16 min: 0–20% B, 16–22 min: 20% B, 22–40 min: 20–40% B, 40–46 min: 40% B, 46–65 min: 40–60% B; flow-rate, 1 ml/min. Peaks: 1 = D-Asp; 2 = L-Asp; 3 = L-Glu; 4 = D-Glu; 5 = D-Ser; 6 = L-Ser; 7 = D-Thr; 8 = L-His; 9 = D-His; 10 = LThr; 11 = Gly; 12 = L-Arg; 13 = D-Arg; 14 = D-Ala; 15 = L-Ala; 16 = L-Tyr; 17 = D-Tyr; 18 = L-Val; 19 = D-Met; 20 = L-Met; 21 = D-Val; 22 = D-Phe; 23 = L-Phe; 24 = L-Ile; 25 = D,L-Lys; 26 = D-Ile; 27 = D-Leu; 28 = L-Leu. Each peak corresponds to 5 ng of free amino acid.

intensely fluorescent products suitable for separations on reversed-phase columns, as expected. After completion of the present study, similar results were reported by Aswad[26], who used acetylcysteine, and by Buck and Krummen[27], who used N-*tert.*-butyloxycarbonyl-L-cysteine. However, our conditions for reaction and separation, which produced excellent resolution of amino acids, are different from those described in these reports. In addition, the present reaction was successfully applied to the pre-column derivatization for chiral resolution and the post-column derivatization of amino acids. This paper deals with the details of the precolumn derivatization for chiral resolution and the post-column derivatization for improved amino acid analysis.

The excitation and fluorescence spectra of the reaction mixture of L-alanine with the OPTA–AcCys reagent were approximately similar to those obtained from alanine, OPTA and mercaptoethanol. Consequently, it was anticipated that OPTA–AcCys reagent would also give a thiosubstituted isoindole derivative, N-alkyl-2-[S-(N-acetyl-L-cystein)]-yl isoindole, as shown in Fig. 1. The procedure for pre-column derivatization was therefore established as described in the Experimental section. As AcCys should be kept from exposure to alkali in order to preserve its optical purity, it was dissolved in pure methanol, together with OPTA, and mixed with alkaline borate solution prior to use. The fluorescence intensities of amino acids, mixed with the OPTA–AcCys–borate reagent, reached their maxima within 1 or 2

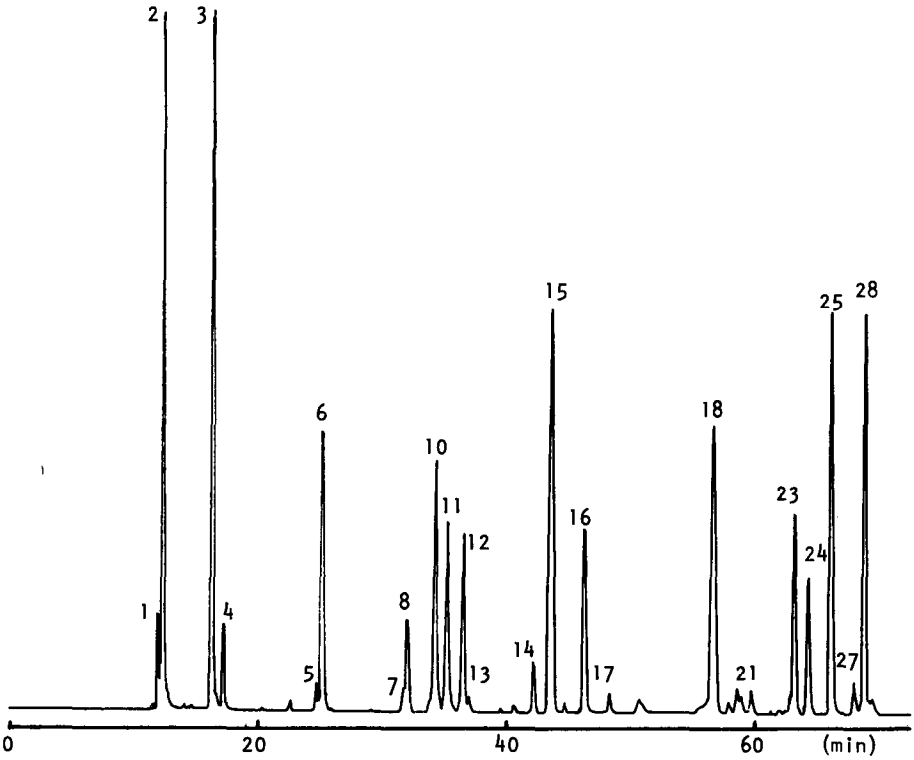

Fig. 3. Chromatogram of OPTA–AcCys derivatives of amino acids in a hydrolysate of bovine serum albumin. Chromatographic conditions and peaks as in Fig. 2.

min and the fluorescence was stable for 20 to 30 min. Even the fluorescence of the glycine derivative, which is the most susceptible to autoxidation[28], was stable for 20 min. This indicates that the AcCys derivatives of amino acids are much more stable than their mercaptoethanol or ethanethiol derivatives.

Fig. 2 shows a typical chromatogram of standard samples of common protein amino acid enantiomers on an ODS column with gradient elution. All, except D,L-lysine were satisfactorily resolved within 70 min. The same procedure was employed for the analysis of an acid hydrolysate of bovine serum albumin (in Fig. 3). Small peaks of D-isomers were observed, showing that L-amino acids were racemized during the acid hydrolysis.

Next, we examined the application of the OPTA–AcCys reagent in place of the conventional OPTA–mercaptoethanol or OPTA–ethanethiol reagents for amino acid analysis. Imino acids, such as proline or sarcosine, which do not react directly with the OPTA–thiol reagent, were detected by means of preliminary sodium hypochlorite oxidation[19,29] to generate primary amino groups. The sensitivity of proline was much lower than that of other amino acids when 2-mercaptoethanol was

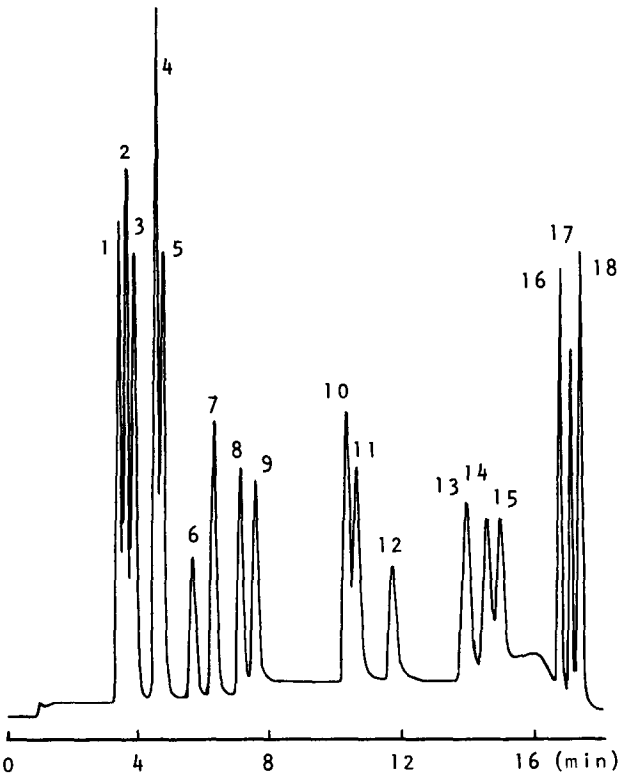

Fig. 4. SDS ion-pair chromatographic separation of standard amino acids detected by post-column derivatization with OPTA–AcCys reagent. Column, Hypersil ODS (particle size 3 μm, 50 × 6 mm I.D.) at 50°C; mobile phase, (A) 40 m*M* SDS, (B) acetonitrile; gradient, 0–5 min: 0–25% B, 5–11 min: 25–30% B, 11–13 min: 30–60% B; flow-rate, 1.5 l/min. Peaks: 1 = Asp; 2 = Ser; 3 = Glu; 4 = Gly; 5 = Thr; 6 = NH_3; 7 = Ala; 8 = Pro; 9 = Tyr; 10 = Val; 11 = Met; 12 = Cys; 13 = Ile; 14 = Phe; 15 = Leu; 16 = His; 17 = Lys; 18 = Arg. An amount of 0.5 nmol of each amino acid was injected.

TABLE I

REPRODUCIBILITY OF RETENTION TIME, PEAK AREA AND PEAK HEIGHT

250 pmol of each amino acid was injected (n = 8).

Amino acid	*Coefficient of variation (%)*		
	Retention time	*Peak area*	*Peak height*
Asp	0.042	0.16	0.02
Ser	0.042	0.43	0.22
Glu	0.045	0.35	0.30
Gly	0.024	0.57	0.50
Thr	0.037	0.72	0.34
Ala	0.042	0.40	0.29
Pro	0.041	0.14	0.31
Tyr	0.045	0.37	0.20
Val	0.048	0.55	0.37
Met	0.047	1.05	0.61
Cys-	0.053	0.75	0.81
Ile	0.119	0.46	0.56
Phe	0.144	0.67	0.60
Leu	0.146	0.84	0.45
His	0.034	1.53	1.44
Lys	0.054	2.28	1.03
Arg	0.020	1.96	0.96

used. Preliminary experiments proved[30] AcCys to be the thiol agent that gives the most intense fluorescence on reaction with imino acids oxidized with sodium hypochlorite. The excellent sensitivity of the present method in the determination of imino acids may be due to the high stability of the cysteinyl isoindole against oxidation. As a result, the simultaneous determination of amino and imino acids at the same level of sensitivity was achieved.

The present post-column detection system was applied to the reversed-phase ion-pair chromatography of protein amino acids. SDS was used as a counter-ion[31], together with acetonitrile for gradient elution. The concentration of SDS in the mobile phase and the column temperature, to which the separation is very sensitive, were set at 40 mM and 50°C, respectively. The reaction coil length for the sodium hypochlorite oxidation and the fluorescence development were determined by the ion-exchange method[30]. Fig. 4 shows a chromatogram of standard protein amino acids. Seventeen amino acids were adequately separated in 17 min. The gradient programme was run for 13 min, and then the column was re-equilibrated for 7 min with 40 mM SDS solution for the next sample injection. Accordingly, only 20 min were required for the analysis of a single sample. This was about one third of the time required for a conventional ion-exchange separation. The detection limit of amino or imino acid was *ca.* 5 pmol per injection (signal-to-noise ratio 3). The reproducibility of this procedure was suitable for the routine analysis of protein hydrolysate samples. Coefficients of variation of the retention times, peak heights, and peak areas of all amino acids examined are listed in Table I. In addition, since AcCys is a white, crystalline powder that has scarcely no smell, it is also superior to con-

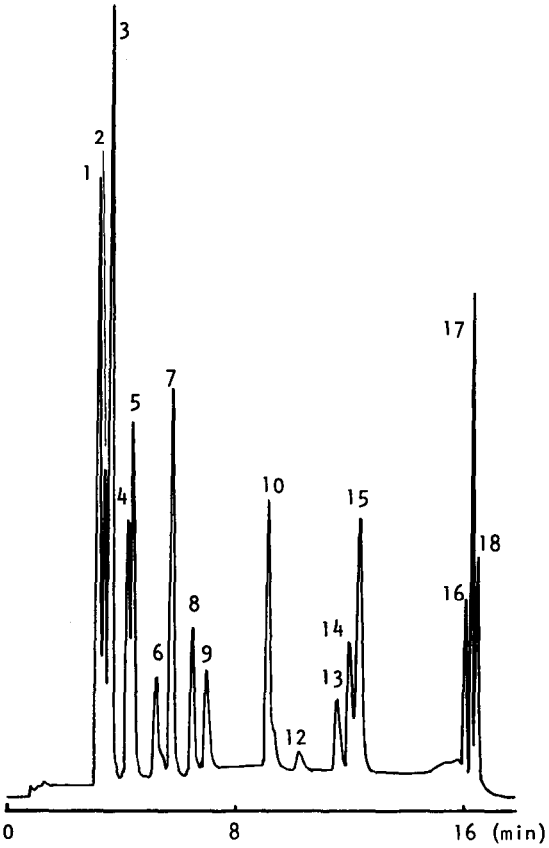

Fig. 5. SDS ion-pair chromatographic separation of amino acids in a hydrolysate of bovine serum albumin. Conditions and peaks as in Fig. 4.

TABLE II

AMINO ACID COMPOSITION OF HYDROLYZED BOVINE SERUM ALBUMIN

All values normalized to alanine.

Amino acid	*Found*	*Known*
Lys	1.28	1.28
His	0.37	0.34
Arg	0.50	0.51
Asp + Asn	1.15	1.20
Glu + Gln	1.69	1.73
Thr	0.74	0.58
Ser	0.61	0.61
Pro	0.61	0.63
Gly	0.33	0.35
Ala	1.00	1.00
Val	0.78	0.69
Ile	0.30	0.23
Leu	1.33	1.27
Tyr	0.41	0.37
Phe	0.57	0.54

ventional thiol compounds in tractability. The amino acid analysis described above was also applied to a hydrolysate of bovine serum albumin (Fig. 5). The composition of amino acids, based on the chromatographic data, was satisfactorily correlated with that in the literature[32] (Table II), the coefficient of correlation value was 0.977.

ACKNOWLEDGEMENTS

We are grateful to Ms. A. Toyama, Ms. Y. Hiraga, Ms. C. Yamauchi and Ms. E. Kayaba for their technical assistances. Part of this work was supported by a grant-in-aid for scientific research (No. 60771897) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1 M. Roth, *Anal. Chem.*, 43 (1971) 880.
2 J. R. Benson and P. E. Hare, *Proc. Natl. Acad. Sci., U.S.A.*, 72 (1975) 619.
3 W. Voelter and K. Zech, *Biochem. Biophys. Res. Commun.*, 82 (1978) 1104.
4 B. R. Larsen and F. G. West, *J. Chromatogr. Sci.*, 19 (1981) 259.
5 B. N. Jones and J. P. Gilligan, *J. Chromatogr.*, 266 (1983) 471.
6 M. H. Joseph and P. Davies, *J. Chromatogr.*, 277 (1983) 125.
7 T. P. Davis, C. W. Gehrke, C. W. Gehrke, Jr., T. D. Cunningham, K. C. Kuo, K. O. Gerhardt, H. D. Johnson and C. H. Williams, *J. Chromatogr.*, 162 (1979) 293.
8 L. D. Mell, Jr., A. R. Dasler and A. B. Gustafson, *J. Liq. Chromatogr.*, 1 (1978) 261.
9 Y. Tsuruta, K. Kohashi and Y. Ohkura, *J. Chromatogr.*, 146 (1978) 490.
10 F. Perini, J. B. Sadow and C. V. Hixon, *Anal. Biochem.*, 94 (1979) 431.
11 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
12 H. Furukawa, *Chem. Pharm. Bull.*, 23 (1975) 1625.
13 T. Nambara, S. Ikegawa, M. Hasegawa and J. Goto, *Anal. Chim. Acta*, 101 (1978) 111.
14 S. Yang, H. Gelboin, J. Weber, V. Sankaran, D. Fischer and J. Engel, *Anal. Biochem.*, 78 (1977) 520.
15 J. Goto, M. Hasegawa, S. Nakamura, K. Shimada and T. Nambara, *J. Chromatogr.*, 152 (1978) 413.
16 J. Goto, N. Goto, A. Hikichi, T. Nishimaki and T. Nambara, *Anal. Chim. Acta*, 120 (1980) 187.
17 N. Nimura, A. Toyama and T. Kinoshita, *J. Chromatogr.*, 316 (1984) 247.
18 T. Kinoshita and N. Nimura, *Jap. Pat.*, 147079/83.
19 Y. Ishida, T. Fujita and K. Asai, *J. Chromatogr.*, 204 (1981) 143.
20 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
21 N. Nimmura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
22 A. J. Sedman and J. Gal, *J. Chromatogr.*, 278 (1983) 199.
23 J. Gal, *J. Chromatogr.*, 307 (1984) 220.
24 K. J. Miller, J. Gal and M. M. Ames, *J. Chromatogr.*, 307 (1984) 335.
25 S. S. Simmons and D. F. Johnson, *J. Am. Chem. Soc.*, 98 (1976) 7098.
26 D. W. Aswad, *Anal. Biochem.*, 137 (1984) 405.
27 R. H. Buck and K. Krummen, *J. Chromatogr.*, 315 (1984) 279.
28 V.-J. K. Svedas, I. J. Galaev, I. L. Borisov and I. V. Berezin, *Anal. Biochem.*, 101 (1980) 188.
29 P. A. St. John, *Aminco Lab. News*, 31 (1975) 1.
30 N. Nimura, T. Kinoshita, M. Fujiwara and Y. Ishida, in preparation.
31 T. Hayashi, H. Tsuchiya and H. Naruse, *J. Chromatogr.*, 274 (1983) 318.
32 M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, National Biomedical Foundation, Washington, DC, 1976, Vol. 5, Suppl. 2.