Preparation of [B23-D-alanine]des-(B25–B30)-hexapeptide-insulin by a combination of enzymic and non-enzymic synthesis

You-shang ZHANG, Qiu-ping CAO, Zhi-gang LI* and Da-fu CUI
Shanghai Institute of Biochemistry, Academia Sinica, 320 Yueyang Road, Shanghai, China

(Received 5 August 1983/Accepted 26 September 1983)

Des-(B25–B30)-hexapeptide-insulin with B23-glycine replaced by D-alanine was prepared by a combination of enzymic and non-enzymic syntheses. The purified product was homogeneous in polyacrylamide-gel electrophoresis and could be crystallized. The biological activity *in vivo* of crystalline [B23-D-Ala]des-(B25–B30)-hexapeptide-insulin was determined as 58% of that of standard pig insulin (27 i.u./mg).

It was reported previously that des-(B25–B30)-hexapeptide-insulin with B23-glycine replaced by D-alanine was still biologically active (Chu *et al.*, 1973). [B23-D-Ala]des-(B25–B30)-hexapeptide-insulin was first prepared by non-enzymic semi-synthesis, but a homogeneous product was not obtained because of side reactions. The preparation of homogeneous des-(B25–B30)-hexapeptide-insulin was later achieved by enzymic semi-synthesis (Cao *et al.*, 1981). However, the high optical specificity of the enzyme excludes its direct application in peptide synthesis involving D-amino acid residues, and special manipulations are needed to overcome this difficulty. In the present study, a combination of enzymic and non-enzymic syntheses was devised. The first step is the specific enzymic conversion of the carboxy group of arginine in des-(B23–B30)-octapeptide-insulin into acyl hydrazide. The second step is the conversion of hydrazide into azide followed by non-specific coupling with D-Ala-Phe-OBu^t. After deprotection and purification, [B23-D-Ala]des-(B25–B30)-hexapeptide-insulin is obtained in crystalline form. The specific activation of the arginine carboxy group in des-(B23–B30)-octapeptide-insulin was first reported by Canova-Davis & Carpenter (1980).

Experimental and results

Des-(B23–B30)-octapeptide-insulin, (Msc)₂-des-(B23–B30)-octapeptide-insulin and D-Ala-Phe-OBu^t were prepared as reported previously (Cui *et al.*, 1983). A 100 mg portion of (Msc)₂-des-(B23–B30)-octapeptide-insulin was dissolved in 5.5 ml of 2 M-

hydrazine solution, pH 7.0, and 4.5 ml of dimethylformamide. The mixture was incubated at 37°C for 24 h. Trypsin was added before and after 2 and 6 h of incubation, the total amount of trypsin being 10 mg. After incubation, the mixture was dialysed against 10% (v/v) acetic acid to remove hydrazine and dimethylformamide, and any precipitate formed was removed by centrifugation. The dialysis residue was freeze-dried, dissolved in 1 M-acetic acid and passed through a Sephadex G-50 (fine grade) column (2 cm × 150 cm) with 1 M-acetic acid as eluent. The main peak, consisting of a mixture of (Msc)₂-des-(B23–B30)-octapeptide-insulin and (Msc)₂-des-(B23–B30)-octapeptide-insulin-NHNH₂, was freeze-dried and weighed 90 mg. A 50 mg portion of this product was purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column (0.8 cm × 20 cm) with salt-gradient elution [initial buffer 150 ml of 0.05 M-Tris/HCl buffer, pH 7.4, containing 40% (v/v) propan-2-ol; final buffer 150 ml of initial buffer containing 0.15 M-NaCl] Fig. 1). The A₂₈₀ ratio between peak I, (Msc)₂-des-(B23–B30)-octapeptide-insulin-NHNH₂, and peak II, (Msc)₂-des-(B23–B30)-octapeptide-insulin, was 7:3. After desalting through Sephadex G-25 and freeze-drying, 26 mg of the former and 10 mg of the latter were recovered.

Preliminary experiments showed that the crude product of (Msc)₂-des-(B23–B30)-octapeptide-insulin-NHNH₂ obtained from enzymic synthesis followed by gel filtration could be used without further purification. Therefore this material was used in the following steps. A 30 mg portion of crude (Msc)₂-des-(B23–B30)-octapeptide-insulin-NHNH₂ was dissolved in 0.2 ml of dimethylformamide, and 0.055 ml of 1 M-HCl in dimethylformamide was added. The mixture was cooled to –20°C, and 0.01 ml of t-butyl nitrite, 10-fold diluted with di-

Abbreviations used: OBu^t, t-butoxy; Msc, methanesulphonylethoxycarbonyl.

* Present address: Shanghai Institute of Plant Physiology, Shanghai, China.

Fig. 1. Purification of $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-NH $_2$ by ion-exchange chromatography

For experimental details see the text. —, A_{280} ; — — —, concn. of NaCl.

Fig. 2. Purification of $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-D-Ala-Phe-OBu t by ion-exchange chromatography

For experimental details see the text. —, A_{280} ; — — —, concn. of NaCl.

methylformamide, was added. After 15 min, 0.01 ml of triethylamine was added. Also, 0.11 mmol of D-Ala-Phe-OBu t (previously treated with an equivalent amount of triethylamine) was dissolved in 0.1 ml of dimethylformamide. After the addition of 0.02 ml of triethylamine, the mixture was cooled to -20°C . The above two mixtures were mixed and kept at 4°C for 3 days. Anhydrous diethyl ether was added, and the dried precipitate was dissolved in 1.5 ml of the

Fig. 3. Polyacrylamide-gel electrophoresis at alkaline pH of, from left to right, [B23-D-Ala]des-(B25-B30)-hexapeptide-insulin, $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-D-Ala-Phe-OBu t and $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-NH $_2$

For experimental details see the text.

above-mentioned initial buffer and dialysed against the same buffer for 2 h. The supernatant was purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column ($0.5\text{ cm} \times 16\text{ cm}$) with salt-gradient elution (100 ml of final buffer to 100 ml of initial buffer as mentioned above) (Fig. 2). Peak I was $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-D-Ala-Phe-OBu t and peak II $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin. After desalting through Sephadex G-25 and freeze-drying, 18 mg of the former and 8 mg of the latter were recovered.

A 16 mg portion of $(Msc)_2$ -des-(B23-B30)-octapeptide-insulin-D-Ala-Phe-OBu t was treated with 1.5 ml of anhydrous trifluoroacetic acid at 25°C for 30 min, vacuum-evaporated to dryness and washed with anhydrous diethyl ether. Polyacrylamide-gel electrophoresis showed that the reaction was complete, and the product was dissolved in 0.65 ml of dioxan/methanol/water (6:1:7, by vol.). Then 0.09 ml of 2 M-NaOH was added to the mixture at 0°C . After 2 min, 0.2 ml of acetic acid was added and the solution passed through a Sephadex G-50 column ($1\text{ cm} \times 50\text{ cm}$). A yield of 12 mg of [B23-D-Ala]des-(B25-B30)-hexapeptide-insulin was obtained. A 1 mg sample of this material was dissolved in 0.4 ml of 0.01 M-HCl and dialysed against a citrate buffer containing 25% acetone [2% (w/v) citric acid, 50 ml; 6.5% (w/v) zinc acetate, 1 ml; acetone, 25 ml; adjusted to pH 5.7–5.8 with NaOH and diluted to 100 ml]. Crystals appeared upon standing. The biological activity *in vivo* of crys-

talline [B23-D-Ala]des-(B25-B30)-hexapeptide-insulin was determined by mouse convulsion assay as 58% of that of standard pig insulin (27 i.u./mg). The amino acid composition of purified [B23-D-Ala]des-(B25-B30)-hexapeptide-insulin was determined as: Asx, 3.3 (3); Glx, 7.0 (7); Thr, 1.0 (1); Ser, 2.8 (3); Gly, 3.8 (4); Ala, 2.2 (2); Val, 4.2 (4); Ile, 2.0 (2); Leu, 6.3 (6); Tyr, 2.8 (3); Phe, 1.9 (2); His, 2.0 (2); Arg, 1.0 (1).

Discussion

The above experiments have shown that a combination of enzymic and non-enzymic syntheses can be used in peptide-bond formation involving D-amino acid residues. The overall yield of [B23-D-Ala]des-(B25-B30)-hexapeptide-insulin from (Msc)₂-des-(B23-B30)-octapeptide-insulin was about 40%. [B25-D-Ala]des-(B25-B30)-hexapeptide-insulin prepared by this method was homogeneous in polyacrylamide-gel electrophoresis (Fig. 3) and could be crystallized. When B23-glycine in des-(B25-B30)-hexapeptide-insulin was replaced by D-alanine, its biological activity *in vivo* increased

from 40% (Cui *et al.*, 1983) to 58%, as compared with insulin. This apparent increase might be due to its resistance to enzymic degradation.

The synthetic method reported in the present paper may also find applications in peptide-bond formation involving some other amino acid residue, e.g. proline, that is resistant to proteinase catalysis.

We thank Hui-xi Shen in the Shanghai Biochemical Pharmaceutical Factory for the bioassay and De-ming Chen and Jia-ping Wang for the amino acid analysis.

References

- Canova-Davis, E. & Carpenter, F. H. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 107–115, Walter de Gruyter, Berlin and London
- Cao, Q. P., Cui, D. F. & Zhang, Y. S. (1981) *Nature (London)* **292**, 774–775
- Chu, S. C., Li, K. T., Tsao, C. P., Chang, Y. S. & Lu, T. H. (1973) *Sci. Sin. (Engl. Ed.)* **16**, 71–78
- Cui, D. F., Cao, Q. P., Zhu, S. Q., Zhang, X. T. & Zhang, Y. S. (1983) *Sci. Sin. (Engl. Ed.)* **26**, 248–254