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# Expression of Monomeric Insulin Precursor in *Pichia pastoris* and Its Conversion into Monomeric B27 Lys Destripeptide Insulin by Tryptic Hydrolysis

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**Abstract** Monomeric B27 Lys destripeptide insulin (B27 Lys DTrI) was designed and produced from its precursor expressed in *Pichia pastoris* through tryptic hydrolysis instead of the less efficient tryptic transpeptidation. The monomeric B27 Lys DTrI precursor (MIP) was purified from a cultured medium of *P. pastoris* by a combination of hydrophobic, size-exclusion, and ion-exchange chromatography. The purified MIP was converted, by tryptic hydrolysis, to B27 Lys DTrI, which was then purified by ion-exchange chromatography to homogeneity as assessed by native gel electrophoresis, HPLC, amino acid composition, and electrospray mass-spectrometric analysis. B27 Lys DTrI exhibited superior monomeric properties in size-exclusion chromatography. The yield of MIP was 200 mg per liter of culture, and the overall yield of purified B27 Lys DTrI from the crude MIP was 70%. The *in vivo* biological activity of B27 Lys DTrI as determined by the mouse convulsion assay was 21 U/mg, identical to that obtained by semisynthesis.

**Key words** monomeric B27 Lys destripeptide insulin; tryptic hydrolysis; *Pichia pastoris*

Since the discovery of insulin by Banting and Best, diabetics have been treated successfully with animal insulin and recombinant human insulin. To achieve improved glucose control, novel insulins with faster action have been developed by protein engineering [1,2]. While *in vivo* insulin is secreted into the bloodstream rapidly, injected insulin takes 30 min or more to reach its highest level in the blood. The slow absorption of injected insulin is due to the self-association of insulin monomers into dimers and hexamers. To accelerate the absorption process, monomeric forms of insulin with decreased association tendency have been engineered. For example, monomeric Humalog with swapped B28 Pro, B29 Lys, and NovoRapid with B28 Pro replaced by Asp, are available commercially. In our early studies, despentapeptide insulin (DPI), a truncated form of insulin that is missing residues 26–30 from its B-chain (B26–30) was found to be monomeric and biologically active [3]. Similarly, monomeric destetrapeptide insulin (DTI, human insulin with B27–30 truncated) was prepared from its precursor expressed in *Saccharomyces cerevisiae* through tryptic transpeptidation at B22 Arg in the presence of a synthetic tetrapeptide [4]. Later, semisynthetic B27 Lys DTrI (human insulin with B28–30 removed and B27 Thr replaced by Lys) was shown to be active and monomeric [5]. With a Lys residue at the C-terminus of the B-chain, this form of insulin could be obtained from its precursor through tryptic hydrolysis instead of tryptic transpeptidation. Tryptic hydrolysis is much more efficient than tryptic transpeptidation, with the additional advantage that no synthetic tetrapeptide is needed.

The expression of the B27 Lys DTrI precursor was

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carried out in the *Pichia pastoris* system that we have previously used to successfully produce the human insulin precursor in large quantities [6].

## Materials and Methods

### Materials

Zinc-free insulin, desoctapeptide insulin (DOI, human insulin with B23–30 removed), DPI, and B28 Lys,B29 Pro insulin were prepared in our lab. The single chain insulin precursor (SCI) was a gift from Shenglongda Biotechnology Company. The culture medium reagents were from Oxoid. The restriction enzymes, T4 ligase, dNTPs and *Taq* polymerase were from TaKaRa. The insulin RIA kit was from the Shanghai Institute of Biological Products. The XAD-7 resin, TPCK-trypsin, and BSA were from Sigma. The Sephadex G-25, SP Sepharose fast flow and Superdex 75 HR 10/30 columns were from Amersham. The G418 sulfate was from Calbiochem-Novabiochem.

### Strains, media and high-density fermentation

The *E. coli* strain DH5α was used for cloning. The *P. pastoris* strain GS115 (His 4) and plasmid pPIC9K were from Invitrogen. The YPD, MD and MM plates, the YPG, YPM (1% yeast extract, 2% polypepton, 3% methanol), basal salt medium (BSM) and trace element solution (PTM1) were prepared according to *P. pastoris* protocols [7]. High-density fermentation was performed in a Bioengineering KLF 2000 fermentor according to *P. pastoris* protocols with minor modifications.

### Purification of monomeric B27 Lys DTrI precursor

The cultured medium was centrifuged at 6000 rpm for 10 min and the supernatant was analyzed by 15% native polyacrylamide gel electrophoresis (PAGE) run at pH 8.3 according to the method of Gabriel [8] to determine the concentration of monomeric B27 Lys DTrI precursor (MIP). An XAD-7 hydrophobic column was used to adsorb the MIP from the cultured medium according to the method used by Wang *et al.* [6]. The concentrated solution was eluted from the XAD-7 column and loaded onto a Sephadex G-25 column. The crude MIP was then eluted with 1 M acetic acid. The eluted protein solution was purified by SP Sepharose fast flow ion-exchange chromatography to obtain highly purified MIP.

### Tryptic hydrolysis of MIP

MIP was dissolved in 0.1 M potassium phosphate buffer (pH 7.5) to a concentration of 5 mg/ml. TPCK-trypsin was added to produce a concentration of 0.025 mg/ml. The reaction mixture was incubated at 30 °C for 1 h. Hydrolysis was stopped by lowering the pH to 4.0, and B27 Lys DTrI was purified by SP Sepharose fast flow chromatography.

### Protein estimation

The protein concentration was determined by ultraviolet absorption at 280 nm. The absorbance for insulin is 1.0 at a concentration of 1 mg/ml and a path length of 1 cm, whereas the absorbance values for DPI, B28 Lys,B29 Pro insulin, and B27 Lys DTrI are 0.88, 1.01 and 1.07, respectively [9].

### Bioassay

A semi-quantitative mouse convulsion assay was used to analyze *in vivo* biological activity. For each dosage, ten male ICR mice (fasted; body weight 20–22 g) were injected subcutaneously and kept in a 35 °C chamber. Their responses were observed according to the "Chinese Pharmacopoeia" (1995).

### Self-association assay

Self-association was determined by size-exclusion chromatography [2,4] using a Superdex 75 HR 10/30 column. MIP was eluted with phosphate-buffered saline (pH 7.4) at a flow rate of 0.8 ml/min. The distribution coefficient $K_D$, inversely related to molecular weight, was plotted *vs.* protein concentration as **Equation (1)**,

$$K_D = \frac{V_r - V_o}{V_c - V_o} \quad (1)$$

where $V_r$ is the retention volume, $V_o$ is the void volume, and $V_c$ is the total bed volume. Molecular homogeneity, $F_s$, was measured by symmetry factor as **Equation (2)**,

$$F_s = \frac{W_{0.05h}}{2A} \quad (2)$$

where $W_{0.05h}$ is the band width at 0.05 peak height and $A$ is the width of the first half peak at 0.05 peak height.

## Results

### Construction of the expression vector pPIC9K/MIP

The DNA encoding MIP was chemically synthesized according to the codon usage in *P. pastoris* [10] (**Fig. 1**), and cloned into the plasmid pPIC9K between *Eco*RI and

```
    5′gaattcaag ttc gtc aac caa cac ttg tgt ggt tcc cac
                 F   V   N   Q   H   L   C   G   S   H
ttg gtc gag gct ttg tac ttg gtc tgt ggt gaa aga ggt ttc
 L   A   E   V   L   Y   L   V   C   G   E  R  G   F
ttc tac aag gct gct aag ggt atc gtc gaa caa tgt tgt acc
 F   Y  K   A   A  K   G   I   V   E   Q   C   C   T
tcc atc tgc tcc ttg tac caa ttg gag aac tac tgt aac tag
 S   I   C   S   L   Y   Q   L   E   N   Y   C   N   *
gcggccgc 3′
```

**Fig. 1 Nucleotide and amino acid sequence of MIP**

The 5′ end and 3′ end are *Eco*RI site and *Not*I site, respectively.

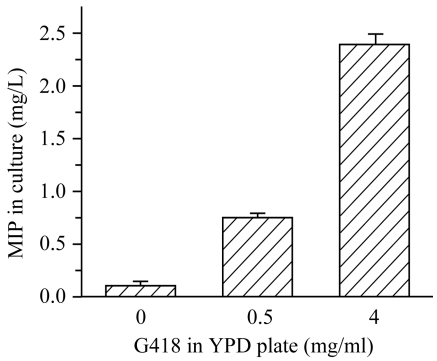


**Fig. 2 MIP expression levels of Mut$^+$ clones to different concentrations of G418**

*Not*I sites to obtain pPIC9K/MIP. The secretory protein has the following structure: [α-MFL-KR]-EAEAYVEFK-[insulin B-chain$^{1–21}$]-RGFFYKAAK-[insulin A-chain$^{1–21}$], where KR is a dibasic endoprotease-processing site in *P. pastoris*. The α-mating factor leader sequence (α-MFL) directed the secretion of MIP, and a removable spacer EAEAYVEFK increased the yield of the precursor in *P. pastoris* [11].

## Screening of high expression clones

The 5 μg plasmid pPIC9K/MIP was linearized by cutting with *Bgl*II and then transferred to an ice-cold 4-mm electroporation cuvette containing *P. pastoris* GS115 cells. After incubation in an ice bath for 5 min, the cells were pulsed twice at 2.4 kV, 5.3 ms each time, using the Finnigan MATLCQ ESI-MS instrument. The transformed cells were plated onto MD plates for histidine selection. A total of 1500 single colonies were cultured in 2 ml YPD medium for 24 h at 30 °C, then 2 μl culture was grown on the YPD plates containing 0.5, 1, 2, and 4 mg/ml G418 for *in vivo* multiple insert screening. Eighty G418-resistant colonies were inoculated onto both MD plates and MM plates. Forty Mut$^+$ colonies grown on the MM plate were further screened. Single Mut$^+$ colonies were cultured in 2 ml YPD medium for 48 h at 30 °C, then in YPM medium for 72 h. After dilution 400-fold with PBS containing 0.5% BSA, the MIP concentration in the supernatant was determined by radioimmunoassay (**Fig. 2**). Clone 5 was found to have the highest expression level that remained stable for eight generations. Clone 5 was therefore used in the 2-liter scale fermentation.

## Fermentation

Twenty milliliters of YPG in a 100-ml flask was inoculated with strain 5 and cultured in a shaking incubator for 24 h at 30 °C. Then 20 ml culture was added to 200 ml YPG in a 500-ml flask and cultured for 10 h to an $A_{600}$ of 4.0. The culture was added to 2 l BSM and 8 ml PTM1 in a 3.3-liter fermentor. The temperature, pH, and dissolved oxygen (DO) were set at 29 °C, 5.0 and 30%–35%, respectively. The first step was a glycerol batch phase to generate cell mass. After about 20 h, glycerol was completely exhausted, and the DO reading rose sharply. In the second step, 50% glycerol containing 0.25% PTM1 was fed for 4 h at a rate of 13 ml/h per liter of culture. The feeding rate was then ramped up to 21 ml/h per liter of culture. A DO spike was performed at 0.5, 1, 2, 3, and 4 h to ensure that glycerol was not accumulating. After 4 h, glycerol feeding was terminated. For the third step, methanol (containing 0.2% PTM1) was added at a feeding rate of 3 ml/h per liter of culture and ramped up to 12 ml/h per liter of culture within 8 h. DO was maintained at 20%–30%. The culture was harvested when the wet cell weight reached 400 mg/ml (**Fig. 3**). Analysis of the culture

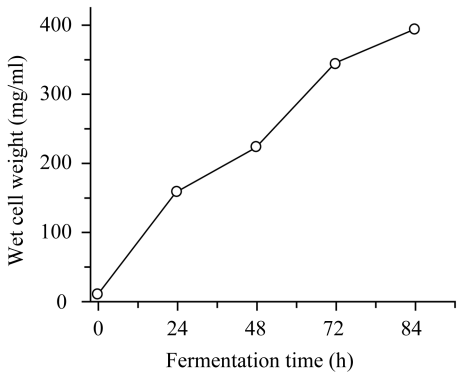


**Fig. 3 Wet cell weight increase in the high-density fermentation of clone 5**

supernatant by using 15% native PAGE run at pH 8.3 showed two major bands representing MIP and MIP with a spacer (**Fig. 4**). The total concentration of MIP was 200 mg per liter of culture as assessed by Coomassie brilliant blue staining (**Fig. 4**).

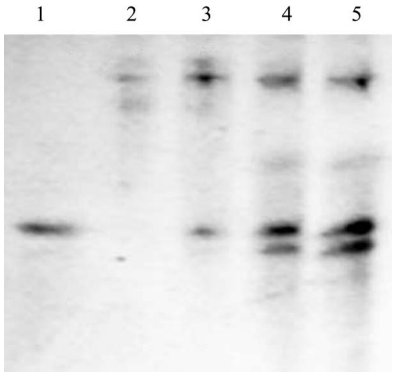


**Fig. 4 Expression of MIP as shown by native PAGE**

1, SCI (2 μg); 2–5, 20 μl supernatant of the culture grown for 24, 48, 72, and 84 h, respectively.

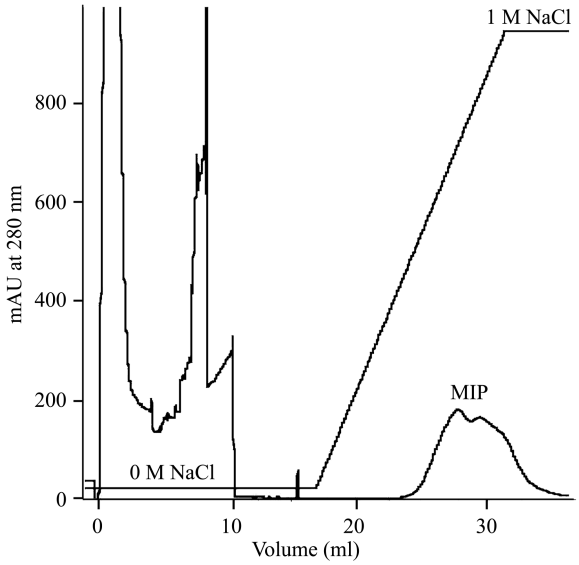


**Fig. 5 SP Sepharose fast flow ion-exchange chromatography of MIP**

After washing the column with pH 4.0, 0.05 M acetate initial buffer, MIP was eluted with a linear gradient of NaCl from 0 to 1 M in the initial buffer. The absorbance of the eluent was monitored at 280 nm.

## Purification of MIP

Kjeldsen *et al.* [11] showed that the N-terminal extension of the secreted insulin precursor is partly cleaved by aspartyl protease 3 in *P. pastoris*. Similarly, in the present study, MIP was secreted with the N-terminal extension partly cleaved. Through XAD-7 hydrophobic chromatography, MIP was isolated from the culture supernatant. Lipid-like substances that formed in the course of fermentation [12] were not removed by the XAD-7 column, so the eluted product contained about 30% proteins and 70% lipid-like substances. The product was further purified by Sephadex G-25 and SP Sepharose fast flow chromatography (**Fig. 5**).

## Conversion of MIP into B27 Lys DTrI through tryptic hydrolysis

The time course of tryptic hydrolysis was monitored by native PAGE run at pH 8.3 (**Fig. 6**). The following optimal conditions were used: desalted MIP (5 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.5) was hydrolyzed with TPCK-trypsin at 30 °C for 60 min at an enzyme/substrate weight ratio of 1:200. The reaction mixture was adjusted to pH 4.0 with acetic acid and loaded onto an SP Sepharose fast flow column to remove unprocessed MIP and byproduct DOI (**Fig. 7**). The overall yield of B27 Lys DTrI purified from the crude MIP obtained by XAD-7 hydrophobic chromatography was 70%.

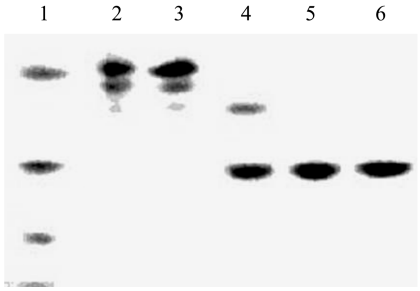


**Fig. 6 Native PAGE (pH 8.3) of MIP and B27 Lys DTrI**

1, SCI, insulin, and DOI, from cathode to anode; 2, MIP purified by XAD-7 column; 3, MIP purified by SP Sepharose fast flow column; 4 and 5, MIP digested by TPCK-trypsin for 30 and 60 min; 6, B27 Lys DTrI purified by SP Sepharose fast flow column.

## Characterization of B27 Lys DTrI and its *in vivo* biological activity

The purified B27 Lys DTrI was homogeneous as

assessed by native PAGE run at pH 8.3 (**Fig. 6**, lane 6) and HPLC (**Fig. 8**). The molecular weight determined by electrospray mass spectroscopy was 5509.0 (**Fig. 9**), the calculated value being 5508.3. The amino acid composition listed below was consistent with the theoretical value: R 1.07 (1), K 0.9 (1), H 2.16 (2), F 3.09 (3), Y 4.0 (4),

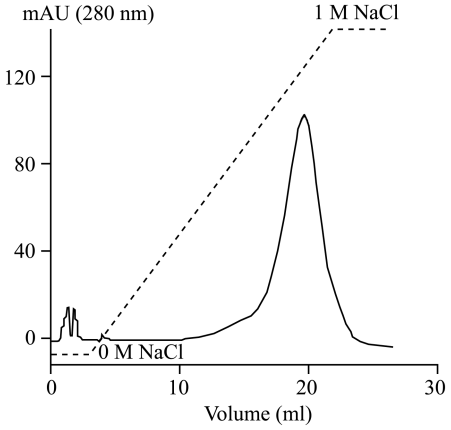


**Fig. 7 Purification of B27 Lys DTrI by SP Sepharose fast flow chromatography**

After washing the column with pH 4.0, 0.05 M acetate initial buffer, MIP was eluted with a linear gradient of NaCl from 0 to 1 M in the initial buffer. The absorbance of the eluent was monitored at 280 nm.

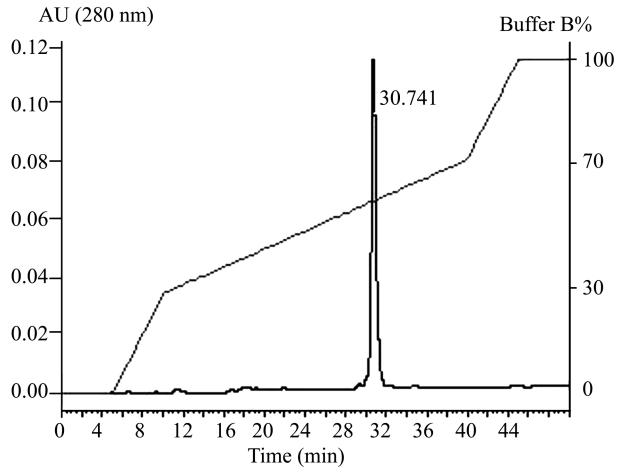


**Fig. 8 HPLC analysis of purified B27 Lys DTrI**

Chromatographic conditions: C8 column (4.6 mm×250 mm); solvent A, 0.1% TFA; solvent B, 70% acetonitrile/0.1% TFA, gradient 30% to 70% solvent B in 10–40 min; flow rate, 1 ml/min.

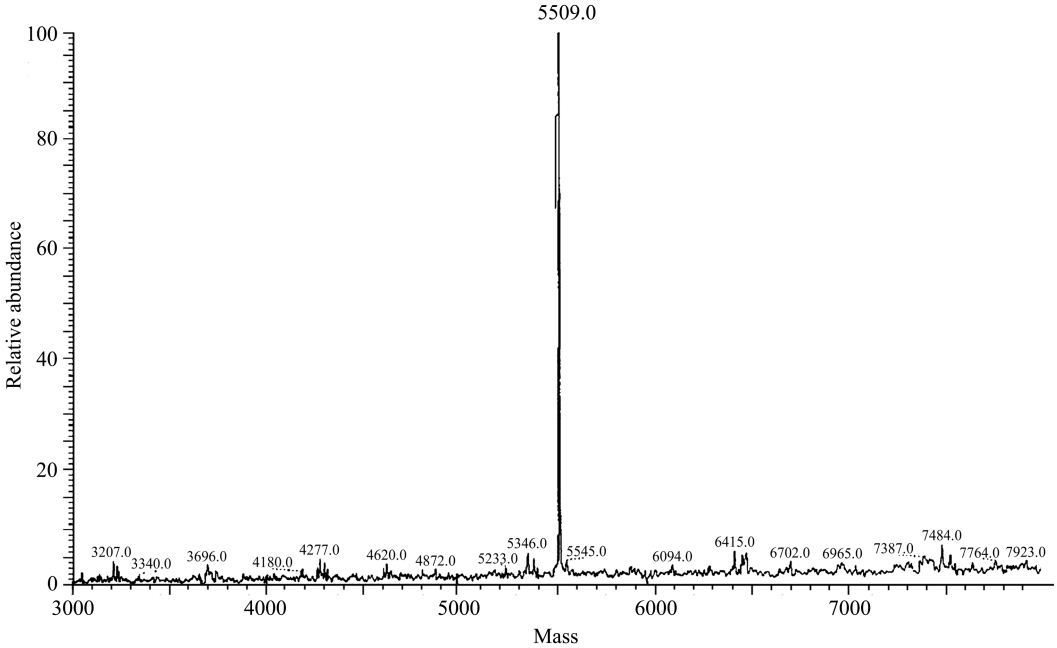


**Fig. 9 Electrospray mass-spectrometric analysis of B27 Lys DTrI using the Finnigan MATLCQ ESI-MS instrument**

Spray voltage was 4.25 kV, and capillary temperature was 200 °C.

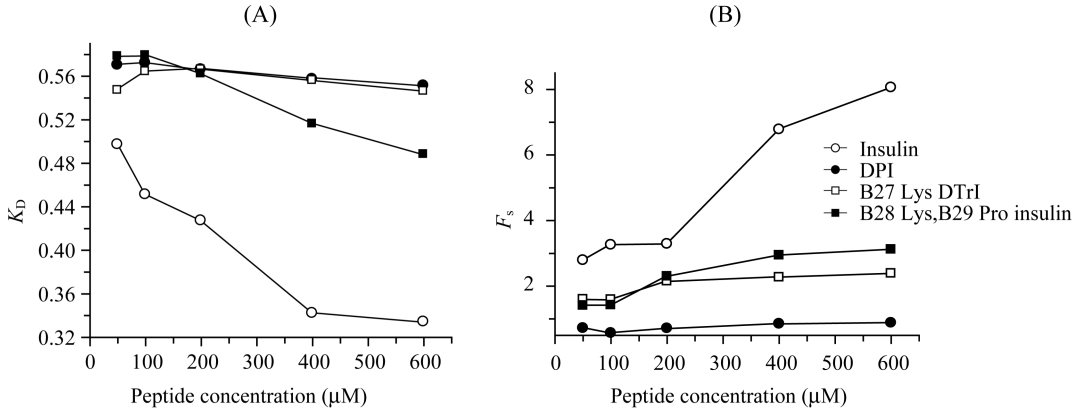


**Fig. 10 Effect of protein concentration on $K_D$ (A) and $F_s$ (B) of insulin, DPI, B27 Lys DTrI and B28 Lys,B29 Pro insulin**

L 5.6 (6), I 1.72 (2), V 3.75 (4), A 1 (1), G 3.7 (4), E/Q 7.6 (7), S 2.1 (3), T 0.82 (1), D/N 2.92 (3), C not determined (6). The *in vivo* biological activity of B27 Lys DTrI as determined by the mouse convulsion assay was 21 U/mg, identical to that of semisynthetic B27 Lys DTrI [5].

### Monomeric behavior of B27 Lys DtrI

The monomeric behavior of zinc-free insulin, DPI, B28 Lys,B29 Pro insulin, and B27 Lys DTrI were compared under the same conditions on a Superdex 75 HR 10/30 column (**Fig. 10**). DPI and B27 Lys DTrI showed better monomeric behavior than B28 Lys,B29 Pro insulin.

## Discussion

*P. pastoris* is suitable for expressing proteins to a high level through high-density fermentation [13]. The expression level of the insulin precursor and DTI precursor in *S. cerevisiae* is less than 40 mg per liter [4,14], whereas the expression level of MIP in *P. pastoris* is 200 mg per liter. Multiple insertions of the MIP gene into the *P. pastoris* chromosome, together with high-density fermentation, resulted in high-level expression of MIP. At the same cell density (wet cell weight 400 mg/ml), the concentration of MIP in the culture was 200 mg per liter while that of the insulin precursor was 500 mg per liter [6]. The lower expression level of MIP may be due to its monomeric nature [15,16].

In the treatment of diabetes mellitus, monomeric insulins with improved pharmacokinetic profiles have been developed. DTI was prepared from its precursor using tryptic transpeptidation, but the yield was very low [4], whereas the yield of B27 Lys DTrI from MIP through tryptic hydrolysis was much higher. The preparation of B27 Lys DTrI in *P. pastoris* using tryptic hydrolysis instead of tryptic transpeptidation is therefore a new and more efficient way to produce monomeric insulin.

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