Preparative and Analytical Separations by High-Performance Liquid Chromatography and Capillary Electrophoresis of the Products from the Reaction of Insulin with *exo*-3,6-Epoxy-1,2,3,6-tetrahydrophthalic Anhydride

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An anion-exchange high-performance liquid chromatographic method using a TSKgel DEAE-2SW column and zone electrophoresis using a fused-silica capillary are described for the preparative and analytical separations of the products of the reaction between insulin and *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (ETPA), an amino protective reagent. Four stable products were identified having *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalyl group(s) on insulin at the i) Gly(A1), ii) Gly(A1) and Phe(B1), iii) Gly(A1) and Lys(B29), and iv) Gly(A1), Phe(B1) and Lys(B29) sites, together with four labile products produced by the reaction of ETPA with the non-amino groups of the stable products. The usefulness of capillary electrophoresis was demonstrated in the evaluation of the effect of the molar ratios of anhydride to insulin on the production of the insulin derivatives.

Keywords HPLC; capillary electrophoresis; insulin modified; amino blocking; dicarboxylic anhydride; *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride

Several dicarboxylic anhydrides, such as the anhydrides of 3,4,5,6-tetrahydrophthalic,^{1,2)} succinic,^{3–5)} citraconic^{6–8)} and maleic^{9,10)} acids can provide proteins with carboxyl negative charges in neutral media^{1,11)} and have been used for studies of protein dissociation^{9,12)} and hybridization^{3,8)} and to provide reversible^{1,6,10,13)} or irreversible^{3–5,14)} protection of protein amino groups. The compound, *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (ETPA),¹⁵⁾ which has been used for the reversible protection of amino groups in lysozyme,¹³⁾ should react with insulin to introduce *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalyl (ETP) group(s) at the free amino group(s) located on its Gly(A1), Phe(B1) and Lys(B29) residues (Fig. 1). Separation of the products (ETP-insulins) resulting from the reaction of insulin with ETPA is unsatisfactory when a conventional anion-exchange chromatographic method is used. Although slab-gel or tube-gel electrophoresis is able to separate the products, long migration times are required.

This paper describes the use of anion-exchange high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) for the simple preparative and rapid analytical separation of ETP-insulins.

MATERIALS AND METHODS

Materials and Apparatus ETPA and Sephadex G-25 were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Pharmacia LKB (Uppsala, Sweden), respectively. Lyophilized porcine insulin was prepared as previously reported.¹⁶⁾ Other chemicals were of reagent grade. Molecular membrane tubes (Spectra/Por 3, molecular weight cut-off approximately 3500, Spectrum Medical Ind., Los Angeles, U.S.A.) were used for dialysis, which was carried out at 4 °C. Absorbance was measured using a Hitachi 150-20 spectrophotometer equipped with semi-micro quartz cells (1 ml).

HPLC System and Its Operation Preparative anion-exchange HPLC was performed on a TSK gel DEAE-2SW column (300 × 7.8 mm i.d., Tosoh, Tokyo, Japan) at ambient temperature (25–28 °C) using 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 (100-μl loop) and a Tosoh UV-8 model II spectrophotometer operating at 280 nm. The mobile phase consisted of a mixture of 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea and 0.5 M NaCl. A 20-min isocratic elution without NaCl was followed by a 70-min linear gradient elution with 0 to 0.5 M NaCl, at a flow rate of 1.4 ml/min. The column was regenerated by washing with the initial eluant for 20 min.

CZE System and Its Operation The CZE equipment consisted of an Applied Biosystems Model 270A capillary electrophoresis system (Foster City, CA, U.S.A.) equipped

Fig. 1. Structures of ETP-Insulins (Stable Species)

Gly(A1)-ETP-insulin: $R_1 = \text{ETP}$, $R_2 = R_3 = \text{H}$; Gly(A1), Phe(B1)-diETP-insulin: $R_1 = R_2 = \text{ETP}$, $R_3 = \text{H}$; Gly(A1), Lys(B29)-diETP-insulin: $R_1 = R_3 = \text{ETP}$, $R_2 = \text{H}$; Gly(A1), Phe(B1), Lys(B29)-triETP-insulin: $R_1 = R_2 = R_3 = \text{ETP}$.

with a fused-silica capillary tube (72 cm in length and 50 μm i.d.) and a UV detector operating at 200 nm. Electrophoretic runs were made at a constant applied potential of 20 kV (278 V/cm) under an electric current of 23 μA . The capillary tube was maintained at 30 °C. A 1.0-s vacuum injection (approximately 4 nl) of sample was carried out and 25 mM sodium phosphate buffer (pH 7.0) was used as the migration buffer. Under these conditions, ETP-insulins moved towards the negative electrode (the grounded outlet). Each run was followed by a 3-min rinse of the capillary with 0.2 M NaOH then a 6-min rinse with phosphate buffer.

Reactions at Different Molar Ratios of ETPA to Insulin To lyophilized insulin (1.0 mg, 167 nmol) dissolved in 1 ml 50 mM Na phosphate buffer (pH 7.5) was added 10.5–105 μl of 50 mM ETPA (3 to 30-fold molar excess with respect to insulin) in dried acetonitrile at 20 °C with stirring. The mixture was then stirred at 20 °C for 1 h. A 100- μl portion of the resulting mixture was subjected to HPLC. The mixture was diluted 4-fold with water and a portion (approximately 4 nl) of the mixture was used for CZE. Peak areas on the electropherogram were used to obtain the ratios for the production of ETP-insulins.

Preparation of ETP-Insulins Equal volumes (1 ml) of mixtures obtained following the 6-h reaction of 6- and 30-fold molar excesses of ETPA (50 mM in acetonitrile) with 1 ml of 1 mg/ml insulin in 50 mM sodium phosphate buffer (pH 7.5) at 20 °C were combined to obtain a mixture (mixture A) which contained as many species of ETP-insulins as possible. A 100- μl portion of this mixture was subjected to HPLC. The HPLC procedure was repeated and the respective protein fractions were combined and dialyzed 3 times against 1 l of water, then lyophilized to give the dry ETP-insulins. The sites of the ETP groups introduced at the free amino groups of insulin were determined from the amino acid analyses of the ETP-insulins using the 1-fluoro-2,4-dinitrobenzene method.^{16,17)}

Four stable ETP-insulins (all colorless powders, peaks

2, 4, 5 and 8 in Fig. 2) were obtained. ϵ ($\text{M}^{-1} \text{cm}^{-1}$) at 280 nm in 50 mM Na–K phosphate buffer (pH 7.0), and ϵ at 200 nm in 25 mM Na phosphate buffer (pH 7.0) in parentheses: Gly(A1)-ETP-insulin (peak 2, 5.4×10^3 , 3.2×10^4), Gly(A1), Phe(B1)-diETP-insulin (peak 4, 5.5×10^3 , 3.3×10^4), Gly(A1), Lys(B29)-diETP-insulin (peak 5, 5.5×10^3 , 3.3×10^4), and Gly(A1), Phe(B1), Lys(B29)-triETP-insulin (peak 8, 5.4×10^3 , 3.4×10^4).

RESULTS AND DISCUSSION

Separation of ETP-Insulins by HPLC and CZE The preparative separation of the ETP-insulins was achieved by anion-exchange HPLC on a TSKgel DEAE-2SW column following gradient elution with sodium chloride and a 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea. Figure 2 shows an example of a chromatogram obtained with mixture A as described above (preparation of ETP-insulins). Four stable species (represented by peaks 2, 4, 5 and 8) and four labile species of ETP-insulins (derivatives of the stable ETP-insulins described later, represented by peaks 3, 6, 7 and 9) were simultaneously separated (Fig. 2, Table I). In the absence of urea, ETP-insulins could not be separated at sodium chloride concentrations higher than about 0.3 M. In the absence of sodium chloride, ETP-insulins were completely retained by the column. When a pH 7.5 phosphate buffer was used as mobile phase, peaks 3 and 4 and peaks 8 and 9 were overlapped. Since ETP-groups on the insulins could be partially removed even in slightly acidic medium (pH 6.5, 20 °C), an acidic mobile phase could not be used.

The ETP-insulins in mixture A could also be separated rapidly by CZE (Fig. 3). The peaks due to the ETP-insulins in the electropherogram were identified by co-electrophoresis with the ETP-insulins obtained by preparative HPLC.

When the pH of the running buffer was 6.5–9.0, the applied electric current ranged from 19 to 31 μA at 20 kV (Fig. 4). At pH 7.0, an adequate separation of ETP-insulins was achieved within 6.5 min. A single analytical run could

Fig. 2. Chromatogram of the Reaction Mixture of Insulin with ETPA

Peaks: 1, intact insulin; 2, Gly(A1)-ETP-insulin; 3, Gly(A1)-ETP-insulin derivative; 4, Gly(A1), Phe(B1)-diETP-insulin; 5, Gly(A1), Lys(B29)-diETP-insulin; 6, Gly(A1), Phe(B1)-diETP-insulin derivative; 7, Gly(A1), Lys(B29)-diETP-insulin derivative; 8, Gly(A1), Phe(B1), Lys(B29)-triETP-insulin; 9, Gly(A1), Phe(B1), Lys(B29)-triETP-insulin derivative; 10, peak due to ETPA; 11, unknown.

TABLE I. Location of ETP Group(s) Introduced into the Insulin Molecule and the Numbers of Glycyl, Phenylalanyl and Lysyl Residues in the Chromatogram Peaks (Fig. 2) after Dinitrophenylation

Peak number ^{a)} Location of ETP group(s) on A- or B-chain free amino group	The number of amino acid residues					
	Gly		Phe		Lys	
	Theoretical	Found ^{b)}	Theoretical	Found ^{b)}	Theoretical	Found ^{b)}
Porcine insulin ^{c)}	4	4.04	3	2.93	1	1.00
1 None	3	3.01	2	1.97	0	0.03
2 A1	4	3.82	2	2.16	0	0.12
3 A1, # ^{d)}	4	3.91	2	2.21	0	0.08
4 A1, B1	4	3.93	3	2.92	0	0.08
5 A1, B29	4	3.79	2	2.12	1	0.70
6 A1, B1, # ^{d)}	4	3.86	3	2.90	0	0.07
7 A1, B29, # ^{d)}	4	3.88	2	2.14	1	0.88
8 A1, B1, B29	4	3.93	3	2.78	1	0.88
9 A1, B1, B29, # ^{d)}	4	4.28	3	3.00	1	1.02

a) For peak numbering, see Fig. 2. b) Calculation based on six leucyl residues per porcine insulin molecule. c) Not subjected to dinitrophenylation. d) Unknown modification occurred.

Fig. 3. Electropherogram of the Reaction Mixture of Insulin with ETPA

Peaks are numbered as in Fig. 2.

be completed within 15.5 min, including regeneration of the capillary.

Following repeated measurements ($n=5$) of Mixture A, using CZE, the relative standard deviations of the migration times and areas for the respective peaks did not exceed 0.28% and 3.0%, respectively.

Characterization of ETP-Insulins The number of amino acid residues in the ETP-insulins after dinitrophenylation and the location of the ETP group(s) are shown in Table I. The amino acid analyses of the labile components represented by peaks 3, 6, 7 and 9 were almost identical to those for peaks 2, 4, 5 and 8, respectively. When the labile components were allowed to stand in neutral and slightly alkaline solution (pH 7.0–8.0) at 23 °C for 6 h, approximately 50% conversion to the respective stable components was obtained. All these changes were accompanied by the loss of negative charges from the molecules of the labile species, since the stable species were less mobile than their labile counterparts. When the stable component represented by peak 8 (Gly(A1),

Fig. 4. Effect of the Applied Electric Current and pH on the Migration Times of ETP-Insulins in CZE

Curves are numbered as in Fig. 2.

Phe(B29), Lys(B29)-triETP-insulin) was treated with ETPA, as described for the preparation of ETP-insulin using the same reaction conditions at a molar ratio of ETPA to insulin of 6, the component represented by peak 9, a labile species, was produced. This labile species was not produced by treatment in the same solvent in the absence of ETPA. This demonstrates that the stable component represented by peak 8 (the triETP-insulin that has no free amino group) was modified by reaction with ETPA. Thus, the modification occurred on functional groups other than amino groups. The site of the modification (possibly a phenolic hydroxyl group of tyrosyl residues^{3,5,13}) remains unclear.

The components represented by peaks 2, 4, 5 and 8, characterized as Gly(A1)-ETP-, Gly(A1), Phe(B1)-diETP-, Gly(A1), Lys(B29)-diETP- and Gly(A1), Phe(B1), Lys(B29)-triETP-insulins, respectively, were stable for at least 6 h at 23 °C and for 3 months or longer at –20 °C

Fig. 5. Effect of the Molar Ratio of ETPA to Insulin on the Introduction of ETP Group(s) into Insulin Following Reaction for 60 min at pH 7.5 and 20 °C

Curves are numbered as in Fig. 2.

in neutral and slightly alkaline pHs (7.0–8.0).

Assessment of the Number and Site(s) of the ETP Group(s) Introduced into Insulin The molar absorptivities of the stable species of ETP-insulins were used for the corresponding labile species of ETP-insulins, since the ϵ values of the molar absorptivities at 280 or 200 nm for the mono-, di- and tri-ETP insulins were virtually identical, implying that the ETP group made only a small contribution to the ϵ values. The effect of the molar ratio of ETPA to insulin, calculated on the basis of the peak areas and molar absorptivities of the products, on the production of ETP-insulins was evaluated by means of CZE separation (Fig. 5). Gly(A1), Phe(B1)-diETP-insulin was the main product even at low molar ratios (3–9), and was formed even at high molar ratios where the production

of the labile Gly(A1), Phe(B1), Lys(B29)-triETP-insulin derivative was predominant.

In conclusion, we have demonstrated that a combination of the HPLC and the CZE can be helpful tools for the preparative and analytical separations of ETP-insulins. CZE should be suitable, with minor changes in the electrophoretic conditions, for the rapid analytical separation of various products, resulting from the reaction of insulin with various dicarboxylic anhydrides.

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