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A new strategy for recovery of two peptides without Glu employing glutamate-specific endopeptidase from *Bacillus licheniformis*

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ABSTRACT

The difficulty in the purification of bioactive peptide limited its application in food, drug and cosmetic industry. Here we report a new strategy for the recovery of two peptides employing glutamate-specific endopeptidase from *Bacillus licheniformis* (GSE-BL), which shows strong specificity for Glu residue. Human glucagon and human beta-defensin-2 (HBD-2) were peptides without Glu residue, and Glu residue was introduced between affinity tag and target peptide as recognition site of GSE-BL. Tagless human glucagon with the same HPLC retention time as native human glucagon and mature HBD-2 with antibacterial activity and cytotoxicity were obtained after GSE-BL treatment. This strategy has great potential in the recovery of bioactive peptide without Glu residue, thus facilitating large scale preparation of peptide and widening the application of bioactive peptide.

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1. Introduction

With the development of bioinformatics, gene manipulation technique and proteomics, more and more proteins and peptides have been produced employing recombinant technique. Affinity tags such as His, Strep, FLAG and GST tags were fused with recombinant proteins or peptides to facilitate the expression and purification, or to protect target protein, or to increase the solubility thereby improving the yield of target protein [1,2]. However, application of affinity tags also suffers from some drawbacks such as decreasing the enzymatic activity, altering the structure of target protein and increasing the toxicity and immunogenicity [3–5]. Thus, proteases including blood coagulation factor Xa [6,7], enterokinase [8,9], thrombin [10], Rhinovirus 3C [11,12], Tobacco etch virus (TEV) protease [13] and more recently a self splicing element intein [13–15] were employed to remove affinity tags. Ideal protease for cleavage of affinity tags should possess the following characteristics: (i) high specificity for target sequence in order to prevent non-specific cleavage, (ii) high cleavage efficiency, (iii) broad pH and temperature adaptability, (iv) easy to be removed after the cleavage, and (v) can be obtained at low cost. However, some current commercially available proteases were associated with the problems of material cost, low efficiency, buffer and temperature sensitivity, non-specific cleavage [16] and removal of proteases [17].

Glutamate-specific endopeptidase from *Bacillus licheniformis* (GSE-BL), which belongs to serine protease family, has strong specificity and high catalytic efficiency for α-carboxyl of Glu residue [18], the presence of secondary structure especially α-helix could decrease the catalytic efficiency of GSE-BL toward Glu residue [19,20], and GSE-BL has been employed to remove His-tag of recombinant *Vitreoscilla* hemoglobin (VHb) without destructing the native secondary structure of VHb, thus improving the function of VHb [21]. However, application of GSE-BL in removing affinity tags of target protein also suffers from shortcomings such as demand for information about structure of target protein, accuracy control the duration and condition of protease treatment. Some important peptides could be found without Glu residues in amino acid sequence, thus GSE-BL could be used to remove affinity tags fused with those peptides with the advantages of high efficiency, specific cleavage, low demand for buffer and temperature and easy to be removed after cleavage.

Human glucagon can stimulate the liver glycogenolysis, thus enhancing blood sugar levels, which can be utilized in the treatment of hypoglycemia and cardiogenic shock [22]. Human glucagon has

Abbreviations: GSE-BL, glutamate-specific endopeptidase from *Bacillus licheniformis*; HBD, human beta defensin.

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Table 1
Primers and DNA fragments for the amplification of human glucagon and HBD-2.

Nucleotide	Sequence (5′ → 3′)
Glucagon I	CACTCTCAGGGTACCTTCACTTCCGACTACTCTAAA TACCTGGACTCCCGT
Glucagon II	TACCTGGACTCCCGT CGTGCTCAGGACTTCGTTCAGTGGCTGATGAACACC
P_F for glucagon	CCG*GAATTC*CACCACCACCACCACCACGGTGGTAGTGGTGGT-GAACACTCTCAGGGTACC
P_R for glucagon	GCTCCTTACAGCATTGAATGCGTGATTGGT
P_F for HBD-2	GGAATTC*CATATG*CACCACCACCACCACCAC ATGCGTGTTCTGTATCTG
P_R for HBD-2	ATAAGAAT*GCGGCCGC*TTAGGTGTTCATCAGCCACTGAAGT

been expressed in yeast with low yield <10 mg/L [23,24], and the purification protocol was intricate and time-consuming. It has also been reported that human glucagon was fused with γ-interferon and expressed in *Escherichia coli* with yield of 12 mg/L [22]. Human beta defensin-2 (HBD-2) is a kind of cysteine-rich and cationic antimicrobial peptide, which has broad application in food and biomedical industry [25]. Recombinant HBD-2 fused with His-tag have been expressed in *E. coli* [26] and cell-free system [2], CNBr was used to cleave the affinity tag, which was environmentally hazardous. HBD-2 fused with GST tag was also expressed in *E. coli* and the recombinant protein was cleaved by thrombin with duration of 16 h, and GST tag was separated from tagless HBD-2 by ion exchange chromatography, which was inefficient and complicated [17]. Therefore, a strategy to obtain human glucagon and HBD-2 with high yield and high efficiency is urgently needed.

In the present study, we report a new strategy employing GSE-BL to obtain tagless human glucagon and HBD-2 with relative high yield and high purity, Glu residue was introduced between affinity tags and peptide as recognition site of GSE-BL. This new strategy will provide a new method for the mass production of tagless peptide without Glu residue, thus promoting the application of peptide in food, cosmetic and biomedicine industry.

2. Materials and methods

2.1. Materials

Protease GSE-BL with His tag was prepared in our laboratory [20]. *E. coli* strain DH5α and BL21 (DE3), pPIC9K vector and *Pichia pastoris* strain KM71 were purchased from Invitrogen (Carlsbad, CA, USA). Enzymes for DNA manipulation were purchased from Fermentas (MBI Fermentas, USA). pET-22b(+) vector was purchased from Novagen (Madison, WI, USA). Immunoblotting grade affinity purified His-tag Monoclonal antibody was purchased from Abcam (Cambridge, UK), SuperSignal West Pico chemiluminescent kit and BCA protein assay kit were purchased from Thermo Scientific (San Jose, CA, USA). Synthesized human glucagon was purchased from Sigma–Aldrich (St. Louis, Missouri, USA). HP nickel ion affinity column was purchased from GE (GE Healthcare, Munich, Germany). Primers listed in Table 1 were synthesized by Invitrogen Technologies (Shanghai, China). Genes encoding human glucagon and HBD-2 was synthesized by Generay (Shanghai, China).

2.2. Cloning of human glucagon and HBD-2 genes

Human glucagon gene was amplified from human glucagon fragment I and fragment II with oligonucleotides that appended a 5′-*EcoRI* restriction site following a hexa-His-encoding gene and 3′-*NotI* restriction, the PCR product was digested with *EcoRI* and *NotI*, then ligated with pPIC9K digested with *EcoRI* and *NotI*. The resultant product was transformed into *E. coli* strain DH5α. Positive clone was confirmed by double digestion and sequencing. The plasmid pPIC9K-glucagon was electroporated (2 kV/cm) into *P. pastoris* yeast strain KM71 [27]. The genome of randomly picked clone was extracted using yeast genome extraction kit (Qiangen, USA, Valencia, CA), and colony PCR was performed to confirm that pPIC9K-glucagon was integrated with genome of KM71 strain.

HBD-2 gene was amplified by PCR from synthesized new HBD-2 gene (Glu residue was added between mature HBD-2 and HBD-2 pro-peptide according to original HBD-2 Gene (GenBank accession no. AY155577) using forward primer that appended a 5′-*NdeI* restriction site following a hexa-His-encoding gene and reverse primer that appended a 3′-*XhoI* restriction site immediately following the stop codon. The PCR product was digested with *NdeI* and *XhoI* followed by ligation with pET22b digested with the same restriction enzymes. The resultant product was transformed into *E. coli* strain DH5α, and double digestion and sequencing were performed to confirm that pET22b-HBD-2 was successfully reconstructed. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3).

2.3. Expression and purification of glucagon and HBD-2

Positive clone containing recombinant pPIC9K-glucagon was grown in buffered minimal glycerol-complex (BMGY) medium for 24 h and induced by the addition of 1% methanol (v/v) every 24-h interval. After 72 h of induction, the supernatant after centrifugation at 4 °C, 12,000 × *g* for 20 min was loaded into 5 mL nickel affinity column (His Trap HP, GE), recombinant human glucagon fused with N-terminal hexa-His tag was eluted by buffer with different concentrations of imidazole. Samples were collected before and after methanol induction and then analyzed on Tricine-SDS-PAGE.

Reconstruct plasmid pET22b-HBD-2 precursor was transformed into *E. coli* strain BL21 (DE3) and grown in LB medium. HBD-2 precursor was induced by the addition of 1 mM IPTG when the OD600 was about 0.4, the bacteria were harvested after incubation at 37 °C for 4 h, then the cells were sonicated by ultrasonic crasher in lysis buffer (20 mM Tris–HCl, 500 mM NaCl, pH 8.0) for 15 min. The supernatant after centrifugation at 4 °C, 12,000 × *g* for 15 min was loaded onto 1 mL column of nickel affinity (His Trap HP, GE), and HBD-2 precursor was eluted with 20 mM Tris–HCl supplemented with 500 mM NaCl and different concentrations of imidazole at the flow rate of 2.0 mL/min. The eluted solutions were analyzed on Tricine-SDS-PAGE.

2.4. Removal of His-tag and propeptide

The concentrations of proteins in eluted solutions were confirmed using a BCA kit, then the eluted solutions containing recombinant peptide were dialyzed with 20 mM Tris–HCl (pH 8.5), following by GSE-BL treatment at a molar ratio of 1:100 (E:S) at 37 °C for different duration tests. The resultant product after protease treatment was loaded onto 1 mL nickel affinity column (His Trap HP, GE) and eluted by buffer with imidazole. The flow through solution was collected and analyzed by Trincine-SDS-PAGE and Western blot.

2.5. Western blot

Tricine-SDS-PAGE was carried out on 16.5% (w/v) polyacrylamide gel with urea. After electrophoresis, peptides were electro-blotted onto PVDF membrane by wet transfer instrument (Bio-Rad) in 80 V for 30 min. The membrane was incubated in 5% BSA dissolved in TBST (50 mM Tris–HCl, containing 100 mM NaCl, 0.05% Tween-20, pH 7.5) at room temperature for 3 h. His-tag monoclonal primary antibodies were diluted in 5% BSA with 1500 fold dilution, and then incubated with membrane at 4 °C overnight. Then the membrane was washed three times in TBST with 20 min interval at room temperature to remove unbound antibodies. Immunological binding was visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) by following the instruction of manufacturer.

2.6. Identification of human glucagon and HBD-2

Tagless human glucagon and synthesized human glucagon were analyzed by 16.5% Tricine-SDS-PAGE and HPLC. Human glucagon was loaded onto a Waters C_{18} column with 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). Elution was performed with 35% solvent B for 10 min at a flow rate of 0.5 mL/min.

Staphylococcus aureus and *Pseudomonas aeruginosa* were cultured in LB medium for 16 h, HBD-2 precursor and mature HBD-2 after GSE-BL treatment at concentration of 0, 25, 50, 100 and 200 μg/mL was added, and at last OD600 was detected after 12 h to confirm the bacteriostasis of mature HBD-2. The inhibition rate was defined as the decrease of absorbance at 600 nm divided by the original absorbance at 600 nm before HBD-2 treatment [28].

Dendritic cells collected from mice femur and HCT-8 Cells were adjusted to 1×10^5 cells/mL, then HBD-2 precursor and mature HBD-2 with different concentrations were added to the cells and equal volume of corresponding buffer was added as blank control, and HBD-2 precursor was defined as negative control, while 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added after 72 h, and OD490 was detected using a microplate device to confirm the cytotoxicity of mature HBD-2 [29].

3. Results

3.1. Cloning of human glucagon and HBD-2

Two bands corresponding to pPIC9K and human glucagon were detected on 1.5% agarose gel after digestion of recombinant plasmid with *EcoRI and NotI* (Fig. 1A), demonstrating that human glucagons gene was successfully inserted into pPIC9K vector. Result of colony PCR templated with yeast genome could demonstrates that recombinant plasmid pPIC9K-glucagon was integrated into genome of

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