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ACHROMOBACTER PROTEASE I-CATALYZED CONVERSION OF PORCINE INSULIN INTO HUMAN INSULIN

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SUMMARY

We have established a procedure for converting porcine insulin into human insulin using a serine protease from Achromobacter lyticus M497-1 which shows unique specificity against lysine residues on the carboxyl side of the splitting point. Desalanine-(B30)-insulin (DAI) was prepared by digestion of porcine insulin with Achromobacter protease. The coupling between DAI and Thr-OBu^t was performed by the same enzyme at pH 6.5 with a large excess of the amine component (Thr-OBu^t) in the presence of high concentrations of organic co-solvents. The highest yield was 85% by 20 h reaction at 37°C. The synthesized [Thr-OBu^t-B30]-insulin was isolated, then deprotected with trifluoroacetic acid in the presence of anisole to obtain semisynthetic human insulin.

Morihara and Oka (1-4) have studied the peptide bond synthesis catalyzed by various proteases. This method has been used (5,6) for the semisynthesis of human insulin, in which trypsin is used as a catalyst for the coupling of des-octapeptide-(B23-B30)-insulin (DOI) with a synthetic octapeptide corresponding to positions B23-B30 of human insulin or of desalanine-(B30)-insulin (DAI) with Thr-OBu^t. DOI or DAI was prepared by digestion of porcine insulin with trypsin or carboxypeptidase A, respectively. The enzymatic method has many advantages, in particular, better yields and simple operation, over the chemical methods originally used by Ruttenberg (7) and Obermeier and Geiger (8).

Masaki et al. (9) have recently shown that Achromobacter lyticus M497-1 can produce a serine protease which shows a

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unique specificity only against lysine residues at the carboxyl side of the splitting point in peptide or protein substrates. We therefore thought that Achromobacter protease might be more useful than trypsin in the replacement of alanine-B30 with threonine in porcine insulin, i.e., for semisynthesis of human insulin. The present study was undertaken to clarify this.

MATERIALS AND METHODS

Porcine insulin (lot 1FJ91, 26.2 U/mg) was kindly supplied by Eli Lilly & Co. A homogeneous preparation of protease I of Achromobacter lyticus M497-1 was a kind gift of Drs. T. Masaki and M. Soejima. Thr-OBu^t was prepared according to the usual method. DAI was obtained by digestion of porcine insulin with Achromobacter protease I (E/S = 1/100, in weight) for 24 h at pH 8.3 and 37°C. The product was isolated by column chromatography on Sephadex G-50 using 0.5 M acetic acid the eluant. Amino acid analysis (theoretical values in parentheses) gave: Lys 1.00 (1), His 2.01 (2), Arg 0.80 (1), Asp 3.21 (3), Thr 2.09 (2), Ser 3.04 (3), Glu 6.74 (7), Pro 1.15 (1), Gly 4.07 (4), Ala 1.07 (1), Val 2.77 (4), Ile 1.15 (2), Leu 6.05 (6), Tyr 4.08 (4), and Phe 3.00 (3).

The coupling of DAI and Thr-OBu^t was determined quantitatively using reverse-phase liquid chromatography (LC) as described previously (6; refer to Fig. 2, b and c). Protease activity was determined using casein as substrate (10). The activity was expressed by the absorbancy at 670 nm. Ninhydrin value was examined at 570 nm. LC was performed with a Waters Model M-6000A (1.0 ml/min), in which the absorbance at 220 nm was monitored with a JASCO UVIDEC-100. A Hitachi KLA Model 5 amino acid analyzer was used for amino acid analysis. Hydrolysis was done with 6 N HCl for 20 h at 110°C. SDS-polyacrylamide gel electrophoresis was performed at pH 8.8 in a 10% gel by the technique of Weber et al. (11). Bioassay of hypoglycemic activity was done using male DS/Shi mice weighing 25 to 30 g. Insulin dissolved in saline (0.1 ml) was administrated through a tail vein. After 45 min, the blood glucose level was determined using a "Blood Sugar-GOD-Perid-Test" kit (Boethringer Mannheim).

RESULTS AND DISCUSSION

Table I shows the coupling of DAI with Thr-OBu^t catalyzed by Achromobacter protease I, which indicates that the optimum pH is at 6.5-7.0 and a large excess of the amine component (Thr-OBu^t) is essential in the presence of high con-

Table I
Coupling between DAI and Thr-OBu^t catalyzed by Achromobacter protease[a]

[DAI] (mM)	[Thr-OBu^t] (M)	[E] (μM)	pH	Yield (%)
5	0.05	10	6.5	27
5	0.1	10	6.5	50
5	0.3	10	6.5	73
5	0.5	10	6.5	80
10	0.3	10	6.5	60
10	0.5	10	6.5	85
5	0.5	10	5.3	60
5	0.5	10	6.0	73
5	0.5	10	7.0	80
5	0.5	10	8.0	42
5	0.5	10	8.5	28
5	0.5	1	6.5	80
5	0.5	0.3	6.5	67
5	0.5	0.1	6.5	70

[a] The reaction mixture contained McIlvaine buffer (30%, v/v; pH 5.3-7.0) or 0.3 M Tris-maleate buffer (pH 8.0 and 8.5), 60% solvent (a mixture of ethanol and dimethylformamide, 1/1 by volume), and the other components being described in the table, which was kept for 20 h at 37°C. The pH was adjusted as seen in the table.

centrations of organic co-solvents. The highest yield was 85% by 20 h reaction at 37°C.

A preparative-scale experiment was carried out as follows. Zinc-free porcine insuline (100 mg) (12) was dissolved in 20 ml of 0.1 M NH_4HCO_3 (pH 8.3), to which 0.32 mg of Achromobacter protease I was added. After 90 h incubation at 37°C, the reaction mixture was lyophilized. More than 95% of the porcine insulin was converted into DAI. The lyophilized material (corresponding to 10 mM DAI and 7 μM Achromobacter protease) and Thr-OBu^t (195 mg, 0.5 M) were dissolved in a solution containing 0.7 ml of 0.5 M Tris buffer (pH 6.5), 0.5 ml of ethanol, and 0.5 ml of dimethylformamide, and the reaction mixture was kept at 37°C for 20 h. LC showed that 83% of the DAI had been converted into the product.

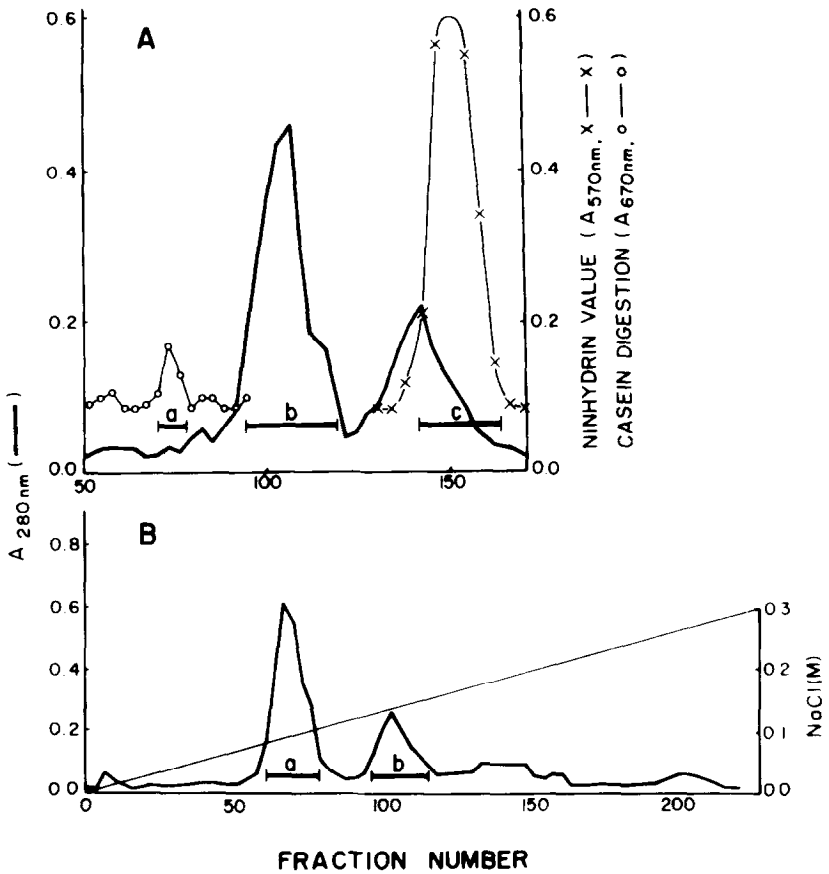

Fig. 1. A, Chromatography on Sephadex G50 (4.2 x 130 cm). Eluted with 0.5 M acetic acid at 4°C. Fraction volume, 11 ml. B, Chromatography on DEAE-Sephadex A25 (2.2 x 22 cm). Equilibrated with 0.01 M Tris buffer (pH 7.5) and 7 M urea. A linear Na^+ gradient at 4°C. Fraction volume, 7.1 ml.

The reaction mixture was applied to a column of Sephadex G-50 (super fine) and eluted with 0.5 M acetic acid. Three fractions corresponding to Achromobacter protease (a), insulin or its derivatives (b), and Thr-OBu^t (c) were successively eluted (Fig. 1A). The fractionated Achromobacter protease and Thr-OBu^t could be reused. The fraction corresponding to insulin or its derivatives was lyophilized, then applied to a column of DEAE-Sephadex A-25 which had been equilibrated with 0.01 M Tris buffer (pH 7.4) and 7 M urea. A linear Na^+ gradient (to 0.3 M NaCl) was performed according to the

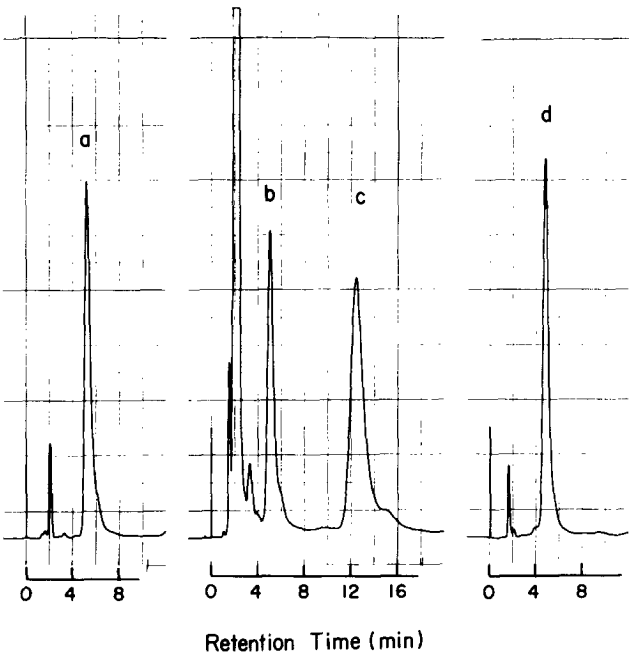

Fig. 2. Liquid chromatogram of porcine insulin (a), porcine DAI (b), [Thr-OBu^t-B30]-porcine insulin (c), and semisynthetic human insulin (d): column, Nucleosil 5C$_{18}$, 0.4 x 20 cm; eluant, 31.5% CH_3CN in 5 mM phosphate buffer (pH 3.0) containing 5 mM n-$BuSO_3Na$ and 50 mM Na_2SO_4; detection at 220 nm.

method of Bromer and Chance (13), as shown in Fig. 1B. The first peak (a, 56 mg) corresponded to [Thr-OBu^t-B30]-insulin and the second one (b, 25 mg) to DAI and/or intact insulin (Figs. 2, 3). The latter materials could be reused.

[Thr-OBu^t-B30]-insulin was deprotected with trifluoroacetic acid in the presence of anisole. The yield was 52 mg (52%). This semisynthetic human insulin was homogeneous according to LC (Fig. 2) and polyacrylamide gel electrophoresis (Fig. 3). Amino acid analysis (theoretical values in parentheses) gave: Lys 1.00 (1), His 1.90 (2), Arg 0.93 (1), Asp 3.21 (3), Thr 3.05 (3), Ser 2.99 (3), Glu 7.23 (7), Pro 1.25 (1), Gly 4.33 (4), Ala 1.25 (1), Val 3.91 (4), Ile 1.58 (2), Leu 6.51 (6), Tyr 3.66 (4), and Phe 3.15 (3). In the bioassay for hypoglycemic activity, no significant

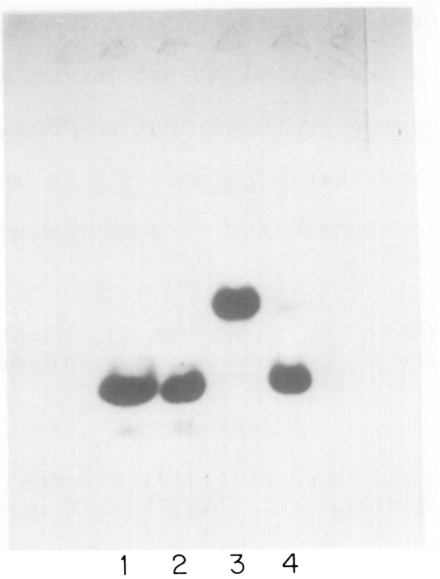

Fig. 3. Polyacrylamide gel electrophoresis of insulins: 1, porcine insulin; 2, porcine DAI; 3, [Thr-OBu^t-B30]-porcine insulin; 4, semisynthetic human insulin.

difference was observed between the semisynthetic human insulin and porcine insulin (Table II).

A similar preparative-scale experiment was carried out using bovine insulin under the same conditions. The product was isolated by the above method, and pure semisynthetic [Thr-OBu^t-B30]-bovine insulin was obtained in 58% yield.

The present study indicates that Achromobacter protease I can be used not only for digestion of porcine insulin to obtain DAI but also for coupling of DAI with Thr-OBu^t to

Table II
Insulin activity of semisynthetic human insulin

Material	Dose (μg/10 g body wt.)	No. of mice	Body wt. (g)	Blood glucose level (mg%)
Saline	--	6	28.2±0.4	129.7±2.9
Porcine insulin	0.25	6	28.2±0.4	83.7±7.5
	1.0	6	28.2±0.4	43.5±6.5
Semisynthetic human insulin	0.25	6	28.3±0.3	73.3±3.9
	1.0	6	28.2±0.4	34.5±4.2

generate human insulin. Cleavage of the Arg(B22)-Gly(B23) bond in DAI or the product might not be expected in the coupling with Achromobacter protease, but might be a problem with trypsin. We can say that Achromobacter protease I is a more useful enzyme than trypsin for preparing semisynthetic human insulin.

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