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SINGLE-COLUMN SEPARATION OF AMINOETHYLCYSTEINE AND OTHER AMINO ACIDS

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SUMMARY

Analytical conditions for aminoethylcysteine were established with the use of a single-column amino acid analyser equipped with a 150 × 4 mm I.D. column packed with Hitachi custom ion-exchange resin 2617 (5 μm). Using three buffer solutions, twenty amino acids including aminoethylcysteine were separated for analysis. This method was applied to the acid hydrolysate produced by aminoethylation of insulin and lysozyme. The results showed that the method is effective not only for the determination of cysteine but also for acquiring better quantitative data on other amino acids.

INTRODUCTION

The sulphydryl enzyme papain is inactivated through oxidation of cysteine to cystine by oxidative cleavage, and the processing of cystine, which has a disulphide bridge (S–S) in its molecule, has remained a major problem in the analysis of amino acid compositions forming proteins. It has resulted in uneven, incomplete reactions between proteinase and various reagents, and has also required the utmost care in acquiring accurate quantitative values for cysteine and cystine, which is one of the components of proteins.

The S–S bond is cleaved mainly by the following two methods. The first is oxidative cleavage with performic acid, the cystine and cysteine forming a cysteic acid, which can then be subjected to quantitative analysis. However, it requires highly sophisticated techniques and is incapable of determing tryptophan. In the other method, the S–S bond is reducibly cleaved by a reducing agent such as 2-mercaptoethanol, with alkylation to prevent further reaction.

The alkylating agents includes monoiodoacetic acid, acrylonitrile, 4-vinylpyridine and ethyleneimine [or N-(indoethyl)trifluoroacetamine]. Each of them produces cysteines such as S-carboxymethylcysteine, S-cyanoethylcysteine, S-pyridylcysteine and S-aminoethylcysteine. Among these, the S-aminoethylcysteine (AEC) resembles

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lysine in structure, and thus makes enzyme cleavage possible at the AEC position in addition to the lysine or arginine position. This feature greatly facilitates the determination of the arrangement of amino acid components.

In amino acid component analysis, it has been reported that AEC cannot be separated by the conventional method[1]; it can be separated with a basic column using the dual-column method[2,3], and in this instance the analysis should be carried out twice on acidic neutral components and basic components.

It is necessary to have as much sample as possible in order to achieve a high sensitivity for such trace components. Considered from this viewpoint, the single-column method must be regarded as a very efficient method because it requires only half the amount sample.

EXPERIMENTAL

Materials and reagents

S-2-Aminoethyl-L-cysteine hydrochloride, porcine insulin crystals and lysozyme egg white (crystallized six times) were purchased from Sigma (St. Louise, MO, U.S.A.), Shimizu Pharmaceuticals (Tokyo, Japan) and Seikagaku Kogyo (Tokyo, Japan), respectively. Ethyleneimine, purchased from Sogo Pharmaceuticals (Kanagawa, Japan), was used after being distilled. We also purchased urea and 2-mercaptoethanol, of special grade for biochemical analysis, from Nakarai Chemicals (Kyoto, Japan).

Mercaptoethanesulphonic acid purchased from Pierce (Chester, U.K.). All other reagents were of analytical-reagent grade from Wako (Tokyo, Japan).

TABLE I

PREPARATION OF BUFFER SOLUTIONS FOR STANDARD PROTEIN HYDROLYSATE ANALYSIS

Parameter	*Buffer*			
	(1) PH-1, 0.2 M Na	*(2) PH-3, 0.2 M Na*	*(3) PH-4, 1.2 M Na*	*PH-RG (regenerating), 0.2 M Na*
Distilled water	700 ml	700 ml	700 ml	700 ml
Sodium citrate dihydrate	7.74 g	15.7 g	26.67 g	–
Sodium hydroxide	–	–	–	8.0 g
Sodium chloride	7.07 g	2.92 g	54.35 g	–
Citric acid monohydrate	17.7 g	10.50 g	6.10 g	–
Ethanol	60 ml	–	–	–
Benzyl alcohol	–	10.0 ml	5.0 ml	–
Thiodiglycol	5 ml	5 ml	–	–
Brij-35*	4 ml	4 ml	4 ml	4 ml
pH (nominal)	3.3	4.3	4.9	–
Total volume	1 l	1.1 l	1 l	1 l
Caprylic acid	0.1 ml	0.1 ml	0.1 ml	0.1 ml

* Used as dissolved at a ratio of 25 g/100 ml. (Dissolve by heating.)

Chromatographic conditions

We used a Hitachi Model 835 high-speed amino acid analyser[4]. The column (150 × 4 mm I.D.) was packed with Hitachi custom ion-exchange resin 2617 (5 μm). The analytical temperature was set at 40–50°C (stepwise).

Table I shows the eluents used. Aminoethylinsulin was separated into A and B chains with a Hitachi Model 655 high-performance liquid chromatograph with a 150 × 4 mm I.D. column packed with Hitachi Gel 3063 (ODS-silica, 5 μm, 300 Å pore size).

The gradient elution method was adopted with eluents A and B, where A is 0.1% trifluoroacetic acid (TFA) and B is 0.1% TFA–19.9% water–80% isopropyl alcohol–acetonitrile (70:30, v/v).

Sample preparation

Aminoethylation. A 20-mg amount of porcine insulin (3.5 μmol) was dissolved in 1.7 ml of 0.7 *M* Tris–HCl buffer (pH 8.6) containing 0.2% EDTA and 8 *M* urea for aminoethylation. Nitrogen was purged from the insulin, followed by addition of 110 μl (1.47 mmol) of 2-mercaptoethanol, then the solution was reduced at 40°C for 4 h. A total of 420 μl (8.0 mmol) of ethyleneimine was added to the insulin in three separate portions and the solution was incubated at 30°C for 1 h. The pH was then adjusted to 3.0 with acetic acid, followed by dialysis with 0.01 *M* acetic acid and lyophilization, giving 16.4 mg of aminoethylinsulin (AE-insulin). Lysozyme was also aminoethylated[5] as for insulin.

Hydrolysis. Insulin, AE-insulin, A and B chains of AE-insulin and AE-lysozyme were hydrolysed with 6 *M* hydrochloric acid 110°C for 22 h. In order to recover

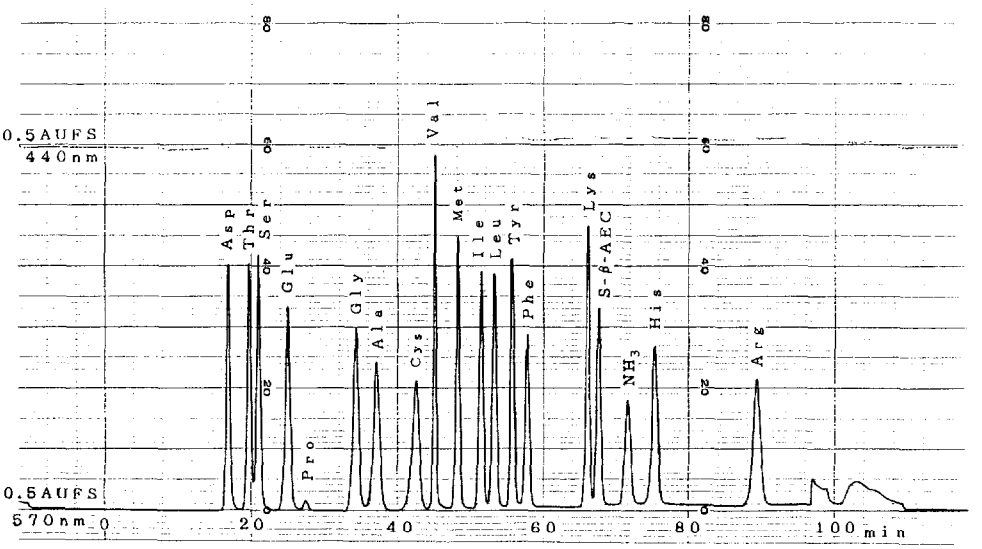

Fig. 1. Analytical chromatogram for amino acids containing AEC. Column: 150 × 4 mm I.D., Hitachi custom ion-exchange resin 2617. Temperature programme: 50°C to 40°C (50 min) to 50°C (60 min). Flow-rates: 0.225 ml/min buffer solution with 2650 NH_3 trap column, 0.3 ml/min ninhydrin solution. Buffer change: PH-1 (0–16 min), PH-3 (16–34 min), PH-4 (34–80 min), PH-RG (80–92 min), PH-1 (92–120 min).

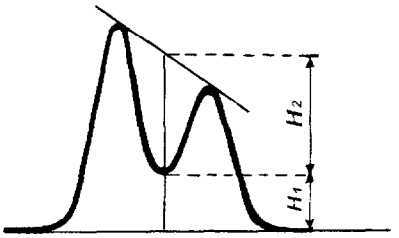

Fig. 2. Calculation of resolution. Resolution (%) = $[H_2/(H_1 + H_2)] \cdot 100$, where H_1 = depth of valley between two adjacent peaks and H_2 = average height of two adjacent peaks.

the tryptophan of AE-lysozyme, it was hydrolysed with mercaptoethanesulphonic acid at 110°C for 22 h.

RESULTS AND DISCUSSION

Separation of AEC and other amino acids

Fig. 1 shows the separation of lysine and AEC by adding 10% ethanol to the third buffer solution and measuring the difference in concentration of the resin surface. The calculation method shown in Fig. 2 indicated that the resolution of lysine and AEC is as high as 94%. The resolution can be further improved by adding 10% ethanol to the second buffer solution. However, the results for isoleucine–leucine and cystine–valine, etc., become poor.

If ornithine is present in the hydrolysate to be used as a sample, *e.g.*, gramicidine, it overlaps with lysine, and no separation will occur under those conditions (10% ethanol). Fig. 3 shows the shift of the elution point of the basic amino acid when 5% water, ethanol and isopropyl alcohol are added to the remaining 5%.

Determining the resolution of Orn–Lys, Lys–AEC and Trp–Arg, the separation of Orn–Lys was the highest (97%) when water was added, and those of Lys–AEC and Trp–Arg were as high as 69% and 94%, respectively, when isopropyl alcohol was added. The test also indicated that all rates of separation are relatively good when isopropyl alcohol is added. Fig. 4 shows the analytical chromatograms of amino acids containing AEC.

When isopropyl alcohol, ethanol, dioxane or tetrahydrofuran (THF) are added in amounts up to 10% to the third buffer solution, Orn and Lys are not separated, *i.e.*, the resolution of Lys and AEC is as high as 94% when 10% ethanol is added.

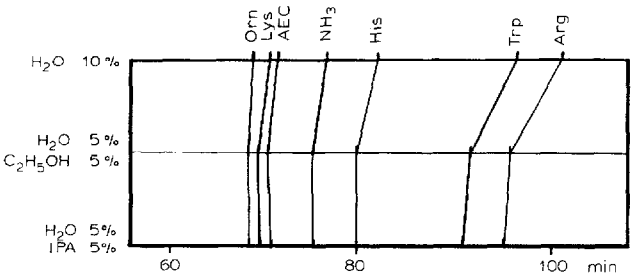

Fig. 3. Shift of basic amino acids on adding alcohols.

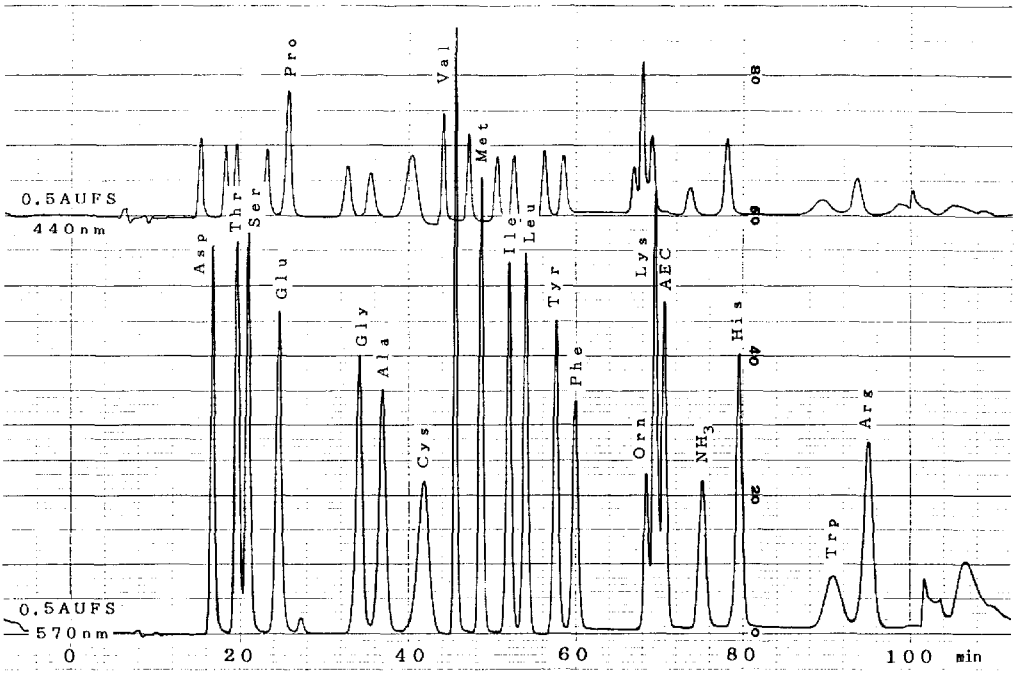

Fig. 4. Analytical chromatograms of amino acids containing Orn, AEC and Trp.

When 10% THF is added the resolution is relatively high (89%), but "leading" is seen on the arginine peak so that this peak overlaps with that of histidine because tryptophan is eluted too fast.

Application to protein

The A and B chains of 2.7 mg of AE-insulin were separated by reversed-phase high-performance liquid chromatography (HPLC) and each was then hydrolysed for amino acid analysis. Fig. 5 shows a chromatogram of 270 μg of AE-insulin obtained by reversed-phase HPLC.

Table II shows the analytical results for amino acid compositions obtained on insulins that had been aminoethylated and not modified by the aminoethylation.

Fig. 6 shows the analytical chromatogram for amino acids present in AE-insulin. In the non-aminoethylated insulin the Ile and Val values are considerably lower than the theoretical values. This indicates that hydrolysis for 22 h at 110°C is not sufficient to hydrolyse them completely. The incomplete separation can be demonstrated by the appearance of unknown peaks other than amino acids, which appear to be partially hydrolysed peptides.

On the other hand, the Ile and Val values of AE-insulin are very close to the theoretical values although the insulin had been hydrolysed under the same conditions. This indicates that the insulin was hydrolysed almost completely by aminoethylation, giving smaller peptides, without applying the conventional external insertion method.

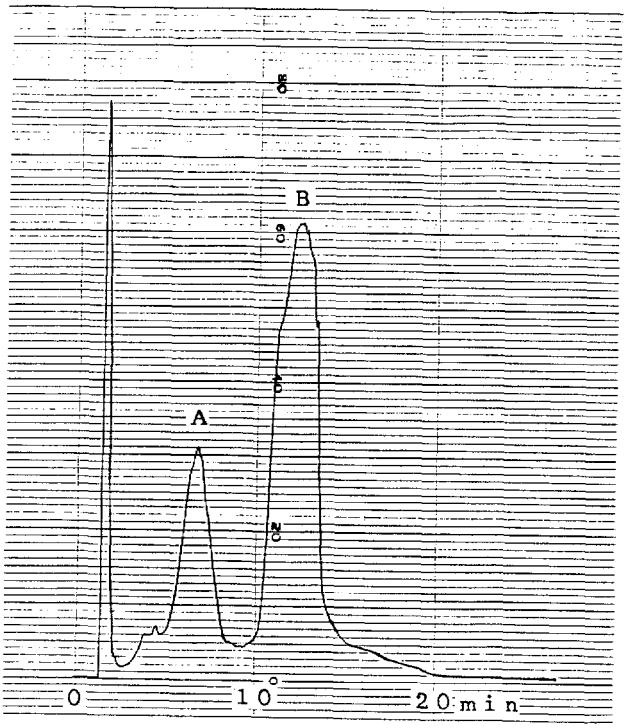

Fig. 5. Chromatogram of (A) A chains and (B) B chains in aminoethylated insulin (270 μg). Column: 150 × 4 mm I.D., Hitachi Gel 3063. Flow-rate: 1.0 ml/min. Eluent: (A) 0.1% TFA in water; (B) 0.1% TFA–19.9% water–80% isopropyl alcohol–acetonitrile (7:3), gradient elution.

TABLE II

ANALYTICAL RESULTS FOR AMINO ACIDS IN INSULIN

Amino acid	*Aminoethylinsulin*				*Insulin*	
	Analytical		*Theoretical*		*Analytical*	*Theoretical*
	A chain	*B chain*	*A chain*	*B chain*		
Asp	2.1	1.1	2	1	3.0	3
Thr	1.0	1.0	1	1	2.1	2
Ser	1.8	1.1	2	1	2.7	3
Glu	4.0	3.1	4	3	6.3	7
Pro	–	1.3	0	1	0.9	1
Gly	0.6	3.0	1	3	4.4	4
Ala	0.0	2.1	0	2	2.1	2
Cys	–	–	0	0	2.0	6
Val	0.7	2.7	1	3	2.2	4
Ile	1.6	0.1	2	0	0.8	2
Leu	2.1	4.1	2	4	5.3	6
Tyr	1.9	1.9	2	2	3.8	4
Phe	0.0	2.6	0	3	3.4	3
Lys	–	0.9	0	1	1.1	1
AEC	3.2	1.8	4	2	–	0
His	–	1.6	0	2	1.6	2
Arg	–	1.0	0	1	1.0	1

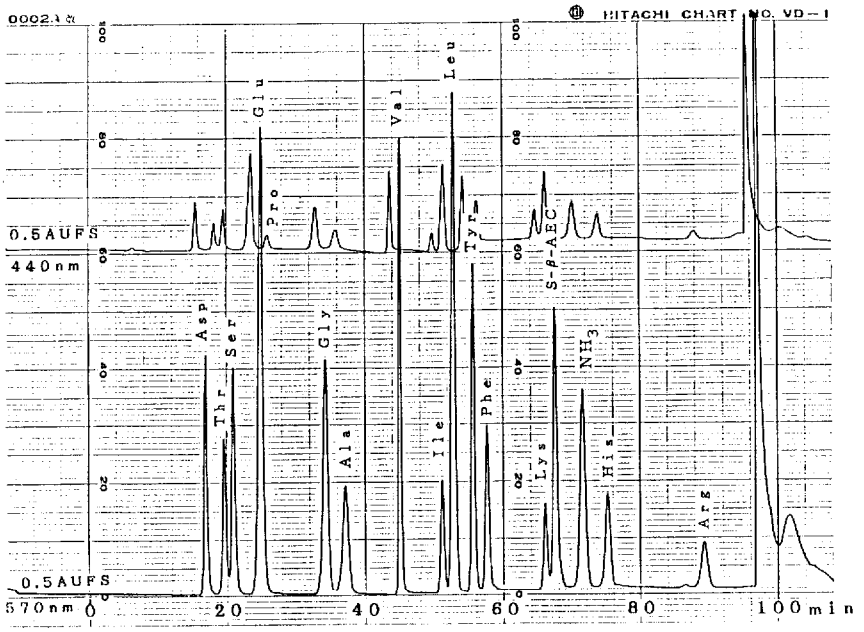

Fig. 6. Amino acid analysis of AE-insulin.

TABLE III

ANALYTICAL RESULTS FOR AMINO ACIDS IN AE-LYSOZYME HYDROLYSATE

Amino acid	*AE-lysozyme hydrolysate*		
	6 N HCl	*Mercaptoethanesulphonic acid*	*Theoretical*
Asp	21.0	21.0	21
Thr	7.0	7.0	7
Ser	9.8	8.9	10
Glu	5.2	5.2	5
Pro	2.1	1.1	2
gly	12.3	12.9	12
Ala	12.3	12.2	12
Val	5.1	6.2	6
Met	1.6	1.8	2
Ile	5.2	5.8	6
Leu	8.2	8.2	8
Tyr	3.0	3.2	3
Phe	3.0	3.5	3
Lys	5.3	5.8	6
AEC	7.7	6.0	8
His	1.0	1.4	1
Trp	2.7	5.5	6
Arg	11.0	10.6	11

As can be seen from Table II, the Glu value is low, *i.e.*, the acid was decomposed considerably in the hydrolysis stage.

Table III shows the ratio of amino acid components when AE-lysozyme was hydrolysed with 6 *M* hydrochloric acid and mercaptoethanesulphonic acid. In both instances the values obtained were similar to the theoretical values.

When hydrolysis was performed with mercaptoethanesulphonic acid, the yields of Trp, Ile, Val and Lys were much higher than those of Pro and AEC. Note that the hydrolysis with mercaptoethanesulphonic acid was performed about 1 month later than that with 6 *M* hydrochloric acid, so the aminoethyl group in the AE-lysozyme hydrolysate might have decomposed before performing the hydrolysis. This can be understood by the fact that the concentration of S-β-AEC aqueous solution is reduced by about 20% after 1 month even if it was stored in a refrigerator.

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