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(16) National Science Foundation Graduate Fellow, 1972–1975.

Douglas Balogh, William J. Begley,[15] David Bremner
Matthew J. Wyvratt,[16] Leo A. Paquette*
Evans Chemical Laboratories, The Ohio State University
Columbus, Ohio 43210
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Enzyme-Assisted Semisynthesis of Human Insulin

Sir:

We wish to describe herein the use of trypsin as a catalyst for peptide-bond formation with special reference to the semisynthesis of human insulin. Conversion of porcine insulin into human insulin, which differs from porcine hormone by a single amino acid residue in position B30, has been attempted by Ruttenberg[1] and by Overmeier and Geiger.[2] In these attempts a derivative of deoctapeptide-(B23–B30)-insulin (DOI), derived from porcine hormone by tryptic digestion,[3] was coupled with DCC to a synthetic octapeptide corresponding to the sequence B23–B30 of human insulin. These chemically conducted couplings, however, seem to be of little use for simple and inexpensive production of pure insulin, because of a number of difficulties involved and low yields.[2,4] Now, we have found that, under certain conditions, trypsin can catalyze the coupling between DOI and human octapeptide to lead to the production of human insulin in a moderate yield.

Trypsin catalysis of peptide-bond formation has been little investigated except for such a special case as in a modified soybean trypsin inhibitor.[5] We thus studied the following coupling reaction in homogeneous systems:[6]

$$\text{Boc-X-OH} + \text{H-Val-OBu}^t \overset{\text{E}}{\rightleftharpoons} \text{Boc-X-Val-OBu}^t + \text{H}_2\text{O}$$

X = Lys or Arg; E = trypsin

The coupling was performed with a large excess of the amine component (H-Val-OBu^t) so as to make a substantial shift of equilibrium to the right. The optimum pH for these trypsin-catalyzed syntheses was found to be near 6.5, as seen in Figure 1. The equilibrium of the systems also depends on the concentration of water. Table I clearly shows that within a certain limit the addition of an organic solvent, such as DMF or Me_2SO, to the reaction medium remarkably increases peptide formation.[8] The effect is much greater than that expected from the decrease in water concentration.[9] The addition of organic solvent was also effective in increasing the solubility of both carboxyl (C) and amine (N) components, another important factor which determines the extent of peptide formation. These results prompted us to develop the procedure for semisynthesis of human insulin.

Table I. Effect of Concentration of Organic Solvent on the Yield of Boc-X-Val-OBu^t in Trypsin-Catalyzed Coupling of Boc-X-OH with H-Val-OBu^t (X = Lys or Arg)[a]

X	solvent added	concn of solvent, %	yield of Boc-X-Val-OBu^t, %
Lys	none	0	15
Lys	methanol	10	21
		20	30
		30	39
		40	49
		50	42
Lys	dimethylformamide	10	23
		20	34
		30	49
		40	65
		50	81
		60	79
		70	0
Lys	dimethyl sulfoxide	20	33
		40	68
Lys	tetrahydrofuran	20	30
		40	0
Arg	dimethylformamide	10	20
		20	42
		30	47
		50	75

[a] [Boc-X-OH] = 0.034 M, [H-Val-OBu^t] = 0.34 M, [E] = 1.89 mg/mL, pH 6.5, 25 °C, 20 h.

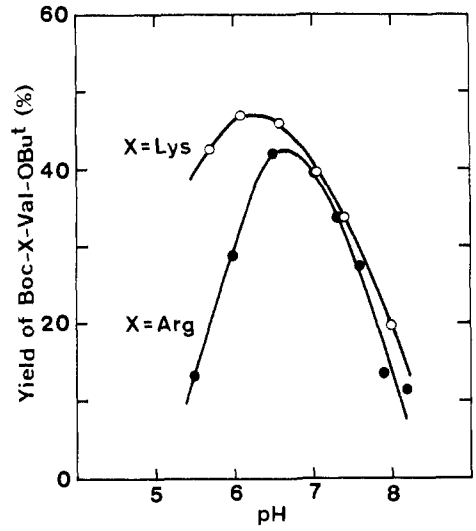

Figure 1. Effect of pH on the yield of Boc-X-Val-OBu^t in trypsin-catalyzed coupling of Boc-X-OH with H-Val-OBu^t- (X = Lys or Arg).[7] [Boc-X-OH] = 0.046 M (X = Lys) or 0.054 M (X = Arg), [H-Val-OBu^t] = 0.49 M (X = Lys) or 0.50 M (X = Arg), [E] = 1.96 mg/mL, [DMF] = 20%, 25 °C, 20 h. The progress curves of the reaction (not shown) revealed that it came to equilibrium within 20 h. The term yield used here represents the extent of peptide formation in such an equilibrium state.

Zinc-free porcine insulin[10] was digested with TPCK-treated trypsin[11] at pH 9–9.5[12] and the resulting DOI was acylated with Boc-N_3, as described for insulin,[13] to give $N^{\alpha A1}$,$N^{\alpha B1}$-$(Boc)_2$-DOI quantitatively. Human octapeptide was synthesized in the form of H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(R_1)-Thr(R_2)-OR_2 (I, R_1 = Boc, R_2 = H; II, R_1 = Boc, R_2 = Bu^t) by the conventional solution method, in which much care was taken to minimize the danger of racemization.[14] The C and N components thus obtained were coupled in a typical experiment as follows.

To a solution of $(Boc)_2$-DOI (100 mg) and octapeptide (I, 200 mg) in a mixture of DMF and 0.25 M Tris buffer (1:1 by volume, 1.2 mL) was added TPCK-trypsin[11] and the reaction mixture (pH 6.5) was incubated at 37 °C for 20 h. The enzyme (10 mg) was added in three portions at time: 0, 2, and 6 h. The concentrations of $(Boc)_2$-DOI and octapeptide were 16.3 and 163 mM, respectively (N/C ratio = 10), and that of enzyme 0.35 mM. At the end of the reaction, LC showed that 58% of the DOI had been converted into insulin. The entire material was applied to a Sephadex LH-20 column and eluted with DMF–0.5 M acetic acid (1:1). The $(Boc)_3$-insulin formed and the $(Boc)_2$-DOI which remained uncoupled were emerged as a single peak. The octapeptide which had been present in a large excess came out as the second peak and was isolated in pure state after rechromatography (160 mg). The material in the first peak was treated for deprotection with TFA at 0 °C for 60 min in the presence of anisole. The product was then chromatographed on a column of Sephadex G-50 (superfine) with 0.5 M acetic acid as eluant. From one of two major peaks

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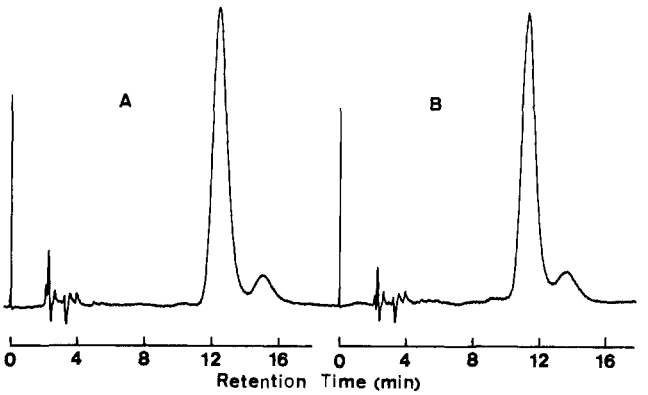

Figure 2. LC of insulins: A, porcine insulin; B, semisynthetic human insulin; column, Nucleosil $5C_{18}$, 0.4 × 20 cm; eluant, 30% CH_3CN in 5 mM tartrate buffer (pH 3.0) containing 5 mM *n*-$BuSO_3Na$ and 50 mM Na_2SO_4; detection, at 220 nm.

the desired human insulin was obtained by lyophilization (51 mg), and from another peak pure DOI was recovered in the unprotected form (37 mg). By rechromatography of the overlapping portion of the two peaks, an additional quantity of insulin was isolated, giving a total yield of 56 mg (49%). Amino acid analysis (theoretical values in parentheses):[15] Lys, 0.97 (1); His, 2.10 (2); Arg, 1.00 (1); $CySO_3H$, 5.75 (6); Asp, 3.03 (3); Thr, 2.92 (3); Ser, 2.87 (3); Glu, 7.10 (7); Pro, 1.32 (1); Gly, 4.08 (4); Ala, 1.07 (1); Val, 3.91 (4); Ile, 1.56 (2); Leu, 6.00 (6); Tyr, 3.02 (4); Phe, 2.88 (3).

Polyacrylamide gel electrophoresis and LC (Figure 2) showed that the semisynthetic insulin obtained above was as pure as crystalline porcine hormone used as starting material.[16] The sufficient purity was further confirmed by crystallization. In the assay for hypoglycemic activity in normal mice, no significant difference was found between the semisynthetic material and bovine insulin.[17] The identity of this material with natural human hormone[18] was evidenced by careful LC. Thus, a simple procedure for conversion of porcine insulin into human insulin by the two-way use of trypsin has been established. The enzymatic method developed for the coupling of DOI with octapeptide has many advantages over the existing chemical methods,[1,2] especially in (1) the reaction, which is highly specific and free of racemization; (2) the starting materials, which can be recovered for reuse; and (3) better yields and simple operation.

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(5) R. W. Sealock and M. Laskowski, Jr., *Biochemistry*, **8**, 3703–3710 (1969).
(6) Abbreviations: Boc = *tert*-butoxycarbonyl, Bu^t = *tert*-butyl, DCC = dicyclohexylcarbodiimide, DOI = deoctapeptide-(B23–B30)-insulin, LC = high pressure liquid chromatography, TFA = trifluoroacetic acid, TPCK = L-1-tosylamido-2-phenylethyl chloromethyl ketone, Tris = tris(hydroxymethyl)aminomethane.
(7) Aliquots from the reaction mixture were acidified, lyophilized, and treated for deprotection with TFA/anisole. After removal of the TFA the residue was analyzed on an amino acid analyzer for Lys and Lys-Val (or Arg and Arg-Val), from which the extent of peptide bond formation was calculated according to $[b/(a+b)] \times 100$ (%), where *a* stands for μmol of Lys (or Arg) and *b* for μmol of Lys-Val (or Arg-Val).
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(14) Octapeptide I: $[\alpha]^{25}_D$ −22.2 ± 0.8° (*c* 0.5, acetic acid). II: $[\alpha]^{25}_D$ −31.1 ± 0.5° (*c* 0.8, acetic acid).
(15) A sample for analysis was hydrolyzed with 6 M HCl at 110 °C for 24 h after periodate oxidation.
(16) For further purification a sample of the semisynthetic insulin was chromatographed on a QAE-Sephadex A-25 column according to Schlichtkrull et al: J. Schlichtkrull, J. Brange, A. H. Christiansen, O. Hallund, L. G. Heding, and K. H. Jørgensen, *Diabetes*, **21** (Suppl. 2), 649–656 (1972). The material isolated from a major peak was found to be homogeneous in electrophoresis and in LC.
(17) Lot 800147, Calbiochem, 27.17 units/mg.
(18) An authentic sample of human insulin (Lot 615-D63-29-C) was kindly supplied by Dr. R. E. Chance, Lilly Research Laboratories.

Ken Inouye,* Kunio Watanabe, Kazuyuki Morihara
Yoshihiro Tochino, Takashi Kanaya
Shionogi Research Laboratory, Shionogi & Co., Ltd.
Fukushima-ku, Osaka 553, Japan

Junji Emura, Shumpei Sakakibara
Peptide Institute, Protein Research Foundation
476 Ina, Minoh, Osaka 562, Japan

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Phosphoranes as Intermediates in the Acid Hydrolysis of Acyclic Phosphonate Esters: Evidence from Oxygen Exchange

Sir:

Convincing evidence places phosphoranes in the pathway for the hydrolysis of cyclic esters of phosphoric, phosphonic, and phosphinic acids.[1,2] However, phosphoranes with small rings form more readily than do their acyclic analogues,[3] so that some doubt had persisted as to whether the hydrolysis of acyclic esters of acids of phosphorus might not proceed by way of trigonal-bipyramidal transition states rather than by way of phosphoranes as intermediates. We now report that, when diphenyl methylphosphonate is heated with acid in 60:40 dimethoxyethane (DME)-water, exchange of oxygen with solvent takes place at ~8% of the rate of hydrolysis. This finding provides strong evidence for a phosphorane intermediate.

Diphenyl methylphosphonate enriched in ^{18}O was prepared by hydrolyzing methyltriphenoxyphosphonium triflate.[4] The labeled ester melted at 35.5–37 °C and showed an identical ^1H NMR spectrum with that of an unenriched sample.[5,6] Exchange during hydrolysis was measured mass spectrometrically; unhydrolyzed ester was recovered by ether extraction. The large increase in the peak at *m/e* 250 (the molecular ion for ester that contains ^{18}O) that occurred during hydrolysis of unlabeled ester with 71% $H_2{}^{18}O$ is shown in Figure 1. The decrease in the peak at *m/e* 250 that occurred during hydrolysis of labeled ester with ordinary water is shown in Figure 2. When the reaction is begun by hydrolyzing unlabeled ester in $H_2{}^{18}O$,

$$k_{ex}/k_h = \ln [1 - (E^*)/(E)]/\ln (E)/(E_0) \qquad (1)$$

where k_{ex} is the rate constant for exchange, k_h is that for hydrolysis, (E) is the total concentration of ester, (E_0) is its initial

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