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Human Proinsulin C-peptide from a Precursor Overexpressed in *Pichia pastoris*

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Abstract In this article we report the production of human proinsulin C-peptide with 31 amino acid residues from a precursor overexpressed in *Pichia pastoris*. A C-peptide precursor expression plasmid containing nine C-peptide genes in tandem was constructed and used to transform *P. pastoris*. Transformants with a high copy number of the C-peptide precursor gene integrated into the chromosome of *P. pastoris* were selected. In high-density fermentation in a 300 liter fermentor using a simple culture medium composed mainly of salt and methanol, the C-peptide precursor was overexpressed to a level of 2.28 g per liter. A simple procedure was established to purify the expression product from the culture medium. The purified C-peptide precursor was converted into C-peptide by trypsin and carboxypeptidase B joint digestion. The yield of C-peptide with a purity of 96% was 730 mg per liter of culture. The purified C-peptide was characterized by mass spectrometry, N- and C-terminal amino acid sequencing, and sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Key words proinsulin; C-peptide; *Pichia pastoris*

The incidence of diabetes is increasing rapidly. At present, approximately 150 million people suffer from this disease worldwide. In diabetic patients, various complications usually occur even with well-controlled blood glucose levels. No efficient medicine is currently available to prevent diabetic complications.

In pancreatic β-cells, C-peptide with 31 amino acid residues is cleaved off from proinsulin and co-secreted with insulin in response to glucose stimulation. Formerly, C-peptide was considered to possess no biological function other than its role in insulin biosynthesis. Recently, it was reported that C-peptide could ameliorate sensory nerve dysfunction, nephropathy, neuropathy [1,2], and impaired myocardial function [3] in patients with type I diabetes lacking endogenous C-peptide. In diabetic rats, C-peptide could prevent vascular and neural dysfunction, nephropathy, glomerular hypertrophy, albuminuria, glomerular hyperfiltration and hippocampal apoptosis [4–7]. It was also reported that C-peptide could stimulate rat renal tubular Na^+-K^+-ATPase activity [8]. These results suggest that C-peptide might be a new medicine to prevent diabetic complications. For pre-clinical and clinical studies, it is necessary to produce human C-peptide on a large scale. It is difficult to express a single C-peptide because of its low molecular weight and its random structure in solution. One strategy is to express it in the form of fusion protein. Another strategy is to express a gene encoding multiple copies of the small peptide [9,10] including the expression of C-peptide in *Escherichia coli* [11].

Here we report the expression of tandemly repeated C-peptide in *Pichia pastoris* and the enzymatic cleavage of the purified expression product to yield human C-peptide.

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Materials and Methods

Construction of plasmid p36DD/PIC9 to express C-peptide precursor

The flow sheet of plasmid construction is shown in **Fig. 1**. A double-stranded C-peptide gene of the sequence encoding C-peptide and an extra C-terminal Lys with 5′ *Bgl*II site and 3′ *Bam*HI site was constructed from six oligonucleotides (**Fig. 2**).

Each oligonucleotide (100 pmol) was phosphorylated in 10 μl reaction mixture containing 0.5 μl T4 polynucleotide kinase (New England Biolabs, Ipswich, USA), 10 nmol ATP and 1 μl buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol). One microliter of each phosphorylated oligonucleotide solution was mixed and incubated at 70 ºC for 10 min with subsequent cooling to room

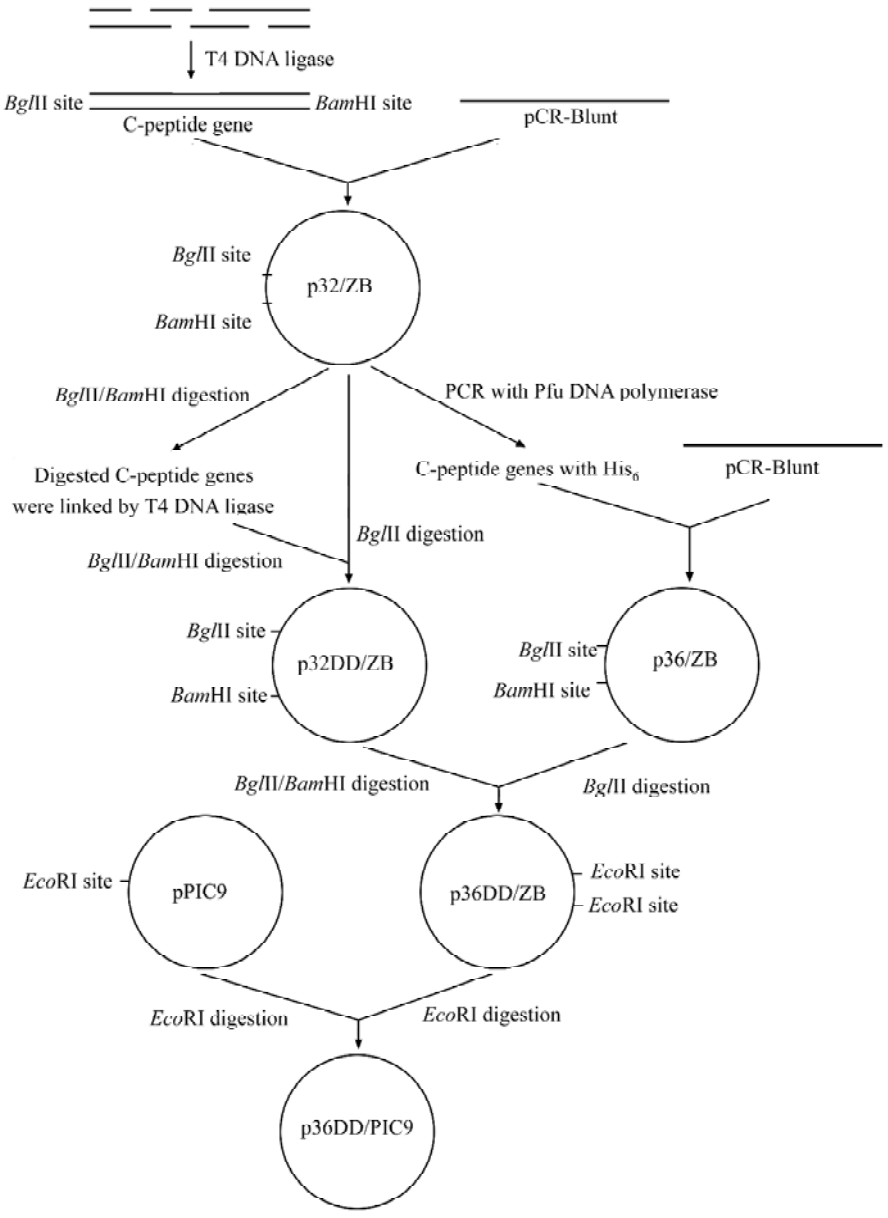

Fig. 1 Flow sheet of p36DD/PIC9 construction

Plasmid p32/ZB contains a single copy of the C-peptide gene; p32DD/ZB contains multiple copies of the C-peptide gene; p36/ZB contains a single copy of the C-peptide gene and the His_6 gene; p36DD/ZB and p36DD/PIC9 contain multiple copies of the C-peptide gene with the His_6 gene.

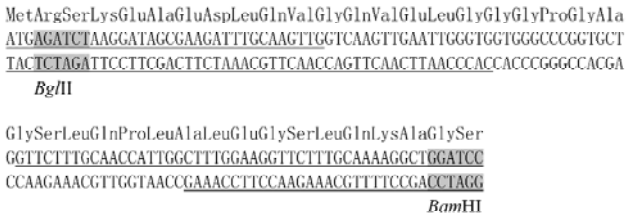

Fig. 2 Sequence of C-peptide gene encoding C-peptide and an extra C-terminal Lys

The 5′ and 3′ oligonucleotides are underlined and the 5′ *Bgl*II site and 3′ *Bam*HI site are shaded.

temperature. T4 DNA ligase (New England Biolabs) was added and the mixture was incubated at room temperature for 2 h. The double-stranded DNA containing the C-peptide gene was ligated with pCR-Blunt (Invitrogen, Carlsbad, USA) to obtain p32/ZB. As 5′ *Bgl*II and 3′ *Bam*HI in the C-peptide gene are isocaudarners, they were used to produce multiple copies of the C-peptide gene. The C-peptide gene in p32/ZB was digested with *Bgl*II/*Bam*HI (New England Biolabs) and joined by T4 DNA ligase to obtain multicopy C-peptide gene, followed by *Bgl*II/*Bam*HI digestion to cleave ligated product with wrong direction. The multicopy C-peptide gene was inserted into p32/ZB, which was linearized by *Bgl*II and digested with bovine small intestine phosphatase (New England Biolabs) to remove the 5′ phosphate group. p32DD/ZB containing the largest number of C-peptide gene was selected by PCR using T7 promoter and M13 reverse as primers. To facilitate characterization and purification, a His_6 tag was added to the C-peptide gene. PCR catalyzed by Pfu DNA polymerase (Promega, Madison, USA) was carried out using p32/ZB as the template and 5′-GGAATTCATGCATCATCATCATC-ATCATAGATCTAAGGAAGCTGAAGAT-3′ and 5′-GGAT-CCAGCCTTTTGCAAAGAACCTTCCAAAG-3′ as the primers. The PCR product was ligated with pCR-Blunt to obtain p36/ZB. *Bgl*II and *Bam*HI digested fragment was cleaved from p32DD/ZB and inserted into the *Bgl*II site of p36/ZB to obtain p36DD/ZB. A 1 kb DNA fragment containing the C-peptide tandem gene was cleaved from p36DD/ZB by *Eco*RI (New England Biolabs) digestion and inserted into the *Eco*RI site of pPIC9 to obtain the expression plasmid p36DD/PIC9. p36DD/PIC9 was confirmed by DNA sequencing and the C-peptide gene number was determined by 1.2% agarose gel electrophoresis after *Eco*RI digestion.

Transformation of *P. pastoris* by p36DD/PIC9

p36DD/PIC9 was linearized with *Sal*I and cloned into *P. pastoris* GS115 (his4; Invitrogen) by electroporation. The His^+ transformants were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). The chromosome DNA was extracted and spotted on a nylon membrane. The high expression transformant C-peptide/pPIC9 with a high copy number of the C-peptide precursor gene integrated into the chromosome was selected using the dot blotting method [12] using the C-peptide tandem gene as the probe.

Shake flask culture of transformant C-peptide/pPIC9

Transformant C-peptide/pPIC9 was grown in 20 ml YPD medium at 30 ºC for 2 d. The cells were collected by centrifugation, resuspended in 10 ml YPM medium (YPD medium with dextrose replaced by methanol) and grown for 3 d. After centrifugation, 10 μl supernatant was analyzed by SDS-PAGE and Western blot using mouse anti-C-peptide antibody (Dakocytomation, Glostrup, Denmark) as the first antibody and horseradish peroxidase-conjugated rabbit anti-mouse antibody (Sino-American Biotech, Shanghai, China) as the second antibody.

High density fermentation

High density fermentation was carried out basically according to *Pichia* protocols [13]. The following media were used. Each liter of the basal salt medium (BSM) contained 40 g glycerol, 27 ml H_3PO_4, 1 g $CaSO_4 \cdot 2H_2O$, 18 g K_2SO_4, 7 g $MgSO_4$, 1.68 g sodium citrate$\cdot 2H_2O$ and 4.0 g KOH. Each liter of the trace element solution (PTM1) contained 6 g $CuSO_4$, 0.08 g KI, 3 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 20 g $ZnCl_2$, 5 ml H_2SO_4, 65 g $FeSO_4 \cdot 7H_2O$, 0.5 g $CoCl_2 \cdot 2H_2O$ and 0.2 g biotin. PTM1 solution was sterilized by filtration. One liter of BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 10% yeast nitrogen base, 0.2% biotin, 0.1 M potassium phosphate buffer, pH 6.0) in a flask was inoculated and cultured for 20 h at 30 ºC. The culture was transferred to 11 liters of BSM medium containing 44 ml PTM1 in a 20 liter fermentor (Bioengineering, Wald, Switzerland) and cultured for 4 h. The culture was added to 100 liters of BSM medium containing 400 ml PTM1 in a 300 liter fermentor (Bioengineering). The pH was adjusted to and maintained at 5 with 30% NH_4OH. After approximately 20–24 h, when glycerol was depleted, 50% glycerol containing PTM1 (12 ml per liter of 50% glycerol) was fed for 4–8 h, followed by 24–48 h methanol feeding (methanol with 12 ml PTM1 per liter of methanol). The supernatant was collected by centrifugation.

Purification of C-peptide precursor by hydrophobic chromatography

NaCl was added to the supernatant to a concentration

of 3 M. The solution was applied to a phenyl Sepharose FF (Amersham Pharmacia Biotech, Piscataway, USA) hydrophobic chromatography column and washed with 20 mM phosphate buffer (pH 7.4) containing 3 M NaCl to remove impurities. The C-peptide precursor was eluted from the column with 20 mM phosphate buffer (pH 7.4) at a flow rate of 60 cm/h for 0.5 column volume.

Enzymatic digestion of C-peptide precursor

The eluted C-peptide precursor at a concentration of approximately 9.5 mg/ml, determined by the Lowry method [14], was digested with trypsin (Sigma-Aldrich, St. Louis, USA) at an enzyme-to-substrate ratio of 1:600 by weight, and carboxypeptidase B (Worthington, Lakewood, USA) at an enzyme-to-substrate ratio of 1:1500 by weight at 30 ºC in the presence of 1 mM $CaCl_2$. Samples were taken at a 1 h interval, adjusted to pH 2–3 by adding HCl, and the enzymatic digestion of C-peptide precursor was monitored by HPLC using a 250 mm Ultrasil C18 column (Beckman, Fullerton, USA) (inner diameter 4.6 mm, particle size 10 μm, pore size 10 nm) and a 2487 HPLC instrument (Waters, Detroit, USA). The column was eluted with 21%–42% (*V*/*V*) acetonitrile gradient containing 0.08% (*V*/*V*) trifluoroacetic acid at a flow rate of 1 ml/min. The A_{230} was measured.

Purification of C-peptide

The digested product was purified by ultrafiltration. The solution retained by the 10 kDa film was equilibrated with 10 mM phosphate buffer (pH 7.4), applied to a Sepharose QFF column (Amersham Pharmacia Biotech) and washed with 10 mM phosphate buffer (pH 7.4) to remove impurities. C-peptide was eluted from the column with the same buffer containing 1 M NaCl at a flow rate of 30 cm/h for two column volume. The A_{230} was measured. The concentration of C-peptide was determined by HPLC using synthetic C-peptide as standard.

Characterization of C-peptide

The C-peptide was identified and characterized by mass spectrometry (Finnigan LCQ; Thermo Electron, San Jose, USA), N- and C-terminal amino acid sequencing (PE-ABI 491A; Applied Biosystems, Foster, USA) and tricine-SDS-PAGE.

Results

Cloning and expression of C-peptide precursor

DNA sequencing and agarose gel electrophoresis of *Eco*RI-digested p36DD/PIC9 (**Fig. 3**) showed that the C-peptide precursor contained nine copies of C-peptide. A transformant with a high copy number of p36DD/PIC9 was selected by dot blotting (**Fig. 4**).

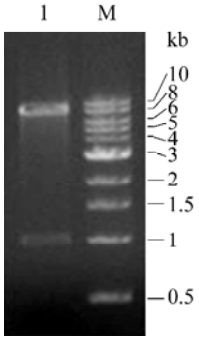

Fig. 3 Agarose gel electrophoresis analysis of plasmid p36DD/PIC9 digested by *Eco*RI

Gel concentration is 1.2%. M, marker; 1, p36DD/PIC9 digested by *Eco*RI.

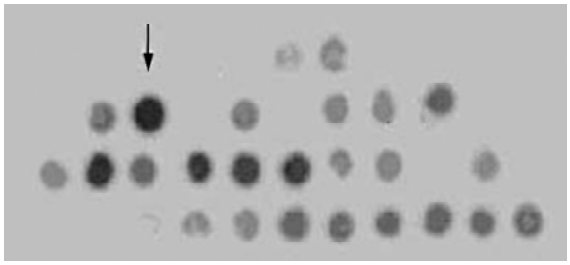

Fig. 4 Screening of multicopy transformant of plasmid p36DD/PIC9 by dot blotting

The arrow shows the high-copy colony.

The expression level of C-peptide precursor in the shake flask estimated by SDS-PAGE reached 100 mg per liter culture (**Fig. 5**). In the high density fermentation using the 300 liter fermentor, the expression level of C-peptide precursor increased with prolonged methanol induction time, and reached 2.28 g per liter culture after 48 h methanol induction as shown by the SDS-PAGE (**Fig. 6**). The Western blot analysis results of C-peptide precursor expressed in the 300 liter fermentor are shown in **Fig. 7**.

Purification of C-peptide precursor

The expressed C-peptide precursor was purified by hydrophobic chromatography that has the advantage of

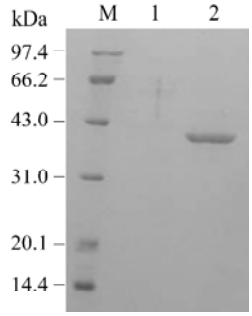

Fig. 5 Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of C-peptide precursor expressed in shake flask
Gel concentration is 10%. M, molecular weight marker; 1, GS115/pPIC9; 2, GS115/p36DD/PIC9.

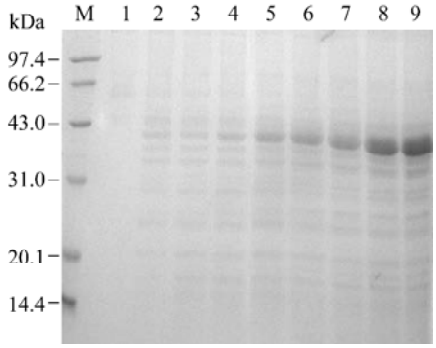

Fig. 6 Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of C-peptide precursor expressed in the fermentor
Gel concentration is 10%. M, molecular weight marker; 1–9, samples taken after 0, 4, 12, 20, 28, 32, 40, 44 and 48 h methanol induction, respectively.

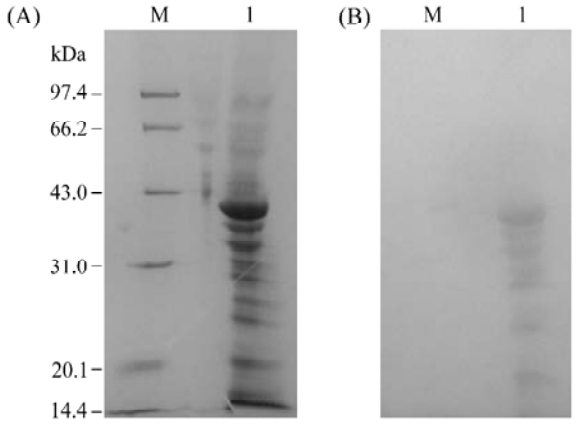

Fig. 7 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot of C-peptide precursor expressed in the fermentor
(A) SDS-PAGE with gel concentration 10%. M, molecular weight marker; 1, samples taken after 48 h methanol induction. (B) Western blot of C-peptide precursor with rabbit anti-C-peptide antibody as the first antibody and horseradish peroxidase-conjugated sheep anti-rabbit antibody as the second antibody.

concentrating the precursor and removing the impurities at the same time. The result is shown in **Table 1**.

Table 1 Purification of C-peptide precursor expressed in *Pichia pastoris* in 100 liters of culture

Step	Total protein (g)	C-peptide precursor (g)	Purity (%)
Supernatant	596	228	38
Phenyl Sepharose FF	160	137	86

Enzymatic digestion of purified C-peptide precursor

The digest of purified C-peptide precursor by trypsin and carboxypeptidase B was analyzed by HPLC, and the result indicated that the precursor was completely digested after 3 h (**Fig. 8**).

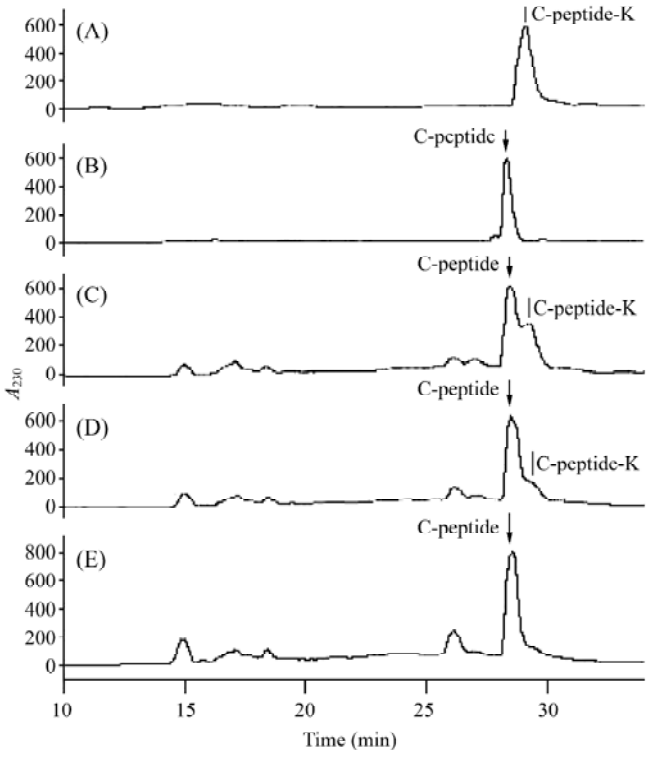

Fig. 8 HPLC analysis of trypsin and carboxypeptidase B digestion
(A) Synthetic C-peptide plus C-terminal Lys. (B) Synthetic C-peptide. (C) C-peptide precursor after 1 h digestion. (D) C-peptide precursor after 2 h digestion. (E) C-peptide precursor after 3 h digestion. Column 4.6 mm×250 mm. Buffer A, 0.1% TFA. Buffer B, 0.08% TFA, 70% acetonitrile. Gradient: 0–5 min, 0% B; 5–10 min, 0–30% B; 10–30 min, 30%–60% B; 30–31 min, 60%–100% B; 31–32 min, 100% B; 32–33 min, 100%–0% B; and 33–35 min, 0% B.

Purification of C-peptide

C-peptide was purified by ultrafiltration and Sepharose QFF chromatography. The result is shown in **Table 2**. C-peptide preparation was analyzed by HPLC before and after Sepharose QFF chromatography (**Fig. 9**). The overall yield of C-peptide preparation with a purity of 96% was 73 g per 100 liters of culture.

Table 2 Purification of C-peptide by ultrafiltration and Sepharose QFF chromatography

Step	Total protein (g)	C-peptide (g)	Purity (%)
Ultrafiltration	87	81	93
Sepharose QFF	73	70	96

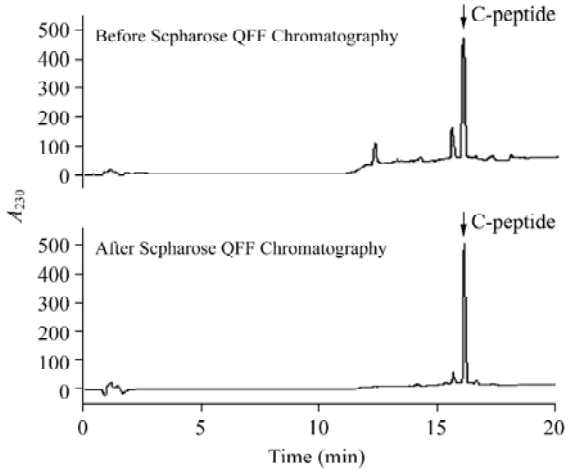

Fig. 9 HPLC analysis of purified C-peptide

Column 4.6×100 mm. Buffer A, 0.1% TFA. Buffer B, 0.08% TFA, 70% acetonitrile. Gradient: 0–5 min, 0% B; 5–10 min, 0–40% B; 10–20 min, 40%–80% B; 20–21 min, 80%–100% B; 21–22 min, 100% B; 22–23 min, 100%–0% B; and 23–30 min, 0% B.

Purified C-peptide was homogeneous in HPLC. Its molecular mass determined by mass spectrometry was 3020.0 Da, in agreement with the theoretical value of 3020.3 Da. N- and C-terminal amino acid sequencing showed its amino acid sequence as E-A-E-D-L-Q-V-G-Q-V-E-L-G-G-G-P-G-A-G-S-L-Q-P-L-A-L-E-G-S-L-Q and C-terminal sequence as Q-L-S-G-E. Amino acid analysis showed that the purified C-peptide had the expected amino acid composition (data not shown).

Discussion

Proinsulin as a precursor in insulin biosynthesis was first discovered by Steiner *et al*. [15]. Subsequently, proinsulin was isolated from crystalline porcine insulin preparation and its amino acid sequence was determined by Chance *et al*. [16]. The C-peptide connecting the B and A chains of insulin will naturally facilitate the right pairing of disulfide bonds in insulin. At first, C-peptide was thought to have no other physiological functions, even though it is hard to imagine why such a long connecting peptide of 31 residues, instead of a short spacer, would be needed to join the C-terminus of the B chain and the N-terminus of the A chain when they are so closely located. In 1997, the prevention of vascular and neural dysfunction in diabetic rats by C-peptide was reported by Eli Lilly Company [4]. Since then, more and more experimental and clinical studies on the beneficial effects of C-peptide on diabetes have been reported. For further pre-clinical and clinical studies, it is essential to have a large amount of human C-peptide. In expressing C-peptide with a random solution structure and easily degraded *in vivo*, we adopted the strategy of joining nine C-peptide genes in tandem to form the C-peptide precursor gene. The recombinant C-peptide precursor was easily converted into C-peptide *in vitro* by joint trypsin and carboxypeptidase B digestion. The expression system we used is *P. pastoris* which gave high expression of many proteins, including insulin, in high density fermentation. The C-peptide precursor expressed in shaken flask was shown by Western blot as a single band (data not shown). However, in high density fermentation, in addition to C-peptide precursor as the main component, degraded fragments were also present, as shown by Western blot (**Fig. 7**). Fortunately, the degradation by trypsin-like digestion occurred after Lys but not in C-peptide, so the degraded products are fragments containing different numbers of C-peptide. These fragments pooled together were easily converted into C-peptide *in vitro* by joint trypsin and carboxypeptidase B digestion. A His_6 tag was added to the C-peptide gene to facilitate characterization and purification. However, it was found later that the His_6 tag was not very efficient for purifying C-peptide precursor, so it was only used to detect C-peptide precursor by Western blot. The overexpressed C-peptide precursor secreted into the cultural medium with a purity of 38% could be easily purified to 86% purity by a single step of hydrophobic chromatography. After enzyme digestion, the C-peptide with a purity of 96% was obtained by ultrafiltration and ion exchange chromatography.

In summary, by using a combination of different effective measures, including the joining of the C-peptide gene in tandem, screening of recombinant *P. pastoris* with a high copy number of the integrated C-peptide precursor gene, and expression of the secreted C-peptide precursor into a simple culture medium in high density fermentation, a high expression level of 2.28 g C-peptide precursor per liter of culture was achieved in a 300 liter fermentor, resulting in a high yield of highly purified C-peptide (73 g per 100 liters of culture with a purity of 96%).

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