Preparation of Fluorescence Labeled Insulins, Sulfobenzoxadiazolyl-insulins, for Fluorescence Immunoassay

Masahiko NANAMI, Kiyoshi ZAITSU* and Yosuke OHKURA

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812, Japan.

Received July 10, 1992

The preparation of insulins labeled with a fluorescent moiety at a definite position (Gly(A1) or Lys(B29)) on the molecule is described. Gly(A1)- and Lys(B29)-S-acetylthioglycolyl-insulins and Gly(A1)-2(or 3)-acetylmercapto-3-carboxypropanoyl-insulin were deacetylated and then reacted with 7-chloro-4-sulfobenzoxadiazole, a fluorogenic reagent, to afford the corresponding fluorescence labeled insulins. The preparative separation of the fluorescence labeled insulins from the insulin derivatives that remained unreacted was carried out by anion-exchange high-performance liquid chromatography on a TSKgel DEAE-2SW column.

Keywords fluorescence labeled insulin; 7-chloro-4-sulfobenzoxadiazole; anion-exchange HPLC; acetylthioglycolyl insulin; acetylmercapto-3-carboxypropanoyl insulin

Fluorescence immunoassay employing fluorescence labeled insulin has been reported as an assay method for insulin in biological samples; randomly fluorescence labeled insulins have been used.^{1,2)} It has been shown that, in the case of enzyme (horseradish peroxidase) labeling of insulin, the labeled position of the insulin molecule influenced the assay sensitivity.³⁾ Therefore, there is a possibility to improve assay sensitivity for insulin provided that fluorescent moiety is labeled at a definite and adequate position of insulin.

The aim of this study is to prepare fluorescence labeled insulins, of which fluorescent moiety is located at a definite position of the insulin, Gly(A1)-S-acetylthioglycolyl (ATG)⁴⁾-, Lys(B29)-ATG- and Gly(A1)-acetylmercapto-3-carboxypropanoyl (AMCP)⁵⁾-insulins (Chart 1) which have protected sulfhydryl (acetylmercapto) groups are reacted after deacetylation with 7-chloro-4-sulfobenzoxadi-

azole (SBD-C1),⁶⁾ a fluorogenic reagent, to yield Gly(A1)-[S-(4-sulfobenzoxadiazol-7-yl)-thioglycolyl] (SBD·TG)-, Lys(B29)-SBD·TG- and Gly(A1)-[S-(4-sulfobenzoxadiazol-7-yl)-2(or 3)-mercapto-3-carboxypropanoyl] (SBD·MCP)-insulins, respectively (Chart 1). The yielded insulins were purified by anion-exchange high-performance liquid chromatography (HPLC).

Experimental

Reagent and Apparatus Lyophilized porcine insulin was obtained as previously described.⁷⁾ Gly(A1)-ATG-, Lys(B29)-ATG- and Gly(A1)-AMCP-insulins and urea solution were prepared as previously described.^{4,5)} Ammonium salt of SBD-C1 was obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of reagent grade. Molecular membrane tubes (Spectra/Por 3; molecular weight cut off, approximately 3500; Spectral Medical Ind., Los Angeles, U.S.A.) were used for dialyses, which were carried out at 4 °C.

Absorbances were measured with a Hitachi 150-20 spectrophotometer

Chart 1. Structures of Insulin Derivatives Studied

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 protein samples in 6M HCl *in vacuo* at 110°C for 24 h.

HPLC System and Its Operation A liquid chromatograph equipped with a Hitachi (Tokyo, Japan) 655 proportioning valve pump for gradient elution, a Hitachi 650-60 recording processor, a Rheodyne 7125 syringe-loading sample injection valve (200- μ l loop), a Tosoh (Tokyo, Japan) UV-8 model II spectromonitor operating at 280 nm and a Hitachi F 1000 fluorescence spectrophotometer was used. The fluorescence spectrophotometer was operated at the excitation wavelength of 380 nm and the emission wavelength of 513 nm.

Preparative anion-exchange HPLC was performed on a TSKgel DEAE-2SW column (300 $\times$ 7.8 mm i.d.; Tosoh) and the column temperature was ambient (20–25°C). An NaCl concentration gradient elution was made using a mixture of eluant A (0.05 M Na-K phosphate buffer (pH 7.0) containing 4 M urea) and eluant B (eluant A containing 1 M NaCl), which was pumped at a flow rate of 1.4 ml/min. The NaCl gradient was executed as follows. 1) For ATG-insulins, eluant A was run for the first 10 min, and then eluant B was increased to 10% linearly during the next 50 min (Figs. 1 and 2). 2) For AMCP-insulin, eluant A was run for the first 5 min and eluant B was increased to 15% linearly during the next 45 min, then the eluant mixture was pumped for a further 10 min (Fig. 3). The used column was regenerated by running eluant A for 30 min.

Size-exclusion HPLC was carried out on a TSKgel G2000-SW column (300 $\times$ 7.8 mm i.d.; Tosoh) and the column temperature was ambient. The mobile phase was 0.05 M Na-K phosphate buffer (pH 7.0) containing 0.9% NaCl, which was pumped at a flow rate of 1.0 ml/min.

Preparation of Gly(A1)-SBD·TG- and Lys(B29)-SBD·TG- insulins To Gly(A1)-ATG- and Lys(B29)-ATG-insulins (both 5 mg, 1.67 μ mol) dissolved in 5 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl (evacuated) was added 1.25 ml of 1.0 M hydroxylamine hydrochloride in the buffer and the mixtures were stirred at 30°C for 60 min, respectively. To the resulting Gly(A1)-thioglycoloyl (TG)- and Lys(B29)-TG-insulin solutions was added 600-times molar excess of SBD-Cl dissolved in 2.5 ml of the buffer at 25°C with stirring. The pHs of the mixtures were adjusted to 9.1 with 5 M NaOH. Then, the mixtures were stirred at 25°C for 30 min. The resulting mixtures were treated by chromatography on a Sephadex G-25 (50 g; Pharmacia LKB Biotechnology, Uppsala, Sweden) column (360 $\times$ 30 mm i.d.) using 1 mM Na phosphate buffer (pH 7.0) to remove any hydroxylamine and SBD-Cl that remained unreacted. The protein fractions were collected and lyophilized. The resulting lyophilizates (both approximately 4.0 mg) were dissolved in 800 μ l of 0.05 M Na₂HPO₄ (pH 9.4), respectively. Portions (200 μ l) of the solutions were subjected to anion-exchange HPLC. The combined individual eluates were dialyzed against 2 l of water (5 times) to remove urea and sodium phosphate, and lyophilized. The lyophilizates were dissolved in 1.0 ml of 0.05 M Na₂HPO₄, respectively, and subjected to chromatography on a Sephadex G-25 (5.6 g) column (360 $\times$ 10 mm i.d.) with 1 mM Na phosphate buffer (pH 7.0) to remove urea that remained unremoved. The respective protein fractions were dialyzed against 2 l of water (5 times) to remove Na phosphate and lyophilized to give Gly(A1)-SBD·TG-insulin (yellow powder, yield 580 μ g) and Lys(B29)-SBD·TG-insulin (yellow powder, yield 440 μ g). The identification of the fluorescent insulins was carried out by size-exclusion HPLC and the method of Levy with minor modifications.^{8,9)}

Preparation of Gly(A1)-SBD·MCP-insulin Gly(A1)-AMCP-insulin (10 mg, 3.33 μ mol) was treated as mentioned above except that the reaction with SBD-Cl was conducted at 20°C for 2 h to yield Gly(A1)-SBD·MCP-insulin (yellow powder, 355 μ g). The identification of the fluorescent insulin was carried out by the above-mentioned method.

Results and Discussion

Gly(A1)-ATG- and Gly(A1)-AMCP-insulins are readily deacetylated with hydroxylamine to give the corresponding sulfhydryl insulins. These sulfhydryl insulins can react with SBD-Cl to give insulins labeled with fluorescent moiety at a definite site. The separation of these fluorescence labeled insulins from the insulin derivatives that remained unreacted, were achieved by anion-exchange HPLC on a DEAE-2SW column.

Figures 1, 2 and 3 depict preparative chromatograms

Fig. 1. Chromatogram Obtained with a Reaction Mixture of SBD-Cl with Deacetylated Gly(A1)-ATG-insulin

Gly(A1)-ATG-insulin (1.0 mg) was treated by the procedure and the mixture was subjected to anion-exchange HPLC. The shaded peak is Gly(A1)-SBD·TG-insulin. Curve a: the concentration of sodium chloride in the eluent in the gradient elution. Peaks: 1, Gly(A1)-SBD·TG-insulin; 2, Lys(B29)-SBD·TG-insulin.

Fig. 2. Chromatogram Obtained with a Reaction Mixture of SBD-Cl with Deacetylated Lys(B29)-ATG-insulin

Lys(B29)-ATG-insulin (1.0 mg) was treated by the procedure and the mixture was subjected to anion-exchange HPLC. The shaded peak is Lys(B29)-SBD·TG-insulin. Curve a: the concentration of sodium chloride in the eluent in the gradient elution.

obtained with the reaction mixtures of Gly(A1)-SBD·TG-, Lys(B29)-SBD·TG- and Gly(A1)-SBD·MCP-insulins, respectively, (injection amount, 1 mg protein each). A gradient elution with NaCl at concentration up to 0.1 or 0.15 M in the mobile phase (Figs. 1, 2 and 3) gave satis-

factory separation. Under the chromatographic conditions for Fig. 1, Gly(A1)-SBD·TG-insulin was separated from Lys(B29)-SBD·TG-insulin which resulted from Lys(B29)-ATG-insulin as an impurity in the Gly(A1)-ATG-insulin preparation.

Figure 4 shows the effect of reaction temperature on the conjugation of Gly(A1)-TG-insulin (curve 1) and Gly(A1)-mercapto-3-carboxypropanoyl (MCP)-insulin (curve 2) with SBD-Cl at the molar ratios of SBD-Cl to the insulin derivatives of both 600 at pH 9.1 for 30 min or 2 h. The temperatures of 25°C and 20°C were selected for the preparation of Gly(A1)-SBD·TG- and Gly(A1)-SBD·MCP-insulins as optima, respectively.

The reactions of Gly(A1)-TG- and Gly(A1)-MCP-insulins with SBD-Cl were complete after 15 min and 1 h, respectively. The reaction times of 30 min and 2 h were chosen for the preparation of Gly(A1)-SBD·TG- and Gly(A1)-SBD·MCP-insulins, respectively. pH 9.1 could afford the maximum yields of Gly(A1)-SBD·TG- and Gly(A1)-SBD·MCP-insulins.

The yields of Gly(A1)-SBD·TG- and Gly(A1)-SBD·MCP-insulins increased by increasing the molar ratio of SBD-Cl to Gly(A1)-ATG- and Gly(A1)-AMCP-insulins,

respectively. At a ratio greater than 600, SBD-Cl deposited in the reaction mixture; the ratio of 600 was chosen in both cases.

Under the optimized conditions, the yield of Gly(A1)-SBD·TG-insulin was approximately four-times that of Gly(A1)-SBD·MCP-insulin. This suggested that ATG-insulin could react with SBD-Cl more advantageously than AMCP-insulin. Thus, Lys(B29)-ATG-insulin was employed for the preparation of fluorescent insulin labeled at Lys(B29) position. The preparation of Lys(B29)-SBD·TG-insulin was carried out under the same conditions as the preparation of Gly(A1)-SBD·TG-insulin.

Identification of the fluorescent insulins was carried out by size-exclusion HPLC and by the modified Levy method. The chromatograms obtained from size-exclusion HPLC of the SBD labeled insulins showed that the retention times (9.6 min) of the SBD labeled insulins were the same time of intact insulin. On amino acid analysis of the acid-hydrolyzed SBD-insulins after deamination, there was no discrepancy between the theoretical and found values of glycyl, phenylalanyl and lysyl residues of the SBD labeled insulins (Table I). The reaction of intact insulin with SBD-Cl did not give any fluorescent insulin. These observations indicated that SBD moiety on the SBD labeled

Fig. 3. Chromatogram Obtained with a Reaction Mixture of SBD-Cl with Deacetylated Gly(A1)-AMCP-insulin

Gly(A1)-AMCP-insulin (1.0 mg) was treated by the procedure and the mixture was subjected to anion-exchange HPLC. The shaded peak is Gly(A1)-SBD·MCP-insulin. Curve a: the concentration of sodium chloride in the eluent in the gradient elution.

Fig. 4. Effect of the Reaction Temperature on the Fluorescence Labeling of Deacetylated Gly(A1)-ATG- and AMCP-insulins

Gly(A1)-ATG- and AMCP-insulins (100 µg each) were deacetylated by the procedure, respectively, and then treated according to the procedure at the molar ratio of SBD-Cl to the insulin of 600 at various temperatures at pH 9.1 for 30 min (curve 1) or 2 h (curve 2). Curves: 1, Gly(A1)-ATG-insulin; 2, Gly(A1)-AMCP-insulin. The fluorescence intensity of Gly(A1)-SBD·TG-insulin at 25°C was taken as 100.

TABLE I. Numbers of Glycyl, Phenylalanyl and Lysyl Residues of the Insulin Derivatives after Deamination

Derivatives	Number of amino acid residues per molecule ^{a)}					
	Gly		Phe		Lys	
	Theoretical	Found	Theoretical	Found	Theoretical	Found
Porcine insulin	3	2.92	2	1.84	0	0.07
Gly(A1)-SBD·TG-insulin	4	4.29	2	1.86	0	0.17
Gly(A1)-SBD·MCP-insulin	4	4.11	2	1.71	0	0.13
Lys(B29)-SBD·TG-insulin	3	2.98	2	1.91	1	1.19
Intact porcine insulin ^{b)}	4	4.11	3	3.02	1	1.04

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

insulins was introduced definitely into the Gly(A1) or Lys(B29) position.

In conclusion, this paper provided a method for the preparation of SBD-insulins labeled at a definite position (Gly(A1) or Lys(B29)) *via* sulfhydryl group on the insulin. The investigation of the fluorescence immunoassay using these SBD-insulins is in progress in our laboratory.

References

- 1) S. D. Lidofsky, T. Imasaka and R. N. Zare, *Anal. Chem.*, **51**, 1602 (1979).
- 2) Y. Yamaguchi, C. Hayashi and K. Miyai, *Anal. Lett.*, **15**, 731 (1982).
- 3) M. Nakayama, K. Zaitzu and Y. Ohkura, *Rinsho Kagaku.*, **18**, 95 (1989).
- 4) M. Nanami, K. Zaitzu and Y. Ohkura, *Chem. Pharm. Bull.*, **40**, 957 (1992).
- 5) M. Nanami, K. Zaitzu and Y. Ohkura, *Chem. Pharm. Bull.*, **40**, 1843 (1992).
- 6) J. L. Andrews, P. Ghosh, B. Ternai and M. W. Whitehouse, *Arch. Biochem. Biophys.*, **214**, 386 (1982).
- 7) K. Zaitzu, H. Hosoya, Y. Hayashi, H. Yamada and Y. Ohkura, *Chem. Pharm. Bull.*, **33**, 1159 (1985).
- 8) D. Levy, *Biochim. Biophys. Acta*, **317**, 473 (1973).
- 9) K. Zaitzu, M. Nakayama and Y. Ohkura, *Chem. Pharm. Bull.*, **36**, 1425 (1988).