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Preparation of [3-(2-Pyridyldithio)propionoyl]insulins and Horseradish Peroxidase–Insulin Conjugates by High-Performance Liquid Chromatographic Separation

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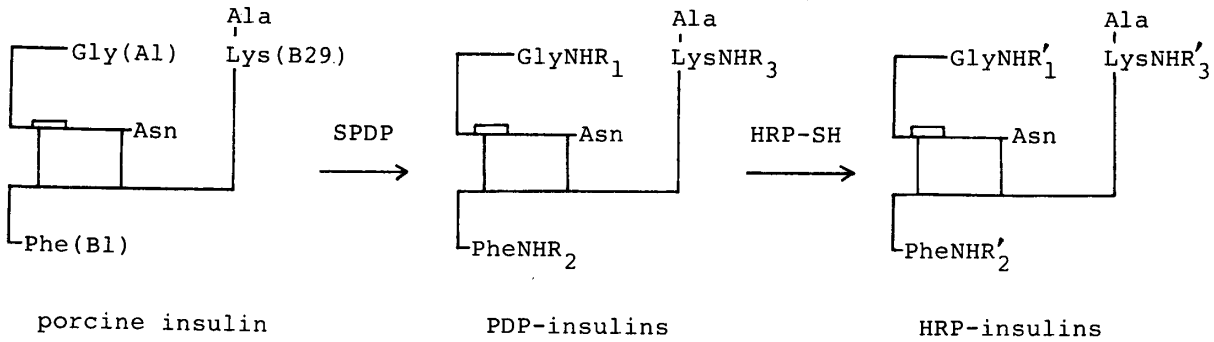
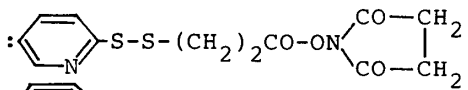
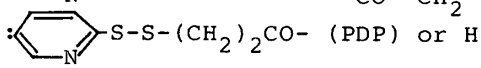
Five [3-(2-pyridyldithio)propionoyl]insulins (PDP-insulins) were separated preparatively from the reaction mixture of porcine insulin with *N*-succinimidyl 3-(2-pyridyldithio)propionate by means of anion-exchange high-performance liquid chromatography on a TSK gel DEAE-SW column. Horseradish peroxidase (HRP)–insulin conjugates (Gly^{A1}–HRP–insulin, Lys^{B29}–HRP–insulin and Gly^{A1}, Lys^{B29}–diHRP–insulin) were prepared by the reaction of thiolated HRP and the corresponding PDP-insulins and purified by gel-permeation high-performance liquid chromatography on a TSK gel G3000SW column.

Keywords——HPLC; [3-(2-pyridyldithio)propionoyl]insulin; horseradish peroxidase–insulin conjugate; *N*-succinimidyl 3-(2-pyridyldithio)propionate; cross-linking reagent; protein conjugate; enzyme label; insulin; horseradish peroxidase

Introduction of 3-(2-pyridyldithio)propionoyl (PDP) group(s) into the insulin molecule by using *N*-succinimidyl 3-(2-pyridyldithio)propionate[1] (SPDP), a heterobifunctional cross-linking reagent, results in several insulin derivatives with one, two and three PDP group(s) (PDP-insulins, Chart 1). These PDP-insulins can be used as starting materials for preparation of analytical probes such as enzyme-labeled insulin for enzyme-immunoassays of insulin and



SPDP : 

R_1, R_2 or R_3 : 

HRP-SH : HRP-NHCOCH(CH_2COOH)-SH or HRP-$NHCOCH_2$CH(COOH)-SH

R'_1, R'_2 or R'_3 : HRP-NHCOCH(CH_2COOH)-S-S-$(CH_2)_2$CO-,
HRP-$NHCOCH_2$CH(COOH)-S-S-$(CH_2)_2$CO-,
PDP or H

Chart 1

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anti-insulin antibody.

This paper describes the preparative separation of PDP-insulins by using anion-exchange high-performance liquid chromatography (HPLC), and the use of gel-permeation HPLC for the purification of horseradish peroxidase (HRP; EC 1.11.1.7)-labeled insulins (HRP–insulins: Gly^{A1}–HRP–insulin, Lys^{B29}–HRP–insulin and Gly^{A1}, Lys^{B29}–diHRP–insulin) obtained by the reaction of the corresponding PDP-insulins with thiolated HRP (HRP–SH) (Chart 1).

Experimental

Materials——SPDP was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). HRP (285 purpurogallin units/mg, type VI) was from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Lyophilized porcine insulin was prepared[2] from monocomponent insulin solution (Insulin Novo Actrapid MC, Novo Ind., Copenhagen, Denmark). Aqueous urea was deionized by passing the solution through an Amberlite MB3 (mixed resin; 20—50mesh; Bio-Rad, Richmond, U.S.A.) column. Deionized water was passed through a Milli-QII system (Japan Millipore Ltd., Tokyo, Japan). Other chemicals were of reagent grade.

Apparatus and Its Operation——Anion-exchange HPLC was performed on a TSK gel DEAE-2SW column (300 × 7.8 mm i.d.; Tosoh, Tokyo, Japan) with a Hitachi 655 liquid chromatograph equipped with a 655 proportioning valve pump for gradient elution, a 650—60 recording processor, a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Tosoh UV-8 model II spectromonitor operated at 278 nm. Elution with an NaCl concentration gradient during 50 min was done at a flow rate of 1.4 ml/min with eluant A (0.05 M Na–K phosphate buffer (pH 7.0) containing 4 M urea) and eluant B (eluant A containing 0.5 M NaCl): the initial eluant, a mixture of eluants A and B (9 : 1, v/v), was run for 15 min and eluant B was increased to 100% linearly during the next 25 min, then eluant B was passed for 10 min. The column was regenerated by washing with the initial eluant for 20 min.

Gel-permeation HPLC was carried out on a TSK gel G3000-SW column (600 × 7.5 mm i.d.; Tosoh) using a mobile phase of 0.05 M Na phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.8 ml/min with a Waters M-45 liquid chromatograph equipped with a Rheodyne 7125 syringe-loading injector valve (200-μl loop) and a Waters 440 absorbance detector operated at 254 and 405 nm.

Amino acid analyses were performed with a Hitachi 835 amino acid analyzer after hydrolysis of protein samples in 6 M HCl *in vacuo* at 110 °C for 24 h. Absorbances were measured with a Hitachi 150-20 spectrophotometer in semimicro quartz cells (10-nm optical path length, 1 ml).

Introduction of PDP Group into Insulin Molecule under Various Reaction Conditions——Lyophilized porcine insulin (1.2 mg, 200 nmol) was dissolved in 0.4 ml of 0.1 M Na phosphate buffer (pH 7.0) and 1- to 30-fold molar excess of SPDP in 100 μl of ethanol was added. The mixture was maintained at 23 °C for 30 min. The resulting mixture was dialyzed against 1 l of water (3times) at 4 °C and subjected to anion-exchange HPLC. The reaction was also performed at different pHs (5, 6, 8, and 9) with a 3-fold molar excess of SPDP.

Preparation of a Mixture of PDP-Insulins and Preparative Separation of PDP-Insulins——Lyophilized porcine insulin (144 mg, 24 μmol) was dissolved in 24 ml of 0.1 M Na phosphate buffer (pH 7.5) buffer A. To this solution, 6 ml of 12 mM SPDP (72 μmol) in ethanol was added under vigorous stirring, and the mixture was stirred at 25 °C for 30 min. The resulting mixture was subjected to chromatography on a Sephadex G-25 column (36 × 6 cm i.d.) with 1 mM Na phosphate buffer (pH 7.0) as the eluant. The protein fraction (a mixture of PDP-insulins and intact insulin) was collected and lyophilized (mixture I, 110 mg). Mixture I (110 mg) was dissolved in 1100 μl of eluant A. A portion (100 μl) of the solution was subjected to anion-exchange HPLC. The eluates from the peaks due to individual PDP-insulins were collected. This chromatographic procedure was repeated 11 times and the combined individual eluates were dialyzed against 2 l of water (4 times) at 4 °C and lyophilized. Gly^{A1}–PDP-insulin (26 mg, UV λ_{max} (in buffer A) nm (ε): 277 (8.62×10^3)), Lys^{B29}–PDP-insulin (11 mg, UV λ_{max} (in buffer A) nm (ε): 277 (8.56×10^3)), Gly^{A1}, Phe^{B1}–diPDP-insulin (9.5 mg, UV λ_{max} (in buffer A) nm (ε): 277 (1.04×10^4)), Gly^{A1}, Lys^{B29}–diPDP-insulin (6 mg, UV λ_{max} (in buffer A) nm (ε): 277 (1.04×10^4)) and Gly^{A1}, Phe^{B1}, Lys^{B29}–triPDP-insulin (10 mg, UV λ_{max} (in buffer A) nm (ε): 278 (1.19×10^4)) were obtained. The method used to identify these PDP-insulins is described below.

Deamination and Identification of PDP-Insulins and Intact Insulin——This was achieved by the method of Levy[3] with minor modifications as follows: each sample (0.5 mg) was dissolved in 0.5 ml of 0.5 M Na acetate buffer (pH 4.5). To each solution, 3 M sodium nitrate (2 ml) was added, and the mixture was stirred at 25 °C for 6 h. To the resulting solution, 0.75 ml of 1 M ammonia was added and the whole was dialyzed against 1 l of water (3 times) and lyophilized. The location of PDP group(s) was confirmed from the data obtained by amino acid analysis of an acid hydrolyzate of the deaminated sample.

Preparation of HRP–SH——HRP–SH was prepared with *S*-acetylmercaptosuccinic anhydride as described previously.[4] The number of sulfhydryl groups in the HRP–SH was 1.45 when measured by the 4,4′-dithiodipyridine method.[5]

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Preparation of HRP–Insulins——Each PDP-insulin (Gly^{A1}–PDP-insulin, Lys^{B29}–PDP-insulin or Gly^{A1}, Lys^{B29}–diPDP-insulin) (750 nmol) was dissolved in 2.5 ml of HRP–SH solution [125 nmol, containing 0.1 M Na phosphate buffer (pH 6.0), 0.05 M NaCl and 5 mM ethylenediaminetetraacetic acid·2Na] and the pH of the mixture was adjusted to 8.0 with 2.5 M NaOH (evacuated) under stirring at 30 °C. The mixture was kept at 30 °C for 1 h. The resulting mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The resulting powder was dissolved in 100 μl of 0.05 M Na phosphate buffer (pH 7.0) containing 0.9% (w/v) NaCl and subjected to gel-permeation HPLC. The eluate corresponding to the peak of HRP–insulin was collected, and dialyzed against 1 l of water (3 times) at 4 °C. The dialyzate was lyophilized and stored at −20 °C. Gly^{A1}–HRP–insulin (3.5 mg), Lys^{B29}–HRP–insulin (2.5 mg) and Gly^{A1}, Lys^{B29}–diHRP–insulin (0.4 mg) were obtained.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)——The electrophoresis of HRP–insulins, HRP and insulin was carried out using the discontinuous buffer system described by Laemmli[6] with 5% acrylamide stacking gel and 10% acrylamide separating gel. Samples were boiled for 1 min in the absence of thiol compounds in 10 mM Tris–HCl buffer (pH 6.8) containing 1% SDS and 20% glycerol prior to application on slab gel of 1-mm thickness. The gels were stained for 1 h in 0.025% Coomassie brilliant blue R-250 (Sigma Chemicals Co.) in 50% methanol containing 10% acetic acid, destained overnight in 25% methanol containing 7% acetic acid and photographed.

Results and Discussion

Porcine insulin has two α-amino (Gly^{A1} and Phe^{B1}) groups and one ε-amino (Lys^{B29}) group.[7] SPDP can react with the amino group(s) at the *N*-succinimidyl ester side to yield the protein having PDP group(s) (Chart 1), and the PDP groups introduced into the protein can react with thiol group(s) of another protein. In this study, HRP–SH was conjugated through the PDP group to give HRP–insulin conjugate(s) by thiol–disulfide interchange reaction (Chart 1).[1,8]

When one PDP group is introduced onto an amino group of the insulin molecule, one positive charge is lost. The more PDP groups are introduced into insulin molecule, the more strongly the insulin is negatively charged. Therefore, PDP-insulins should be separatable by anion-exchange HPLC.

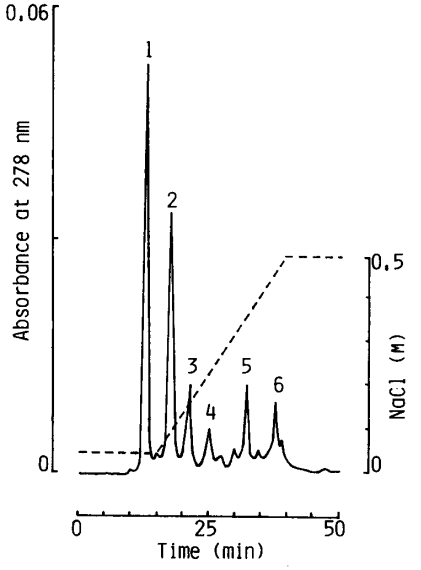

Fig. 1. Separation of a Mixture of PDP-Insulins and Intact Insulin

Mixture I (0.38 mg) dissolved in eluant A (100 μl) was injected into the chromatograph. Peaks: 1, intact insulin; 2, Gly^{A1}–PDP-insulin; 3, Lys^{B29}–PDP-insulin; 4, Gly^{A1},Phe^{B1}–diPDP-insulin; 5, Gly^{A1},Lys^{B29}–diPDP-insulin; 6, Gly^{A1},Phe^{B1},Lys^{B29}–triPDP-insulin. Chromatographic conditions: see the text. Dotted line: sodium chloride concentration in the eluant.

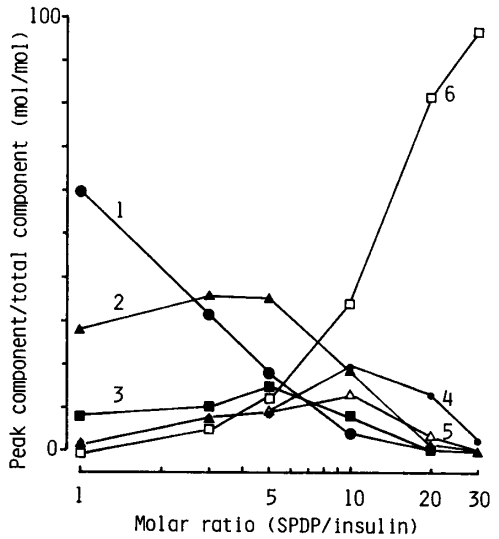

Fig. 2. Effect of the Molar Ratio of SPDP to Insulin on the Introduction of PDP Group(s) into the Insulin Molecule at pH 7

Curves: 1, intact insulin; 2, Gly^{A1}–PDP-insulin; 3, Lys^{B29}–PDP-insulin; 4, Gly^{A1},Phe^{B1}–diPDP-insulin; 5, Gly^{A1},Lys^{B29}–diPDP-insulin; 6, Gly^{A1},Phe^{B1},Lys^{B29}–triPDP-insulin. Reaction conditions: see the text.

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TABLE I. Numbers of PDP Groups Introduced into the Insulin Molecule and Amino Acid Residues of the Peak Components in the Chromatogram (Fig. 1) after Deamination

Peak number[a]	Location of PDP group	Number of PDP groups (group/molecule)		Number of amino acid residue[c] (residue/molecule)					
				Gly		Phe		Lys	
		Theoretical	Found[b]	Theoretical	Found	Theoretical	Found	Theoretical	Found
1	None	0	0	3	3.01	2	2.04	0	0.15
2	Gly(A1)	1	1.2	4	3.86	2	1.97	0	0.18
3	Lys(B29)	1	0.9	3	3.09	2	1.94	1	0.93
4	Gly(A1), Phe(B1)	2	1.9	4	4.23	3	2.85	0	0.12
5	Gly(A1), Lys(B29)	2	1.9	4	4.00	2	2.12	1	0.93
6	Gly(A1), Phe(B1), Lys(B29)	3	3.1	4	4.15	3	2.87	1	0.95
Intact porcine insulin		0		4		3		1	

a) For peak numbering, see Fig. 1. *b*) Determined by the method of Carlsson *et al.*[1] based on the measurement of absorbance at 343 nm which is due to 2-thiopyridone released from PDP-insulins by adding dithiothreitol. *c*) Based on 6 residues of leucine per one porcine insulin molecule.

As shown in Fig. 1, insulin and five PDP-insulins among seven possible PDP-insulins were separated successfully. Elution with a sodium chloride concentration gradient was employed for separation of the PDP-insulins and insulin.

The number of PDP group(s) introduced in the individual peak components was determined by the method of Carlsson *et al.*[1] (Table I). The location of the PDP group(s) was ascertained by analyzing the number of amino acid residues after deamination of amino group(s) free from PDP followed by acid hydrolysis of the peak components (Table I).

The effect of the molar ratios of SPDP to insulin on the introduction of PDP groups into the insulin molecule was examined. As shown in Fig. 2, at the molar ratio of SPDP to insulin of 3, five PDP-insulins were formed during a 30-min reaction at 23 °C. When the reaction was carried out at the molar ratios of 20 and 30, and at pH 7.0, Gly^{A1}, Phe^{B1}, Lys^{B29}–triPDP-insulin was predominantly formed.

No evidence for the production of Phe^{B1}–PDP-insulin or Phe^{B1}, Lys^{B29}–diPDP-insulin under any of the reaction conditions examined was obtained.

The reaction of HRP–SH with an approximately 6-fold molar excess of Gly^{A1}–PDP-insulin, Lys^{B29}–PDP-insulin and Gly^{A1}, Lys^{B29}–diPDP-insulin gave Gly^{A1}–HRP–insulin, Lys^{B29}–HRP–insulin and Gly^{A1}, Lys^{B29}–diHRP–insulin, respectively. These HRP–insulins were separated successfully by gel-permeation HPLC. Separation of HRP–SH from HRP–insulin by gel-permeation HPLC is difficult. Thus we used a 6-fold molar excess of PDP-insulins over HRP–SH. Figure 3(a—c) shows typical chromatograms of the mixtures obtained in the preparation of individual HRP–insulins. HRP–insulins corresponding to Gly^{A1}, Phe^{B1}–diPDP-insulin and Gly^{A1}, Phe^{B1}, Lys^{B29}–triPDP-insulin were not prepared in this study.

The SDS-PAGE data indicated molecular weights of 46000 for both Gly^{A1}–HRP–insulin and Lys^{B29}–HRP–insulin and 86000 for Gly^{A1}, Lys^{B29}–diHRP–insulin, in accordance with the theoretical values. Another band was also observed in the SDS-PAGE of Gly^{A1}–HRP–insulin at the molecular weight of 52000. This may indicate the existence of an HRP–insulin (1 : 2) conjugate. The use of HRP–SH having a mean content of SH groups of 1.45 mol is consistent with the production of the 1 : 2 conjugate. Gly^{A1}–HRP–insulin could be obtained in high purity by repeated HPLC.

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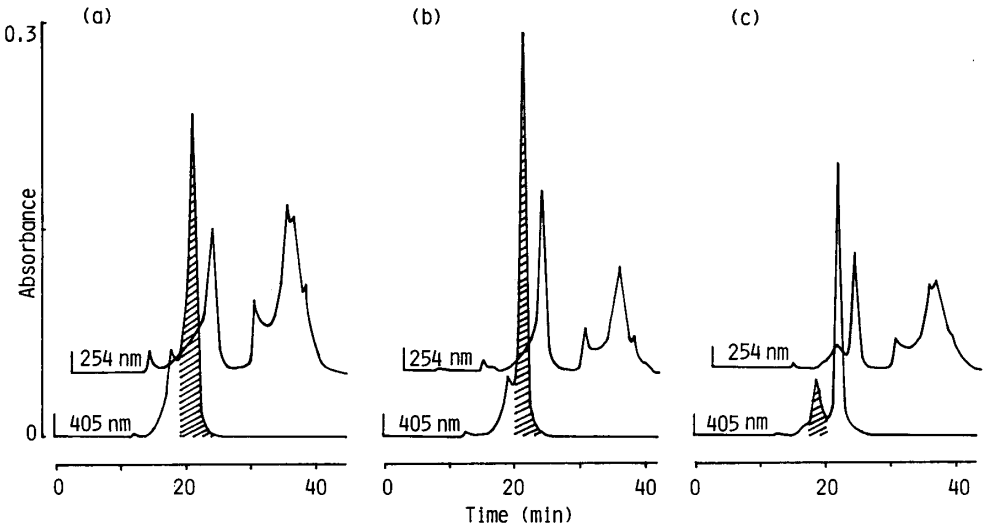

Fig. 3. Separation of Gly^{A1}–HRP–Insulin (a), Lys^{B29}–HRP–Insulin (b) and Gly^{A1},Lys^{B29}–diHRP–Insulin (c)

Reaction conditions: see the text. Eluates from hatched peaks were collected.

The reaction of Gly^{A1}, Phe^{B1}, Lys^{B29}–triPDP-insulin with HRP–SH did not give triHRP–insulin, but a small amount of diHRP–insulin; the two HRP-labeling sites on the insulin could not be identified. Gly^{A1}, Lys^{B29}–diHRP–Phe^{B1}–PDP-insulin is thought to be most improbable, since glycine A1 and lysine B29 are located quite close to one another (10 Å) in the three-dimensional crystal structure of insulin[9] and the molecular shape is compactly globular[10] and does not change dramatically in solution.[11] However, the possibility of the formation of Gly^{A1}, Lys^{B29}–diHRP–Phe^{B1}–PDP-insulin can not be completely ruled out because of the definite formation of Gly^{A1}, Lys^{B29}–diHRP–insulin from Gly^{A1}, Lys^{B29}–diPDP-insulin.

In conclusion, this paper demonstrates the utility of anion-exchange HPLC and gel-permeation HPLC for the preparative separation of PDP-insulins and HRP–insulins, respectively. Such an approach to the preparation of protein conjugates is desirable because preparative HPLC can give pure analytical probes. The use of pure probes in analytical work is very important to obtain highly reproducible results. Another method of conjugation between insulin and HRP has been reported using *N*-(bromoacetamido-*n*-propionoyloxy)succinimide. In this method, citraconylation of amino groups (Gly^{A1} and Phe^{B1}) of insulin and decitraconylation with acid after the introduction of a bromoacetamido-*n*-propionoyl group at the ε-amino group of lysine B29 were employed.[4] In the present method, on the other hand, the PDP group(s) was directly introduced into intact insulin to form several PDP-insulins.

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