Effect of Spacer Groups Connecting Insulin with Fluorophore in Gly(A1)-sulfobenzoxadiazole-Labeled Insulins on the Immunoreactivity toward Anti-insulin Antibody

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The immunoreactivity (specific binding) of three Gly(A1)-sulfobenzoxadiazole-labeled insulins which have different spacer groups, Gly(A1)-[3-(4-sulfobenzoxadiazol-7-ylthio)propanoyl]-insulin, Gly(A1)-[2-(4-sulfobenzoxadiazol-7-ylthio)acetyl]-insulin, Gly(A1)-[3-carboxy-2(or 3)-(4-sulfobenzoxadiazol-7-ylthio)propanoyl]-insulin, toward an anti-porcine insulin monoclonal antibody was investigated, where solid-phase fluoroimmunoassay technique was utilized. The immunoreactivities of three labeled insulins were 2.1, 1.8 and 1 in that order.

Keywords sulfobenzoxadiazolyl insulin; spacer group; fluoroimmunoassay; porcine insulin; solid-phase antibody

We recently described that the immunoreactivity of Gly(A1)-[2-(4-sulfobenzoxadiazol-7-ylthio)acetyl] (SBD·TA)-insulin, a fluorescence labeled insulin, toward an anti-porcine insulin monoclonal antibody was superior to that of Lys(B29)-SBD·TA-insulin.¹⁾ This finding implies that the fluorescence labeled position on the insulin molecule is one of the factors affecting the immunoreactivity of the labeled insulins, which reflected directly the assay sensitivity in the fluoroimmunoassay of insulin. Spacer groups connecting insulin with fluorophore should also influence the immunoreactivity. We wish to report herein the immunoreactivity of three species of Gly(A1)-SBD-labeled insulins which have different spacer groups, Gly(A1)-[3-(4-sulfobenzoxadiazol-7-ylthio)propanoyl] (SBD·TP)-insulin, newly synthesized in this study, Gly(A1)-SBD·TA-insulin²⁾ and Gly(A1)-[3-carboxy-2(or 3)-(4-sulfobenzoxadiazol-7-ylthio)propanoyl] (C·SBD·TP)-insulin²⁾ (Chart 1), toward

the anti-insulin antibody.

Experimental

Reagents and Apparatus Lyophilized porcine insulin,³⁾ Gly(A1)-SBD·TA- and Gly(A1)-C·SBD·TP-insulins²⁾ and anti-porcine insulin monoclonal antibody coated beads¹⁾ were prepared as described previously. *N*-Succinimidyl *S*-acetylthiopropionate (SATP)⁴⁾ and ammonium salt of 7-chloro-4-sulfobenzoxadiazole (SBD-Cl)⁵⁾ were purchased from Molecular Probes Inc., (Eugene, Oregon, U.S.A.) and Dojindo Lab. (Kumamoto, Japan), respectively. Other chemicals were of reagent grade. UV and fluorescence spectral measurements were carried out as described previously.^{1,6)}

HPLC System and Its Operation A preparative anion-exchange HPLC system and its operating conditions were almost identical with those reported previously.^{2,6)} For the purification of Gly(A1)-acetylthiopropionyl (AcTP)-insulin, the mobile phase was 50 mM phosphate buffer (pH 6.0, 4 M urea) and the flow rate was 1.4 ml/min. For the purification of Gly(A1)-SBD·TP-insulin, an NaCl concentration gradient elution was made using eluent A (50 mM phosphate buffer (pH 7.0), 4 M urea) and eluent B (eluent A containing 1 M NaCl) (flow rate, 1.4 ml/min); eluent A was run for the first 5 min, and eluent B was linearly increased to 10% during the next 55 min, then a mixture of eluents A and B (9:1, v/v) was pumped for 10 min.

Preparation of Gly(A1)-SBD·TP-insulin To lyophilized porcine insulin (20.0 mg, 3.33 μmol) dissolved in 20 ml of 0.1 M Na phosphate buffer (pH 6.0) was added five 200 μl portions of 16.7 mM SATP (total 16.7 μmol) in dried dimethylformamide (DMF) at intervals of 1 min during 4 min at 0 °C with vigorous stirring, and the stirring was continued for another 6 min. The resulting mixture was chromatographed on a Sephadex G-25 (50 g; Pharmacia, Uppsala, Sweden) column (360 × 30 mm i.d.) using 1 mM Na phosphate buffer (pH 7.0) to remove any SATP that remained unreacted. The protein fraction was collected and lyophilized. The lyophilizate (17 mg) was dissolved in 2.0 ml of 50 mM Na₂HPO₄ (pH 9.4). Portions (200 μl) of the solution were subjected to preparative anion-exchange HPLC. The eluate from the largest peak (retention time, 21.4 min) was treated to remove urea and salt as described previously⁶⁾ to give Gly(A1)-AcTP-insulin (colorless powder, yield 3.22 mg, UV λ_{max} (nm) (0.1 M Na phosphate buffer (pH 7.5)) (ε); 275.7 (7.15 × 10³)). The position of an AcTP group was determined as described previously.⁷⁾

To Gly(A1)-AcTP-insulin (3 mg, 0.5 μmol) dissolved in 3 ml of 0.1 M Na phosphate buffer (pH 7.5, 0.1 M NaCl, evacuated) was added 0.75 ml of 1.0 M hydroxylamine hydrochloride in the buffer, and the mixture was stirred at 30 °C for 1 h. To the resulting Gly(A1)-thiopropionyl-insulin solution was added a 600-times molar excess of SBD-Cl dissolved in 1.5 ml of the buffer at 30 °C with stirring. After adjusting the pH to 9.3 with 5 M NaOH, the mixture was stirred at 30 °C for 1 h and then chromatographed on a Sephadex G-25 column as mentioned above to remove any hydroxylamine and SBD-Cl that remained unreacted. The protein fraction was collected and lyophilized. The resulting lyophilizate (3.2 mg) was dissolved in 1.2 ml of 50 mM Na₂HPO₄ (pH 9.4). Portions (200 μl) of the solution were subjected to the preparative anion-exchange HPLC. The eluate from the largest peak (retention time, 54.6 min) was treated to

Chart 1

remove urea and salt as described previously²⁾ to give Gly(A1)-SBD·TP-insulin (yellow powder, yield 70 μ g, UV λ_{max} (nm) (0.1 M Na phosphate buffer (pH 7.5)) (ϵ); 276.0 (7.79×10^3)). The position of a SBD·TP group was confirmed by the method previously described.²⁾

Assay Procedure A solid-phase fluoroimmunoassay was performed as described previously¹⁾ using 167 nM solutions of the three Gly(A1)-SBD-insulins.

Results and Discussion

The reaction conditions of porcine insulin with SATP described under Experimental could afford the maximum yield of Gly(A1)-AcTP-insulin. Gly(A1)-AcTP-insulin, after removal of the acetyl group with hydroxylamine, was reacted with SBD-Cl. The resulting crude Gly(A1)-SBD·TP-insulin was purified by preparative anion-exchange HPLC with a sodium chloride concentration gradient elution.

Gly(A1)-SBD·TP-insulin solution in 0.1 M phosphate buffer (pH 7.5) had the fluorescence excitation (maximum, 380 nm) and emission (maximum, 513 nm) spectra and intensity which were identical with those of Gly(A1)-SBD·TA- and Gly(A1)-C·SBD·TP-insulins in the buffer at an equimolar concentration (167 nM). When these SBD-labeled insulins were bound to the anti-porcine insulin monoclonal antibody coated beads, no fluorescence spectral shift was found.

The fluorescence intensities increased with increasing amounts of the SBD-insulins bound to the beads; the intensities obtained in the absence and presence of excess insulin reflected the total (specific and non-specific) binding and non-specific binding, respectively. The specific bindings (immunoreactivity; total binding deducted by non-specific binding) of Gly(A1)-SBD·TP-, Gly(A1)-SBD·TA- and Gly(A1)-C·SBD·TP-insulins were 2.1, 1.8 and 1, respectively, at a concentration of 167 nM when examined by the assay procedure. The observations indicated that the spacer groups affected the immunoreactivity of the SBD-labeled insulin and that the presence of carboxymethyl or a carboxyl group in TA or TP moiety caused the reduction in immunoreactivity.

The standard curves for porcine insulin constructed according to the assay procedure are shown in Fig. 1. The relative standard deviations ($n=5$ each) were 2.4–12.1%, 1.8–9.2% and 3.9–10.3% in the determinable ranges 0.83–16 pmol/tube, 1.6–16 pmol/tube and 8.3–16 pmol/tube for Gly(A1)-SBD·TP-, Gly(A1)-SBD·TA- and Gly(A1)-C·SBD·TP-insulins, respectively. The detection limits of insulin were 0.83 pmol/tube for Gly(A1)-SBD·TP-insulin, 1.6 pmol/tube for Gly(A1)-SBD·TA-insulin and 8.3 pmol/tube for Gly(A1)-C·SBD·TP-insulin. The limit

Fig. 1. Standard Curves for Porcine Insulin

Porcine insulin was treated according to the assay procedure. Curves: 1 = Gly(A1)-SBD·TP-insulin; 2 = Gly(A1)-SBD·TA-insulin; 3 = Gly(A1)-C·SBD·TP-insulin. Each plot represents the mean value of 5 determinations. The fluorescence intensity obtained with Gly(A1)-SBD·TP-insulin in the absence of porcine insulin was taken as 100.

was defined as the amount of insulin giving a fluorescence intensity in the absence of insulin (at zero insulin concentration in Fig. 1) subtracted by the intensity corresponding to twice the standard deviation at zero insulin concentration. The assay sensitivity obtained with Gly(A1)-SBD·TP-insulin was 2-times higher than that with Gly(A1)-SBD·TA-insulin, and one order of magnitude greater than that with Gly(A1)-C·SBD·TP-insulin. The assay sensitivities directly reflected the immunoreactivities of the SBD labeled insulins.

In conclusion, this study is the first to confirm that spacer groups in fluorescence labeled insulins affected the immunoreactivity of the labeled insulin toward anti-insulin antibody.

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