Cloning, expression and purification of recombinant human proinsulin C-peptide in *E. coli*

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Abstract **Aim**: To obtain recombinant human proinsulin C-peptide, a novel expression vector pEDCC was constructed to facilitate the expression and purification of C-peptide. **Methods**: Gene fragments encoding a truncated asparaginase fragment mutant, native C-peptide, a hinge fragment of human IgG_1, an extra acid-labile dipeptide and a basic-amino-acid-riched octopeptide were introduced in turn into plasmid pET28a. The fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide was expressed effectively as inclusion bodies after induced by lactose and partially purified by means of washing and ethanol fractionation. After being hydrolyzed, the polypeptide PKRKRKKSRNGSGR-C-peptide was liberated from the fusion partner. The N-terminal tetradecapeptide extension of C-peptide was subsequently cleaved by trypsin and removed by DE52 column. **Results**: The nucleotides sequence of plasmid pEDCC was confirmed to be identical with that of designed fusion protein. Recombinant human proinsulin C-peptide was obtained with high purity after purification. **Conclusion**: Employing truncated asparaginase as the fusion partner and basic-amino-acid-riched octopeptide to modulate isoelectric point is an effective approach to produce recombinant human proinsulin C-peptide.

Key words recombinant human proinsulin C-peptide; gene fusion; expression and purification; asparaginase; trypsin

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重组人源胰岛素原C肽在大肠杆菌中的克隆、表达与纯化

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摘 要 **目的**：构建一种新型表达载体（pEDCC），表达并纯化得到人源胰岛素原C肽。**方法**：将编码截短的门冬酰胺酶突变体（ansB-C），天然C肽，人IgG_1铰链区（hinge），额外的酸敏感二肽（DP）以及富含碱性氨基酸的8肽（KRKRKKSR）的核苷酸序列依次分别插入pET28a载体中，构建表达载体pEDCC。在乳糖的诱导下，融合蛋白ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide以包涵体形式高效表达。融合蛋白经洗涤和乙醇分级沉淀纯化后，通过酸水解将PKRKRKKSRNGSGR-C-peptide释放出来。C肽N端14肽用胰蛋白酶切割，通过DE52柱与C-peptide分离。**结果**：构建的表达载体pEDCC序列正确，融合蛋白经分离纯化得到了高纯度的重组人源胰岛素原C肽。**结论**：以截短的门冬酰胺酶作为融合伙伴，并以富含碱性氨基酸的8肽调节等电点是一种生产重组人源胰岛素原C肽的有效方法。

关键词 重组人源胰岛素原C肽；基因融合；表达与纯化；门冬酰胺酶；胰蛋白酶

C-peptide, a cleavage product from the processing of proinsulin to insulin, has been considered to possess little if any biological activity other than its participation in the assembly of the two-chain insulin structure. However, during the past decade, numerous investigations involving humans and animals studies have suggested that C-peptide, although not influencing blood sugar control[1], might play a role in a broad range of

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molecular and physiological activities, indicating that it is a hormonally active endogenous peptide. C-peptide replacement in insulin dependent diabetes mellitus (IDDM, type 1 diabetes) patients and animal models of type 1 diabetes was accompanied by augmented blood flow in several tissues[2-4], diminished glomerular hyperfiltration, reduction of urinary albumin excretion[5], and prevention of glomerular hypertrophy and mesangial matrix expansion[6]. Nociceptive sensory neuropathy[7], nodal and paranodal degeneration[8], cognitive dysfunction and hippocampal apoptosis[9] can likewise be prevented by C-peptide administration. Moreover, C-peptide may have beneficial effects on cavernosal smooth muscle relaxation[10] and reduce the risk of infection in diabetes mellitus[11]. Some data suggested that C-peptide transduction may crosstalk with the insulin signaling pathway[12]. Nevertheless, its molecular mechanism of action is not as yet fully understood. So continued experimental research and clinical trials are necessary to establish whether C-peptide may prove to be a bioactive peptide of importance in the treatment of the long-term inexorable progression of diabetes-related complications. Hence, the need for efficient large-scale production strategies for the human proinsulin C-peptide has arisen. Gene fusion and inclusion body strategies are very useful in the expression of small polypeptides and result in increased expression levels, improved product stabilization and/or efficient purification. However, gene fusion introduces a problem into the downstream processing scheme, due to the fact that the fusion partner should be cleaved off and removed. A number of enzymatic methods have been investigated for this purpose, and most of them are too expensive to be available to the large-scale production. Trypsin, which can cleave the peptide bond between a basic amino acid (e.g. Arg, Lys) and an arbitrary amino acid with the exception of Pro, is inexpensive, readily available and has been used extensively to digest protein to small peptides. Human proinsulin C-peptide does not contain any basic amino acid, suggesting that trypsin may be useful in liberating the native C-peptide.

An asparaginase C-terminal fragment from *E. coli* was tailored to serve as a fusion partner for C-peptide. The truncated asparaginase fragment whose unique acid-labile aspartyl-prolyl bond in the amino acid sequence was mutated into aspartyl-alanyl bond, termed as ansB-C, is easily expressed to high levels in BL21(DE3) and accumulates in inclusion bodies[13]. A linker consisting of a hinge fragment (CPPCPAP) of human IgG_1 (226-232/226′-232′) which is known to act as a flexible linker and can form two interchain disulfide bond was introduced into fusion partner to improve the fusion protein stability. An extra dipeptide aspartylproline containing an acid-labile aspartyl-prolyl bond and a basic-amino-acid-riched octopeptide was in turn inserted between the ansB-C moiety and C-peptide. The fusion protein was cleaved with HCl at first, and then treated with trypsin to yield desired C-peptide.

1 Materials and methods

1.1 *Materials*

Vector pET-28a(+) was obtained from Novagen; pfu DNA polymerase was purchased from Promega; all restriction endonucleases, T4 DNA ligase, DNA marker and protein marker were products of Fermentas; CM-cellulose (CM52) and DEAE-cellulose (DE52) were obtained from Whatman; trypsin (TPCK-treated) was purchased from Sangon.

1.2 *Construction of the plasmid encodind ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide*

DNA fragment encoding ansB-C and aspartyl-proline dipeptide linker was generated by PCR with forward primer 5′-CCCCATGGATACGCCATTCGATGTC TC-3′ (with *Nco* Ⅰ site) and reverse primer 5′-CCGAAGCTTACGGATCCGCGTACTGGTTGAAGATCTG TTGGATTTGCTGCGCATC-3′ (with *Hin*d Ⅲ and *Bam*H Ⅰ sites) from the chromosome of *E. coli*. The PCR product was digested by *Nco* Ⅰ and *Hin*d Ⅲ, and subsequently inserted into downstream of the T7 *lac* promoter of the linearized pET28a. The recombinant plasmids were transformed into *E. coli*. BL21(DE3), and the resultant colonies were screened by kanamycin resistance and PCR-screening technique, and then confirmed by sequencing.

Next, the minigene encoding DPNGSGR-C-peptide was synthesized by two-step PCR using the following oligonucleotides: the first step forward primer A: 5′-AAGCTGAAGACCTGCAGG TTGGTCAGGTTGAACTGG GTGGCGTTCCTGG-3′; the first step reverse primer B: 5′-AAGA GCCAGCGGCTGCAGAGAACCAGCGCCAGG ACCGCCACCCAGTTC-3′; the second step forward primer C:5′-GGGGGGATCCGCCGAATGGTTCTGGCCG TGAAGCTGAAGACCTGC AGGTTG-3′(with *Bam*H Ⅰ site); the second step reverse primer D: 5′-AAAAAAGCTTACTGCA GGGAGCCTTCAAGAGCCAG CGGCTGCAGAGAAC-3′(with *Hind* Ⅲ site). The PCR product was double-digested by *Bam*H Ⅰ and *Hind* Ⅲ, and inserted into linearized pED, previously digested with the same enzymes. The recombinant plasmids were transformed into BL21(DE3). Thereafter primers C and D were used to screen the positive transformant and then sequenced as above. The resulting plasmid was denoted pEDC1.

Then, the expression vector pEDC1 was modified by add-on PCR using the following oligonucleotides. Firstly, the forward primer WUP flanking the *Nco* Ⅰ site 5′-ACTTTAAGAAGGAGATATACCA-3′ and the add-on reverse primer WDOWN 5′-AAACGGATCCGGTGCAGG GCACGGCGGGCAAGTTTGCGTCAGAGCCAG-3′ were used to introduce the hinge fragment of human IgG_1 into fusion partner. The expression vector was termed as pEDC. A screening primer WSHAI (5′-GGATCCGGTG CAGGGCACGG-3′) that only had annealing sites in positive recombinant but not in plasmid pEDC1 was designed to identify the recombinant clone. Secondly, the add-on forward primer CUP (5′-GGGGGATCCGAAA CGTAAACGTAAAAAAAGCCGTAATGGTTCTGGCCGTG AA-3′) and the reverse primer D were used to insert the encoding sequence of octopeptide(KRKRKKSR) which originated from exon6 of VEGF183[14] between the encoding sequences of fusion partner and NGSGR-C-peptide, resulting in the isoelectric point (pI) of the small polypeptide be modulated to 9.99 (calculated by the software of ProtParam in the web site of http://cn.expasy.org), while those of the fusion partner and the fusion protein is 4.65 and 7.93, respectively. The polypeptide purification process can be facilitated significantly; the expression vector was denoted pEDCC. Similarly, another specific screening primer CHS (5′-AAACGTAAACGTAAAAAAAG-3′) was also designed to identify the positive transformant. Diagram of pEDCC was described in Fig.1.

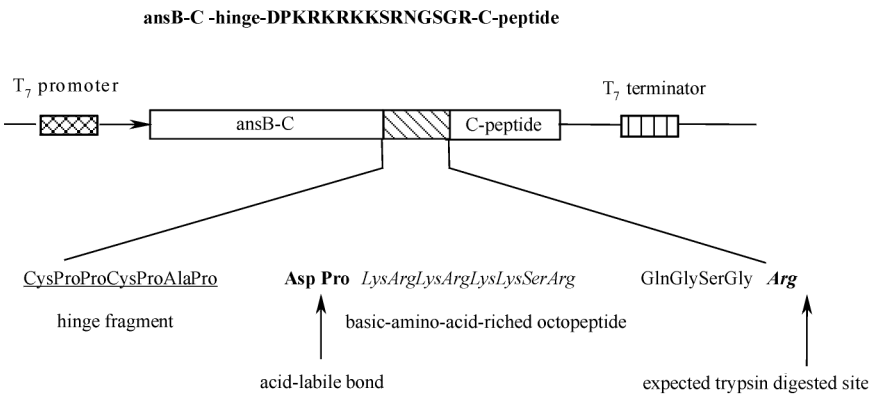


Fig.1 Diagram of ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide

1.3 *Optimize the expression conditions of fusion protein*

A single transformed colony containing pEDCC was used to inoculate 10 mL LB medium (containing 50 μg/mL Kanamycin) and was grown overnight at 37 ℃ with shaking at 200 r/min. Aliquots containing 1 mL of the seed culture were transferred to 50 mL fresh LB medium in two 250 mL shake flasks. Both cultures were grown with 200 r/min shaking at 37 ℃. 1 mL samples were removed from two flasks at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 11, 12 h after inoculation. Each sample was diluted by 2 mL distilled water and used to measure A_{600}. When A_{600} reached 0.6

~0.8, one culture was induced by addition of 0.5 mL of 500 mmol/L sterilized lactose solution, and extra 1 mL fluids were sampled hourly from this culture after induction. These samples were centrifuged. The pellets were resuspended and analyzed by 15% SDS-PAGE described by LaemmLi[15].

1.4 *Expression of the fusion protein*

The recombinant strain harboring pEDCC was inoculated into LB medium containing 50 μg/mL Kanamycin and was grown overnight in a 37 ℃ shaker. The seed was inoculated to fresh medium at the ratio of 1∶50. Lactose was added to a final concentration of 5 mmol/L when A_{600} reached 0.6 ~ 0.8. Cells were subsequently grown for another 7 h and harvested by centrifugation. The expression level of the fusion protein was detected by SDS-PAGE.

1.5 *Purification of the fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide*

Recombinant cells were suspended in cell splitting buffer (50 mmol/L Tris-Cl, pH 8.0; 0.5% Triton-X 100; 0.2 mg/mL lysozyme and 0.01 mg/mL DNase) by stirring for over 10 h at 37 ℃. After centrifugation at 12 000 r/min for 20 min, the precipitation was resuspended with inclusion body washing buffer (50 mmol/L Tris-HCl, pH 8.0; 0.2% Triton-X 100) and then distilled water, respectively. After washing, the final inclusion body pellets were suspended in inclusion body dissolving buffer (50 mmol/L Tris-HCl, pH 8.0; 8 mol/L urea) and stirred for at least 6 h at room temperature and centrifuged. The supernatant was carefully removed and added with equivalent volume of cold anhydrous ethanol and kept at −20 ℃ for 0.5 h. After centrifugation, the supernatant was added another equivalent volume of cold ethanol of initial supernatant at −20 ℃ for 0.5 h for further precipitation of impurities. After removing the sediment, another volume cold ethanol of initial supernatant was added to the supernatant again to precipitate the fusion protein. All sediments samples were taken for analysis by SDS-PAGE.

1.6 *Preparation of PKRKRKKSRNGSGR-C-peptide*

The fusion protein was dissolved in 40 mmol/L HCl to 6% and incubated at 45 ℃; samples (80 μL) was removed at 24, 48, 72 h to determine the optimum hydrolysis time. Samples were analyzed by a novel SDS-PAGE method[16].

The cleavage reaction was terminated by addition of 0.5 mol/L NaOH to adjust to pH 8.2. The resultant solution was filtrated through CM52 column that had been pre-equilibrated with 5 mmol/L $NH_3 \cdot H_2O$-NH_4Cl, pH 8.2. The column was washed with three column volumes (CV) of the equilibration buffer and then eluted with 20 mmol/L $NH_3 \cdot H_2O$. The elution was collected and lyophilized. Samples were taken for SDS-PAGE as described above.

1.7 *Processing of PKRKRKKSRNGSGR-C-peptide turned into C-peptide*

Lyophilized PKRKRKKSRNGSGR-C-peptide was solubilized in 100 mmol/L PB, pH 7.5 (containing 0.1% Tween 20). Trypsin (TPCK treated) was added to an enzyme/substrate ratio of 1∶200. PKRKRKKSRNGSGR-C-peptide was subjected to enzymatic processing for 1, 2 and 3 h respectively. After 3 h, the reaction mixture was loaded on DE52 column pre-equilibrated with 5 mmol/L PB, pH 6.0. The column was washed with 3 CVs of the equilibration buffer and then eluted with 10 mmol/L glycine-HCl buffer, pH 2.6. The elution containing C-peptide was pooled and sampled for SDS-PAGE analysis.

2 Results and Discussions

2.1 *Construction of the recombinant expression vector*

The native proinsulin C-peptide is not suitable for being expressed in bacterial cells directly. So according to the bias for preferred codons of *E. coli*, we designed four primers to synthesize the minigene (133 bp) encoding DPNGSGR-C-peptide by two-step PCR. After being digested by restriction endonucleases, the gene was inserted into pED to construct pEDC1 successfully. Then, two add-on PCRs were carried on to introduce the nucleotide sequences of the hinge fragment (CPPCPAP) and the octopeptide (KRKRKKSR) into pEDC1, respectively. The agarose gel electrophoresis of all PCR-amplified fragments was shown in Fig.2.

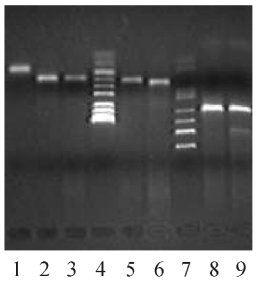
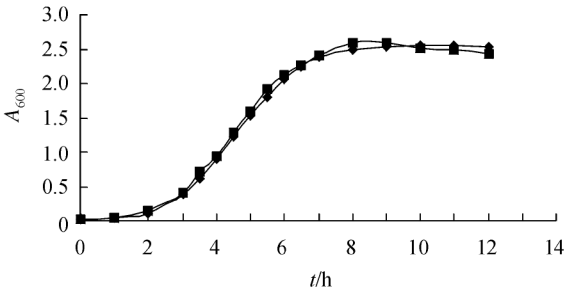


Fig.2 Agarose gel (1.7%) electrophoresis of PCR products
1: PCR products amplified by primer A and B (77 bp); 2: PCR products amplified by primer C, D with the template of lane 1 (133 bp); 3: Screening PCR products of pEDC1 (133 bp); 4: pUC19 DNA/ *Msp* I (*Hpa* II) Marker (501, 404, 331, 242, 190, 147, 111, 67 and 34 bp); 5: Screening PCR products of pEDCC (137 bp); 6: PCR products amplified by primer CUP, D and template pEDC (153 bp); 7: pBR322 DNA/ *Alu* I Marker (908, 659, 521, 403, 281, 257, 226 and 100 bp); 8: PCR products amplified by primer WUP, WDOWN and template pEDC1 (396 bp); 9: Screening PCR products amplified by primer WUP, WSHAI and template pEDC (392 bp)

pET28a was chosen to express the fusion protein, due to the strong power for high-level expression of recombinant protein and the two-stage cultivation property. Moreover, pET28a contains a more stringent promoter-T7 *lac* promoter-which has a *lac* operator sequence just downstream of the T7 promoter[17]. Adoption of the more stringent promoter and two-stage cultivation strategy could decrease the toxicity of fusion protein to the host and increase the plasmid stability. Therefore, the fusion gene has been stably cloned and effectively expressed.

2.2 *Optimize the expression conditions of fusion protein*

Growth curves of the recombinant strain harboring plasmid pEDCC had been drawn as Fig.3. From the curves, we can see A_{600} reached 0.708 after inoculated for 3.5 h and the bacteria have been in stationary phase after inoculated for 11 h. The expression of fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide was induced by adding lactose to a final concentration of 5 mmol/L in the culture after inoculated 3.5 h. The two curves are close to superposition, suggesting that the addition of lactose solution in the culture had innocuous effects on host cell growth. Fig.4 showed that the expression level increased with time of culturing bacterial cells in LB medium after induction. Therefore, we cultured the engineered bacteria for 7 h post-induction for harvesting more fusion proteins.



—◆—uninduced sate; —■—induced state

Fig.3 Growth curves of the recombinant clone harboring plasmid pEDCC

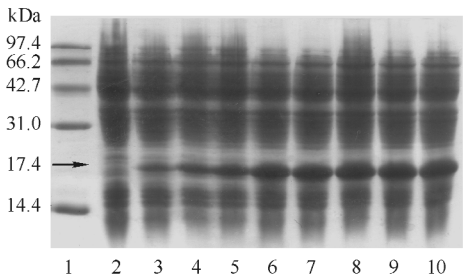


Fig.4 Expression level of the fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide
1: Proteins marker; 2: Proteins from BL21 (DE3) with plasmid pEDCC without induction; 3～10: Proteins from BL21 (DE3) with plasmid pEDCC after induction for 1, 2, 3, 4, 5, 6, 7, 8 h, respectively

2.3 *Purification of the fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide*

The fusion protein ansB-C-hinge-DPKRKRKKSRNGSGR-C-peptide was highly expressed as inclusion bodies. It seemed easy to partially purify by cell disruption, washing and ethanol fractionations, with the yield about 3% of the total wet bacteria weight (Fig.5).

In this study, a delicate controversy between quality and quantity has been found. When two volumes cold anhydrous ethanol was added for precipitating impurities for 0.5 h, the supernatant still had a few impurities, while keeping it overnight, the target fusion proteins had few impurities, but a lot of fusion protein had coprecipitated with impurities. In respect that the pI of PKRKRKKSRNGSGR-C-peptide is 9.99, the remnant impurities can be easily removed, so the output is payed more attention than purity.

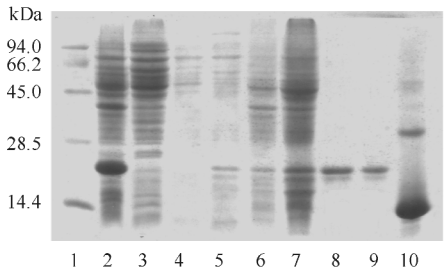


Fig.5 Samples of the purify process of the fusion protein under reducing condition

1: Proteins marker; 2: Total proteins of cells harboring plasmid pEDCC; 3: Supernatant of the cells lysed in the cell splitting buffer after centrifugated; 4: Supernatant of the cells resuspended in the inclusion body washing buffer after centrifugated; 5: Supernatant of the cells resuspended in distilled water after centrifugated; 6: Sediments precipitated by single volume of ethanol; 7: Sediments precipitated by double volumes of ethanol; 8, 9: Sediments precipitated by triple volumes of ethanol; 10: Lysozyme (Sigma)

2.4 *Preparation of PKRKRKKSRNGSGR-C-peptide*

The fusion protein ansB-C-hinge-DPKRKRKKSR NGSGR-C-peptide only had unique acid-labile aspartyl-prolyl bond, theoretically, it would be divided into two parts: fusion partner (ansB-C-hinge-D) and polypeptide (PKRKRKKSRNGSGR-C-peptide) by low concentration acid. In fact, as the non-specific hydrolysis existed, the fusion protein could be cleaved into other parts. Fig.6 showed that hydrolysis for 72 h was appropriate for this fusion protein. After hydrolysis, the solution pH was adjusted to 8.2 and filtrated through CM52 column. Here, 20 mmol/L $NH_3 \cdot H_2O$ was used to elute PKRKRKKSRNGSGR-C-peptide, when lyophilized, ammonia would volatilize from the elution to bring forth facilitation of the purification process.

2.5 *Processing of PKRKRKKSRNGSGR-C-peptide changed into C-peptide*

Trypsin cleaved PKRKRKKSRNGSGR-C-peptide of each peptide bond C-terminal to Arg and Lys residues to get native C-peptide. To investigate when the trypsin treatment reached completion, the PKRKRKKSRNGSGR-C-peptide was subjected to enzymatic processing for 1, 2 and 3 h respectively, and it was concluded that PKRKRKKSRNGSGR-C-peptide was completely cleaved after 3 h treatment (data not shown). Then the solution pH was adjusted to 6.0 and filtrated through DE52 column. The elution from column was collected and analyzed by SDS-PAGE (Fig.7). Throughout the experiment, C-peptide existed as a single bond in SDS-PAGE. Chymotrypsin, a less specific endopeptidase than trypsin, is present to varying degrees in commercial preparations of trypsin. TPCK can react with the histidine 57 residue at the active site of chymotrypsin, thus inhibiting the enzyme. To prevent nonspecific cleavage, we used trypsin (TPCK-treated) to liberate the native C-peptide.

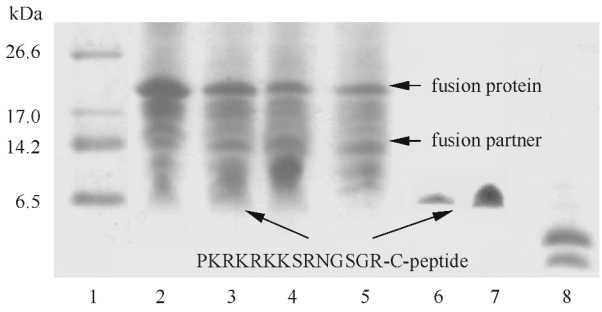


Fig.6 SDS-PAGE analysis of PKRKRKKSRNGSGR-C-peptide

1: Protein marker; 2 ~ 4: Samples cleaved for 24, 48 and 72 h; 5: Effluent from CM-52; 6, 7: Elution from CM-52, i.e., PKRKRKKSRNGSGR-C-peptide; 8: Insulin

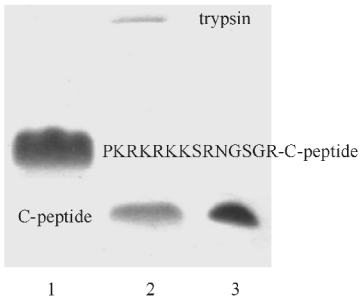


Fig.7 SDS-PAGE analysis of C-peptide

1: Purified PKRKRKKSRNGSGR-C-peptide; 2: Reaction mixture after 3 h treatment by trypsin, the upper band is trypsin and the lower band is C-peptide; 3: Purified C-peptide

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为了交流药学服务工作的经验,促进药学服务工作深入开展和药学事业快速发展,中国药学会医院药学专业委员会和《药学服务与研究》杂志社决定召开第二届全国药学服务与研究学术会议。

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