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High-Performance Liquid Chromatographic Separation of Citraconylinsulins and Preparation of $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin

KIYOSHI ZAITSU, HIROYUKI HOSOYA, YOHJI HAYASHI, HIDENORI YAMADA and YOSUKE OHKURA*

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

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A method for the mutual separation of six citraconylated porcine insulins and intact insulin from the reaction mixture of insulin with citraconic anhydride is described, based upon anion-exchange high-performance liquid chromatography on a TSK IEX-540 DEAE column. The method permits the preparative isolation of citraconylinsulins. The optimal reaction conditions for the preparation of $Gly^{A1}Phe^{B1}$-dicitraconylinsulin, a useful starting material for the preparation of Lys-B29 modified insulin derivatives, were also investigated.

Keywords——citraconylinsulin; ion-exchange high-performance liquid chromatography; $Gly^{A1}Phe^{B1}$-dicitraconylinsulin; insulin modified; citraconylinsulin amino acid analysis; insulin; citraconic anhydride

Various derivatives of insulin have been prepared to elucidate the effects of modification on the biological and immunological activities of the hormone; in porcine insulin, for example, α-amino groups of Gly-A1 and Phe-B1 and ε-amino group of Lys-B29 have been modified. Derivatization reactions have mainly been performed directly with intact insulin and the products have been separated by chromatography on a column of an ion-exchanger such as DEAE-Sephadex.[1] These reactions tend to provide a low yield of the desired derivative and to require time-consuming trial-and-error trials to find suitable reaction conditions. To prepare insulin derivatives of interest, therefore, the use of modified insulin having definite reactive amino group(s) is preferable.

Citraconylation of amino group(s) in a protein other than a definite reactive amino group(s) has been used for the protection of amino group(s) because the citraconyl groups can easily be removed at acidic pH and no racemization occurs.[2] The citraconylation of insulin was described only briefly by Dixon *et al.*[3] and the reaction was applied in the course of the preparation of Lys^{B29}-acetylinsulin by Zahn *et al.*;[4] more elaborate studies are required to provide a basis for the general use of citraconylation of insulin.

This paper describes the preparative and analytical separation of intact insulin, Phe^{B1}- and Lys^{B29}-citraconylinsulin, $Gly^{A1}Phe^{B1}$-, $Phe^{B1}Lys^{B29}$- and $Gly^{A1}Lys^{B29}$-dicitraconylinsulin and $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin by anion-exchange high-performance liquid chromatography (HPLC) on a TSK gel IEX—540 DEAE column. $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin may be useful as a starting material for the preparation of various derivatives of insulin because it has only one reactive amino group at the Lys-B29 position which can be conjugated with various compounds, including chromophores, fluorophores, enzymes and radio-active substances. Such conjugates (Lys^{B29}-monosubstituted insulins) should be useful as powerful probes in various biological and immunological studies since they have nearly the same biological and immunological activities as intact insulin.[5] Thus, the optimum reaction conditions for the preparation of $Gly^{A1}Phe^{B1}$-dicitraconylinsulin were also investigated.

Experimental

Materials and Instrumentation——Deionized and distilled water was used. Lyophilized porcine insulin was prepared from monocomponent insulin solution (Insulin Novo Actrapid MC, Novo Ind., Copenhagen, Denmark) (50 ml) by gel chromatography on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (36 × 6 cm i.d.) using 0.05 M NH_4HCO_3 as the eluent, followed by lyophilization. Citraconic anhydride and urea were obtained from Nakarai Chemicals (Kyoto, Japan). Aqueous urea was deionized to remove cyanate ion, which causes carbamylation of insulin,[6] by passing the solution through an Amberlite MB 3 (mixed resin; 20—50 mesh; Bio-Rad, Richmond, U.S.A.) column. The solution was stored in a refrigerator at 4 °C and used within 2 d. Dioxane and Et_2O were distilled and passed through a column packed with neutral alumina (Bio-Rad), respectively, to remove peroxides, which were checked with Peroxide Test (Japan Merck, Tokyo, Japan). Other chemicals were of reagent grade.

Preparative (300 × 7.5 mm i.d.) and analytical (300 × 4 mm i.d.) anion-exchange HPLC columns of TSK gel IEX—540 DEAE were obtained from Toyo Soda (Tokyo, Japan). A Shimadzu LC-3A high-performance liquid chromatograph was used, equipped with a syringe-loading sample injector (injection volume, 20—100 μl) and a Toyo Soda UV-8 model II spectrophotometer (10 nm spectral bandwidth, 10 mm optical path length, 8 μl cell volume). Amino acid analyses were performed with a Hitachi 835 amino acid analyzer. Ultraviolet (UV) spectra and absorbances were measured in 10 mm quartz cells with a Hitachi 150-20 spectrophotometer.

Preparation of a Mixture of Citraconylinsulins for the Separation Study——Lyophilized porcine insulin (100 mg, 17.3 μmol) was suspended in 13 ml of water and the pH of the suspension was adjusted to 10.5 by adding a small amount of 5 M NaOH (approximately 25 μl) under vigorous stirring. To the resulting clear solution, 35 μl of 1 M citraconic anhydride solution (35 μmol) in dioxane was added. Immediately thereafter, 0.05 M AcOH (approximately 1.25 ml) was added dropwise during 5 min to bring the pH to 7.0. The above procedure was carried out at 4 °C. Immediately after the addition of the AcOH, the mixture was subjected to gel chromatography on a Sephadex G-25 column (36 × 6 cm i.d.) with 0.05 M NH_4HCO_3 as the eluent. The protein fraction was collected and lyophilized. A mixture of citraconylinsulins and intact insulin (mixture I, 70 mg) was obtained.

Separation of Citraconylinsulins and Intact Insulin in Mixture I——This was done by HPLC on a preparative column (preparative HPLC). Elution with an NaCl concentration gradient during 60 min was done at the flow rate of 1.4 ml/min with a starting eluent (60 ml) consisting of 0.05 M Na–K phosphate buffer (pH 7.0) containing 0.05 M NaCl and 4 M urea, and a running eluent (60 ml) whose components were the same as those of the starting eluent except that the NaCl concentration was 0.5 M. The used column was regenerated by passing the running eluent for 20 min and then the starting eluent for 20 min at the flow rate described above.

HPLC was repeatedly carried out with 1 mg of mixture I dissolved in 35 μl of the starting eluent. The eluate was monitored at 276 nm. The individual eluates corresponding to the peaks of citraconylinsulins and insulin were subjected to Sephadex G-25 column (40 × 2.7 cm i.d.) chromatography with 0.05 M NH_4HCO_3 as the eluent, and the protein fractions were lyophilized. Each of the lyophilized protein fractions was subjected to amino acid analysis.

Mixture I was also subjected to HPLC on an analytical column (analytical HPLC). The HPLC conditions were the same as for preparative HPLC except that the flow rate was 0.7 ml/min and 30-ml portions of the starting and running eluents were employed. The used column was regenerated in the same way as described above except for the flow rate, 0.7 ml/min.

Amino Acid Analysis of Citraconylinsulins——Amino acids in citraconylinsulins and intact insulin were analyzed by the method of Schroeder *et al.*[7] with slight modifications as follows. A 2.0 ml aliquot of 2% (v/v) 1-fluoro-2,4-dinitrobenzene (FDNB) solution in EtOH was added to the lyophilized protein fraction (1 mg) dissolved in 1 ml of 15% (w/v) Na_2CO_3 under vigorous stirring, and the mixture was stirred at 20 °C for 6 h in the dark. The EtOH in the mixture was evaporated off *in vacuo*. The residual aqueous solution was washed with 4 ml of Et_2O to remove excess FDNB, acidified with approximately 50 μl of concentrated HCl and again washed twice with 4 ml of Et_2O. The resulting mixture (which contained material that clotted on addition of HCl) was concentrated to dryness *in vacuo*. The residue was hydrolyzed in 1.5 ml of 6.0 M HCl at 110 °C for 24 h. The hydrolyzate was concentrated to dryness *in vacuo*, the residue was dissolved in 0.7 ml of 0.02 M HCl, and 0.1 ml of the solution was subjected to amino acid analysis. Intact insulin was hydrolyzed without being treated with FDNB and the hydrolyzate was subjected to amino acid analysis.

Procedure for Testing Various Reaction Conditions fot the Formation of $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin——A solution of 56 μg—1.68 mg (0.5—15 μmol) of citraconic anhydride (molar ratio of citraconic anhydride to insulin, 1—30) dissolved in 100 μl of dioxane was added in 20-μl portions to lyophilized porcine insulin (3 mg, 0.5 μmol) dissolved in 2 ml of 0.05 M Na phosphate buffer (pH 7.0) during 5 min at 4 °C with vigorous stirring. The concentration of insulin in the reaction mixture was 0.25 mM. Immediately after each addition, 50 μl of the reaction mixture was subjected to analytical HPLC. The areas of the peaks in the chromatogram were used for quantification.

With a molar ratio of 6 and a 5-min reaction period at 4 °C, the effect of pH (6, 8 and 9) on the citraconylation was examined in the same way as described above.

Preparation of $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin——Citraconic anhydride (16.8 mg, 150 μmol, 6-fold molar excess over insulin) dissolved in 5 ml of dioxane was added in 100-μl portions to lyophilized porcine insulin (144 mg,

25.0 μmol) dissolved in 100 ml of 0.05 M Na phosphate buffer (pH 7.0) during 5 min with stirring. Each half of the resulting mixture was chromatographed on a Sephadex G-25 column (36 × 6 cm i.d.) with 0.05 M NH_4HCO_3 at a flow rate of approximately 1.7 ml/min. The protein fraction was lyophilized to give a mixture of citraconylinsulins and intact insulin (112 mg).

The mixture (100 mg) was dissolved in 500 μl of the phosphate buffer containing 0.05 M NaCl. A portion (50 μl) of the solution was subjected to preparative HPLC. The eluates from the peaks due to individual citraconylinsulins were collected. This procedure was repeated 10 times and the combined individual eluates were chromatographed again on a Sephadex G-25 column in the same way as described above. Each protein fraction was collected and lyophilized.

$Gly^{A1}Phe^{B1}$-dicitraconylinsulin (40 mg, UV λ_{max} (in 0.1 M Na-phosphate buffer, pH 7.5) nm (ε): 275 (7010)) was obtained along with Phe^{B1}-citraconylinsulin (8 mg, UV λ_{max} (in 0.1 M Na-phosphate buffer, pH 7.5) nm (ε): 276 (6280)) and $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin (19 mg, UV λ_{max} (in 0.1 M Na-phosphate buffer, pH 7.5) nm (ε): 275 (7420)).

Regeneration of Insulin from $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin——Lyophilized $Gly^{A1}Phe^{B1}$-dicitraconylinsulin (1 mg) dissolved in 1 ml of 0.1 M AcOH (adjusted to pH 3.5 with pyridine) was left to stand at 23 °C for various periods (1—145 h). Portions (40 μl, corresponded to 6.67 nmol of insulin) of the resulting mixtures taken at standing times were subjected to preparative HPLC.

Results and Discussion

In the preparative and analytical HPLC, phosphate buffer of pH 7.0 was used as one of the components of the eluent because citraconyl groups in citraconylinsulins were eliminated in acidic pH, and a neutral or weakly acidic eluent was preferable for the HPLC column used. When the separation of mixture I was carried out by isocratic elution on the analytical column with 0.05 M Na–K phosphate buffer (pH 7.0) containing 0.05 M sodium chloride and 4 M urea, the peaks were considerably broadened. At the sodium chloride concentration of 0.2 M, mono-, di- and tricitraconylinsulins and intact insulin were eluted within 9 min with insufficient separation, and more negatively charged compounds than $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin (described later), if present in the sample, could not be eluted. The elution of these negatively charged compounds could be done with the phosphate buffer containing 0.5 M sodium chloride and 4 M urea.

On the basis of the above observations, elution with a sodium chloride concentration gradient as described in Experimental was employed for simultaneous separation of various citraconylinsulins. The absence of urea in the starting and running eluents caused prolonged elution times and peak broadening. The most satisfactory separation was achieved in the presence of 4 M urea.

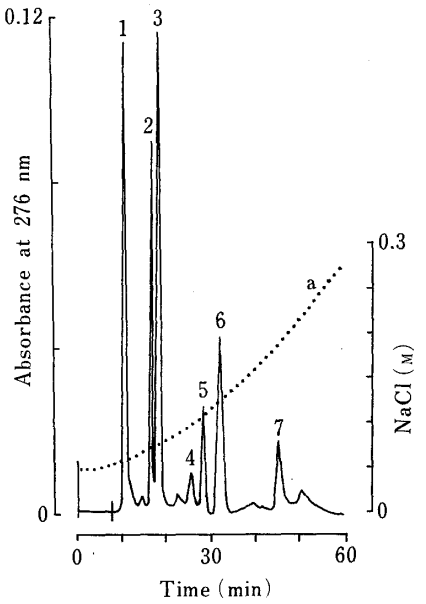

Fig. 1. Preparative Separation of Citraconylinsulins and Intact Insulin

Chromatograms were obtained under the conditions described in Experimental. Curve a: the concentration of sodium chloride in the eluent in the gradient elution. Mixture I (1 mg, obtained according to the procedure for the preparation of the mixture of citraconylinsulins for the separation study) dissolved in the starting eluent (35 μl) was injected. Peaks: 1, intact insulin; 2, Phe^{B1}-citraconylinsulin; 3, Lys^{B29}-citraconylinsulin; 4, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin; 5, $Phe^{B1}Lys^{B29}$-dicitraconylinsulin; 6, $Gly^{A1}Lys^{B29}$-dicitraconylinsulin; 7, $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin.

TABLE I. Amino Acid Analyses of Intact Insulin and the Components of the Peaks in the Chromatogram (Fig. 1) after Dinitrophenylation

Peak number[a]	Location of citraconylated amino group(s)	Number of amino acid residues					
		Gly		Phe		Lys	
		Theoretical	Found[b]	Theoretical	Found[b]	Theoretical	Found[b]
Intact porcine insulin[c]		4	4.03	3	3.08	1	1.05
1	None	3	3.02	2	2.05	0	0.05
2	Phe-B1	3	3.01	3	2.98	0	0.03
3	Lys-B29	3	3.10	2	2.24	1	0.95
4	Gly-A1, Phe-B1	4	4.07	3	2.99	0	0.01
5	Phe-B1, Lys-B29	3	3.01	3	3.20	1	1.00
6	Gly-A1, Lys-B29	4	4.00	2	2.10	1	1.04
7	Gly-A1, Phe-B1, Lys-B29	4	4.03	3	3.20	1	1.09

a) For peak numbering, see Fig. 1. *b*) The values were calculated from the data obtained with leucine. *c*) Without dinitrophenylation.

The conditions of the reaction of insulin with citraconic anhydride were investigated in order to obtain various citraconylinsulins for the separation study. At the molar ratio of citraconic anhydride to insulin of 2, 6 kinds of citraconylinsulins (except Gly^{A1}-citraconylinsulin) out of 7 possible kinds of citraconylinsulins were formed during a 5-min reaction when the pH of the reaction medium was varied from 10.5 to 7.0. At higher pH, $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin was predominantly formed. Therefore, this pH change was required to obtain the 6 citraconylinsulins simultaneously. The reaction mixture (mixture I) was subjected to anion-exchange HPLC on the preparative and analytical columns. A typical chromatogram obtained by preparative HPLC of mixture I is shown in Fig. 1. The chromatogram obtained by analytical HPLC of mixture I showed an elution pattern almost identical with that obtained in the preparative HPLC: the retention times of the peaks in analytical HPLC were smaller than those in preparative HPLC by 2—5 min. The eluates from individual peaks in the chromatogram were lyophilized after the removal of salts and urea by gel chromatography on a Sephadex G-25 column.

Amino acid analyses of the individual lyophilized materials were carried out after dinitrophenylation with FDNB. When intact insulin was treated with FDNB and hydrolyzed, there was a loss of one residue each of glycine, phenylalanine and lysine. For example, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin lost only one residue of lysine on dinitrophenylation followed by the hydrolysis (Table I). This indicates that the ε-amino group of the lysine residue (Lys-B29) in $Gly^{A1}Phe^{B1}$-dicitraconylinsulin was present.

To optimize the preparation of $Gly^{A1}Phe^{B1}$-dicitraconylinsulin, the effect of the molar ratio of citraconic anhydride to insulin on the citraconylation was examined according to the procedure already described. The result obtained in the reaction at pH 7.0 is shown in Fig. 2. At the molar ratio of 6 and at pH 7.0, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin could be obtained on a preparative scale.

When the reaction was carried out at molar ratios of 2 and 30, Phe^{B1}-citraconylinsulin and $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin were predominantly formed, respcetively. At the molar ratio of 30, an unknown compound (retention times in the analytical and preparative HPLC were 45 and 50.5 min, respectively) which had a more negative charge than $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin was formed (Fig. 2, curve 5).

The reaction at pH 8.0 or 9.0 and at the molar ratio of 6 gave the tricitraconylinsulin

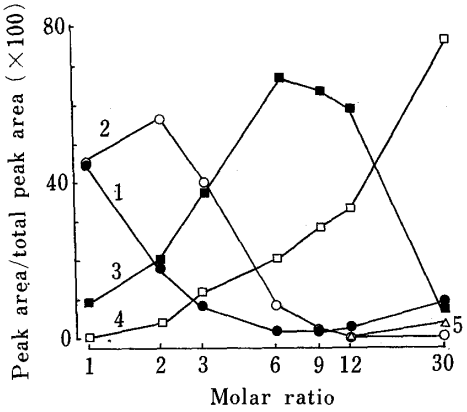

Fig. 2. Effect of the Molar Ratio of Citraconic Anhydride to Insulin on the Citraconylation at pH 7.0

For the reaction conditions, see Experimental. Curves: 1, intact insulin; 2, Phe^{B1}-citraconylinsulin; 3, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin; 4, $Gly^{A1}Phe^{B1}Lys^{B29}$-tricitraconylinsulin; 5, a more negatively charged compound.

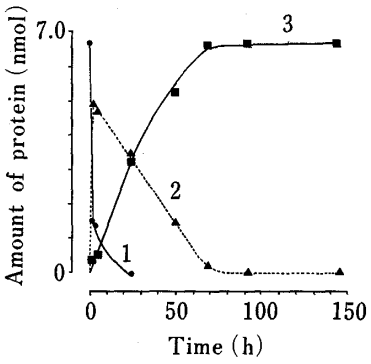

Fig. 3. Time-Course of Regeneration of Insulin from $Gly^{A1}Phe^{B1}$-Dicitraconylinsulin

The dicitraconylinsulin (1 mg) was treated according to the procedure for the regeneration of insulin. Curves: 1, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin; 2, Phe^{B1}-citraconylinsulin; 3, insulin.

and 2 kinds of more negatively charged compounds. The retention times of the latter two compounds were 45 and 53 min under the conditions of the analytical HPLC, respectively. The structures of these compounds are not known. At pH 6.0, $Gly^{A1}Phe^{B1}$-dicitraconylinsulin was mainly produced but a large amount of insulin remained unreacted.

The regeneration of insulin from $Gly^{A1}Phe^{B1}$-dicitraconylinsulin could be achieved by treating the solution in 0.1 M acetic acid (pH 3.5) at 23 °C for 100 h (Fig. 3).

In conclusion, we have developed a method for the separation of 6 kinds of citraconylinsulins by anion-exchange HPLC. The method was effectively applied for the preparation of $Gly^{A1}Phe^{B1}$-dicitraconylinsulin, which should be applicable to the preparation of various insulin derivatives useful in biological and immunological investigations.

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References

1) D. G. Lindsay and S. Shall, *Biochem. J.*, **121**, 737 (1971).
2) M. Z. Atassi and A. F. S. A. Yabeeb, *Methods Enzymol.*, **25-B**, 546 (1972).
3) H. B. F. Dixon and R. N. Perham, *Biochem. J.*, **109**, 312 (1968).
4) H. Zahn, D. Brandenberg and H. G. Gattner, *Diabetes*, **21**, 468 (1972).
5) D. G. Lindsay and S. Shall, *Eur. J. Biochem.*, **15**, 547 (1970).
6) G. R. Stark, W. H. Stein and S. Moose, *J. Biol. Chem.*, **235**, 3177 (1977).
7) W. A. Schroeder and J. Legette, *J. Am. Chem. Soc.*, **75**, 4612 (1953).