0273-2289/99/76/0107/$12.00

Separation and Characterization of Trypsin and Carboxypeptidase B–Digested Products of Met-Lys-Human Proinsulin

Zhi-Hong Yang, Xin Du, and Jian-Guo Tang*

National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China, E-mail: jt@pul2.lib.pku.edu.cn

Received September 3, 1997; Accepted September 10, 1998

Abstract

Met-Lys-human proinsulin could be converted into insulin in vitro with the treatment of trypsin and carboxypeptidase B (CPB). Under less effective conditions, the enzymatic reaction does not proceed perfectly, and two main bands have been identified by native-polyacrylamide gel electrophoresis (PAGE) analysis. These two main products were thus separated and purified by DEAE-Sephadex A25 chromatography in a Tris-isopropanol system with an NaCl gradient. The isopropanol and NaCl were removed by a second DEAE-Sephadex column. Native-PAGE, mass spectrometric, and amino acid composition analyses indicate that one fraction of these two major products contains human insulin and desB30-insulin and that the other fraction is a mixture of human insulin analogs, which have one more basic amino acid than human insulin owing to the unsuitable amount of proteases, especially the lack of CPB. Furthermore, both receptor binding assay and radioimmunoassay have been utilized for the activity determination, and both fractions display almost full biological activity with porcine insulin as the standard. Present results provide further evidence for the quality control of recombinant human insulin production.

Index Entries: Insulin analog; proinsulin; enzymatic conversion; MS analysis; activity.

Introduction

Insulin has long been used to treat diabetes and has been one of the most successful biotechnological products. Since the late 1980s, recombinant DNA techniques have played a significant role in insulin manufacturing

*Author to whom all correspondence and reprint requests should be addressed.

and marketing *(1)*. Two major methods have been used in insulin production in the *Escherichia coli* system. One is to express gene in the fusion protein form to stabilize insulin protein *(2,3)*, which has a short half-life in the host cell *(4,5)*. The other is to express its precursor directly in the inclusion body form as has been shown by directly expressing inverted A-C-B proinsulin *(6)*. Both methods are limited by either the relatively low yield or the difficulty and complexity of downstream processing *(2–6)*.

It has been suggested that the use of Met-Lys-human proinsulin is a good way to obtain insulin owing to both its direct and high-level expression of proinsulin gene in a very short induction time in *E. coli* and its simplified procedure for target protein purification *(7)*. However, some by-products are presented in the enzyme-digested mixture and these need to be elucidated, although we assume that these by-products are insulin analogs. In this article, we focus on the study of only one major by-product band on native-polyacrylamide gel electrophoresis (PAGE) during the conversion of Met-Lys-human proinsulin to insulin.

Materials and Methods

Separation and Purification of Human Insulin and Its Analog

The crude Met-Lys-human proinsulin fraction was pooled after Sephadex G50 separation as described previously *(7)* and was adjusted to pH 3.0 with diluted HCl. Solid NaCl was added to a saturation of 12% to salt out proinsulin protein. The pellet of proinsulin was collected by centrifugation and washed with water at pH 5.3. Then it was dissolved in 0.08*M* Tris-HCl buffer, pH 7.5, and trypsin (9900 U/mg, Sigma, St. Louis, MO) and carboxypeptidase B (CPB) (180 U/mg, stored in 0.1*M* NaCl at –20°C), with enzyme/substrate ratios of 1:100 and 1:400, respectively, were added *(8)*. The mixture was incubated at 37°C for 30 min and cooled down immediately. Isopropanol was added to a final concentration of 40% to terminate the reaction. The enzyme-digested products were then loaded onto a DEAE-Sephadex A25 column and eluted with 0.05*M* Tris-HCl, 40% isopropanol at pH 7.5, with a linear NaCl gradient from 0.02 to 0.10*M* *(7)*. Fractions of human insulin and their analogs were pooled separately and adjusted to pH 9.0. Each of the products was loaded onto a second DEAE-Sephadex A25 column equilibrated with 0.05*M* Tris-HCl, 40% isopropanol at pH 9.0. NaCl and isopropanol were removed by eluting the column with water at pH 9.0. Finally, the column was washed with 0.05*M* HCl to obtain purified protein. The enzyme-digested proinsulin and the purified products were analyzed by native-PAGE. Human insulin showed a purity of up to 97%.

Characterization of Human Insulin and Its Analog

The molecular weight of human insulin and its analog were determined by mass spectrometry. The amino acid composition analysis was carried out as described previously *(9)*. Both receptor binding and radio-

immunoassays were utilized for the determination of biological activity *(9)*. Placental receptor was prepared following the method of Yoko et al. *(10)*. Radioimmunoactivity assay was carried out with the kit offered by Higrade Biotechnology Center (Beijing, PRC) according to the procedure enclosed.

Results and Discussion

Conversion of Met-Lys-Human Proinsulin to Human Insulin and Separation of the Products

The crude Met-Lys-human proinsulin could be converted into human insulin by the treatment of trypsin and CPB. Although the amount of by-products could be reduced by controlling the amount of enzyme and the reaction time, by-products could not be avoided completely. This is also the case for conversion of both proinsulin *(11)* and mini-proinsulin (B-Lys-Arg-A) *(12)*. Both Frank et al. *(11)* and Thim et al. *(12)* utilized trypsin and CPB for the conversion of recombinant proinsulin and mini-proinsulin to recombinant insulin. During their studies, they had to keep the proteases at a certain amount to reduce the formation of by-products. B31Arg-insulin, B31Arg-B32Arg-insulin, desB30Thr-insulin, desoctapeptide (B23-30)-insulin are the major by-products *(11,12)*. In our case, as has been shown previously by both anion-exchange chromatography on DEAE-Sephadex A25 and native-PAGE analyses *(7)*, there is a main by-product with either less negative or more positive charges than native insulin. This by-product could be converted into insulin with increasing amounts of trypsin; thus, it is highly valuable for investigation. Figure 1 shows the result of DEAE-Sephadex A25 separation of trypsin and CPB-digested products of Met-Lys-human proinsulin. Both human insulin and analog fraction could be separated. Another DEAE-Sephadex A25 chromatography was introduced to remove isopropanol and NaCl in the fraction, as shown in Fig. 2. The purified proteins were analyzed by native-PAGE, as illustrated in Fig. 3. Both human insulin and its analog give good purity.

Characterization of Human Insulin and Its Analog

Table 1 shows the amino acid composition analysis of both human insulin and its analog. The data are quite in agreement with those of standard human insulin, except for minor differences. Figure 4 shows the result of mass spectrometric analysis for both human insulin and its analog. Peak 2A ($M + H^+ = 5803.7$) in human insulin fraction (Fig. 4B) is almost identical to standard human insulin ($M + H^+ = 5808$). Peak 1A ($M + H^+ = 5959.3$) in analog fraction (Fig. 4A) is 155.6 Daltons heavier than peak 2A. These data therefore indicate that peak 1A has one more Arg (M = 156.2) than human insulin (peak 2A). Peak 1A is in fact a mixture of two components—B31Arg-insulin and B65Arg-insulin—as a result of the failure of the cleavage by both trypsin and CPB. Peak 1B ($M + H^+ = 6062.1$) is a small peak and is 258.4 Daltons heavier than peak 2A. These data indicate that Met-Lys (M = 259.4)

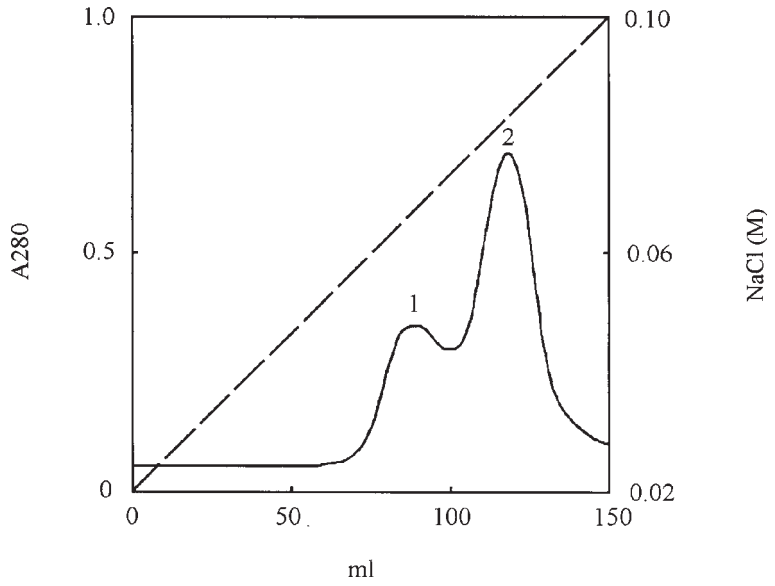

Fig. 1. DEAE-Sephadex A25 separation of the digested products of Met-Lys-human proinsulin by trypsin and CPB. The column (1 × 9 cm) was eluted with 0.05*M* Tris-HCl, 40% isopropanol, pH 7.5, with a linear NaCl gradient of 0.02–0.1*M* in a total volume of 150 mL. Peaks 1 and 2 represent human insulin analog and human insulin, respectively.

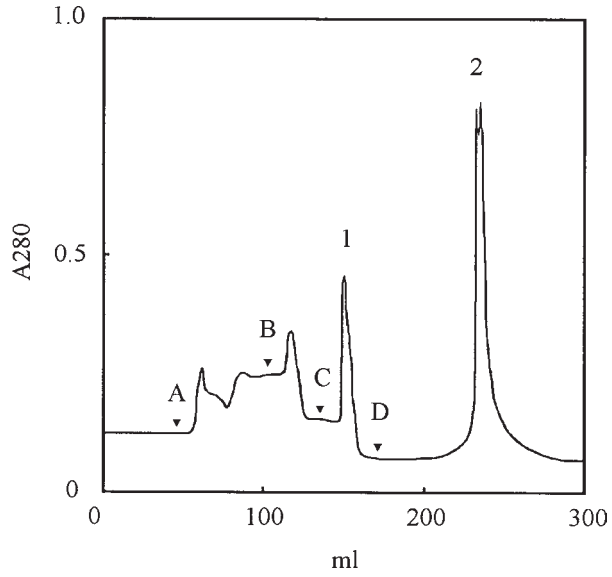

Fig. 2. DEAE-Sephadex A25 chromatography to remove NaCl and isopropanol in human insulin fraction. The column (1 × 9 cm) was equilibrated with 0.05*M* Tris, 40% isopropanol, pH 9.0. After loading the protein sample, it was washed with water to remove isopropanol and NaCl. Human insulin was finally eluted out by 0.05*M* HCl. Peak 1 indicates the mixing up of water with isopropanol; peak 2 represents human insulin fraction. **(A)** Loading sample; **(B)** equilibrating with 0.05*M* Tris, 40% isopropanol, pH 9.0; **(C)** washing with water; **(D)** eluting with 0.05*M* HCl.

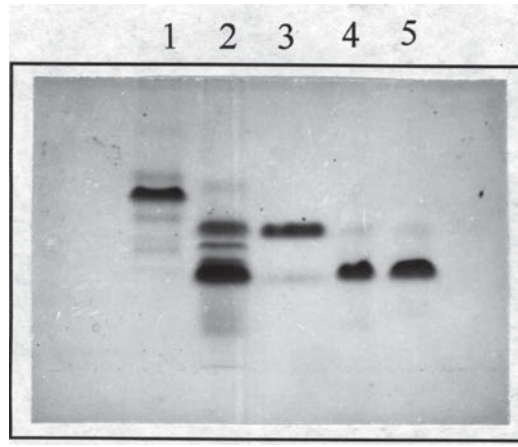

Fig. 3. Electrophoretic analysis of the purified Met-Lys-human proinsulin and its products after treatment with trypsin and CPB on 15% polyacrylamide gel. (1) Crude Met-Lys-human proinsulin; (2) Met-Lys-human proinsulin after treatment with trypsin and CPB; (3) human insulin analog; (4) human insulin; (5) porcine insulin as standard.

Table 1
The Amino Acid Composition Analysis of Human Insulin and Its Analog

	Expected	Standard human insulin	Human insulin analog	Human insulin
Asp	3	3.4	3.1	3.4
Thr	3	3.2	1.9	2.3
Ser	3	3.0	3.0	3.0
Glu	7	8.2	8.0	8.4
Gly	4	4.4	3.8	4.0
Ala	1	1.2	1.2	1.3
Val	4	3.1	3.1	3.7
Met	0	0.0	0.4	0.0
Ile	2	2.1	1.7	1.4
Leu	6	6.6	4.6	5.6
Tyr	4	4.2	2.4	3.2
Phe	3	3.4	2.1	2.6
Lys	1	1.1	1.1	1.0
His	2	2.2	1.4	1.7
Arg	1	1.1	1.0	0.9

may exist on this analog and that this minor analog is Met-Lys-insulin. The existence of Met-Lys-insulin is owing to the unsuccessful cleavage of Met-Lys-human proinsulin by trypsin. The fact that the amino acid composition of insulin analog fraction has some Met whereas insulin does not also supports this conclusion (Table 1). It is difficult to distinguish B31Arg-insulin, B65Arg-insulin, and Met-Lys-insulin by the analyses of native-PAGE and

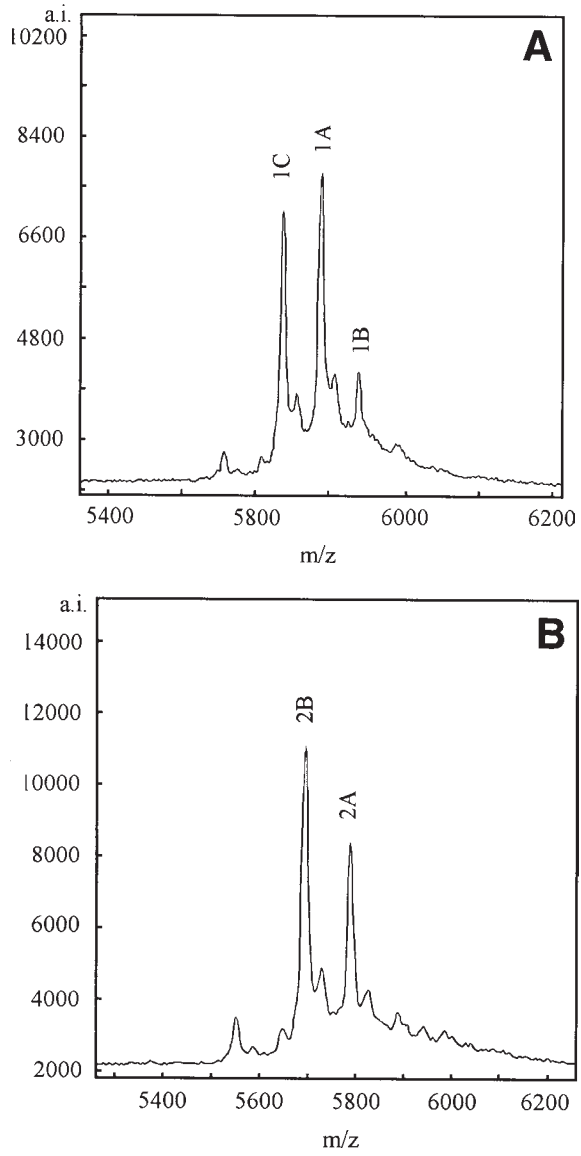

Fig. 4. Mass spectrometry analysis of the purified proteins. **(A)** Human insulin analog fraction; **(B)** human insulin fraction.

anion-exchange chromatography because these three human insulin analogs show no charge difference. But these three human insulin analogs do show slower mobility than native insulin owing to one more positive charge, as evidenced by native-PAGE. Peaks 1C and 2B are 101.0 and 102.1 Daltons lighter than peaks 1A and 2A, respectively. So peaks 1C and 2B represent desB30Thr derivatives (Thr, M = 102.1) of B65Arg-insulin (in peak 1A) and insulin (in peak 2A). Amino acid composition supports this assumption in that both human insulin and its analog fractions have less Thr than standard

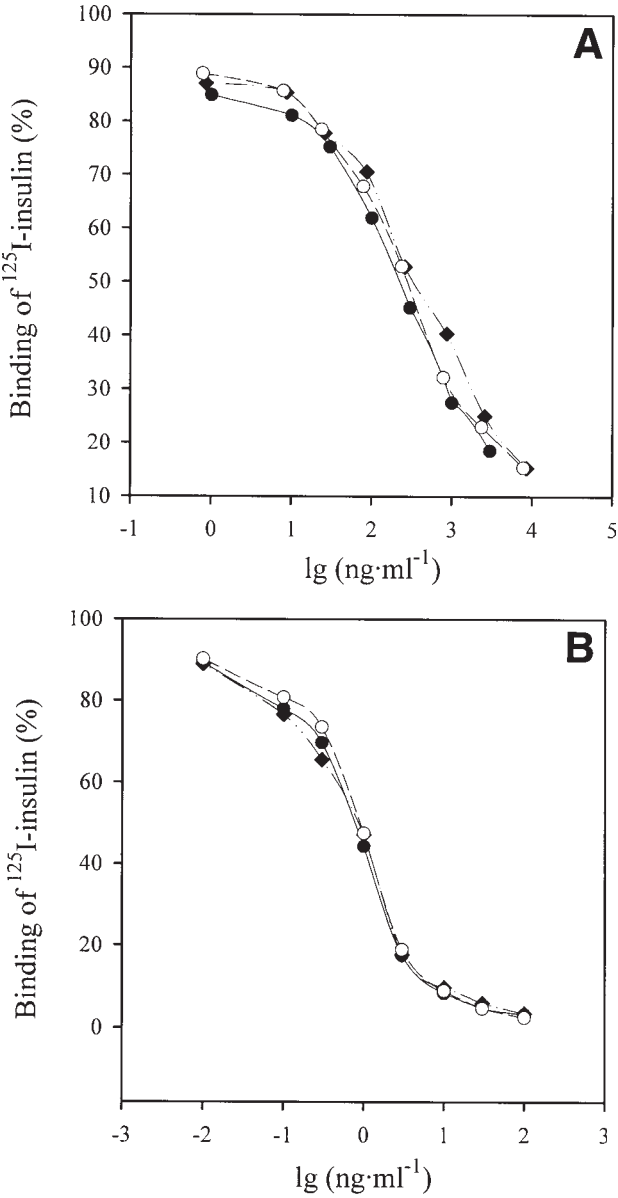

Fig. 5. Biological activity analysis of human insulin and its analog. **(A)** Receptor binding assay of human insulin (○) and its analog (◆) with porcine insulin (●) as standard; **(B)** radioimmunoassay of human insulin (○) and its analog (◆) with porcine insulin (●) as standard.

human insulin. This indicates the lack of CPB presented in the reaction mixture. As has been described previously, the presence of CPB may prevent the trypsin cleavage that converts insulin to desB30-insulin *(8,11)*.

Receptor binding assay shows that both human insulin and its analog fraction show similar activity with porcine insulin as standard (Fig. 5A).

This indicates that the surplus basic amino acid, and thus the consequent surplus positive charge, has almost no effect on the interaction of insulin with its receptor. Radioimmunoassay demonstrates that the surplus basic amino acid does not affect the epitome of human insulin either (Fig. 5B). It is suggested that the analogs are still active insulin derivatives and can be regarded as insulin protein in clinical trial if present in small amounts, just as Arg-insulin and insulin ester in crystallized bovine or porcine insulin. The present data provide some useful information for the large-scale manufacture of recombinant human insulin in our system *(7)*.

Acknowledgments

This work was supported in part by grants from both the National Funds of Distinguished Young Scholars of China (39525008) and the National Natural Science Foundation of China (39500032) to J. G. Tang. The authors thank Hongsheng Yuan for the amino acid composition analysis, Xiaoyu Pan for mass spectrometric analysis, and Higrade Biotechnology Center for its generous offer of ^{125}I-insulin.

References

1. Bristow, A. F. (1993), *TIBTECH* **11,** 301–305.
2. Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakvra, K., and Riggs, A. D. (1979), *Proc. Natl. Acad. Sci. USA* **76,** 106–110.
3. Sung, W. L., Yao, F. L., Zahab, D. M., and Narang, S. A. (1986), *Proc. Natl. Acad. Sci. USA* **83,** 561–565.
4. Talmadge, K. and Gilbert, W. (1982), *Proc. Natl. Acad. Sci. USA* **79,** 1830–1833.
5. Shen, S. H. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 4627–4631.
6. Heath, W. F., Belagaje, R. M., Brooke, G. S., Chance, R. E., Hoffmann, J. A., Long, H. B., Reams, S. G., Roundtree, C., Shaw, W. N., Slieker, L. J., Sundell, K. L., and DiMarchi, R. D. (1992), *J. Biol. Chem.* **267,** 419–425.
7. Chen, J. Q., Zhang, H. T., Hu, M. H., and Tang, J. G. (1995), *Appl. Biochem. Biotechnol.* **55,** 5–15.
8. Kemmler, W., Peterson, J. D., and Steiner, D. F. (1971), *J. Biol. Chem.* **246,** 6786–6791.
9. Tang, J. G., Wang, C. C., and Tsou, C. L. (1988), *Biochem. J.* **255,** 451–455.
10. Yoko, F. Y., Song, C., Yoshikazu, S., and Keiichi, I. (1983), *J. Biol. Chem.* **258,** 5045–5049.
11. Frank, B. H., Pettee, J. M., Zimmerman, R. E. and Burck, P. J. (1981), in *Peptides, Synthesis, Structure and Function* (Proc. 7th Am. Pept. Symp.), Rich, D. H. and Gross, E., eds., Pierce Chemical, Rockford, IL, pp. 729–738.
12. Thim, L., Hansen, M. T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G., and Fiil, N. P. (1986), *Proc. Natl. Acad. Sci. USA* **83,** 6766–6770.