Rapid preparation of human insulin and insulin analogues in high yield by enzyme-assisted semi-synthesis

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Reaction conditions are described that permit the enzyme-assisted semi-synthetic replacement of residue B30 of pig insulin (or of an analogue) to proceed in very high yield in 2 h or less. Immobilized trypsin may be used as catalyst, and excess amino acid ester may be recycled after a simple extraction. Alanine-B30 may be replaced by a variety of nucleophiles, including threonine *O*-*t*-butyl ether *t*-butyl ester, in which case the yield of crude product is about 99%. De-protection of the B30 threonyl ester analogue of insulin thus formed then affords human insulin in an overall yield of about 92%, based on pig starting material. The product has full biological potency, as determined by depression of blood glucose concentration in rats, and showed the expected behaviour on radioimmunoassay.

The only difference between the amino acid sequence of human insulin and that of pig insulin is that the former has threonine at position B30 whereas the latter has alanine. The fact that the difference is so small, and that it occurs at so exposed a site, has led to a great deal of work on the semi-synthetic conversion of pig insulin into human insulin. For instance, residue Ala-B30 of pig insulin can be removed by trypsin, by cleavage either at Arg-B22 or at Lys-B29. The sequence of human insulin can be formed from the resulting truncated insulins, in the first case by coupling back the appropriate octapeptide of the human insulin sequence, and in the second by coupling back threonine. In both cases coupling must be limited to the α -carboxy group of the B-chain of the truncated derivative, yet attempts to achieve this by reversible chemical protection of all its other carboxy groups have been fraught with difficulty (Obermeier, 1978). If protection of carboxy groups is avoided, however, chemical coupling of an octapeptide fragment to desoctapeptide-B23–B30-insulin yields a mixture, from which the desired product may be isolated with a yield of 10–15% (Obermeier & Geiger, 1976). Another means of achieving selective coupling is by use of proteinases under conditions that lead them to catalyse the synthesis, rather than the hydrolysis, of peptide bonds. It is reasonable to expect that the enzymic coupling should be simpler to carry out and would do less damage to the protein than a route involving many chemical operations. Table 1 lists

the principal published examples of the use of endoproteinases in this regard. Exopeptidases have also been used, but so far have given more complex mixtures of products (see, e.g., Breddam *et al.*, 1982). Although trypsin can cleave both after residue B22 and after residue B29, Table 1 shows that it is possible to find conditions under which its action is confined to the peptide bond between residues B29 and B30, without it being necessary to block the side chain of residue B22. This specificity is shown both in the coupling of threonine esters to des-Ala-B30-insulin that had been previously isolated, or in the catalysis of direct exchange between a threonine ester and the Ala-B30 of intact insulin.

It will be seen from Table 1 that, in order to obtain high yields, the hormone has had to be exposed to the reaction conditions for excessively long periods of time. The present paper describes a method for a significantly more rapid conversion of pig insulin into human insulin with high efficiency. In addition, we briefly discuss the use of our method in the production of analogues of insulin.

Materials and methods

Unless otherwise specified, reagents were of analytical grade and were used without further purification.

Conversion of pig insulin into human insulin: method A

Zinc-free pig insulin was prepared by extensive dialysis of a solution of pig insulin (Mono-

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Table 1. *Methods for the enzyme-assisted semi-synthesis of human insulin*

Abbreviations: Boc₂DOI, [*N*^α-Bu¹OCOGly-Al, *N*^α-Bu¹OCOPhe-BI]-des-octapeptide-B23-B30-insulin; DAI, des-Ala-B30-insulin; DAI*, [1,2-dihydroxycyclohex-1,2-ylene-Arg-B22]-des-Ala-B30-insulin; Octa(Boc), Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Bu¹OCO)-Thr; T, trypsin; A, *Achromobacter* proteinase I; I, pig insulin; DMF, *NN*-dimethyl-formamide; DMA, *NN*-dimethylacetamide; THF, tetrahydrofuran; BD, butane-1,4-diol; DMSO, dimethyl sulphoxide.

Starting material	Amino component	Enzyme	Solvent system	Temp. (°C)	Coupling time (h)	Coupling yield (%)	Yield after de-protection (%)	Reference
Boc ₂ DOI	Octa(Boc)	T	DMF/H ₂ O	37	20	58	49	Inouye <i>et al.</i> (1979)
DAI*	ThrOBu ^t	T	DMF/EtOH/H ₂ O	37	20	71	50	Morihara <i>et al.</i> (1979a)
DAI	ThrOBu ^t	T	DMF/EtOH/H ₂ O	37	20	73	41	Morihara <i>et al.</i> (1979b)
DAI	ThrOMe	T	DMF/H ₂ O	38	20	67	40	Gattner <i>et al.</i> (1980)
DAI	ThrOBu ^t	A	DMF/EtOH/H ₂ O	37	20	83	52	Morihara <i>et al.</i> (1980)
I	ThrOBu ^t	T	DMF/H ₂ O		20	50–60		Jonczyk & Gattner (1981)
					4	55		
					24	55		
I	ThrOMe	T	DMA/H ₂ O	12	48	97		Markusen (1981)
I	ThrOBu ^t	T	DMA/H ₂ O	37	24	80		Markusen (1981)
I	ThrOMe	T	THF/H ₂ O	12	4	75		Markusen (1981)
I	Thr(Bu ¹)OBu ^t	T	BD/DMSO/H ₂ O	37	2	99*	92	Present paper, method A

* This value represents actual recovery of material (see Fig. 1 for purity). In other cases the value given represents a conversion efficiency as determined by e.g. high-pressure liquid chromatography, and the corresponding yield of product was lower where recorded.

component; Novo Industri, Copenhagen, Denmark; 10mg/ml in deionized 8M-urea) against acetic acid (1%, v/v) at 4°C followed by freeze-drying. A 20mg portion of the product was suspended in 270μl of a buffered solvent system prepared by first dissolving Tris hydrochloride (reagent grade; Sigma Chemical Co., St. Louis, MO, U.S.A.) in dimethyl sulphoxide (puriss.; Fluka, Buchs, Switzerland) to a concentration of 1.5M, and then adding 2vol. of butane-1,4-diol (purum; Fluka) to 1 vol. of the Tris hydrochloride/dimethyl sulphoxide solution, and finally adding the amino component of the coupling, L-threonine *O*-*t*-butyl ether *t*-butyl ester [Thr(Bu¹)OBu^t; free base, liquid; Bachem, Bubendorf, Switzerland]; 62.3mg of ester was added to 660mg of Tris hydrochloride/dimethyl sulphoxide/butane-1,4-diol. To this insulin suspension was added 36μl of a solution prepared by dissolving 3mg of bovine trypsin [L-1-chloro-4-phenyl-3-tosyl-aminobutan-2-one ('TPCK')-treated; Worthington Biochemical Corp., Freehold, NJ, U.S.A.] in 50μl of distilled water. The insulin dissolved as soon as the trypsin solution was mixed in. A 0.5μl spot of the coupling solution placed on dry external indicator paper (Duotest 3.5–6.8 range; Macherey und Nagel, Düren, West Germany) gave an immediate pH indication of 5.9–6.2, and within 15–30s the same spot gave an indicated pH of 6.5–6.8. The reaction mixture was covered and incubated at 37°C for 2h, at which point the reaction was stopped by chilling the vessel to 0–4°C and adding 300μl of 100% acetic acid, with mixing. The product,

[Thr(Bu¹)OBu^t-B30]-insulin, was isolated by dilution of the reaction mixture with 300μl of acetic acid (1%, v/v), followed by gel filtration through a column (80cm × 2.6cm diam.) of Sephadex G-50 (fine grade; Pharmacia, Uppsala, Sweden) eluted with 1% acetic acid. Adsorption of product on the column was minimized by previous passage of batches of insulin (2–10mg) until quantitative recovery was obtained; this procedure need only be performed once for each batch of Sephadex. The appropriate fractions were pooled and freeze-dried; the yield was 20.1mg (99%, based on pig insulin starting material). De-protection was accomplished by dissolving 19.9mg of the insulin ester in 2ml of redistilled trifluoroacetic acid containing 1mg of L-tryptophan and maintaining the solution at 22°C for 45min. After concentration of the solution to about 0.5ml on the rotary evaporator, the product was precipitated with 15ml of sodium-dried diethyl ether and isolated by centrifugation. The pellet was washed by resuspension in a further 15ml of diethyl ether, re-centrifuged, dried in a vacuum desiccator and then subjected to gel filtration as before. The freeze-dried product weighed 18.5mg. This represents a yield of 93% for the de-protection step, and 92% for the process as a whole (coupling and de-protection).

Samples of product were hydrolysed (6M-HCl at 180°C for 24h *in vacuo*) for amino acid analysis on an LKB 4000 analyser.

Immunoreactive insulin was determined by a standard radioimmunoassay technique with use of

the charcoal separation method (Herbert *et al.*, 1965). Pig insulin (Novo) was used as the standard in the assay with guinea-pig anti-(pig insulin) serum (generously given by Dr. H. H. Schone, Farbwerke Hoechst, Frankfurt, West Germany).

To assay the product *in vivo*, the stock solution of semi-synthetic human insulin was diluted on the basis of its immunoreactive insulin concentration to give a solution containing 8 µg/ml. Rats were then injected with 0.1 ml of this solution per 100 g body wt. (i.e. a final dose of 8 µg/kg according to the immunoreactivity), and the cumulative depression of plasma glucose concentration was read off a standard curve (Halban *et al.*, 1981).

Conversion of pig insulin into human insulin: method B

A 40.6 mg portion of zinc-free insulin (prepared by dialysis, as in method A, of sodium insulin from Boots Co., Nottingham, U.K.) was taken up in 600 µl of a mixture of butane-1,4-diol saturated (at 22°C) with Tris hydrochloride, and Thr(Bu^t)OBU^t (220:23, v/v). To this was added 40 µl of a solution of trypsin in water (7.51 mg in 60 µl), with mixing. After incubation for 2 h at 37°C, the reaction was quenched and the product was obtained by gel filtration as described for method A. The yield was 38 mg (91%). De-protection of 21.46 mg of insulin ester with trifluoroacetic acid at 22°C for 1 h (no tryptophan as scavenger) afforded 20.4 mg of semi-synthetic human insulin. This represents a yield of 97% for the de-protection, and an overall yield (coupling and de-protection) of 88%.

Conversion of pig insulin into human insulin: method C

A small column (30 mm × 2 mm diam.) was packed with trypsin attached to cross-linked beaded agarose (Sigma Chemical Co.; lot 48C-76301) and rinsed through with 0.5 ml of acetic acid (1%, v/v) followed by a mixture of saturated (at 22°C) Tris hydrochloride in butane-1,4-diol, and water 1000:67 (v/v). This was followed by a mixture of saturated Tris hydrochloride in butane-1,4-diol/water/Thr(Bu^t)OBU^t (223:15:23, by vol.), which was continued until the pH of the eluent rose from an indicated value of about 4 to 5.3. At this point 2 mg of zinc-free pig insulin dissolved in 30 µl of the threonine ester solution was applied to the column and was rinsed in with two 30 µl volumes of the buffered threonine ester solution in butane-1,4-diol/water. Pressure is required to induce the viscous solution to flow through the column at an appreciable rate. After 90 min at 22°C, the product was flushed from the column by applying the mixture of Tris hydrochloride/butane-1,4-diol, and water 1000:67 (v/v).

Conversion of pig insulin into human insulin: method D

To 2 mg of des-Ala-B30-insulin (prepared essentially as described by Schmitt & Gattner, 1978) was added 30 µl of buffered threonine ester solution (see method A) followed by 4 µl of aqueous trypsin solution (125 mg/ml). After mixing and incubation at 37°C for 2 min, cellulose acetate electrophoresis demonstrated an almost quantitative coupling yield.

Conversion of des-Ala-B30-insulin into [Lys(Bu^tOCO)OBU^t-B30]insulin

A 72.4 mg portion of N^ε-Bu^tOCO-L-lysine t-butyl ester acetate (Bachem) was dissolved in 400 µl of a mixture of 1.5 M-Tris hydrochloride in dimethyl sulphoxide, and butane-1,4-diol (1:2, v/v). A 20 mg portion of des-Ala-B30-insulin was suspended in 300 µl of this solution, and to it was added 40 µl of water containing 2.4 mg of trypsin. The apparent pH was 5.6–5.9. Incubation for 2 h at 37°C followed by quenching and gel filtration as in method A afforded 19.5 mg of [Lys(Bu^tOCO)OBU^t-B30]insulin.

Results and discussion

Possible variations in experimental conditions

Solvent. The satisfactory range for the final percentage by volume of butane-1,4-diol was between about 80% and 96% (no correction made for volume changes on making up the mixtures). If glycerol was substituted for the diol, coupling yields fell to about half their original value. On the other hand, up to one-third of the diol could be replaced by dimethyl sulphoxide with little effect on coupling efficiency: the resulting mixture is a better solvent for some possible substrates. It seems to be important to add the water last and (except when using immobilized enzyme) to add the trypsin only at this stage.

Concentration. The ratio between trypsin and insulin was usually between 1:8 and 1:10 (w/w), but the reaction still proceeds rapidly at a ratio of 1:50. A ratio of 1:4 or 1:5 gave faster coupling. We have successfully used insulin concentrations in the range 4–12 mM. The upper limit is close to that of the solubility of the insulin. The molar excess of amino component was normally about 40-fold. At a concentration of des-Ala-B30-insulin of 3.5 mM and a concentration of AlaOBU^t of 30, 60 and 120 mM, the coupling yields, as judged by cellulose acetate electrophoresis, were approx. 50, 75 and 90% respectively. These lower coupling efficiencies nonetheless correspond to a greater mass of product (for a fixed amount of AlaOBU^t), and, provided that the product can be easily separated from starting material, such conditions represent a method of maximizing the mass of product obtainable with a scarce amino component. Tris is not necessary at

all; the coupling of Thr(Bu^t)OBu^t with insulin proceeds normally if the pH is adjusted with acetic acid instead of Tris hydrochloride.

Temperature. The range between 22°C and 42°C was found best. The reaction took approx. 20h to go to completion at 4°C and did not take place at all at 60°C.

pH. It was not convenient to use a glass electrode because of the small volumes involved. The external indicator that we employed did not allow us to put a reliable numerical value to the pH, but it was adequate for control of reaction conditions. The optimum indicated range is pH 5.3–5.6. At an indicated pH of 4.7, the coupling reaction is noticeably slower, and an indicated pH of more than 6 gave products having electrophoretic mobilities consistent with cleavage and subsequent coupling at Arg-B22.

Recycling of amino acid ester. This has been examined for the case of AlaOBu^t. After coupling, excess ester may be extracted into ethyl acetate from the salt peak eluted from the Sephadex G-50 column (see method A above). To do this, the pooled fractions are adjusted to a pH above neutrality with 1M-NaOH and then to pH 11.0 by addition of 1M-Na₂CO₃ and 1M-NaOH. After three extractions with ethyl acetate, the amino acid ester is back-extracted into 2M-HCl and freeze-dried as the hydrochloride (as the acetate, it is volatile). Recovery is greater than 70% even when working on a small scale (1400nmol of ester). Ester recovered in this way may be coupled again directly without impaired efficiency. However, if diethyl ether (analytical grade; Merck, Darmstadt, West Germany) is used in place of the ethyl acetate, recovered ester does not couple well; this is in spite of a negative starch/iodide test for peroxides in the ether.

Insulin analogues. Insulin analogues containing a variety of groups at position B30 may be prepared by the above techniques. When coupling molecules that may require very rapid reaction to avoid damage to trypsin or insulin (e.g. cystamine, which may contain some cysteamine), more satisfactory results are obtained by coupling to des-Ala-B30-insulin rather than using pig insulin as substrate. Fig. 1(a) shows that the degree of coupling of Lys(Bu^tOCO)OBu^t to des-Ala-B30-insulin is effectively quantitative. It is of interest to note that various molecules (e.g. AlaOBu^t) may be rapidly and almost quantitatively coupled to des-octa-peptide-B23–B30-insulin, suggesting that the lack of cleavage (and coupling) at Arg-B22 observed when insulin or des-Ala-B30-insulin is used as substrate is a kinetic and not a thermodynamic phenomenon.

Human insulin. Methods A–D represent rapid, convenient and (in terms of insulin) efficient routes to semi-synthetic human insulin. Method C in particular lends itself to larger-scale operations, and

Fig. 1. *Electrophoretograms of insulin and insulin analogues on cellulose acetate*

Samples are: (a) (i) des-Ala-B30-insulin; (ii) [Lys(Bu^tOCO)OBu^t-B30]insulin; (b) (i) pig insulin; (ii) [Thr(Bu^t)OBu^t-B30]insulin produced by method A; (iii) de-protected semi-synthetic human insulin; (c) (i) pig insulin; (ii) [Thr(Bu^t)OBu^t-B30]insulin produced by method B; (iii) de-protected semi-synthetic human insulin; (d) (i) pig insulin; (ii) [Thr(Bu^t)OBu^t-B30]insulin produced by method C. The electrophoresis was performed on strips of cellulose acetate in a urea-containing borate buffer, pH 8.0, staining with Ponceau S, as described previously (Rose *et al.*, 1981). FF indicates the position reached by the dye Cyanol FF, applied at the origin. The coupling mixture may be applied directly to cellulose acetate for convenience in monitoring the progress of the reaction; alternatively, a sample of the appropriate freeze-dried pooled fraction from the Sephadex G-50 separation of the coupling mixture may be applied.

has the advantage, besides efficient use of trypsin, of requiring only dialysis, and not gel filtration, after the coupling reaction. Figs. 1(b)–1(d) indicate the purity of the intermediate insulin ester. The coupling reaction is seen to have proceeded effectively quantitatively: uncoupled material is present only in trace amounts. Fig. 1 also demonstrates the degree of homogeneity of semi-synthetic human insulin obtained after de-protection. Amino acid analysis showed 2.72 residues of threonine (uncorrected) and 1.11 residues of alanine per molecule. The theoretical values for human insulin are 3 and 1 respectively. An analysis of the appropriate freeze-dried pooled fraction from the Sephadex G-50 separation of a coupling mixture was performed by high-pressure liquid chromatography. The resulting chromatogram (Fig. 2) shows a very high yield of human insulin ether ester, confirming results obtained by electrophoresis on cellulose acetate. A similar analysis of purified de-protected human

Fig. 2. High-pressure liquid-chromatographic analysis of the pooled insulin/insulin ether ester fraction

For this analysis the sample of pig insulin was coupled in the presence of $\text{Thr}(\text{Bu})\text{OBU}^t$ (1 M), with trypsin as catalyst. After 2 h at 37°C , the coupling mixture was acidified and the product was separated from trypsin and small molecules by gel filtration on Sephadex G-50 as described in the text. Conditions for high-pressure liquid chromatography: separation of human insulin ether ester from remaining insulin was achieved by reversed-phase high-pressure liquid chromatography on a $45\text{ mm} \times 4.6\text{ mm}$ -diam. column packed with $5\text{ }\mu\text{m}$ -pore-size Ultrasphere ODS (Beckman). The eluent contained sodium butanesulphonate (5 mM), NaH_2PO_4 (5 mM), Na_2SO_4 (50 mM) and acetonitrile (25%, v/v). On injection, a linear gradient of acetonitrile was started, running from the original value of 25% to 60% (v/v) over a 10 min period. A flow rate of 1.5 ml/min was used throughout. The absorbance of the eluate was monitored at 214 nm. The absorbance scale was divided by 5 for a short period in order to keep the ester peak on scale. The elution positions of human insulin ether ester and of pig insulin were established in separate runs with authentic materials applied separately and as a mixture.

Fig. 3. High-pressure liquid-chromatographic analysis of semi-synthetic human insulin

A sample of human insulin ether ester, prepared enzymically, was purified from remaining pig material by ion-exchange chromatography on DEAE-Sephadex A-25 (see the text). After deprotection with trifluoroacetic acid, the product was subjected to ion-exchange chromatography under similar conditions. Conditions for high-pressure liquid chromatography were as indicated in Fig. 2 legend.

glucose concentration in rats, and the sample was found to be fully active by both of these criteria. Semi-synthetic human insulin (1 mg/ml) gave a value of 1.1 mg/ml by radioimmunoassay, with linear dilution against the pig insulin standard. In the assay *in vivo*, semi-synthetic human insulin gave a cumulative depression of blood glucose concentration of 248, compared with 240 for the pig insulin control (for an explanation of these units, see Halban *et al.*, 1981); the difference is not significant. Our coupling methods are thus able to produce biologically active human insulin in high yields and in a short time. If it is necessary to remove the traces of uncoupled material evident in Fig. 1, this is easily done on DEAE-Sephadex A-25 by using 7 M-urea/100 mM-Tris/HCl buffer, pH 8.4, with a gradient of 0–200 mM-NaCl.

insulin is shown in Fig. 3. The semi-synthetic human insulin prepared by method A was tested both by radioimmunoassay and by depression of blood

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References

- Breddam, K., Widmer, F. & Johansen, J. T. (1982) *Eur. Patent Application* 81303383.4
- Gattner, H. G., Danho, W. & Naithani, V. K. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 117–123, W. de Gruyter, Berlin and New York
- Halban, P. A., Berger, M., Gjinojci, A. & Renold, A. E. (1981) *Diabetes Care* **4**, 238–243
- Herbert, V., Lau, K. S., Gottlieb, C. V. & Bleicher, S. J. (1965) *J. Clin. Endocrinol. Metab.* **25**, 1375–1384
- Inouye, K., Watanabe, K., Morihara, K., Tochino, Y., Kanaya, T., Emura, J. & Sakakibara, S. (1979) *J. Am. Chem. Soc.* **101**, 751–752
- Jonczyk, A. & Gattner, H. G. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1591–1598
- Markussen, J. (1981) *U.K. Patent Application* GB 2 069502 A
- Morihara, K., Oka, T., Tsuzuki, H., Inouye, K. & Sakakibara, S. (1979a) in *Peptides: Structure and Biological Function* (Gross, E. & Meienhofer, J., eds.), pp. 617–620, Pierce Chemical Co., Rockford
- Morihara, K., Oka, T. & Tsuzuki, H. (1979b) *Nature (London)* **280**, 412–413
- Morihara, K., Oka, T., Tsuzuki, H., Tochino, Y. & Kanaya, T. (1980) *Biochem. Biophys. Res. Commun.* **92**, 396–402
- Obermeier, R. (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E. & Di Bello, C., eds.), pp. 201–211, Academic Press, London, New York and San Francisco
- Obermeier, R. & Geiger, R. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 759–767
- Rose, K., Drake, C. S., Rees, A. R. & Offord, R. E. (1981) *Biochem. J.* **195**, 765–768
- Schmitt, E. W. & Gattner, H.-G. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 799–802