Preparation of Acetylmercapto-3-carboxypropanoyl Insulins Using Preparative High-Performance Liquid Chromatography on an Anion-Exchange Column

Masahiko NANAMI, Kiyoshi ZAITSU and Yosuke OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812, Japan. *Received January 29, 1992*

A method for the preparation of insulin derivatives which have protected sulfhydryl group(s) at definite site(s) on the molecule is described. Porcine insulin reacts with *S*-acetylmercaptosuccinic anhydride to afford four species of insulin derivatives that have 2 (or 3)-acetylmercapto-3-carboxypropanoyl group(s) at i) Gly(A1), ii) Gly(A1) and Phe(B1), iii) Gly(A1) and Lys(B29), and iv) Gly(A1), Phe(B1) and Lys(B29) positions. The derivatives are efficiently separated in a preparative scale by anion-exchange high-performance liquid chromatography on a TSKgel DEAE-2SW column. The four derivatives are all readily deacetylated with hydroxylamine to give the corresponding sulfhydryl insulin derivatives.

Keywords acetylmercapto-3-carboxypropanoyl insulin; thiol preparation; *S*-acetylmercaptosuccinic anhydride; porcine insulin; high-performance liquid chromatography; anion-exchange chromatography; preparative scale

In order to improve sensitivity in enzyme- and fluoroimmunoassays of insulin and insulin antibodies, it is significant to prepare porcine insulin conjugates of enzymes or fluorophores which are introduced into definite site(s) on the three free amino groups (Gly(A1), Phe(B1), Lys(B29)) on the insulin molecule.[1]

Insulin derivatives which have moiety(s) containing sulfhydryl group(s) at definite site(s) on the molecule should react with enzyme(s) and fluorophore(s) substituted with halogenated moiety(s) or maleimide moiety(s) to yield insulin conjugates. In the previous paper, we reported five *S*-acetylthioglycoloyl insulins as insulin derivatives having protected sulfhydryl group(s) at the definite site(s), of which the protecting group (acetyl) is removable when required.[2] However, a small difference in negative charge between *S*-acetylthioglycoloyl groups in the insulin derivatives did not allow a satisfactory separation of the derivatives in a preparative scale (injection amount, more than approximately 2 mg of a mixture of the derivatives) in high-performance liquid chromatography (HPLC) on an anion-exchange column.

This paper described the preparation of other insulin derivatives bearing protected sulfhydryl group(s), 2(or 3)-acetylmercapto-3-carboxypropanoyl (AMCP) insulins (Fig. 1), of which the acetyl group is eliminable under mild conditions in the presence of hydroxylamine. AMCP-insulins have carboxyl group(s) and the negative charge of the group(s) serves to increase the difference in net charge between the derivatives in a weakly acidic medium, which allows for easy mutual separation of AMCP-insulins by anion-exchange HPLC. Porcine insulin is reacted with *S*-acetylmercaptosuccinic anhydride (*S*-AMS),[3] a cross-linking reagent, to introduce AMCP group(s) to the amino group(s). The reaction mixture is subjected to preparative HPLC on an anion-exchange column to afford four species of AMCP-insulins, Gly(A1)–AMCP-insulin, Gly(A1), Phe(B1)–diAMCP-insulin, Gly(A1), Lys(B29)–diAMCP-insulin, and Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin.

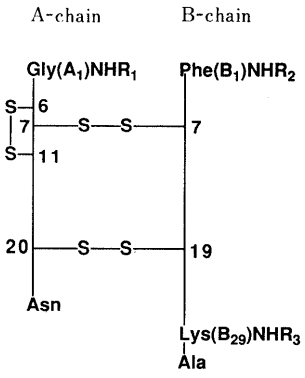

Fig. 1. Schematic Structures of AMCP-Insulins

Gly(A1)–AMCP-insulin: $R_1 = CH_3COSCH(CH_2COOH)CO$ or $CH_3COSCH(COOH)CH_2CO$ (AMCP), $R_2 = R_3 = H$; Gly(A1), Phe(B1)–diAMCP-insulin: $R_1 = R_2 =$ AMCP, $R_3 = H$; Gly(A1), Lys(B29)–diAMCP-insulin: $R_1 = R_3 =$ AMCP, $R_2 = H$; Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin: $R_1 = R_2 = R_3 =$ AMCP.

Experimental

Reagent and Apparatus Lyophilized porcine insulin was prepared from a monocomponent insulin solution (Insulin Novo Actrapid MC; Novo Ind., Copenhagen, Denmark) as previously described.[4] *S*-AMS and urea were obtained from Nacalai Tesque (Kyoto, Japan). Other chemicals were of a reagent grade. Deionized water was passed through a Milli-QII system (Japan Millipore, Tokyo, Japan). An aqueous urea solution was deionized as previously described,[4] to eliminate the cyanate ion, an impurity in urea, which caused the carbamylation of insulin.[5] Molecular membrane tubes (Spectra/Por 3; molecular weight cutoff, approximately 3500; Spectral Medical Ind., Los Angeles, U.S.A.) were used for dialyses, which were normally carried out at 4 °C.

Absorbances were measured with a Hitachi 150-20 spectrophotometer using semimicro quartz cells (1 ml). Amino acid analyses were performed with a Hitachi C-8500 amino acid analyzer equipped with a Hitachi D-2850 chromato-integrator after hydrolysis of the protein sample in 6 M hydrochloric acid *in vacuo* at 110 °C for 24 h.

HPLC System and Its Operation A Hitachi 655 liquid chromatograph equipped with a proportioning valve pump for gradient elution, a Hitachi 650-60 recording processor, a Rheodyne 7125 syringe-loading sample injection valve (200-μl loop) and a Tosoh UV-8 model II spectromonitor operating at 280 nm was used. The column was TSKgel DEAE-2SW (300 × 7.8 mm i.d.; Tosoh, Tokyo, Japan) and the column temperature was ambient (20—25 °C). Elution with an NaCl concentration gradient during 60 min was used at a flow rate of 1.4 ml/min with a mixture of eluent A (0.05 M Na–K phosphate buffer (pH 6.0) containing 4 M urea) and eluent B (eluent A containing 1 M NaCl). The NaCl gradient elution was performed as follows. The initial eluent was 100% eluent A. The proportion of eluent B was increased to 20% linearly during 40 min, then a mixture of eluents A and B (8 : 2, v/v) was pumped for 20 min (for the gradient curve, see Fig. 2). The used column was regenerated by washing with eluent A for 20 min.

Procedure for the Investigation of Reaction Conditions To lyophilized porcine insulin (2.0 mg, 333 nmol) dissolved in 1.0 ml of 0.1 M Na phosphate buffer (pH 6.0, 7.0, 8.0 and 9.0) was added 1—30-times molar excess of *S*-AMS dissolved in 50 μl of dried *N*,*N*'-dimethylformamide (DMF) at 0—37 °C with stirring. The mixture was continually stirred at 0—37 °C

for 1—120 min. Then, a portion (200 μl) of the resulting mixture was subjected to HPLC.

Preparation of AMCP-Insulins To lyophilized porcine insulin (40.0 mg, 6.67 μmol) dissolved in 20 ml of 0.1 M Na phosphate buffer (pH 7.0) was added dropwise 1.0 ml of 0.1 M *S*-AMS solution (0.1 mmol) in dried DMF at 30 °C with vigorous stirring, and the mixture was continually stirred at 30°C for 60 min. The resulting mixture was treated by a chromatography on a Sephadex G-25 (50 g; Pharmacia LKB Biotechnology, Uppsala, Sweden) column (360 × 30 mm i.d.) using 1 mM Na phosphate buffer (pH 7.0) to remove any *S*-AMS which remained unreacted. The protein fraction was collected and lyophilized. The resulting lyophilizate (45 mg) was dissolved in 800 μl of 0.1 M Na phosphate buffer (pH 7.0). Portions (200 μl) of the solution were subjected to HPLC. The combined individual eluates were dialyzed against 2 l of water (5 times) to remove urea and Na phosphate, and lyophilized. The lyophilizates were dissolved in 4.0 ml of 0.1 M Na phosphate buffer (pH 7.0), respectively, and subjected to chromatography on a Sephadex G-25 (5.6 g) column (360 × 10 mm i.d.) with 1 mM Na phosphate buffer (pH 7.0) to remove any remaining urea. The respective protein fractions were dialyzed against 2 l of water (5 times) to remove Na phosphate, then lyophilized to give AMCP-insulins (all colorless powder) [yield (mg), UV λ_{max} (nm) (in 0.1 M Na phosphate buffer (pH 7.0)) and ε, in that order, in parentheses]; Gly(A1)–AMCP-insulin (5.95, 276.0, 7.24×10^3); Gly(A1), Phe(B1)–diAMCP-insulin (9.24, 276.0, 7.72×10^3); Gly(A1), Lys(B29)–diAMCP-insulin (0.52, 275.0, 7.69×10^3); Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin (1.26, 275.5, 8.44×10^3). Intact insulin (1.02 mg) was recovered. The position of AMCP groups of the insulin derivatives was determined by the method of Levy[6] with minor modifications as previously described.[7]

Deacetylation of AMCP-Insulins and Determination of the Sulfhydryl Group in Deacetylated AMCP-Insulins To an AMCP-insulin (1.0 mg, 167 nmol) dissolved in 1.0 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl (evacuated) was added 250 μl of 1.0 M hydroxylamine hydrochloride solution in the buffer, and the mixture was stirred at 30 °C for 60 min. The resulting 2(or 3)-mercapto-3-carboxypropanoyl insulin solutions were immediately subjected to the determination of the sulfhydryl group in the insulins by the 4,4′-dithiodipyridine method.[8]

Results and Discussion

The AMCP group caused the respective insulins to give not only a different net charge value in a weakly acidic medium, but also fairly high solubility. Thus, the mutual separation of AMCP-insulins was attained by anion-exchange HPLC on a TSKgel DEAE-2SW column using a slightly acidic phosphate buffer (pH 6.0) containing 4 M urea. In the absence of urea, AMCP-insulins were intensely retained on the column. Sodium chloride was also required for a satisfactory and rapid separation of the insulins: a gradient elution with salt concentrations up to 0.2 M in the mobile phase (Fig. 2) was employed. When a phosphate buffer of pH 7 was used as a mobile phase, the mutual separation could not be attained in a preparative scale. Figure 2 shows a chromatogram of a reaction mixture obtained by treating insulin with a 15-times molar excess of *S*-AMS at pH 7 at 30 °C for 1 h according to the procedure for the investigation of reaction conditions. The pattern of the chromatogram for the reaction mixture of insulin in a preparative scale (injection volume, 10 mg protein) was almost identical to that given in Fig. 2.

The position(s) of AMCP group(s) in the AMCP-insulins was determined by deaminating the insulins and by measuring the number of glycyl, phenylalanyl and lysyl residues according to a modified Levy method[6] (Table I); there was no discrepancy between the theoretical and found values of the amino acid residues. Although *S*-AMS has been used as a reagent for introducing AMCP moiety(s) to proteins,[9-11] the position of the acetylmercapto group (2- or 3-position in the moiety) still remains unclear; in the study, the position was again unidentified.

Peaks for other possible AMCP-insulins, Phe(B1)– and Lys(B29)–AMCP-insulins and Phe(B1), Lys(B29)–diAMCP-insulin, were not found. The components of small

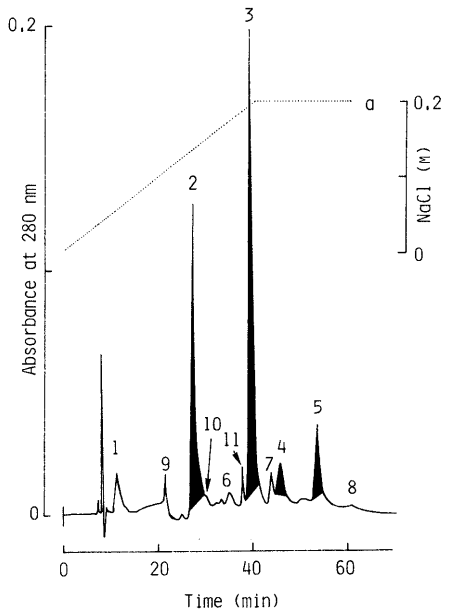

Fig. 2. Chromatogram Obtained with a Reaction Mixture of Insulin with *S*-AMS

Insulin (2.0 mg) was treated using the reported procedure (for details see the text) and the mixture was subjected to anion-exchange HPLC. The shaded peaks are AMCP-insulins. Curve a: the concentration of sodium chloride in the eluent in the gradient elution. Peaks: 1, insulin; 2, Gly(A1)–AMCP-insulin; 3, Gly(A1), Phe(B1)-diAMCP-insulin; 4, Gly(A1), Lys(B29)–diAMCP-insulin; 5, Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin; 6—8, unknown insulin derivatives; 9—11, reagent blank.

TABLE I. Numbers of Glycyl, Phenylalanyl and Lysyl Residues of the AMCP-Insulins after Deamination

Position of AMCP moiety	Number of amino acid residues[a]					
	Gly		Phe		Lys	
	Theoretical	Found	Theoretical	Found	Theoretical	Found
Porcine insulin	3	3.06	2	2.14	0	0.15
Gly(A1)	4	3.93	2	2.19	0	0.03
Gly(A1), Phe(B1)	4	4.07	3	3.07	0	0.13
Gly(A1), Lys(B29)	4	4.03	2	2.06	1	1.01
Gly(A1), Phe(B1), Lys(B29)	4	4.03	3	2.97	1	0.93
Intact porcine insulin[b]	4	3.98	3	3.04	1	1.01

a) Calculated based on six leucyl residues per porcine insulin molecule. *b*) Not subjected to deamination.

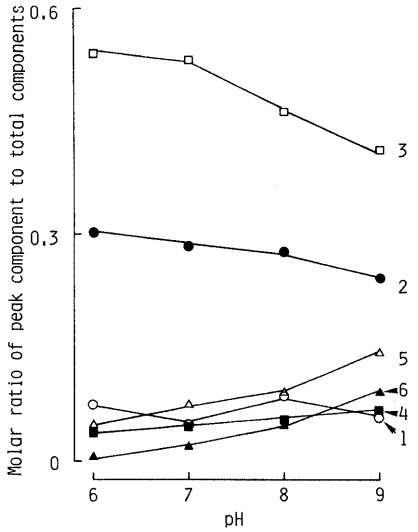

Fig. 3. Effect of pH in the Reaction Mixture on the Formation of AMCP-Insulins

Insulin (2.0 mg) was treated according to the reported procedure at a molar ratio of *S*-AMS to insulin of 15 at 30 °C at various pHs for 1 h. Curves: 1, insulin; 2, Gly(A1)–AMCP-insulin; 3, Gly(A1), Phe(B1)–diAMCP-insulin; 4, Gly(A1), Lys(B29)–diAMCP-insulin; 5, Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin; 6, total yields of unknown insulin derivatives.

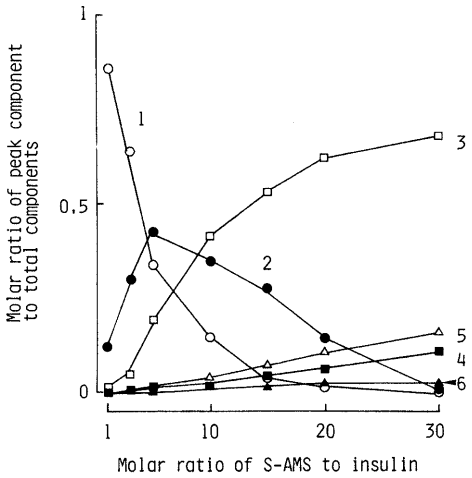

Fig. 4. Effect of the Molar Ratio of *S*-AMS to Insulin on the Formation of AMCP-Insulins

Insulin (2.0 mg) was treated as in Fig. 3 but at various molar ratios of *S*-AMS to insulin at pH 7.0. For curves see Fig. 3.

peaks (peaks 6—8 in Fig. 2) were thought to be due to compounds that occurred by the reaction of *S*-AMS with some group(s) other than the amino groups in the insulin molecule; the component for peaks 6, 7 and 8 showed similar determination values of amino acids to those of Gly(A1)–AMCP-insulin, Gly(A1), Phe(B1)–diAMCP-insulin and Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin, respectively. Reaction site(s) of *S*-AMS in insulin other than the amino groups could not be identified successfully.

The value of pH in the reaction affected the formation of the AMCP-insulins (Fig. 3). In the reaction at a molar ratio of *S*-AMS to insulin of 15 at 30 °C for 1 h, the yields of Gly(A1), Lys(B29)–diAMCP-insulin and Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin (both minor products) increased with increasing pH, but those of Gly(A1)–AMCP-insulin and Gly(A1), Phe(B1)–diAMCP-insulin (both major

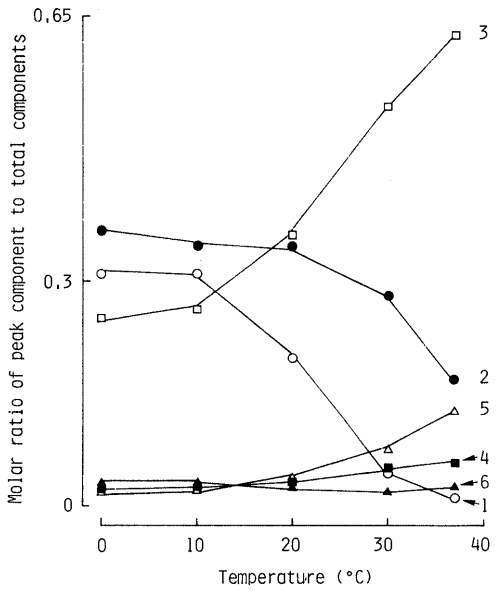

Fig. 5. Effect of the Reaction Temperature on the Production of AMCP-Insulins

Insulin (2.0 mg) was treated as in Fig. 3 but at various temperature under pH 7.0. For curves see Fig. 3.

Table II. Number of Sulfhydryl Groups Generated from AMCP-Insulins by Deacetylation

Position of AMCP moiety	Number of sulfhydryl groups per porcine molecule[a]	
	Theoretical	Found
Porcine insulin	0	0.00
Gly(A1)	1	0.99
Gly(A1), Phe(B1)	2	1.88
Gly(A1), Lys(B29)	2	1.91
Gly(A1), Phe(B1), Lys(B29)	3	2.92

a) Determined by the 4,4'-dithiodipyridine method.[8]

products) decreased. These results implied that *S*-AMS reacted predominantly with the ε-amino group of Lys(B29) residue at a higher pH, and that lower pHs enhanced the reactivity of amino groups of Gly(A1) and Phe(B1) residues with *S*-AMS, possibly because of low values of pK_a for the amino groups of Gly(A1) and Phe(B1) residues. The total yields of unknown insulin derivatives (peaks 6—8 in Fig. 2) also increased with increasing pH. pH 7 was employed for the preparation of the AMCP-insulins.

Figure 4 shows the effect of the molar ratio of *S*-AMS to insulin on the formation of the AMCP-insulins at 30 °C for 1 h at pH 7. The reaction at the molar ratios of 5—10 provided the maximum production of Gly(A1)–AMCP-insulin, and the production of the other three AMCP-insulins increased with increasing molar ratio. At a molar ratio greater than 30, *S*-AMS was deposited in the reaction mixture. The total yields of the unknown derivatives (peaks 6—8 in Fig. 2) slightly increased with an increasing molar ratio. The same was also true for the reactions at pHs 6, 8 and 9. The molar ratio of 15 was chosen for the preparation of the AMCP-insulins.

The production of Gly(A1), Phe(B1)– and Gly(A1), Lys(B29)–diAMCP-insulin and Gly(A1), Phe(B1),

Lys(B29)–triAMCP-insulin was enhanced by a rising reaction temperature in the range of 0—37 °C (Fig. 5). On the contrary, however, the yield of Gly(A1)–AMCP-insulin decreased with a rising temperature (Fig. 5). These facts suggest that the AMCP moiety is first introduced into insulin at the Gly(A1) position, then to the Phe(B1) and/or Lys(B29) positions. The temperature of 30 °C, which was selected in the procedure, could afford the AMCP-insulins in relatively high yields.

The same tendency as that mentioned above was observed in the effect of the reaction time (1 min—2 h); the formation of Gly(A1), Phe(B1)– and Gly(A1), Lys(B1)–diAMCP-insulins and Gly(A1), Phe(B1), Lys(B29)–triAMCP-insulin increased up to at least 2 h, and that of Gly(A1)–AMCP-insulin reached a maximum after 5 min, then decreased as the reaction time progressed. The total yield of the unknown insulin derivatives was minimum (2%) up to 2 h, but after 6 h the total yield reached an extremely high value (32.6%). The reaction time of 1 h was selected in the procedure.

A perfect deacetylation of the AMCP-insulins could be achieved under mild conditions using hydroxylamine without reduction of the disulfide bonds in an insulin molecule (Table II).

In conclusion, this paper provided a method for the preparation of AMCP-insulins having protected sulfhydryl group(s) at definite site(s) on the molecule, which could be separated in a preparative scale more readily than *S*-acetylthioglycoloyl-insulins. AMCP-insulins should be useful for the preparation of insulin conjugates with informative substances such as enzymes and fluorophores.

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