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Accelerated Article

Preparation of Tyr-C-Peptide from Genetically Altered Human Insulin Precursor

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ABSTRACT

C-peptide radioimmunoassay (C-peptide RIA) is widely used in determination of pancreatic B-cell secretion activity. ^{125}I labeled Tyr-C-peptide is indispensable in C-peptide RIA kit. Herein we discuss a way of obtaining recombinant Tyr-C-peptide. Arg32Tyr human proinsulin mutant (R32Y-proinsulin) gene was constructed by site-directed mutagenesis and overexpressed in *Escherichia coli*. Purified R32Y-proinsulin was converted to insulin and Tyr-C-peptide by trypsin and carboxypeptidase B codigestion. Tyr-C-peptide was isolated through reverse-phase HPLC (RP-HPLC) and identified by C-peptide RIA and amino acid analysis.

Index Entries: Tyr-C-peptide; human proinsulin; C-peptide RIA.

INTRODUCTION

During the process of insulin secretion, proinsulin is cleaved into insulin and C-peptide by specific endopeptidase. Insulin and C-peptide are secreted from pancreatic B-cells in an equimolar ratio (*1*). Hence, circulating C-peptide level reflects the B-cell secretion activity. Compared

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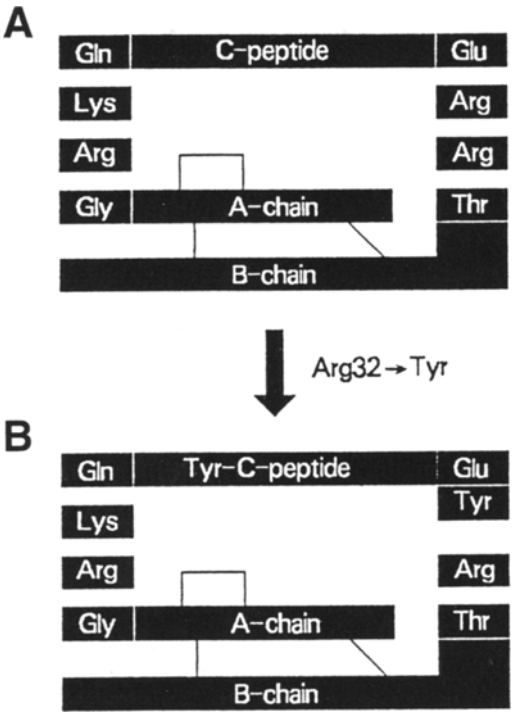

Fig. 1. Diagrammatic representation of the structures of human proinsulin (**A**) and R32Y-proinsulin (**B**).

to insulin measurements, C-peptide measurements are preferable in clinical applications because C-peptide has a much slower metabolic clearance rate and does not crossreact with insulin antibodies generated after exogenous insulin treatment (2).

Radioimmunoassay has long been applied in determining the C-peptide concentration in blood or urine. Tyr-C-peptide, which can be labeled by ^{125}I, is required in the assay as a radioactive tracer (3). Tyr-C-peptide is usually prepared via chemical synthesis (2). To generate Tyr-C-peptide as a by-product during the production of recombinant human insulin (4,5), we constructed a human proinsulin mutant, where the Arg32 was changed to Tyr. Thus, Tyr-C-peptide instead of natural human C-peptide was released after the enzymatic conversion of proinsulin to insulin (Fig.1).

MATERIALS AND METHODS

Oligonucleotide Primer, Gene, and Vector

Oligonucleotide primer (5'CTTCAGCTTCATAACGCGTCTTGG3') was synthesized by ABI 381A DNA synthesizer and was purified by polyacrylamide gel electrophoresis. Human proinsulin gene was synthesized

by Wu et al. (*6*) and was cloned in bacterial phage M13 between *Eco*RI and *Bam*HI. Expression vector pBV220, constructed by Zhang et al. (*7*), contains P_RP_L promotor, pUC8 polycloning site, rrnBT1T2 termination signal, and ampicilin resistance and λcIts857 repressor genes.

Mutagenesis and Plasmid Construction

Oligonucleotide-mediated mutagenesis on human proinsulin gene was carried out according to Kunkel's method (*8*). Mutation site was sequenced by Sanger's dideoxy method. The mutant gene was cut off from M13 and cloned into pBV220 between *Eco*RI and *Bam*HI site. Recombinant plasmid was examined by restriction analysis and transformed into competent $CaC1_2$-treated *E.coli* DH5α cells.

Fermentation

Feed-batch fermentation was accomplished using LEM's benchtop fermenter. Three liters of 2YT + M9 broth containing 100 mg/mL ampicilin plus 1 mL of antifoam was incubated with 300 mL of bacterial culture grown overnight at 30°C. Cells were grown at 30°C while 20% glucose plus 20% yeast extract solution was added and air-flow increased to maintain the exponential growth phase. After A600nm of the culture reached at 30, the temperature was shifted to 42°C to induce protein synthesis for 2 h. Cells were harvested by centrifugation. The expressed products were analyzed by 15% SDS-polyacrylamide gel electrophoresis.

Isolation and Purification of R32Y-Proinsulin

Eighty grams of *E. coli* cells (wet weight) were suspended in 300 mL of STET (0.05 mol/L Tris-HCl, pH 8.0, 0.05 mol/L EDTA, 5%[v/v] Triton X-100, 8% [w/v] sucrose) and cells were disrupted by sonication. The granules were collected by centrifugation at 10,000*g*, 4°C, and resuspened in 50 mL of lysis buffer (8 mol/L Urea, 1 mmol/L DTT, 0.1 mol/L Tris-HCl, pH 7.6, 1 mmol/L EDTA, and 0.2 mol/L NaCl) for an overnight dissolve/denaturation at 10°C. The solution was then added 80 mg DTT and incubated at 37°C for 2 h. The reduced peptides were precipitated from the denaturant and redissolved into 2 L of 0.05 mol/L Gly-NaOH (pH 10.8) for 48 h of renaturation. Renatured products were concentrated by ultrafiltration and loaded onto a Sephadex G50 (fine) column (3 × 100 cm), eluted with 0.05 mol/L Gly-NaOH (pH 10.8). The peaks containing the desired peptides were collected and lyophilized. Sixty milligrams R32Y-proinsulin could be obtained from 80 g wet cells. Purified R32Y-proinsulin was examined by 10% polyacrylamide gel electrophoresis and amino acid composition analysis.

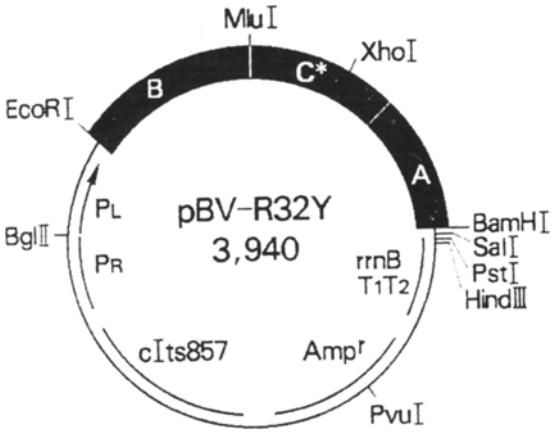

Fig. 2. Restriction map of plasmid pBV-R32Y.

Proinsulin Conversion and Tyr-C-Peptide Isolation

Ten milligrams of R32Y-proinsulin was treated with 0.1 mg trypsin (Sigma Cat. #T7418, 13,000–20,000 BAEE/mg) and 0.05 mg carboxypeptidase B (prepared according to ref. *9*) in 1 mL of 0.08 mol/L Tris-HCl, pH 7.5, for 30 min at 37°C. The reaction mixture was injected (0.1 mL per injection) into an 8 × 300 mm C18 preparative column equilibrated with 0.1% aqueous CF_3COOH. The peptides were eluted with a gradient program (20 min of 0–25%, 15 min of 25–50%, and 5 min of 50–100% CH_3CN in 0.1% aqueous CF_3COOH, 1.5 mL/min). Eluted peptides were collected (0.5 min/tube) and analyzed by C-peptide RIA (according to manufacturer's prescription). The fraction with C-peptide radioimmunoactivity was further identified by amino acid analysis.

RESULTS AND DISCUSSION

Construction and Expression of R32Y-Proinsulin Gene

Three base pairs were altered in the human proinsulin gene (*see* Material and Methods: bases underlined in the sequence of the primer). Besides the mutation of the Arg 32 to Tyr, the codon of Thr 30, ACC, was replaced by ACG to generate an *Mlu*I site and thus facilitate the screening of mutation. The expression plasmid, pBV220 with mutant gene, was designated as pBV-R32Y (Fig. 2).

The expression of R32Y-proinsulin in the pBV-R32Y-containing cells was assessed by 15% SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, lane D, a major protein band corresponding to R32Y-proinsulin (approx 10,000 dalton) was detected and estimated by densitometric scanning to be about 8% of the total cellular protein. The expressed protein was proven to be in the form of inclusion bodies (Fig. 3, lane E).

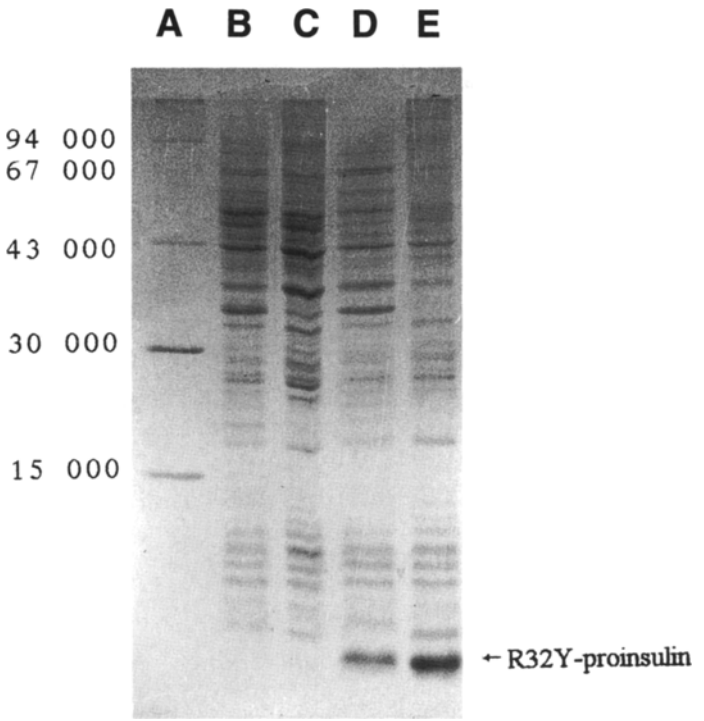

Fig. 3. 15% SDS-PAGE analysis of expression products. Lane A, protein molecular weight markers; B, total proteins of cells containing pBV220; C, pellet of B after sonication and centrifugation; D, total proteins of cells containing pBV-R32Y; E, pellet of D after sonication and centrifugation.

Purification of R32Y-Proinsulin

Sonication and centrifugation were used to isolate inclusion bodies from the bacteria. Conventional methods, such as denaturation/reduction and renaturation/oxidation, and gel filtration chromatography, were applied to purify the overexpressed protein from inclusion bodies (*10*).

Purified R32Y-Proinsulin was identified with polyacrylamide gel electrophoresis (Fig. 4) and amino acid composition analysis (Table 1). As the mutation causing one net charge decreased, the mutant molecule migrated more rapidly than the native molecule did. However, their tryptic digestion products (Fig. 4, lanes B and D) were identical and proved to be des-B30-insulin, which had the same electrophoretic mobility with porcine insulin (Fig. 4, lane E). The homogeneity of the bands shown in Fig. 4, lanes A and B, indicated that the replacement of Arg 32 with Tyr did not influence the protein refolding or the correct formation of disulfide linkages.

Table 1
The Amino Acid Analysis of R32Y-Proinsulin and Tyr-C-Peptide

Amino acid	R32Y-Proinsulin		Tyr-C-Peptide	
	Expected	Found	Expected	Found
Asx	4	4.6	1	0.6
Thr	3	3.5	0	0.1
Ser	5	4.9	2	2.6
Glx	15	16.6	8	8.3
Pro	3	3.2	2	2.2
Gly	11	10.8	7	6.6
Ala	4	4.1	3	3.0
Cys	6	7.0	0	0
Val	6	5.6	2	2.2
Met[b]	1(0)[a]	1.3	0	0
Ile	2	2.5	0	0.1
Leu	12	11.1	6	5.7
Tyr	5(4)[a]	4.9	1(0)[a]	1.2
Phe	3	3.5	0	0.1
Lys	2	2.0	0	0
His	2	2.0	0	0
Arg	3(4)[a]	3.2	0	0

[a]Numbers in parentheses indicate the amount of amino acid in wild type.
[b]N-Met-Proinsulin expressed owing to insufficient processing of overexpressed protein.

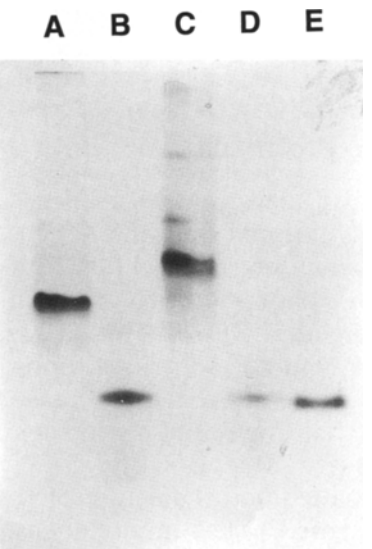

Fig. 4. Identification of R32Y-proinsulin by 10% PAGE. Lanes A, R32Y-proinsulin; B, R32Y-proinsulin/trypsin; C, human proinsulin, D, human proinsulin/trypsin; E, pocine insulin.

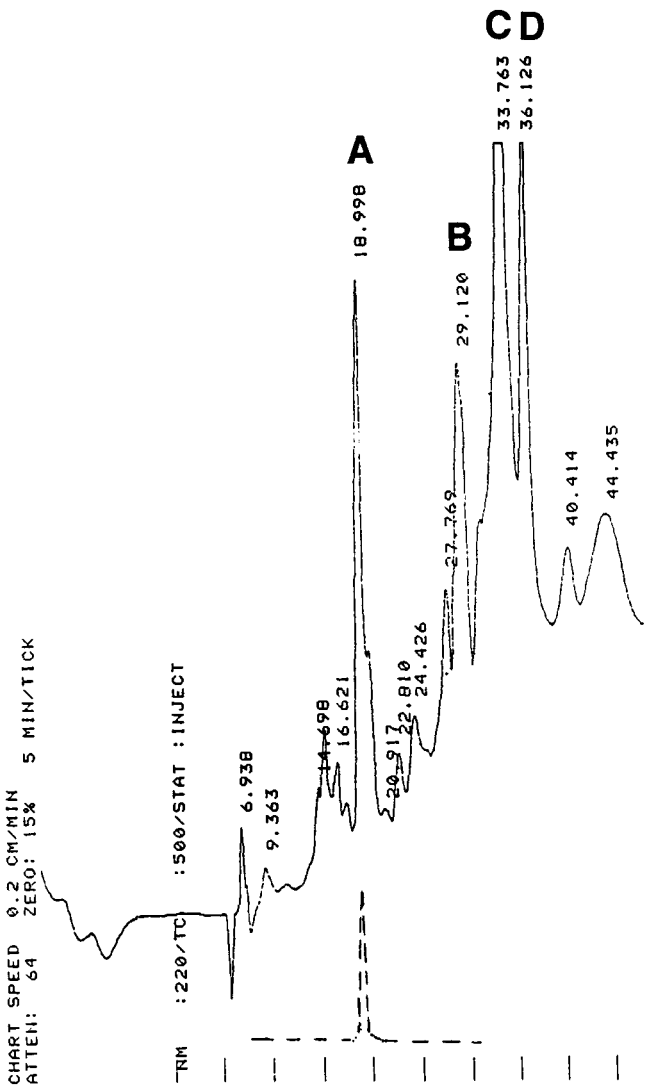

Fig. 5. Separation of Tyr-C-peptide by preparative reverse-phase HPLC (8 × 300 mm C18 column). According to ref. *11*, peaks A, B, C, and D can be confirmed: A, C-peptide; B, DOP-Insulin; C, Arg-Insulin; D, Insulin & Des-B30-Insulin. ——, A 220nm; ------, C-peptide activity detected by C-P RIA.

Isolation of Tyr-C-Peptide

The conversion reaction was carried out by the use of trypsin and carboxypeptidase B. The enzyme digest was followed by reverse-phase HPLC (Fig. 5). Peak A was identified as the Tyr-C-peptide based on the results of C-peptide RIA and amino acid analysis. The chromatogram was comparable to the similar work done by Heath et al. at Lilly research laboratories (*see* Fig. 8 and Table 3 in ref. *11*). The peaks A, B, C, and D in our work (Fig. 5) were corresponding to peaks 9, 14, 16, and 17, respectively,

in Heath's article, except that the fraction of Arg-insulin we obtained by RP-HPLC was relatively larger, owing to probable insufficient activity of carboxypeptidase B prepared by ourselves.

The results of our study illustrate a more economical way of preparing the Tyr-C-peptide, comparing to chemical synthesis. Further work has to be done to remove the N-terminal methionine of R32Y-proinsulin, which could not be efficiently processed in the form of inclusion bodies (*see* Table 1). An N-*Lys*-proinsulin gene has been constructed, resulting in Met-*Lys*-proinsulin expression and easier removal of N-methionine during enzymatic transformation reaction (*12*). The same addition of lysine at the N-terminal of R32Y-proinsulin will make the mutant insulin precursor more adaptable for the production of recombinant human insulin as well as Tyr-C-peptide.

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