Journal of Sciences, Islamic Republic of Iran 22(2): 105-110 (2011) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Synthesis and Production of Sweet-Tasting Protein in *E. coli* and Purification by Amylose Resin

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Received: 19 January 2011 / Revised: 18 May 2011 / Accepted: 3 July 2011

Abstract

A sweet water-soluble protein that reminds stable over wide ranges of temperature and pH, Brazzein has various applications. Its tastes like cane sugar but have no calories. However, the extraction of brazzein from its natural source is expensive and not applicable. In this study we used recombinant DNA technology to provide an alternative option for cheaper mass production of brazzein. A brazzein gene was designed, synthesized by using oligonucleotids and cloned into the pBlueScript vector. The synthetic fragment linked to the C-terminal of Maltose-binding protein (MBP) and Glutathion-S-Transferase (GST) for protein expression. The recombinant protein was expressed as a soluble form after induction by IPTG. The fusion protein was purified by amylose resin column and detected by SDS-PAGE. The best yields were achieved by producing brazzein as a fusion with MBP. MBP-brazzein system permits large-scale functional expression and purification of recombinant soluble proteins, providing a basis for the future study of structure and function of brazzein.

Keywords: Brazzein; Sweet-tasting protein; Recombinant protein

Introduction

Most proteins and peptides are tasteless, whereas some proteins have a sweet taste and stimulate putative receptors in human palate [1, 2]. In this century, eight high potency sweet proteins have been found in west Africa and tropical area plants, namely, thaumatin, monellin, mabinlin, miraculin, neoculin, pentadin, egg white lysozyme and finally brazzein [3-5]. Two proteins, miraculin and neoculin are taste-modifying proteins that have the property of converting a sour taste to a sweet taste [6, 7]. They differ in molecular size and in the intensity of their sweetness (500-4000 times sweeter than sucrose on a weight basis). However, no common similarity or structural homology have been determined in these proteins [8]. These natural proteins have been found in the fruit of rain forests and the indigenous people have frequently used them to sweeten their food [9]. Experiments are in progress to modify taste proteins by adding various genes or mutations in a

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way similar to what happens in nature [10, 11].

Sweet proteins have the potential to replace artificial sweeteners [10]. Chemical sweeteners like Saccharin and Aspartame have typical accompanying effect such as undesirable weight gain, heart failure, bladder cancer and diabetes [12, 13]. Frequent consumption of high-sugar foods alters mouth pH and damages teeth. Some of these problems can be changed by greater use of other sweeteners that have more intense sweet taste, but with lower calorie [6]. In recent years, there has been an increasing demand for natural sweetener.

Among the natural proteins, brazzein, is composed of a single chain of 54 amino acids (about 6.4 kDa), which makes it the smallest known sweet taste protein [9]. The brazzein fold, which contains one alpha-helix and three strands of antiparallel beta-sheet, does not resemble that of other sweet-tasting proteins [14]. Brazzein has no carbohydrate and similarity to sucrose, but is 2000 times sweeter than sucrose solution on a weight-weight comparison [15]. Since brazzein is derived from a protein and produces no increase in blood sugar, it is considered safe for diabetics. Brazzein has a special profile of heat and pH stability, its sweet taste is undiminished after incubation at 100° C for a period of 4 hours in the pH range of 2.5 to 8.0. This may be the result of four intramolecular disulfide bonds [9, 15]. These characteristics make brazzein and other natural sweeteners a very good alternative to sucrose [16]. The solid-phase peptide synthesis of brazzein was performed using the Fmoc strategy. The obtained synthetic form was 500 times sweeter than sucrose. The D enantiomer of brazzein was synthesized, but its powder was tasteless [12].

The extraction of sweet tasting protein from the natural source is a highly complex, exclusive, time-consuming process and the producing plants appear to have difficulty bearing fruit outside the natural habitats [17, 18]. From a technical point of view, any protein can be obtained either by extraction from its natural source or through recombinant DNA technology [12, 19]. Most of sweet proteins were initially discovered at laboratories or universities. There have been attempts to produce sweet-proteins in recombinant organisms, none of which has reached the market [20].

In this report, we used a method based on the MBP and GST fusion system with strong promoters to more efficiently to produce of brazzein in *E. coli* and isolate sufficient quantities. The major advantages of MBP fusion system provide a higher yield, enhance the solubility, improve the proper folding and purity of the target protein on a large scale. We chose *E. coli* for gene synthesis and protein production, because of its easy genetic handling and rapid production. *E. coli* is the dominant prokaryotic system for industrial gene expression to high yields [21].

Materials and Methods

Synthesis and Amplifying Brazzein Gene

The brazzein gene was synthesized by ligating two different oligonucleotids per strand F1; 5'-ATGGACA AATGTAAAAAAGTATACGAAAACTACCCGGTA TCCAAATGTCAGCTGGCAAACCAGTGTAACTAC GACTGTAAACTGGACAAACACGCTCG-3' and F2; 3'-TGCTGACATTTGACCTGTTTGTGCGAGCAAG GCCACTTACGAAGATGCTGCTTTTTGCATTGGA CGTCACGTAGACGCTGATGACGCTTATGATT-5'. This fragment was designed on the basis of the amino acid sequence of brazzein for expression. In addition, a starting codon (Met) was introduced before the first sequence. The nucleotide sequence was divided in to two partially overlapping oligonucleotids. These oligonucleotids were annealed at 65°C to create DNA double strands and were filled with klenow fragment enzyme (Invitrogen, San Diago, USA).

This fragment was designed on the basis of the amino acid sequence of brazzein. This fragment was amplified by PCR using *pfu* DNA polymerase (Promega, USA) with two primers 5′-CGCGGATCC ATGGACAAATGTAAAAAAGT-3′ and 5′-CGCGGA TCCTTAGTATTCGCAGTAG-3′ contains BamH1 enzyme site. Amplification was performed on a Corbet thermal cycler (Corbet, model: CG1-96, Australia) 4 min at 95°C for initial denaturation step, followed by 33 cycles of 30 sec at 95°C, 45 sec at 59°C, 1 min at 72°C for extension, and finally 7 min at 72°C. PCR product was digested with BamH1 enzyme (Fermentase, Burlington, Germany) and extracted from gel. The purified PCR product was inserted into the pBlueScript/sk (+) vector (Fermentase, Burlington, Germany) and transformed in *E. coli Xl-blue* strain. Transformation of plasmid was done as described by Sambrook *et al.* [22]. The correct DNA sequence was confirmed by automated DNA sequencing using an ABI prism dye terminator cycle sequencing (Applied Biosystems, Macrogen, South Korea), using the M13 primers.

Brazzein Expression

After sequencing, the brazzein fragment was digested with BamH1 enzyme, and extracted from 1.7% low melting agarose gel (Invitrogen, San Diago, USA) by gel extraction kit (Fermentase, Burlington, Germany). This excised fragment was subcloned into

expression vectors (BamH1- digested pMAL-C_2X and pGEX-2T). The pGEX-2T (Amersham Pharmacia, USA) and pMAL-C_2X (New England Biolabs, Beverly, MA, USA) was used for the construction of the expression system. The ligation reaction was transformed into *E. coli Top10F* strain competent cell and dispensed on LB agar plates containing 100 mg/ml ampicillin. Bacterial colonies were screened by agar plate containing X-gal (Roche, Mannheim, Germany) and isopropyl-1-β-D-thiogalactopyranoside (IPTG) to discriminate between recombinant and nonrecombinant plasmid for pMAL vector. Colonies were mass cultured in LB medium supplement with 100 mg/ml ampicillin in an orbital incubator at 37°C with vigorous shaking at 250 rpm [22]. Plasmids containing insert were selected and identified by PCR and automated DNA sequencing using an ABI Prism dye terminator cycle sequencing (Applied Biosystems, Fostercity, USA) for correct orientation fragment with specific vector (Puc-M13 primer) and insert primers.

Recombinant plasmids were transformed to *BL21DE3plysS*. The *BL21DE3 plysS* pMAL-C2X-Br and pGEX-2T-Br were grown to an optical density of 0.5 at 600 nm; IPTG was added to a final concentration of 1 mM at 2, 3 and 4 hours to choose the best time for expression fusion protein before being harvested. The cells were collected by centrifugation (12,000 ×g, 30 sec), and uninduced cultures were also included to compare bacterial protein expression [22]. The pellets were collected and stored at -20°C until used and the expressions were analyzed on 12% SDS-PAGE according to the method Laemmli [23]. Protein samples were diluted in SDS loading buffer and vortex to resuspend cells. These mixtures were heated at 90° C for 10 min. The proteins were then detected by developing the gel with coomassie R-250 staining solution.

Purification of the Fusion Protein

The total bacteria cells were collected by centrifugation at 4000 ×g for 20 min. The pellet was resuspended in cold Tris buffer (Tris 1M PH 7.4, NaCl 11.7gr, EDTA 0.5 M, β-Mercaptoethanol 0.7 ml, sodium azide 1M) and protease inhibitor Cocktail tablet (1 tablet for 25 ml buffer). The cells were frozen at -20°C overnight and then sonicated in short pulses of 15 seconds and an interval of 10 sec between each pulse (about 2 min sonication time on ice). The cells were centrifuges at 9,000 ×g for 30 min. The supernatant was transferred to amylose resin (New England Biolabs, Beverly, MA, USA) and the protein was purified with New England Biolabs kit according to the manufacturers' protocol. Bound fusion protein was eluted with 10 mM maltose (New England Biolabs, Beverly, MA, USA). The yield of the fusion proteins was measured using the Bradford procedure [24] and analyzed on SDS-PAGE.

Results

Gene Synthesis and Cloning

To produce sweet protein, we used a synthetic fragment with 54 amino acid and restriction sites (BamH1) to facilitate the cloning and expression of the protein in bacteria based on the fusion systems. This gene was cloned first into the pBlueScriptIIks/sk(+) and identified by restriction analysis and confirmed by DNA sequencing. This sequencing result reveals which synthetic fragment according to original sequence without deletion or insertion (Fig. 1).

Gene Expression

The synthesized brazzein gene was subcloned into pMAL and pGEX plasmids. To get high yields, the expression vectors pMAL-C2X and pGEX-2T contain the strong Ptac promoter. The major advantage for this particular manner is solubility of the protein product that is required for folding and stability of the protein. The constructed expression vectors were transformed into the *E. coli* strain *BL21DE3plysS*, which has been suggested to give rise to increased expression levels of secreted protein by reduction of proteolysis proteins. When protease inhibitor was added, proteolysis was decreased. The pMAL-Br and pGEX-Br plasmids were constructed by inserting a fragment of the brazzein gene into the BamH1 site of plasmids. The pMAL expression vectors tender an easy means to produce expression protein at high levels and an ordinary clone selection using MBP-lacZα. The pMAL without encoded recombinant protein, MBP-lacZα has a mass of 50.8 kDa. Insertion of brazzein fragment inactivates the β-galactosidase α-fragment activity, and MBP has a mass of 42 kDa. Subsequent of the cloning, the pMAL contains a recombinant protein, named MBP-Br with a molecular weight of 48.4 kDa (Fig. 2).

The brazzein fragment also was subcloned in pGEX-2T downstream and in-fram with the Glutathione-S-Transferase; the pGEX-2T with fragment, named GST-Br has a mass of 32.4 kDa and the pGEX-2T vector with no insert that encoded GST has 26 kDa (Fig. 2). Brazzein constructs were tested at different times of 2, 3 and 4 hours. The fusion protein concentration at 2, 3 and 4 hours after induction with IPTG and culturing for

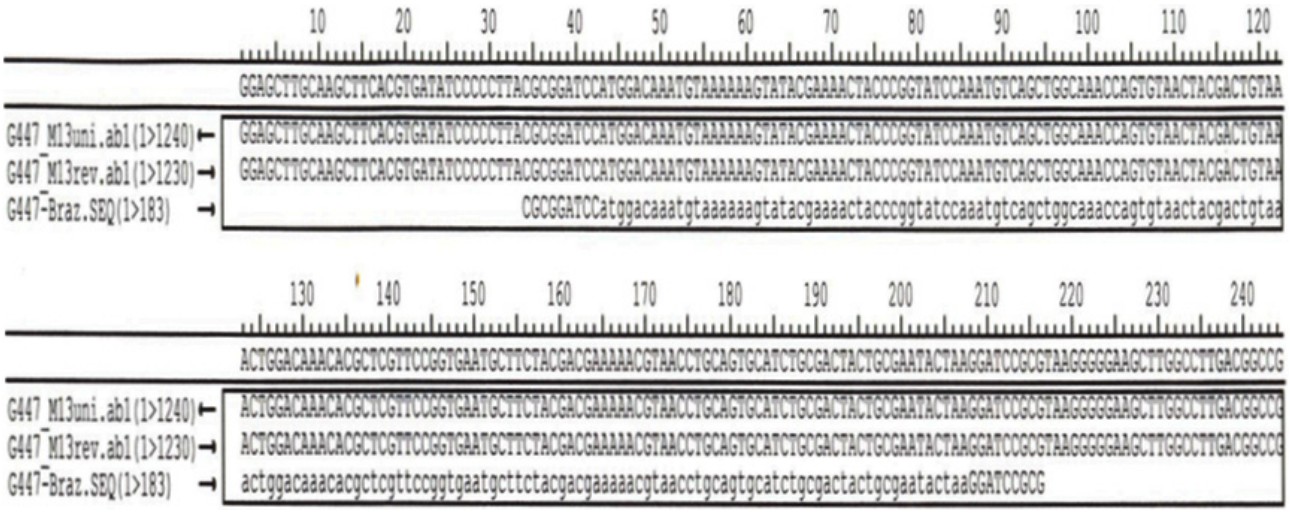

Figure 1. Alignment of synthesized brazzein gene and natural brazzein.

longer periods did not result in a higher yield under equal conditions. Supernatant fraction of cell lysate contained the fusion protein were separated by 12% SDS-PAGE. The amount of GST-Br was lower (about 60 mg/l) than MBP-Br (180 mg/l).

Purification of Recombinant Protein

MBP fusion system was used for purifying a protein with one-step amylose resin column procedure and fusion protein was eluted with maltose in physiological buffer. The final yields were 860 µg/ml MBP-Br and 480 µg/ml for MBP (Fig. 3).

Discussion

In this report, we engineered a synthetic gene to express the brazzein molecule and developed the production system in *E. coli*. Brazzein exists in two forms in the ripe fruit: The major form isolated from its natural source has a pyroglutamic acid (pGlu) in the N-terminal (80%) and the minor form without pGlu (des-pGlu1). Sensory analysis shows that the minor form is about twice sweeter than the major form [25]. There have been suggestions that brazzein could be expressed in eukaryotic and prokaryotic such as *E. coli* and in transgenic plants [7].

We used the sequence of the minor form of brazzein as the basis for designing a synthetic gene for protein production in *E. coli* [8]. The synthetic gene coding for brazzein was prepared on the basis of the amino acid sequence and cloned in pBlueScript plasmid. The correct synthetic gene was confirmed by sequencing. The alignment sequencing results correspond to brazzein gene [7]. Based on the native brazzein, site directed mutagenesis was used to identify critical region for brazzein. On the basis of these studies, deletion or insertion of the N-terminus and C- terminus are involved in the sweetness [17].

A synthetic gene for brazzein has been synthesized, but previous expression in *E. coli* gave poor yields [8]. In order to reach the market, brazzein must have a high yield in expression. Expression could be limited by a weak promoter or copy number plasmid or miss-folded protein.

Expression of brazzein in *E. coli* is feasible for research purposes [26]. The generation of recombinant proteins that produce high levels of product are certainly one of the most critical elements required for the development of protein [26]. Brazzein is difficult to express on its own [15], because of its small size. To improve production level, the synthetic brazzein gene was cloned and inserted into the expression vectors: pGEX-2T and pMAL-C2X. The supernatant fraction of cell lysate contained the fusion protein, indicating that it was over expressed. Comparing the expression rate of MBP versus GST fusion protein, we found a higher expression rate of soluble protein using MBP fusion than that for GST fusion. This observation shows that the MBP is much more effective in promoting proper folding of the other partner in a fusion protein. Therefore, production at high yield with less time can be achieved in *E. coli* when the protein is tagged with maltose binding protein. The MBP can increase the solubility of over expressed fusion proteins in bacteria, especially eukaryotic proteins [21]. A spacer sequence

coding for ten aspargine residues between the MBP and the brazzein protein increase the chances that a particular fusion will bind tightly to the amylose resin. The MBP is important in protein purification and can be helpful for stabilizing proteins or enhancing their solubility [27].

GST-Br fusion protein yield was lower than MBP-Br. This is certainly due to positive charges on the surface of brazzein [15]. Research on brazzein suggested that the positive charge is important in the determination of brazzein sweetness [28]. Its sequence contains five Asp, two Arg, four Glu, one His, six Tyr, seven Lys and two Ser, which constitute about 50% of the total number of residues [25]. Charged and polar residues are the critical for the sweetness of brazzein, monelline and thaumatin [29]. In some cases, GST fusion proteins are totally or partly soluble. It is not clear which factors are responsible for insolubility, but in several cases insolubility of GST fusion proteins were associated with the presence of hydrophobic regions. Some insoluble fusion proteins either contain many charged residues or are larger than 100 kDa. GST fusion proteins are also commonly used in studies on protein-DNA interactions and protein-protein interactions.

The brazzein gene was cloned (Assadi-Porter 2000) into the C-terminus of the modified SNase in the conventional pET vectors [8]. These vectors were transformed into *E. coli* strain BL21 *(DE3)/pLysS*. The pET vectors are generally successful for the expression of soluble foreign proteins, such as staphylococcal nuclease, that are larger than 100 amino acids and have low cysteine content. Approximately 50-70% of the 23 kDa fusion protein was expressed in an insoluble form. The fusion protein was refolded after solubilization from inclusion bodies and the level of fusion protein was 130 mg/l [8]. This procedure had several steps and the refolding and oxidation was time consuming. Ubiquitin-based (SUMO) tags have been used to increase expression levels [10].

Previous attempts to express genetically optimized synthetic brazzein fused with GST in yeast S. cerevisiae were unsuccessful [8, 12]. The production of brazzein in transgenic maize was performed [30]. Brazzein gene was synthesized and fused to plant expression vectors such as a constitutive promoter (maize polyubiquitin-like promoter), an embryo-preferred promoter, and an endosperm-preferred promoter. Among them, the highest brazzein expression level was attained in the seed from the line containing an embryo-preferred promoter [12, 30]. However, the yields with this expression system of small proteins containing multiple disulfide bridge like brazzein frequently are low. For that reason, proteins of this kind must be expressed in a eukaryotic system, which is more related to that of the species of origin for the gene [30].

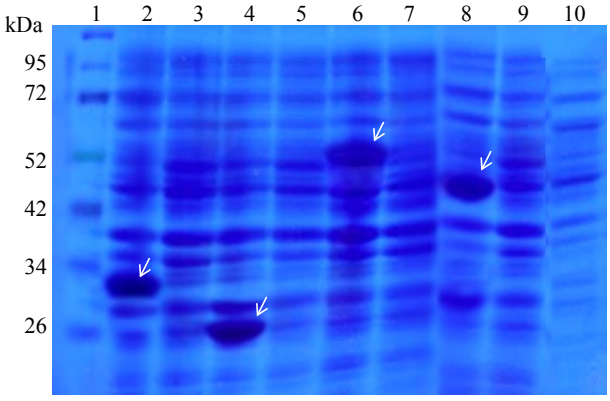

Figure 2. Expression of recombinant protein on 12% SDS-PAGE. *E. coli* strain *BL21DE3PlysS* was transformed with both pGEX-2T and pMAL-c2x following 0.1 mM IPTG induction. Lane1, protein Markers: lane 2, induced bacteria expressing pGEX-Br protein (32 kDa); lane 3, uninduced bacteria expressing pGEX-Br; lane 4, induced bacteria expressing pGEX protein (26 kDa); lane 5, uninduced bacteria expressing pGEX; lane 6, induced bacteria expressing pMAL protein (50.8 kDa); lane 7, uninduced bacteria expressing pMAL; lane 8, induced bacteria culture expressing pMAL-Br (48.4 kDa); lane 9, uninduced bacteria culture expressing pMAL-Br; lane 10, total bacterial of *BL21DE3plysS* without plasmid.

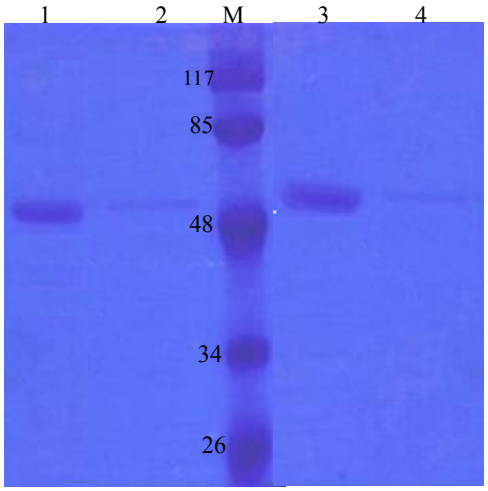

Figure 3. Purification of soluble recombinant MBP-Br and MBP by amylose resin on 12% SDS-PAGE. lane 1, 15µg MBP-Br proteins purified (48.4 kDa); lane 2, 3µg MBP-Br proteins purified (48.4 kDa); lane 3, 15 µg MBP proteins purified (50.8 kDa); lane 4, 3µg MBP proteins purified (50.8 kDa); M, protein molecular Markers.

In order to reach the market, the expressed protein must have the same phenotype as the natural product. Therefore, the biotechnological approach must attain certain yields and toxicological studies will be required. By learning more about its mechanism of action and by developing more potent brazzein, we will be in a better situation to evaluate brazzein as a potential sugar substitute as a major advantage to artificial sweeteners.

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