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Production of Monoclonal Specific Antibody against Brazzein Fusion Protein

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background: The use of a natural sweet protein is important as an attractive alternative to sucrose in the human diet. Rigorous research has identified the use of the artificial sweetener that leads to various diseases, cancer and heart disease. Many functional studies are necessary for examining the structure and biological activity of natural sweeteners such as Brazzein and its receptor targeting. In this report, we produced a panel of monoclonal antibodies against the recombinant brazzein which can be used for functional studies.
Methods: Hybridomas cell fusion technology was used for the production of monoclonal antibody. BALB/c mice were immunized twice with purified fusion proteins with Freund adjuvant. Next, 14 days after the last injection, blood sample was collected from the eye and serum was separated and the antibody titer was detected by enzyme-linked immunosorbent assay (ELISA). The mouse with the highest antibody titers was selected. Seven days before fusion, the selected mouse was boosted with 50 µg antigen into the peritoneum to develop a robust immune response and then

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spleen cells were isolated from the same animal for fusion to myeloma SP2/0 to generate hybridoma cells.
Results: Hybridoma clones were screened by ELISA and their monoclonal antibodies were purified. Specificity and reactivity of the monoclonal antibodies were determined by western blot analysis. We produced a panel of monoclonal antibodies that were highly specific and reacted with Brazzein protein that was detected by western blot analysis.
Conclusion: These monoclonal antibodies bind to the MBP (Maltose binding protein)-containing recombinant protein in western blotting, so this antibody can be a useful probe for tracing proteins in basic research and pharmaceutical applications.

Keywords: Brazzein; fusion protein; monoclonal antibody.

1. INTRODUCTION

Both natural and artificial sweeteners have been proved to be extremely for the human diet. Artificial sweeteners and sugar intake play an important role in the prevalence of some disease such as obesity, diabetes and cardiovascular disease. Recently, there has been an increasing demand for low–calorie sweeteners and healthy natural. Several natural-sweet proteins have been identified and among them brazzein is a small natural protein. Brazzein is a sweet-tasting protein originally isolated and purified from the fresh fruit of *Pentadiplandra brazzeana*, a climbing vine that grows in Cameroon and Zaire [1-2]. Natural brazzein which consists of a single chain [3], is the smallest of the sweet-tasting proteins described to date [4-5]. Two forms of brazzein have been isolated from the fruit [6]. The major form, which has pyroglutamic acid at the N-terminal consists of 54 amino acid residues and the minor form, which is similar to the major form except for the absence of pyroglutamic acid has 53 amino acid residues [7]. Brazzein is 500 times sweeter than sucrose on a weight basis and its sweetness is stable when exposed to elevated temperatures for a period of 4 h [8-9]. The three-dimensional structure of brazzein has been determined by H-NMR spectroscopy [10-11]. Its structure consists of one short alpha helix and a three-strand beta sheet. The C- and N-terminal regions are involved in the sweetness of brazzein and identify a number of residues in brazzein which are important for interaction with the receptor [12]. It is rich in lysine but contains no methionine, threonine or tryptophan [13]. The sweet taste of brazzein can be detected by human, old world monkeys and apes by taste trials. Till now, two T1R2-T1R3 receptor proteins are identified for responsiveness to sweet ligands [14-15]. Sweet proteins interact with the receptor and then induce the receptor activation and sensation of sweetness [16].

Because of its small size, rigidity, stability and solubility in water, brazzein offers advantages over other sweet tasting peptides and proteins system for detailed studies [17-18]. In addition, it is an ideal candidate for a low-calorie sweetener because of its natural origin and favorable physical properties [19-20]. Identification of the sweet-taste receptor and investigation of the interaction between the sweet receptor and sweet compounds like brazzein will be essential for understanding the molecular basis of this sensory transduction pathway [21]. Although the original source and gene sequences described, there is much information about function, receptors, signaling pathway and specific cellular location remains still unclear. In this regard, specific antibodies are necessary to investigate the function of brazzein. An effective antibody against brazzein is not yet available. To understand structural and chemical properties responsible for brazzein, we engineered a synthetic gene to express the brazzein molecule in *E. coli* in our laboratory [22].

In this work, eight stable monoclonal antibodies against fusion protein brazzein were generated and characterized by indirect ELISA and western blot. Thus the mAbs can be used as tools in different molecular techniques [23]. Knowledge of its fundamental properties can be use in basic research and pharmaceutical application.

2. MATERIALS AND METHODS

2.1 Plasmid Construction and Protein Purification

PMAL-Brazzein vector was constructed as described previously in our work in Tehran medical university (we named MBP-Br). The efficient expression of MBP-Brazzein fusion protein was induced by 1 mM IPTG at 37℃ in *E. coli BL21 (DE3)* for 2 h. The fusion proteins were purified from the bacterial lysate by one-step amylase resin purifications described

previously [22]. Protein determination was done by Bradford's dye binding assay and identified by 12% SDS-PAGE [24].

2.2 Preparation of Mouse

In this work, old female BALB/c mice (4-6 week) obtained from the Pasteur Institute of IRAN. The first step, one mouse did not receive any antigenic was selected as a negative control and one mouse received 50 µg diluted MBP was selected as a positive control. Then, four old female BALB/c mice (4-6 week) were immunized by injections intraperitoneally (i.p) with 50 µg purified fusion protein MBP-Br that was diluted in phosphate buffer saline (PBS) with an equal volume of complete freund's adjuvant (100 µL of a 1:1 emulsion of antigen in PBS: adjuvant) and 20 days later, the same mice were boosted again with amount of antigen with incomplete adjuvant. Next, 14 days after the last injection, 70 µL of blood was collected from the eye and serum was separated and the antibody titer of the after injection was compared with the before injection from the same animal as a baseline control for antibody titers of the antiserum by ELISA (1:500, 1:1000, 1:10000 dilution of sera). The mouse with the highest antibody titer was selected for fusion to myeloma cells. Seven days prior to cell fusion, the selected mouse with high titer (1/1000 dilution of sera) was boosted by the i.p route with 50 µg of fusion protein. Pre-immune serum was taken before immunization and one mouse without any antigen were as negative control. All procedures performed in studies involving animals in accordance with the ethical standards.

2.3 Measurement of Humoral Antibody Response

Blood samples were collected by retro-orbital puncture from immunized mice 7 days after the final booster injection and sera were tested for the presence of antibody. Serum antibody titer was determined by ELISA. Purified recombinant protein was individually diluted to a concentration of 10 µg/ml in PBS and 100 µl of each recombinant protein was added to separate wells of micro titer ELISA plates (Nunc, Denmark). Control well was coated with mice generated high serum antibody titer and negative wells were coated with the same mice before injection of antigen. Coated plates were stored at room temperature, blocked for 1h and washed three times with 0.1% BSA in PBS, prior to incubation with sera samples. After adding 100 µl of diluted sample of sera (1:500, 1:1000, 1:10000) to each well, the plate was incubated for 1 h at room temperature and then washed three times with washing buffer. Secondary antibody horseradish peroxidase-labeled conjugates goat anti-mouse IgG (sigma, USA) was added at 1/4000 dilution for 1 h at room temperature. Peroxidase activity was revealed by adding 100 µl to each well solution of 3, 3', 5, 5'-tetra methyl benzidine (TMB) (Sigma, USA) for 20 min in a dark place. The color development reaction was stopped by adding 0.1 ml of 5 M sulfuric acid (H_2SO_4) and the color intensity was read in a micro titer plate ELISA reader at 450 nm. The ratio of the optical density of the negative control was considered as ratio value. Ratio values ≥3 were chosen as positive. Statistical analysis was assessed using SPSS16.0 and Microsoft Excel.

2.4 Monoclonal Antibody Production

SP_2/0 myeloma cells (obtained from the Pasteur Institute of IRAN) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS) (Invitrogen, USA) at 37℃ in a 5% CO_2 incubator one week prior to fusion. Then mouse was euthanized and spleen was removed. All surgical procedures were performed under sterile condition and anesthesia. The spleen cells were fused with the myeloma cell line at a ratio of 3:1 via polyethylene glycol (PEG) (Sigma, USA) method, a material that causes the cell membrane to fuse. The hybridoma cells were suspended in RPMI 1640 media (Invitrogen, USA) supplemented with hypoxantin-aminopterin-thymidine medium (HAT) (Sigma, USA), 20% FBS and a feeder layer from un-immunized mice that distributed to 96-well tissue culture plates and HAT medium were replaced every 2 or 3 days. Subsequently, the micro titer plates were incubated at 37℃ in a 5% CO_2 incubator for two weeks. In HAT medium, only fused cells will grow. Then, clones were cultured in hypoxanthine thymidine (HT) (Sigma, USA) medium for a further two weeks. Hybridoma cell lines producing specific antibodies were subcloned by limiting dilution to ensure the stability of the cell line and were passaged in every 2 or 3 days. Culture supernatants of the cloned cells were collected as the source of monoclonal antibodies. Supernatants from hybridomas were screened 12 days after fusion by ELISA. The positive clones were chosen and transferred to a tissue culture flask. Culture supernatants of SP_2/0 myeloma cells before fusion were collected as a negative control.

2.5 Western Blotting

The specificities of the mAbs were evaluated by western blot analysis. The MBP-Br fusion proteins were electrophoresed on 12% SDS-PAGE (according to the Laemmli method) and transferred onto nitrocellulose membrane (Amersham Pharmacia, USA) by semi-dry blotting technique (Bio-RAD, USA) [25]. Non-specific binding on the membranes was blocked in PBS-T (0.05% Tween 20 in PBS pH 7.3) for 1 h at room temperature (16-24℃). The membranes were trimmed and cut into strips and strips were incubated with supernatants of hybridomas. After incubation, the strips were washed and incubated with 1:4000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG (Sigma, USA) as a secondary antibody for 1 h and washed three times. In the final step, strips were developed with diamino benzidine as a chromogenic substrate and hydrogen peroxidase (H_2o_2). The reaction was stopped by washing with water. In the control experiments, strips were incubated with immunized mice sera. Prestained protein markers were used for both SDS-PAGE and western blotting.

2.6 Preparation and Purification of Monoclonal Antibodies

In order to purify the antibody, we collected all of the supernatant culture and monoclonal antibodies were purified with protein G columns in PBS buffer (pH: 7.2) and antibody isotype determination were determined by the mouse monoclonal antibody isotyping kit (Sigma, USA).

3. RESULTS

BALB/c mice were immunized with MBP-Br fusion protein. Following three booster immunizations with the antigen, the titer of antibody in mice sera were determined using ELISA. All mice have developed antibody titers (1/1000 dilution of sera), detectable even at a 1:10000 dilution of the serum (Fig. 1). The mouse with the highest antibody titer was further boosted i.p and fusion was done to generate hybridomas.

Using cell fusion method, for the first time we screened 38 antigen positive hybridomas and these positive clones were subcloned twice by limiting dilution (Table 1). Stable hybridomas cell lines were grown in 6-well plates. In HAT medium, only fused cells have grown and unfused myeloma cells after 10 days of fusion, were died in the presence of HAT medium. The qualitative and quantitative levels of the secreted antibody in the supernatant were screened by ELISA. Based on ELISA results, eight hybridoma cell lines producing specific mAbs against MBP-Br were selected. These hybridomas were named D7, D9, G6, H4, H9, H11, F2 and F9 respectively (Fig. 2). These positive clones were subcloned twice for one week by limiting dilution in a larger culture flask to obtain a large amount of antibody and their isotype was determined. About 50% of clones produced IgM and 50% produced the IgG1 subclass.

The SDS-PAGE of MBP-Br fusion protein showed a prominent band at molecular weight of 48.4 kDa. The proteins were transferred to the nitrocellulose membrane and the specificity and reactivity of the mAbs against MBP-Br proteins were determined by western blot analysis. Several primary clones and subclones were tested by western blotting to select the specific anti-MBP-Br monoclonal antibody. Western blot analysis showed that monoclonal antibodies D7, D9, G6, H4, H9, H11, F2 and F9 could react to MBP-Br but not reacted to MBP alone and the specificity of these antibodies were better than others. The anti-MBP-Br mAbs detected 48.4 kDa polypeptide band (Fig. 3) and different concentrations of cell lysate were used in western blot analysis and all of them could detect approximately less than 1 ng of antigen in cell lysate. For checking competitive assay we incubated the D9 clone with 50 ug of the purified recombinant protein for 1 hour in suspension (liquid conditions) and were incubated with the membrane containing the purified recombinant protein and then the band was not seen that shows the specificity of antibody (Fig. 4).

4. DISCUSSION

Brazzein as a small sweet–tasting protein has mainly been studied in biochemical properties by Ming and Hellekant [6]. It is difficult to extract Brazzein protein from fresh fruit. Brazzein has been expressed in a wide of variety of vectors, but its expression is limited to host and tags types. There are no reports of successful development of monoclonal antibodies against Brazzein protein in any *in-vitro* system. Therefore, generation of Anti-brazzein antibody would help achieve a better understanding of its function. The goal of this study was to generate and characterize monoclonal antibodies that highly specifically bind to brazzein protein.

Affinity tags can be defined as exogenous amino acid sequences with a high affinity for protein purification and detection. They can be helpful in stabilizing or enhancing their solubility. Sweet-tasting brazzein has been used for the study of sweet taste receptors, signal transduction and for characterization of its biochemical properties [8-9].

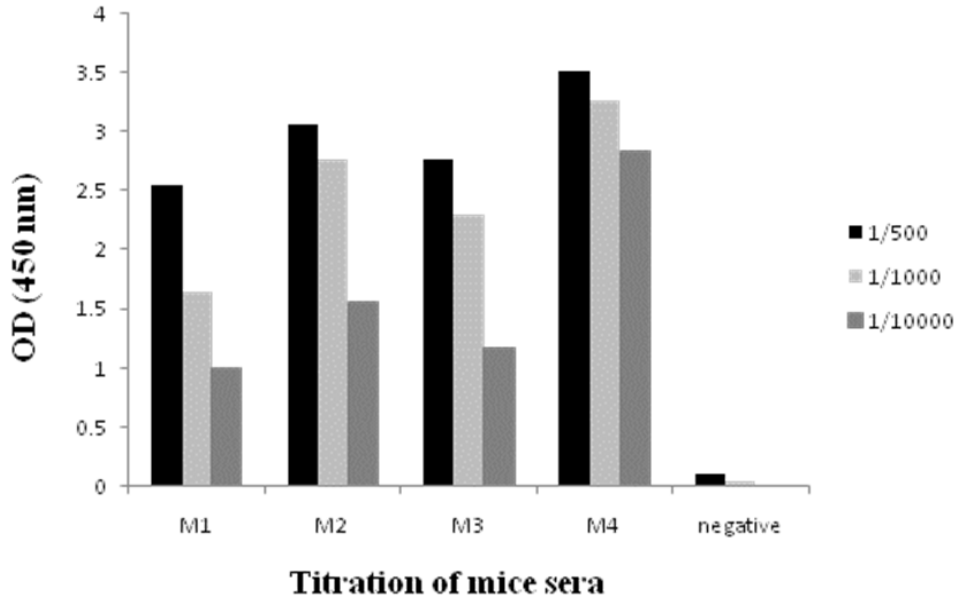

Fig. 1. Four female BALB/c mice were immunized with MBP-Br. Serum antibodies titer (diluted 1:500, 1:1000 and 1:10000) measured by ELISA. Pre-immune serum was taken before immunization and one mouse without any antigenic were as negative control

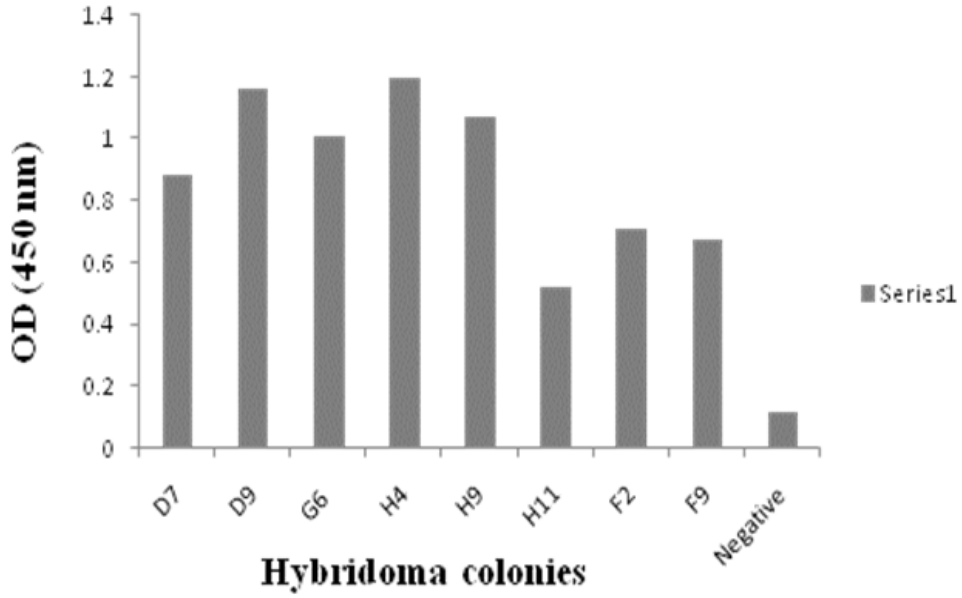

Fig. 2. Titers of stable cell line monoclonal antibodies in hybridoma clones measured by ELISA. Culture supernatants of $SP_2/0$ myeloma cells before fusion were collected as a negative control

Table 1. The presence of antibody in the supernatant of the hybridoma clones were tested 12 days after fusion by ELISA

Number	Hybrid name	OD (450 nm)	Number	Hybrid name	OD (450 nm)
1	P1-A3	3.01	21	P3-B4	1.23
2	P1-A9	2.83	22	P3-D10	1.19
3	P1-B1	2.71	23	P3-F2	0.95
4	P1-B11	2.51	24	P3-G1	1.03
5	P1-B12	3.52	25	P3-H4	1.34
6	P1-E1	2.11	26	P3-G11	1.3
7	P1-F2	2.71	27	P3-H2	1.23
8	P1-F4	2.72	28	P4-G7	1.17
9	P1-G6	2.6	29	P4-H1	1.21
10	P1-E2	2.1	30	P4-H12	1.22
11	P1-H9	2.69	31	P5-G5	1.33
12	P2-A3	2.51	32	P6-D1	1.1
13	P2-A5	2.2	33	P6-D3	1.28
14	P2-A7	0.7	34	P6-D5	1.08
15	P2-A11	0.8	35	P6-D6	1.02
16	P2-B9	0.7	36	P6-D7	1.1
17	P2-F11	0.82	37	P6-D9	1.25
18	P2-H5	1.02	38	P6-H1	1.08
19	P2-H6	0.93	39	Positive control	1.02
20	P2-H10	0.94	40	Negative control	0.01

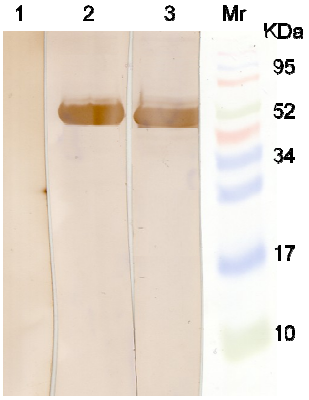

Fig. 3. Supernatant from hybridoma clones were screened by western blot against the recombinant protein MBP-Br. Lane 1 MBP negative control; Lane 2, supernatant from hybridomas clone D7 react with MBP-Br; Lane 3, supernatant from hybridomas clone D9 react with MBP-Br; Mr, Multicolor molecular protein markers

We first constructed the synthetic fragment encoding the amino acid sequence of brazzein by assembling two single strand oligonucleotides and subcloned them into the *E. coli* expression vector pMAL-C2X and pGEX-2T in our laboratory in previous work [22]. MBP is frequently used to attach a signal or carrier which can be used to deliver therapeutic or pharmaceutical proteins. We used MBP for the following features: (a) one-step adsorption purification; (b) a minimal effect on tertiary structure and biological activity; (c) detection and specific removal to produce the native protein; (d) simple and accurate assay of the recombinant protein during purification. The size of the MBP tag included one to some amino acids to complete proteins and in some cases consisted of several subunits that could be attached to either the C- or N-terminus of the protein. MBP tag was purified under its specific buffer conditions. For immunization, the actual timing may vary depending on the purity and nature of the purified antigen used for the immunization. In general, it is difficult to decide on the best fusion system for a specific protein of interest. This depends on qualities of the target protein itself such as stability, hydrophobicity and the application of the protein because brazzein expression is very difficult on its own with a small molecular mass. To improve production level, the synthetic brazzein gene was cloned and inserted into the expression vectors pMAL-C2X. Brazzein expression was obtained in *E. coli* in high yields. The expression of fusion proteins allowed for their convenient purification from other soluble bacterial proteins by amylase resin [26]. The production of this recombinant was recognized in SDS-PAGE, which was then used as an immunogen to produce monoclonal antibodies. The high yield and solubility of maltose-binding proteins are also major effects of high-throughput production [27].

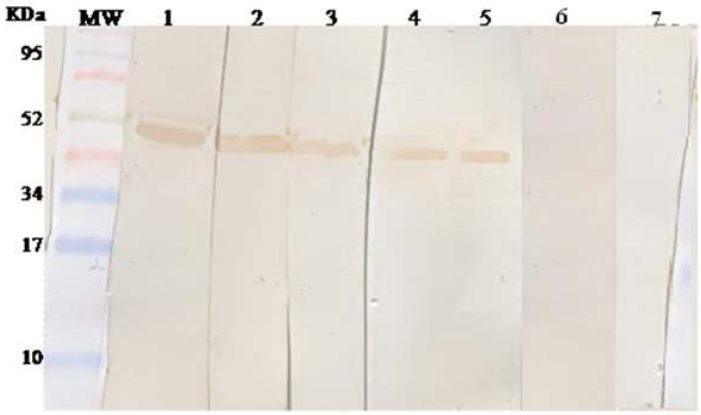

Fig. 4. Western blot analysis of D9 supernatant antibody against different concentration of MBP-Br antigen. MW, Multicolor molecular weight protein markers. Lane1-5, different concentrations of MBP-Br (0.5, 0.08, 0.01, 0.001, 0.0001 µg respectively). Lane 6, the D9 clone incubated with 50 ug of the purified recombinant protein for 1 hour in suspension (liquid conditions) and then incubated with the membrane containing the purified recombinant protein that band is abolished. Lane 7, MBP negative control

To generate monoclonal antibodies specific for MBP-Br, one of the several mice immunized with a protein containing MBP-Br that had higher titer was euthanized and spleen cells were used to fuse with SP2/0 myeloma cells. Usually, an adult animal was been used effectively in the generation of unique monoclonal antibodies. In the first step, BALB/C mice were immunized with 70 µg pure antigens with complete Freund's adjuvant by i.p injection, but all mice died due to the toxicity of pure antigen (this procedure was repeated three times). In the next step, antigen was diluted with PBS and then injected into mice with complete Freund's adjuvant by i.p injection. BALB/c mice developed sufficient serum antibody titer when immunized with MBP-Br, in comparison with before antigen injection. In addition, previous experiment screening with MBP-Br coated on ELISA plates showed mice immunized with MBP-Br generated high serum antibody titer (the serum diluted 1/500, 1/1000, 1/10000). Therefore, MBP is immunogenic and can often be used directly as an antigen in antibody production [27].

As a result, eight hybridoma clones (from 38 clones) secreted antibodies. Eight of these were high titer-antibody measured by ELISA and could be used to detect the Brazzein protein. Most supernatant hybridoma cells were titers higher than 1 at OD 450 nm and the others had 0.5-0.9 at OD 450 nm by ELISA. Western blot analysis was used to detect the specificity of these antibodies to recombinant proteins. The reactivity was observed in western blot using monoclonal antibody against the fusion antigen. All mAb recognized only a band with apparent molecular weight of 48.4 kDa. The results show that all these monoclonal antibodies were highly specific for Brazzein partner of fusion protein and reacted with fusion antigen proteins. We used GST as control and observed that monoclonal antibodies could not detect GST proteins [22]. Different concentrations of cell lysates were used in western blot analysis. Fig. 4 shows that D9 mAb could detect approximately less than 1 ng of 48.4 kDa antigen in a cell lysate. Berlec and colleagues produced polyclonal antibodies against brazzein protein fused to His-tag [28]. Specificity of monoclonal antibodies (recognize only specific epitope) are better than polyclonal antibody (recognize multiple epitopes) and expression of brazzein in MBP can elicit cellular and humoral immune responses.

5. CONCLUSION

The Brazzein protein is useful in research and can be purified and assayed by ELISA or any other immunochemical detection method. Many monoclonal antibodies have been utilized for therapeutic, pharmacy, industry and basic research [29-30]. In this study, we described how to generate a panel of monoclonal antibodies that highly bind to protein and can be utilized for a variety of purposes such as detection, immunoaffinity purification, specific interaction of sweet protein with taste receptor and research.

We recommend using the dual affinity tag to increase the purity and high yield; one tag can be used for purification and the other for detection, then the tag can be removed with a suitable enzyme. In situ production in other bacteria or eukaryotic systems would provide a low-calorie sweet-tasting product.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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