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대장균에서 인체 프로인슐린의 분비 발현 : 프로인슐린 융합체의 고분비 발현과 프로인슐린의 저분비 발현

강 엽
아주대학교 의과학연구소 내분비연구실

Export of Human Proinsulin in *E. coli* : High Export of Proinsulin Fusion Protein but not of Proinsulin Itself

Yup Kang
Laboratory of Endocrinology, Institute for Medical Science, Ajou Universisty, Suwon, Kyungi 441-749, Korea

ABSTRACT

To obtain a correctly folded human proinsulin, export of proinsulin using Staphylococcal protein A signal sequence-mediated secretion pathway has been attempted in *E.coli*. A secretion operon for proinsulin was constructed by consecutively connecting T7 promoter, SPA ribosome binding site, SPA signal sequence gene, and human proinsulin gene. Little immunoreactive proinsulin was detected in the periplasmic space and culture medium, and not even in cytoplasmic space. The qualitative analysis of transcribed proinsulin mRNA and the in vitro transcription/translation experiment suggests that the negligible level of proinsulin export appears to be due to intracellular degradation of proinsulin, rather than due to the blockage during translocation. However, expression of proinsulin fusion protein such as MBP-proinsulin could dramatically increase export of proinsulin in *E.coli*.

Introduction

Human insulin is a phamaceutically important hormone for controlling the blood glucose level in diabetic patients(1). Human insulin is converted from human proinsulin by enzymatic removal of middle C-peptide in secretory granule of pancreatic beta cell(2). Therefore, insulin is composed of two peptide, A-chain and B-chain. Expression of ß-galactosidase-A-chain and ß-galactosidase-B-chain in *E.coli*, in vitro cutting out of ß-galactosidase, and then in vitro reconstitution of these two chain could produce the human insulin, through the so-called recombinant DNA technique (3). However, nowadays, the human insulin is produced from recombinant human proinsulin due to higher refolding yield and due to the development of in vitro conversion method (4). Human proinsulin was also expressed as fusion protein in *E.coli* (5). To increase the final

yield of proinsulin, the size of fusion partner has been reduced (6). Kang and Yoon (7) reported the highest expression yield of proinsulin by using short fusion partner, strong promoter, and good ribisome binding site. However, the expressed proinsulin fusion protein was also accumulated as inclusion body, thus, the processes for denaturation, in vitro cutting out of fusion partner and refolding were still required for obtaining a correctly folded proinsulun. In fact, all these processes are very complex and time-consuming, resulting in a low recovery of correctly folded proinsulin.

Therefore, an extracellular expression has been considered for production of a correctly folded proinsulin itself. There have been several reports on the secretion of rat and human proinsulin using a ß-lactamase promoter, ribosome binding site, and signal sequence in *E.coli*(8, 9). A folded proinsulin activity was detected mainly in periplasmic space. However, the yield of exported proinsulin was very low in all cases (less than 2 ~9ng/mg whole protein), as compared to that of intracellularly expressed proinsulin (250mg/ℓ). In attempts to further increase the yield, the signal sequence was modified(10) and a different promoter and ribosome binding site were used (11). However, the export yield was not significantly affected(12).

Since Staphylococcal protein A (SPA) fusion expression/secretion system is known to be a good system for secretion of human insulin-like growth factor (hIGFI) in *E.coli*, in terms of yield, stability, and folding(13), and since the human proinsulin shares a high similarity in amino acid sequence and a similar three-dimensional structure with hIGFI(14), the export of ZZ-proinsulin (Z : modified B domain of SPA) has been tried. We obtained the highest export yield of proinsulin when its connecting peptide (C-peptide) region was similar in size to that of hIGFI or when most of the connecting peptide region of the proinsulin was deleted(15). Thus, this study was initiated to see whether export of proinsulin itself would be increased by modification of the C-peptide. The SPA signal sequence-mediated proinsulin secretion vector was constructed, the C-peptide region was modified by sequential deletion, and then the export of proinsulin was invetigated by insulin RIA. No clone showing a significant export was selected. Therefore, to know the reason why little proinsulin was exported in *E.coli*, the quantitative and qualitative analysis of transcription and translation for proinsulin was attempted. Finally, the export of proinsulin fusion protein (MBP-proinsulin) was investigated.

Materials and Methods

Materials

All restriction endonucleases, DNA modifying enzymes and T_4 DNA ligase were purchased from GIBCO/BRL (Gaithersburg, MD) and NEB (Beverly, MA) and used as supplier's recommendation. Medium components for bacterial culture were obtained from Difco laboratory (Detroit, MI). All reagents for PAGE (polyacrylamide gel electrophoresis), including acrylamide, bis-acryl amide, ammonium persulfate and TEMED were provided from Bio-Rad (Hercules,CA). For western blotting, nitrocellulose membrane was supplied by MSI (Westborough, CA), and alkaline phosphatase conjugated anti-mouse IgG antibody, bromo-chloro-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were purchased from Sigma (St.Louis, MO). For RNA dot hybridization, nylon membrane was supplied from Bio-Rad and all reagents for hybridization were obtained from Sigma. ^{32}P-dCTP was purchased from NEN /Dupont. Most of general reagents were also obtained from Sigma.

Plasmid and bacteria

The plasmid pTZ18R and pEZZ18 were purchased from Pharmacia Biotech (Piscataway, NJ). pTZ18R-PI, source for proinsulin gene, has been described by Eun et al(16). The pMAL-p2 was obtained from NEB. The pAED4'91 containing T_7 promoter was kindly provided from Dr M. Walsh (University of Calgary). *E.coli* JM109 [F', recA1, supE44, endA1, hsdR17, gyrA96, relA1,

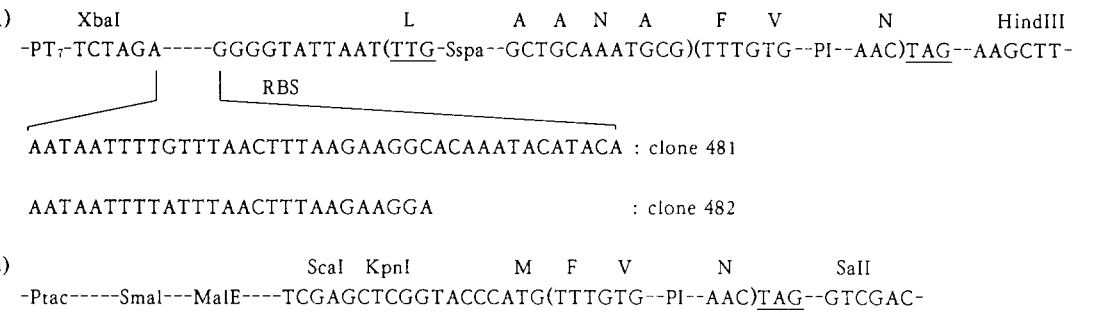

Fig. 1. Schematic diagram of proinsulin export operons and nucleotide sequence of pAED4'91-Sspa-PI (A) and pMAL-p2-PI (B) P: promoter. S:signal sequence. SPA:Staphylococcal protein A. PI: proinsulin. Mal:maltose binding protein. RBS:ribosome binding site. Initiation codon (TTG) and termination codon (TAG) were underlined. Amino acid sequence was denoted alphabetically. The clones (481 and 482) with different size of nucleotides on upstream region of translation initiation sequence were also listed.

thi, △ (lac⁻,proAB)] was used as host for general plasmid manipulation and expression of MBP-proinsulin. *E.coli* HMS262(thr, leu, lacY, thi, supE, hsdR, ton, trx) containing the vector pGP1-2 (17) and *E.coli* BL21 [(lac^{am}, trp^{am}, pho^{am}, mal^{am}, supCts, rpsL, tsx:Tn10, lon delta 100, htpR^{am}), DE3 lysogen, pLYS] were used as host for T_7 expression system(18).

Molecular cloning

Cloning procedure for plasmid preparation, restriction enzyme digestion, modification of DNA and ligation were carried out according to Sambrooks et al(19). Plasmid introduction into *E. coli* was carried out by calcium-dependent transformation(19).

Construction of pAED4'91-Sspa-PIs and pMAL-p2-PIs

The promoter region of SPA was removed out by sequential deletion with Bal31 after digestion of vector pEZZ18 with NotI. Two clones (481 and 482) containing different size between promoter and translation initiation region were selected. The 3'-end of SPA signal sequence gene was processed by FspI digestion of pEZZ18 and by two base deletion with slow Bal31 (IBI. Newheaven, CT). The isolated SPA siganl sequence (Sspa) gene including translation initiation region was incorporated into pTZ18R, resulting in pTZ18R-Sspa. The proinsulin gene was prepared by digestion of pTZ18R-PI with XbaI, three base deletion with Bal31, and HindIII digestion. This proinsulin gene was introduced into 3'-end of the SPA signal sequence to make correct signal sequence cutting site. The resultant plasmid was designated pTZ18R-Sspa-PI. The Sspa-PI was isolated from pTZ18R-Sspa-PI by XbaI and HindIII digestion and inserted into the corresponding site of pAED4'91, resulting in a construction of pAED4'91-Sspa-PI(Fig. 1). The pAED4'91-Sspa-PIs containing modified C-peptide were constructed by AvaI digestion in the middle of C-peptide gene, Bal31 digestion, and self ligation. To investigate the export of the proinsulin fusion protein, a battery of plasmid pMAL-p2-PIs was constructed. The proinsulin genes were isolated from pEZZ18-PI, pEZZ18-PI 4, pEZZ18-PI 9, and pEZZ18-PI 12 (15) by SacI and HindIII digestion and were insereted into the corresponding site of pMAL-p2 vector(Fig. 1).

RNA dot hybridization

Total RNAs were isolated from *E.coli* by the procedure described by Ausubel et al(20). The in vitro transcribed proinsulin RNAs were used as

control. Human proinsulin probe was prepared by random primer labelling method with ^{32}P-dCTP (21). After blotting 2 and 0.4 ug of extracted RNA onto nylone membrane, the membrane was baked and preblocked under prehybridization solution [0.05% NaPPi, 0.5% SDS, 6xSSC (3M NaCl, 0.3M sodium citrate, pH 7.0), 10x Denhardt solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 50ug/ml of salmon sperm DNA, 0.01 M EDTA]. Hybridization was performed by adding the labelled probe and incubating at 65℃ for 16 hours. After sequentially washing the membrane with 2x SSC and 0.2x SSC contaning 0.1% SDS at 55℃ for 30 minutes, the membrane was exposed on X-ray film(22).

In vitro transcription/translation

To investigate the capability of constructed vector (pAED4'91-Sspa-PI) to synthesize the proinsulin precursor, the coupled transcription and translation of proinsulin was performed with *E.coli* cytosolic extract(23). After mixing 15 ul of vector pAED4'91-Sspa-PI (2 ug) and 20 ul of premix solution (Promega BIotech, WI) containing all substrates for transcription and translation, the reaction was started by adding 15 ul of *E.coli* S30 extract (Promega Biotech) and incubating at 37℃ for 1 hour.

Expression and analysis of proinsulin

E.coli HMS262 (pGP2-1) harbouring pAED4' 91-Sspa-PI was grown in 2 YT medium containing 50ug/ml of ampicillin and 20ug/ml of kanamycin. The expression of proinsulin was started by temperature shift from 34℃ to 42℃ at OD_{580} of 0.8 . *E.coli* JM109 containing pMAL-p2-PIs was also grown in the same medium with 50ug /ml of ampicillin. MBP-proinsulin was expressed by addition of IPTG (isopropyl thiogalactopyranoside). Cultured cell and medium were collected 4 hours after induction. Periplasmic proteins were extracted by osmotic extraction method(15). The exported proinsulin in culture medium and periplasmic space was quantitated by insulin radioimmunoassay(15). The immunoreactive proinsulin was detected by western blotting (15) with anti C-peptide antibody and anti A-chain specific monoclonal antibody (AE9D6) (24).

Results and Discussion

Export of human proinsulin using Staphylococcal protein A signal sequence

To produce a correctly folded proinsulin in *E. coli*, an extracellular expression of proinsulin using SPA signal sequence-mediated secretion pathway was tried. The SPA signal sequence has been proved to be functional for secretion of heterologous protein in *E.coli*(13). Our previous report showed that ZZ-proinsulin analogues could be exported through this SPA signal sequence-mediated secretion pathway(15). Two vectors, pAED4'91-Sspa-PI 481 and pAED4'91-Sspa-PI 482, harbouring T_7 promoter, SPA signal sequence gene, and proinsulin gene, but with different distance between promoter and translation initiation site were constructed. As a host, *E.coli* BL21 was firstly selected. This strain contains T_7 RNA polymerase gene in DE3 lysogen and the T7 RNA polymerase gene was regulated by lacUV5 promoter. Furthermore, this strain is deficient in the lon protease and ompT protease, resulting in stable expression of heterologous protein(18). Thus, this strain seemed to be the best strain for extracellulr expression of folded proinsulin under T_7 promoter. However, strangely, all BL21 cells were completely lysed after addition of IPTG, probably due to the action of the cloned lysozyme. In fact, we do not know why lysozyme activity was leaked. One assumption is that the expressed proinsulin precursor affected secretion apparatus located in membrane and induced the leakage of lysozyme. Culture broth did not contain folded proinsulin (Table 1), since insulin RIA did not show any insulin activity in lysed broth. Therefore, we changed to *E.coli* HMS262 strain for extracellular expression of proinulin. However, we did not detect immunoreactive proinsulin activity in both periplasmic extract and

Table 1. Quantitative analysis of proinsulin and fused proinsulin in E.coli

Vector/Host	Induction	Form of insulin	Exported proinsulin[a] (mg/ℓ)
pAED4'91-Sspa-PI481 /HMS262	Heat	Proinsulin	ND[b]
pAED4'91-Sspa-PI482 /HMS262	Heat	Proinsulin	ND
pAED4'91-Sspa-PI /BL21	IPTG	Proinsulin	NT[c]
pMAL-p2-PIc/JM109	IPTG	Proinsulin	46.5
pMAL-p2-PI4/JM109	IPTG	Proinsulin An[d]	ND
pMAl-p2-PI9/JM109	IPTG	Proinsulin An	41.7
pMAL-p2-PI12/JM109	IPTG	Proinsulin An	43.1

a : The amount of immunoreactive exported proinsulin (culture medium and periplasmic fraction). After osmotic extraction, total periplasmic proteins were appropriately diluted with PBS (phosphate buffered saline). The amount of proinsulin, fused proinsulin, or fused proinsulin analogues was determined by quantitation with insulin RIA and multipling the crossreactivity factor (2.5 fold) and molecular ratio (portion of fusion partner)

b : not detected (less than 1 ng/ml)

c : not tested due to cell lysis

d : proinsulin analogue with modified C-peptide.

culture broth (Table 1). Since our previous study showed that the modification of C-peptide in ZZ-proinsulin would modulate the export yield of proinsulin, we modified the C-peptide region in proinsulin gene by sequential digestion of C-peptide gene with Bal31 exonuclease after AvaI cutting. After transformation of *E.coli* HMS262 with thease modified pAED4'92-Sspa-PIs, more than 200 clones containing different sizes of C-peptide were screened by insulin RIA of osmotic extract. However, no clone showed remakable improvement of proinsulin export since all clones showed negligible amount of insulin immunoreactivity.

Investigation of transcription and translation of proinsulin

To determine which step is critical for the export of proinsulin, total cellular proteins isolated

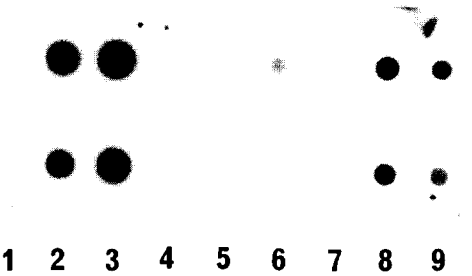

Fig. 2. Autoradiogram of proinsulin RNA. 4ug (upper part) and 0.4ug (lower part) of cellular RNA with (lanes 7, 8, 9) and without (lanes 4,5,6) induction and in vitro transcribed RNA (lanes 1,2,3) were blotted, respectively, and then hybridized with labeled proinsulin cDNA. Radioactivity was exposed on X-ray film. Lanes 1,4,7; Control pAED4'91. Lanes 2,5,8: pAED4'91-Sspa-PI 481. Lanes 3,6,9: pAED4'91-Sspa-PI 482.

from *E.coli* HMS262 containing pAED4'92-Sspa-PI were analysed by western blotting. To detect denatured proinsulin as well as folded proinsulin, monoclonal antibody against C-peptide was used. No immunoreactive proinsulin band appeared in cytoplasmic fraction as well as in periplasmic fraction. In addition, anti A-chain specific monoclonal antibody AE9D6 did not react with any cytoplasmic and periplasmic protein in spite of modification of C-peptide region. This result suggests that the proinsulin is not synthesized under given expression system or an expressed proinsulin is unstable and rapidly degraded. Therefore, to know which assumption is right, the capability of constructed vectors to transcribe and translate proinsulin was investigated. The transcription was analyzed by quantitative comparison of cytosolic proinsulin mRNA after heat induction. As shown in fig. 2, the level of proinsulin mRNA was remarkablly increased after temperature induction. Both vectors, 481 and 482, were able to transcribe the proinsulin mRNA, supporting that the transcription step is

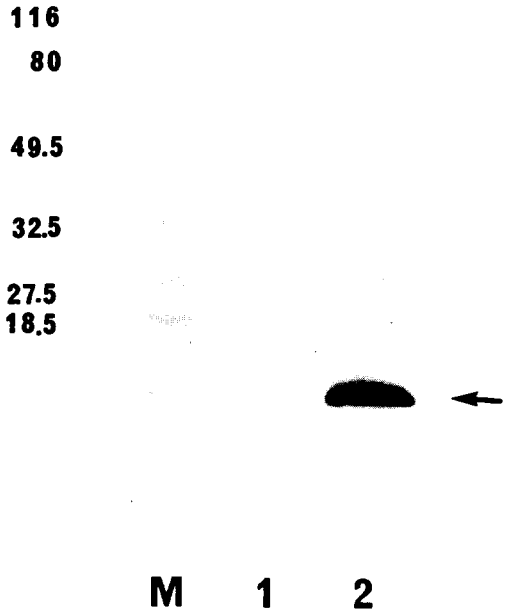

Fig 3. Western blot of in vitro coupled tranascription/translation product. Coupled in vitro transcription and translation of pAED4'91-Sspa-PI 481 was carried out with S30 extract. The translated proteins were separated on 10~15% PAGE and transfered onto a nitrocellulose membrane. The immunoreactive band was detected with anti C-peptide antibody. M: Prestained protein molecular weight standards (kDa) (Bio Rad). Lane 1: Translated product from pAED4'91. Lane 2: Translated product from pAED4'91-Sspa-PI 481.

not problematic for proinsulin export. The capabilty of translation was investigated by coupled in vitro transcription/translation method. The proinsulins were translated in *E.coli* cytosolic extract with all substrates for protein synthesis. The synthesized protein was fractionated on PAGE gel and then an immunoreactive proinsulin band was detected by western blotting with anti C-peptide antibody. Only one distint band appeared in 13-14 kDa region(Fig. 3), sugessting that immunoreactive proinsulin could be translated from our constructed vector. On the basis of this transcription and translation experiment, we concluded that no detection of immunoreactive proinsulin band under western blotting experiment seemed to be due to rapid degradation of translated proinsulin rather than problem of transcription and translation. Hence, the little export of proinsulin appears to be due to limited supply of proinsulin precursor rather than abnormal accumulation or translocation blockage of pro-insulin precursor(12). This result also suggests that instability of target protein in cytoplasm is one cause of defective extracellular expression. In fact, it is well known that most intracellularly expressed eukaryotic proteins are rapidly degraded by *E.coli* intracellular protease(25). Other investigators also reported very low secretion of proinsulin in *E.coli* even when they used different signal sequences(10, 11). Interestingly, intracellular stability of proinsulin was not improved in spite of modification of C-peptide, while stability of ZZ-proinsulin was modulated by C-peptide modification(15). Therefore, proinsulin itself seems to be very susceptible to *E.coli* intracellular protease.

Export of MBP-proinsulin using MBP signal sequence

Our previous result showed that the export yield of ZZ-proinsulin using SPA signal sequence was low (2.1mg/ℓ) and most of translated ZZ-proinsulin was accumulated in cytoplasm. In fact, the low yield of ZZ-proinsulin export seemed to be due to low efficiency of translocation rather than intracellular degradation of translated ZZ-proinsulin. Furthermore, modification of C-peptide would modulate the translocation efficiency as well as intracellular stability. To know whether another proinsulin fusion protein can be exported efficiently, and whether the effect of modification of C-peptide on export is general, export of MBP-proinsulin was tried under MBP promoter and MBP signal sequence. The proinsulin gene (ZZ-proinsulin: low export yield) and modified

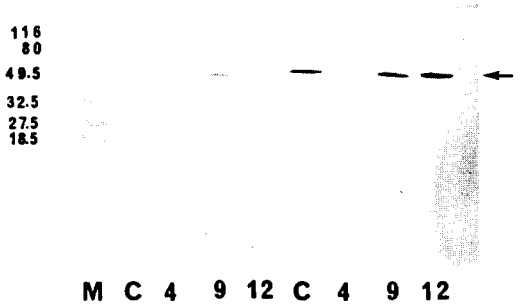

Fig. 4. Western blot of expressed proteins from pMAL-p2-PIs clones E.coli JM109 transformed with pMAL-p2-PIs was grown in 2YT-amp medium. MBP-proinsulin and its analogues were expressed by IPTG induction. Cytosolic fraction and peri-plasmic fraction was separated by osmotic extraction. The proteins were fractionated on 10~15% PAGE. Immunoreactive proinsulins were detected by western blotting with AE9D6 antibody. M:Prestained molecular weight standards (kDa). Left four lanes (C,4,9,12):Cytosolic fraction. Right four lanes (C,4,9,12):Periplasmic fraction. C:Clone pMAL-p2-PI. Lanes 4,9, 12:pMAL-p2-PI analogues containing modified C-peptide (15).

proinsulin gene (ZZ-proinsulin clone 4: no export, clone 9 and clone 12: high export yield) were isolated from each pEZZ-PI and pEZZ-PI analogue plasmid and were inserted into C-terminal end of MBP gene. The MBP-proinsulin was expressed by chemical induction (IPTG) and analysed by western blotting with AE9D6 antibody. Interestingly, the MBP-proinsulin was well exported and the precursor form of MBP-proinsulin was not detected in cytoplasmic fraction (Fig. 4), while most ZZ-proinsulin was accumulated in cytoplasm when the ZZ protein was fused to proinsulin. Furthermore, MBP-proinsulin analogues with modified C-peptide region (Clone 9 and clone 12) also showed high efficiency of export. However, MBP-proinsulin analogue from clone 4 was not detected in both cytoplasm and periplasm, suggesting that there was a rapid degradation in cytoplasm. These experiments suggest that addition of stable fusion partner can improve the export of target protein as well as stability. However, we can not predict which direction, such as degradation, accumulation, or export, the target protein would be driven by modification of mature domain. Exported MBP-proinsulin appeared to have their natural structure of proinsulin, since the exported MBP-proinsulin was detected under non-reducing conditions. In fact, monoclonal antibody AE9D6 fully recognizes the A-chain of insulin, in conjunction with B-chain(23). Furthermore, the insulin antibody used in insulin RIA recognizes only folded insulin or proinsulin. Although exported MBP-proinsulin reached to 46mg/ℓ, proinsulin portion is less than 10mg/ℓ since over 80% of MBP-proinsulin is MBP portion(Table 1). And, in vitro cutting out of MBP is required to obtain proinsulin. Therefore, it seems to be difficult to apply this MBP-proinsulin export system for production of human insulin and further study is needed to apply extracellular expression for production of human insulin.

요 약

자신의 3차구조를 가진 인체 프로인슐린을 얻기 위하여 Staphylococcal 프로테인 A(SPA)의 신호 펩타이드를 이용하여 대장균내에서 분비 발현을 시도하였다. 분비 발현을 위해 T7 프로모터, SPA 리보좀 바인딩 부위, SPA 신호 펩타이드, 프로인슐린 유전자를 연속적으로 연결하여 분비 벡터를 구성하였다. 이 벡터를 대장균에 넣은후 발현을 유도했으나 면역적으로 반응하는 인체 프로인슐린은 배양액이나 페리프라스믹 공간에서 거의 존재하지 않았으며 세포 내에도 존재하지 않았다. 그러나 발현 유도시 세포 내에 프로인슐린 RNA가 급격히 증가하였으며 구성한 벡터는 실험실적으로 프로인슐린을 전사(transcription), 번역(translation) 할 수 있었다. 이는 프로인슐린이 번역 후 급히 세포내에서 분해됨을 의미하며 이로 인해 분비된 프로인슐린을 거의 얻을 수 없게 된 것으로 생각된다. 그러나 프로인슐린의 세포내 안정성을 위해 말토즈 바인딩 프로테

인을 융합짝으로 프로인슐린에 연결한 경우 과량의 분비된 인체 프로인슐린을 검출할 수 있었다.

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