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Expression and purification of beefy meaty peptide in *Pichia pastoris*

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Abstract−We established a gene expression process to produce a flavor peptide in *Pichia pastoris*. The octapeptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala was isolated from beef digested by papain, known as "beefy meaty peptide" (BMP). It was considered a savory seasoning in previous literatures. To produce BMP by microorganisms, BMP expression structures encoding tandem repeats of the octapeptide were designed and cloned in *E. coli* DH5α, then integrated into the *AOX1* gene of *Pichia pastoris* GS115. Clones containing different BMP-gene copies, with 4-copy, 8-copy, 12-copy and 16-copy, were obtained and expressed in *Pichia pastoris* GS115. For the ease of purification, 6×His tag was fused to the C-termini of the peptides. As a result, the fusion peptides were successfully purified by His-tag bind affinity resin. The BMP fusion peptides with expected sizes were secreted from the resulting strains of *P. pastoris* GS115.

Key words: Beefy Meaty Peptide, *Pichia pastoris*, Expression, Purification, Tandem Repeat

INTRODUCTION

The octapeptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, also called "beefy meaty peptide" (BMP), was first reported by Yamasaki and Maekawa [1] and considered a savory seasoning in previous literatures. It was isolated from beef digested by papain. The sequence was further verified by synthesis of the compound and considered to be a meat-like taste octapeptide [2]. The recognition threshold of BMP was confirmed to be 0.533 mM at pH 6.5, which was lower than 1.44 mM reported before [3,4]. The function of BMP is consistent with flavor enhancers such as monosodium glutamate (MSG), inosinic acid (IMP) and guanylic acid (GMP) which are widely used in the foods to enhance their "savory" taste. BMP is stable even after heating at 121 °C for 20 min, suggesting that BMP might be an effective alternative to flavor enhancers such as MSG [5].

To establish a biological producing method to obtain BMP, we tried to clone and express BMP in *Pichia pastoris*. Despite the extensive use of fermentation technique to produce most flavor enhancers, none of the previous studies investigated the use of microorganisms to produce the BMP. The methylotrophic yeast *P. pastoris* has been adapted as an efficient host for expression of heterologous genes. It can grow to very high cell densities (40-50% v/v) and secrete recombinant proteins on minimal medium [6]. Also, it performs many posttranslational modifications of the higher eukaryotes. Here we report the use of *P. pastoris* to produce high quantities of BMP in the form of tandem fusion which is easier to purify and identify. And a new hyperexpressing strain which can produce BMP was successfully constructed, which has the potential to be used in the food industry in the future.

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MATERIALS AND METHODS

1. Strains and Plasmids

Escherichia coli DH5α (Invitrogen) was used as the host strain for plasmid amplification. BMP was inducibly expressed in the host of *P. pastoris* GS115 strain (Mut$^+$, His$^-$) using methanol inducible alcohol oxidase I promoter (P_{AOX1}).

As the tandem repeat of BMP cloning vector pPIC9 (Invitrogen) is a kind of excretion vector, the recombinant protein can be exported to the culture medium facilitating the protein recovery process. The plasmid pPIC9 possesses *HIS4* as the selectable marker. Its *AOX1* promoter is fused to α-MF prepro signal sequence. *Xho* I, *SnaB* I, *Eco*R I, *Avr* II and *Not* I sites are available for insertion of foreign genes. The plasmid pMD 18 simple possesses the ampicillin resistance as selectable marker. The original pMD 18 simple which contained four-copy of BMP expression gene was synthesized by Takara (Dalian, China). Both pPIC9 and pMD 18 simple are maintained and propagated by *E. coli* DH5α host.

2. Reagents

Alkaline phosphatase was purchased from Toyobo (Japan). All restriction enzymes and T4 DNA ligase were purchased from Takara. EZ Spin Column DNA Gel Extraction Kit was purchased from Sangong (Shanghai, China). Its bind affinity purification kit was purchased from Invitrogen (USA).

3. Methods

3-1. Design and Synthesizing of Multicopy BMP Expression Gene

Based on the amino acid sequence of BMP (Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala), we designed the DNA sequence of BMP expression gene 5′-AAGGGTGACGAGGAATCTTT GGCT-3′ according to the optimal codons of *P. pastoris* [7]. To enhance the yield of BMP in *P. pastoris* GS115, the BMP expression gene contained four tandem repeats of the BMP coding sequences. For the pur-

Fig. 1. The construction scheme of synthetical BMP expression gene.

pose of inserting the segment to the expression vector pPIC9, restriction enzyme sites *Eco*R I and *Not* I were included at the two ends. To generate longer BMP repeats, two additional restriction sites, *Xho* I and *Sal* I, were included next to the four copies of BMP genes. In addition, to purify the protein product, a 6×His was fused at the C-terminus followed by a translational stop codon of TAA. The sequence of the whole synthetic gene (Fig. 1) was inserted into the pMD 18 simple.

3-2. Construction of Tandem Repeat BMP Fusion Genes

The original pMD 18 simple, which contains four-copy of BMP expression gene, was digested with *Xho* I and *Sal* I. The 104 bp DNA segment was recovered with EZ Spin Column DNA Gel Extraction Kit. The original pMD 18 simple was linearized with *Xho* I, and then treated with alkaline phosphatase. The recovered BMP fragment was ligated into the vector and introduced into *E. coli* DH5α by electroporation. Resulting transformants grown on LB-agar plate with ampicillin were picked to analyze the sizes of DNA inserts as well as their directions by restriction digests. A series of BMP repeat constructs were obtained and designated as pMD18 simple-mBMP.

3-3. Construction of Expression Vector

pMD18 simple-mBMP was digested with *Eco*R I and *Not* I. DNA digested segments were ligated into the properly linearized pPIC9 vectors. Competent *E. coli* DH5α cells were prepared and transformed with the linearized plasmids by electroporation using a Bio-Rad Gene Pulser. The *E. coli* transformants were validated by PCR using the universal primers *AOX1*, and by digestion of *Eco*R I and *Not* I. Positive transformants were selected on LB plates containing 50 μg/mL of ampicillin.

3-4. Transformation of *P. pastoris*

P. pastoris GS115 (his4) cells were transformed by electroporation with the pPIC9-mBMP linearized by *Sac* I. The recombinants were selected on MD (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) plates for the complement his4 with *HIS4*. Positive transformants were confirmed by PCR amplification of the mBMP using the universal primers *AOX1*.

3-5. Expression of Multicopy BMP in *P. pastoris*

A loop of positive transformants of *P. pastoris* were grown in 500 ml flask with 50 ml BMGY (1.34% YNB, 4×10^{-5}% biotin, 1% glycerol, 1% Yeast Extract, 2% Tryptone, 0.1 M phosphate buffer solution pH 6.1) medium at 28 °C for 2 days until A_{600} reached 10.0. The *P. pastoris* cells were collected by centrifugation at 13,400 ×g, and diverted to BMMY (1.34% YNB, 4×10^{-5}% biotin, 1% methanol, 1% Yeast Extract, 2% Tryptone, 0.1 M phosphate buffer solution pH 6.1) medium, whose volume was the same as BMGY, and were incubated at 28 °C, 180 r·min^{-1}. Methanol (100%) per day was added to a final concentration of 1% (v/v) to induce BMP expression for 70 h.

3-6. Extraction of BMP and Electrophoretic Analysis

The medium of GS115-4BMP, GS115-8BMP, GS115-12BMP, GS115-16BMP from induced cultures was subjected to centrifugation at 2,300 ×g for 5 min. To the 0.2 ml supernatants, 200 μl methanol and 70 μl chloroform were added and mixed. After incubating on ice for 5 min, the admixtures were centrifuged for 5 min at 13,400 ×g. The supernatant was removed and 400 μl methanol was added to the pellet. The admixture was centrifuged for 5 min at 13,400 ×g. The pellets were dried which contained most proteins of the culture supernatant. Tricine-SDS-PAGE was performed according to the method of Schägger and von Jagow [8]. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

3-7. Purification of Recombinant BMP from *P. pastoris*

P. pastoris cells were pelleted by centrifugation at 6,500 ×g for 5 min. The culture supernatant was filtered through a membrane with 0.45 μm pore size.

A chromatography column (2.5-ml resin capacity) was filled by 1 ml His-tag bind resin slurry containing 500 μl resin. When the level of storage buffer drops to the top of the column bed, use 3× volume of sterile deionized water, 5× volume of charge buffer (50 mM $NiSO_4$) and 3× volume of binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) orderly to charge and equilibrate according to the manufacturer's protocol. 10 ml culture supernatant whose pH was adjusted to 7.4 with 5 M NaOH was mixed with 1.43 ml 8× binding buffer. The protein-containing extract was mixed with His-tag bind resin by pipette to incubate for 5 min. The non-bound protein fraction was removed by 10× volume of binding buffer. The fusion peptide of tandem repeat BMP was eluted with 6× volume of wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9) and 6× volume of elution buffer (0.5 M NaCl, 1 M imidazole, 20 mM Tris-HCl, pH 7.9).

3-8. Mass Spectrographic Analysis of 16-copy BMP from *P. pastoris*

GS115-16BMP was selected to ferment, and the electrophoresis strip of 16-copy BMP was analyzed by mass spectrometry. The target protein was reclaimed from the Tris-SDS-PAGE gel. The 16-copy BMP was hydrolyzed into smaller fragments using trypsin, and then the molecular weight of the peptide product was detected by 4700 Proteomics Analyzer (Applied Biosystems). The mass spectrometer was operated under 18 kV accelerating voltage in the reflectron mode with an m/z range of 700-4,000. Peak lists (S/N>10) obtained from MALDI-TOF MS were extracted from raw data and were analyzed by GPS ExplorerTM software (Version 3.5, Applied Biosystems, USA). The peptide spectra data obtained was entered into the database, searching for the corresponding known proteins. So a sequence of testing protein was obtained.

RESULTS

1. Construction of Multicopy BMP Expression Gene

After the ligation and transformation, we selected three different transformants of pMD 18 simple-mBMP which contain 8-copy, 12-copy and 16-copy BMP expression genes (Fig. 2) and was designated as pMD 18 simple-8BMP, pMD 18 simple-12BMP and pMD 18 simple-16BMP, respectively. The copy number of the BMP fusion genes and their insertion directions were determined by digesting the plasmids with *Xho* I and *Sal* I as well as with *Eco*R I and *Not* I. The sizes of the digesting segments were equivalent to the pre-

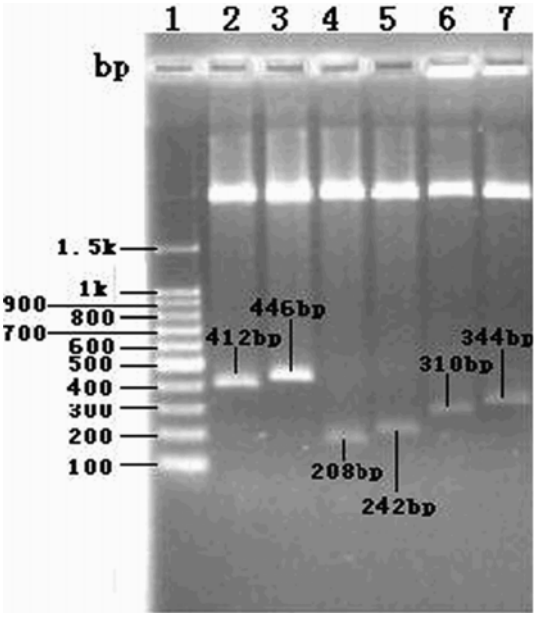

Fig. 2. Digestion patterns of pMD 18 simple-mBMP. Lane 2, 4, 6, the *Xho* I and *Sa*l I digests of pMD 18 simple-16BMP, pMD 18 simple-8BMP and pMD 18 simple-12BMP. Lane 3, 5, 7, *Eco*R I and *Not* I digests of pMD 18 simple-16BMP, pMD 18 simple-8BMP and pMD 18 simple-12BMP.

dicted sizes.

2. Construction of Expression Vector

The segments of DNA that were generated by *Eco*R I and *Not* I digests of pMD 18 simple-4BMP, pMD 18 simple-8BMP, pMD 18 simple-12BMP and pMD 18 simple-16BMP, respectively, were ligated into the same sites of pPIC9 vector. The resulting constructs were named as pPIC9-4BMP, pPIC9-8BMP, pPIC9-12BMP and pPIC9-16BMP, respectively. All the constructs were linearized with *Sac* I and transformed into GS115 by electroporation. The His^+ transformants which were capable of growing on the MD plates were selected, named GS115-4BMP, GS115-8BMP, GS115-12BMP, GS115-16BMP, respectively. The positive transformants were validated by PCR amplification of the BMP fusion genes by the universal primers *AOX1* again (Fig. 3). The sizes of PCR products were equivalent to the theoretic sizes, proving that the tandem repeats BMP expression genes were indeed inserted into the alcohol oxidase1 (*AOX1*) gene of *P. pastoris* GS115.

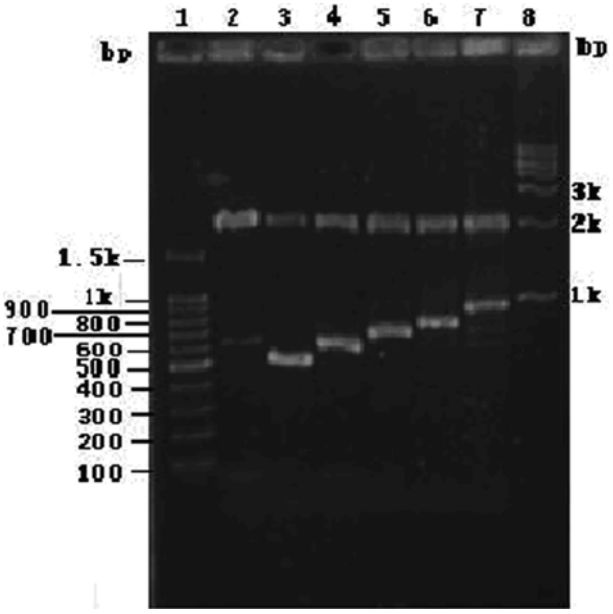

Fig. 3. The amplified segments by PCR of the different positive transformants of GS115. Lane 2, the amplified segments of GS115; lane 3 is the amplified segments of GS115 with the pPIC9 inserted in *AOX1* gene (GS115-pPIC9); lane 4 to 7, the amplified segments of GS115-4BMP, GS115-8BMP, GS115-12BMP, GS115-16BMP, respectively.

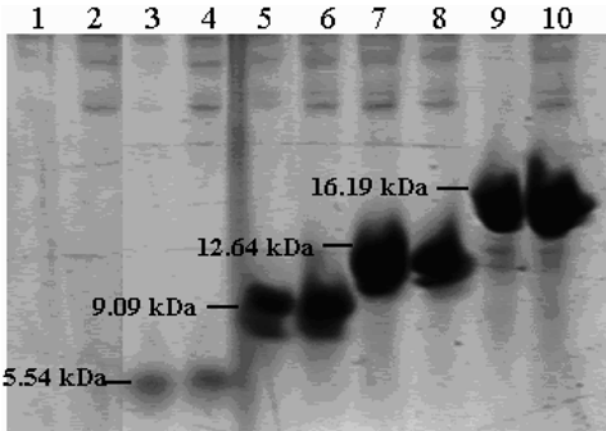

Fig. 4. Electrophoresis gel of the proteins of the ferment liquid supernatant of GS115 and GS115 recombinants. Lane 1, proteins of ferment liquid of GS115; line 2, proteins of GS115-pPIC9; lane 3 and 4, proteins of GS115-4BMP, lane 5 and 6, proteins of GS115- 8BMP; lane 7 and 8, proteins of GS115-12BMP; lane 9 and 10, proteins of GS115-16BMP. This is Coomassie stain.

3. Expression of Multicopy BMP in *P. pastoris*

Tricine-SDS-PAGE was used to separate the proteins (Fig. 4). Compared with the negative control samples, proteins from GS115 and GS115-pPIC9, the recombinant strains produced 4-copy, 8-copy, 12-copy and 16-copy BMP fusions with expected sizes-5.54 kDa, 9.09 kDa, 12.64 kDa, 16.19 kDa.

4. Purification of Recombinant BMP from *P. pastoris*

Considering the yield of BMP expressed by the recombinant of GS115, GS115-16BMP was selected to further purify the 16-copy BMP fusion protein. The 6× His-tag was bound to his-bind resin, and then the whole chain of 16-copy BMP was eluted by 1 M imidazole buffer. The purified 16-copy BMP was confirmed by Tricine-SDS-PAGE (Fig. 5). Combining all of the above, we have constructed a new strain which can secrete 16-copy BMP and we were able to purify the corresponding protein product.

5. Mass Spectrographic Analysis of 16-copy BMP from *P. pastoris*

The theoretical amino acid sequences of the target protein: EAEAYVEFTE(KGDEESLA)4VE(KGDEESLA)4VE(KGDEESLA)4VE(KGDEESLA)4VDH6

In accordance with different degrees of hydrolysis, different peptide molecules were obtained after digestion by trypsin, as shown

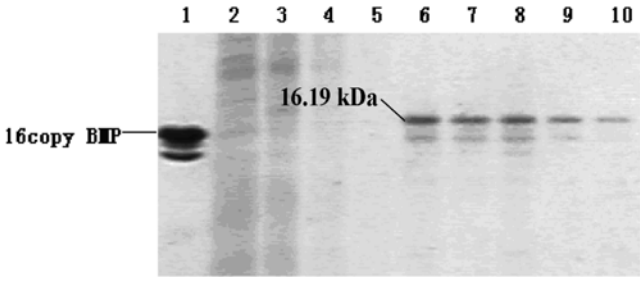

Fig. 5. Electrophoresis gel of the purified 16-copy BMP. Lane 1, total supernatant of GS115-16BMP; lane 2, flow through of the his bind resin; lane 3, 4 and 5, eluate from binding buffer; lane 6 and 7, eluate from wash buffer; lane 8, 9 and 10, eluate from elute buffer, showing most of 16-copy BMP dropped from the resin.

Table 1. The mass spectrographic analysis of BMP digested by Trypsin

The amino acid sequences of peptides	m/z (Theoretical value)
GDEESLAKGDEESLAKGDEESLAVDHHHHHH	3416
EAEAYVEFTEKGDEESLAKGDEESLAK	2974
GDEESLAKGDEESLAKGDEESLAVEK	2735
GDEESLAKGDEESLAVEKGDEESLAK	2735
GDEESLAKGDEESLAVDHHHHHH	2586
GDEESLAKGDEESLAKGDEESLAK	2507
EAEAYVEFTEKGDEESLAK	2145
GDEESLAKGDEESLAVEK	1906
GDEESLAVEKGDEESLAK	1906
GDEESLAVDHHHHHH	1756
GDEESLAKGDEESLAK	1678
EAEAYVEFTEK	1316
GDEESLAVEK	1076
GDEESLAK	848

in Table 1.

The first order mass spectrum of BMP (Fig. 6) was procured by positive ion scan for the hydrolysates. The fragments of corresponding m/z were found from the graph. They were 1906: GDEESLAVEKGDEESLAK, 1757: GDEESLAVDHHHHHH, 1077: GDEESLAV EK, 848.4: GDEESLAK, respectively. According to the breakage rules of peptide bond, four molecular ion peaks were analyzed by the second order mass spectrometry. The ion fragments were in line with the theoretical value. So the results of first order mass spectrum and second order mass spectrum indicated that the protein specific expressed by GS115 16-BMP might be transcribed by exogenous gene which was integrated into the *P. pichia* genome by recombinant DNA technology.

DISCUSSIONS

P. pastoris is a highly successful system for the expression of heterologous genes. Several factors have contributed to its rapid acceptance. First, the promoter is derived from the *AOX1* gene of *P. pastoris* that is uniquely suited for the controlled expression of foreign genes. Second, the strong preference of *P. pastoris* for respiratory growth is a key physiological trait that greatly facilitates its culturing at high-cell densities relative to fermentative yeasts. Third, as an epiphyte, *P. pastoris* has the potential to perform many of the posttranslational modification typically associated with higher eukaryotes. Fourth, in *P. pastoris*, linear vector DNAs can generate stable transformants via homologous recombination between sequences shared by the vector and host genome [9,10]. Such integrants show stronger stability in the absence of selective pressure than plasmids even when present as multiple copies. Finally, proteins can also be secreted into the culture medium using suitable secretion signal. And there are many commercially available plasmids for choice. Up to now, more than 100 different proteins have been successfully produced in *P. pastoris*. The potential advantage of *P. pastoris* ensures the outcome can reach its function, which we expected, so *P. pastoris* was chosen to express BMP.

In previous study, the single copy BMP octapeptides had been

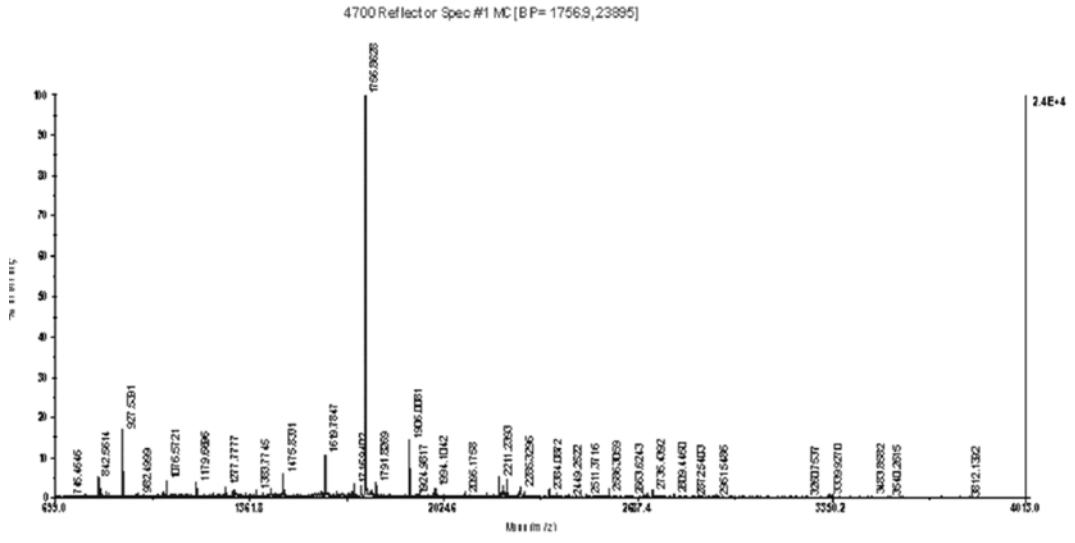

Fig. 6. First order mass spectrum of BMP.

cloned successfully and expressed by *P. pastoris*. But there are some questions with it: the single copy BMP was not prone to purify and its yield was low relatively. Therefore, we constructed the tandem repeat BMP gene with 6× His tag. 6× His tag fusion can make the purification easier, so a simple and high efficiency way can be taken out to produce BMP. This research also designed the tandem repeat expression gene of BMP, ligated it to obtain multicopy BMP expression genes, which can highly enhance the yield of the production. As a small chain of proteins can hardly express from transformants, the strain expressing 16BMP which contains 16 octapeptides enhances the yield of BMP more than four times than GS115-4BMP.

The *P. pastoris* expression system has been widely used to produce a variety of different heterologous proteins. It was reported that the yield of *Aspergillus oryzae* tannase was 72 mg/L [11], and the yield of angiostatin was 108.0 mg/L [12]. The recombinant *P. pastoris* yielded about 900 mg/L procine somatotropin in the shake-flask cultures [13]. In this study, we produced large amounts of BMP in secreted form in the methylotrophic yeast *P. pastoris*. In all, we reached a yield of 160 mg/L of GS115- 16BMP.

The intensity of the umami and/or salty taste of five delicious peptide analogs, Ser-Leu-Ala-Lys-Gly-Asp-Glu-Glu, Ser-Leu-Ala-Asp-Glu-Glu-Lys-Gly, Lys-Gly-Ser-Leu-Ala-Asp-Glu-Glu, Lys-Gly-Asp-Glu-Glu, and Glu-Glu-Asp-Gly-Lys, showed high similarities, despite their chemical structures not being exactly identical [14]. Both thaumatin and monellin were proteinic sweeteners extracted from African berry. And thaumatin expressed from *Aspergillus awamori* showed a sweet taste [15]. Monellin was produced from *Candida utilis*, and the degree of sweet taste of such protein was coordinative with the natural monellin [16]. Their conclusion is the base evidence of the flavor function of the tandem repeats BMP that we produced. Preliminary sensory evaluation of 16BMP carried out in our lab indicated that the peptide had a meaty or savory taste. For production of BMP on large scale, its detailed flavor study (with help of Tianjin Chunfa food ingredients limited company) demonstrated that 16BMP had a palatable taste and could enhance the flavor of beef gravy. A study on the flavor of the 4BMP, 8BMP, 12BMP, 16BMP is in progress, and will be reported successively. To ensure the safety of the taster, we should test the toxicity of the outcome by animal experiment. In conclusion, expression of tandem repeats BMP in *P. pastoris* was successfully expressed and purified.

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