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Expression of the sweet protein brazzein in maize for production of a new commercial sweetener

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Summary

The availability of foods low in sugar content yet high in flavour is critically important to millions of individuals conscious of carbohydrate intake for diabetic or dietetic purposes. Brazzein is a sweet protein occurring naturally in a tropical plant that is impractical to produce economically on a large scale, thus limiting its availability for food products. We report here the use of a maize expression system for the production of this naturally sweet protein. High expression of brazzein was obtained, with accumulation of up to 4% total soluble protein in maize seed. Purified corn brazzein possessed a sweetness intensity of up to 1200 times that of sucrose on a per weight basis. In addition, application tests demonstrated that brazzein-containing maize germ flour could be used directly in food applications, providing product sweetness. These results demonstrate that high-intensity sweet protein engineered into food products can give sweetener attributes useful in the food industry.

Introduction

The existence of naturally derived proteins that possess intrinsic sweetness has been known for many years (reviewed in Faus, 2000). Interest in these proteins has increased with increasing demand for 'low-calorie' sweeteners and food products described as 'natural' and 'healthy' to address the needs of millions of individuals conscious of carbohydrate intake for dietetic and diabetic reasons. This has led to the commercialization of only one member of this family, thaumatin, as a sweetener and flavour enhancer (Witty and Higginbotham, 1994; Faus, 2000). Unfortunately, commercial production of thaumatin, as well as all other sweet proteins, has been limited because the natural sources for all of these proteins are tropical plant species that are difficult to cultivate, and repeated attempts to produce recombinant sweet proteins in microorganisms and transgenic plant systems have failed to yield these proteins at sufficiently high levels to make widespread commercialization economically feasible (Witty and Higginbotham, 1994; Zemanek and Wasserman, 1995; Faus, 2000).

Brazzein is a recently identified protein derived from the African plant, *Pentadiplandra brazzeana* Baillon, that has an intrinsic sweetness 500–2000 times that of sucrose (Ming and Hellenkant, 1994) The brazzein-containing fruit from *P. brazzeana* has been consumed in native regions of tropical Africa because of its sweet properties, where it has been associated with the French name '*l'oubli*', meaning 'forgetting' (Hladick, 1989; Hladick, 1993). This is due to the propensity of native children to become so focused on obtaining more of the delicious ripe fruit that they 'forget' their mothers whilst looking for them. However, limited availability of the fruit and complications associated with large-scale production of the native plant have rendered large-scale production of brazzein from natural sources uneconomical. Therefore, widespread commercial production of brazzein will probably require the transfer of protein expression to a heterologous system by means of recombinant DNA technology. The

suitability of brazzein for recombinant expression has already been demonstrated in *Escherichia coli*, enabling further characterization of the protein's biochemical properties (Assadi-Porter *et al*., 2000a,b).

Brazzein is a 6.5-kDa, single-chain polypeptide with four intramolecular disulphide bridges that enable it to maintain its sweetness profile even after incubation at 80 °C for 4 h (Ming and Hellenkant, 1994; Ming *et al*., 1995). Three forms of the protein differ only at the N-terminal amino acid residue. Type 2 brazzein corresponds to the predicted 54-amino acid translation product containing a glutamine at its N-terminus. This form appears to be short lived as the N-terminal glutamine undergoes natural conversion to pyroglutamate, resulting in type 1 brazzein, and the loss of the N-terminal glutamine (or pyroglutamate) yields the 53-amino acid type 3 brazzein. Only the last two species are detected in the ripe fruit. The sweetness intensity varies between forms, and it has been reported that the type 3 form is twice as sweet as the type 1 form (Izawa *et al*., 1996).

We report here the generation of maize lines with high levels of brazzein protein expressed in the seed. The fractionation of brazzein-expressing corn seed by standard dry-milling procedures resulted in further enrichment of brazzein in germ flour, which was shown to serve as a sweetener in product applications. Furthermore, brazzein purified from seed was up to 1200 times sweeter than sucrose on a per weight basis. These results support maize as an effective, economical expression system for the commercial production of the sweet protein, brazzein.

Results

Expression of brazzein in maize

Strategies for developing maize lines for the commercial production of brazzein were designed with consideration of the goal of producing high-level protein expression coupled with the need to generate expression product structurally and functionally equivalent to the protein from its natural source. Only type 1 and type 3 forms of brazzein are detected in the ripe fruit of *P. brazzeana* (Ming and Hellenkant, 1994), and thus the goal was to produce these forms through a recombinant plant expression system. However, the mechanism that yields these forms *in vivo* probably involves conversion from a type 2 brazzein translation product intermediate (containing a glutamine at its N-terminus) to type 1 and type 3 forms. Thus, expression of an mRNA containing a type 2 brazzein open reading frame was selected to approximate the natural blueprint for expression and to potentially yield predominantly type 1 brazzein protein through *in vivo* conversion of amino-terminal glutamine to pyroglutamate. In addition, to selectively express the type 3 form of brazzein, a molecular genetic approach to mimic the loss of the N-terminal glutamine (or pyroglutamate) was chosen by directly encoding the truncated form of the protein. Therefore, open reading frames that encoded either type 2 or type 3 brazzein were synthesized and fused to various plant regulatory sequences to constitute eight different expression constructs. The open reading frames encoding brazzein were engineered into an expression vector that contained a constitutive promoter (a maize polyubiquitin-like promoter; Hood *et al*., 2003), an embryo-preferred promoter (maize globulin-1; Belanger and Kriz, 1991) or an endosperm-preferred promoter (maize 22-kDa alpha-zein, GenBank AF090447). We tested combinations of promoters, targeting sequences and RNA elements to determine the best parameters for achieving a high level of brazzein expression in maize seed. The combinations included the promoters described above with targeting sites of cell wall and cytoplasm. In addition, for embryo-preferred, cell wall-targeted expression, 5′ untranslated regions (UTRs) from plant viral origin [tobacco etch virus (TEV, GenBank M15239) and maize dwarf mosaic virus (MDMV, GenBank AJ001691)] were used. Figure 1 shows the intraborder region of a typical expression vector, together with the transcription units of the eight constructs used for *Agrobacterium*-mediated transformation of maize. Construct 5 was essentially the same as construct 6, except for the placement of restriction sites to accommodate subsequent subcloning for the creation of constructs 7 and 8. Transformants were selected for herbicide resistance and then pollinated using elite inbred lines for the generation of T_1 (first-generation) seed, which was screened using a brazzein-specific enzyme-linked immunosorbent assay (ELISA) to measure the accumulation of the protein. Results of the highest five single T_1 seeds (representing five separate plants) for each line derived from the eight constructs are shown in Figure 2A. No deviation from normal phenotype on integration of the transgenes was observed in the plants bearing any of the expression constructs. Brazzein protein expression was observed in lines derived from all constructs. Expression detected by ELISA correlated with the presence of a 6.5-kDa immunoreactive band on Western blot analysis using anti-brazzein serum. Results for extracts prepared from seed of constructs 1, 3 (Figure 2B) and 5 (Figure 3B) are shown. The highest brazzein levels were detected in seed from lines engineered to express brazzein forms driven from an embryo-preferred promoter and targeted to the plant cell wall (constructs 2, 5 and 6). Furthermore, both forms of brazzein accumulated to high levels,

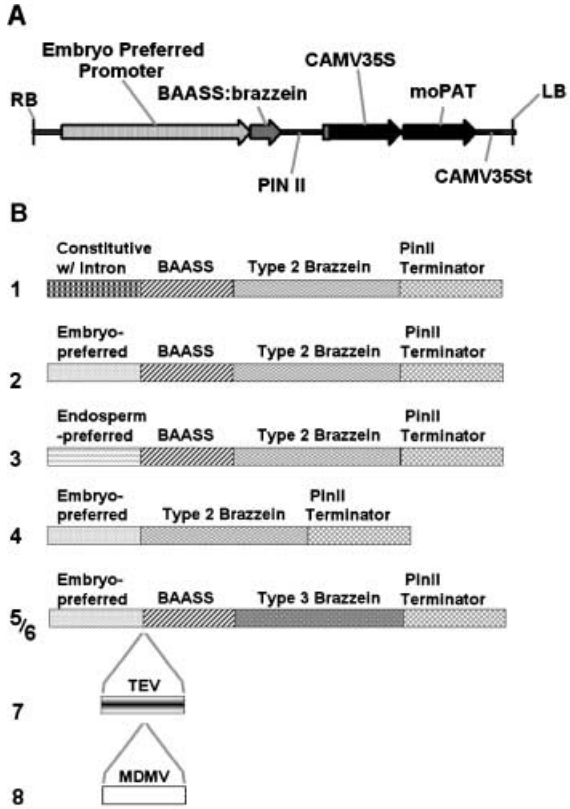

Figure 1 Plant transformation vectors. (A) Map of the intraborder region of the typical transformation construct 2. The positions of the following sequence elements are indicated: promoter type [embryo-preferred and cauliflower mosaic virus 35S (CAMV35S)]; barley alpha-amylase::brazzein coding region (BAASS::Brazzein); potato *Pin*II transcription terminator region; maize-optimized herbicide resistance gene open reading frame (*moPAT*); CaMV terminator (CAMV35St); and left and right border sequences of an *Agrobacterium tumefaciens* Ti plasmid (LB and RB). (B) Plant transcription units (PTUs) used to generate transgenic plants expressing type 2 and type 3 brazzein. The construct numbers are shown to the left of each unit. The promoter type and targeting criteria for each construct are indicated. Constructs 5 and 6 contain the same genetic elements with a change to the placement of engineered restriction sites. The origin of the 5′ untranslated regions of transcripts for constructs 7 and 8 are indicated. BAASS, barley alpha-amylase signal sequence; MDMV, maize dwarf mosaic virus; *Pin*II, protease inhibitor II gene from potato; TEV, tobacco etch virus.

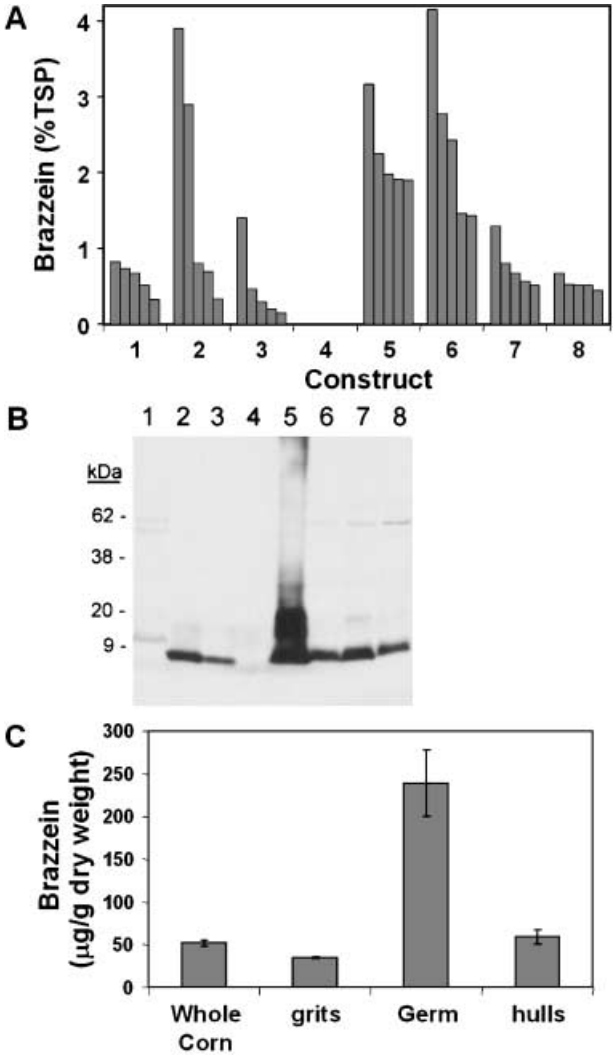

Figure 2 Brazzein protein in maize seed and in fractions resulting from the dry milling of seed. (A) Expression levels of brazzein protein in the highest T_1 seeds from the top five individual plants of the eight vectors used to generate independent transgenic events for brazzein expression in maize. The data for the top five expressing seeds from vectors 1 to 8 were derived from 4, 15, 13, 9, 12, 19, 10 and 7 transgenic events, respectively. TSP, total soluble protein. (B) Immunodetection of brazzein in seed extracts. Extracts from individual seed were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 18% gel, transferred to polyvinylidene difluoride (PVDF), and brazzein was detected by immunoblotting with anti-brazzein antibody as described in 'Experimental procedures'. Lane 1, control corn seed extract (20 μg); lanes 2 and 3, yeast brazzein (100 and 50 pg, respectively); lanes 5–7, extract from construct 1 seed (10 μg); lane 8, extract from construct 3 seed (20 μg). The positions of standard proteins of the indicated molecular weights ($\times 10^{-3}$) are given on the left. (C) Content of brazzein measured in fractions obtained by dry milling of pooled seed derived from construct 5 lines. 'Grits' and 'Germ' correspond to the endosperm- and embryo-rich fractions, respectively, whilst 'hull' represents predominantly seed pericarp. Error bars represent one standard deviation from the mean.

approximately 4% of total soluble protein (TSP) (Figure 2A; constructs 2, 5 and 6), suggesting that subtle differences at the amino-terminal end of the protein are not critical for brazzein protein expression or stability in maize. When 5′ UTR sequences from two plant viruses (TEV and MDMV) were incorporated as the 5′ UTRs of constructs 7 and 8, no increase in protein expression was observed when compared with constructs 5 and 6. Interestingly, only very low expression (≤ 0.005% TSP) was detected in seed from lines in which brazzein expression was targeted to the cytoplasm (construct 4).

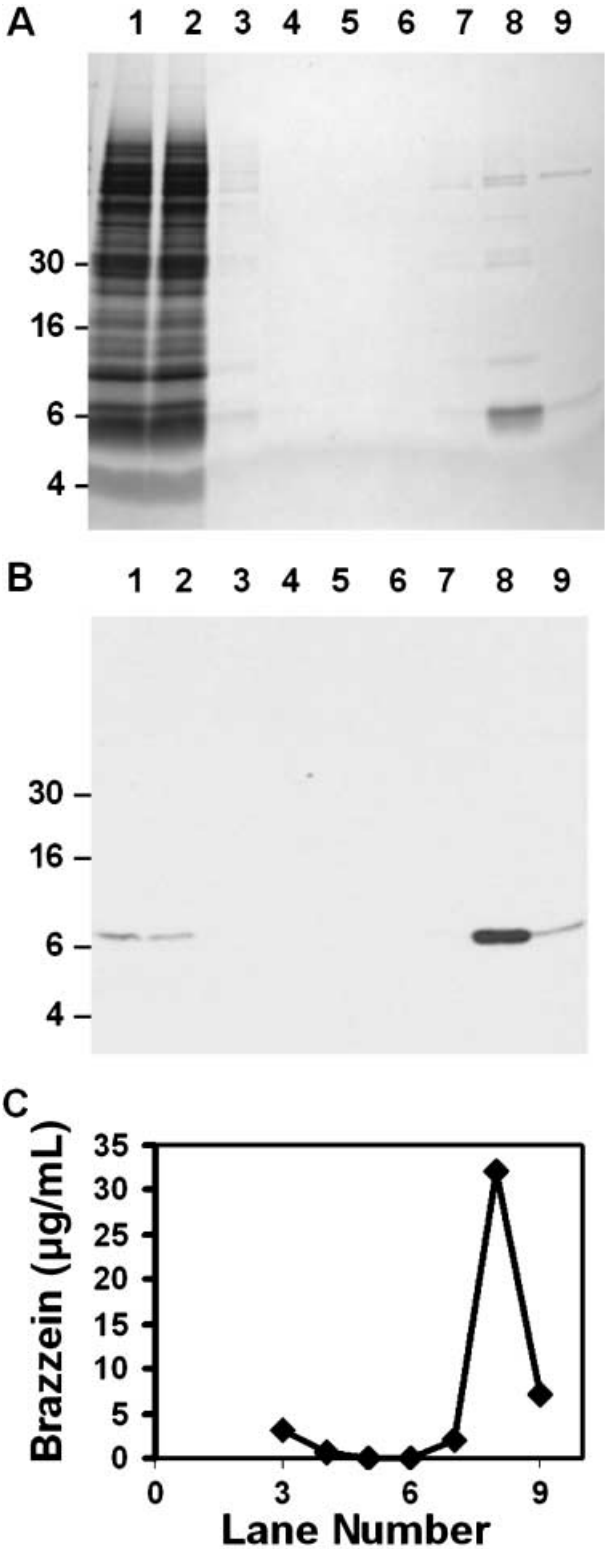

Figure 3 Isolation of type 3 brazzein protein by immunoaffinity chromatography from maize seed bearing construct 5. An extract was prepared from seed of construct 5 lines, and subjected to immunoaffinity chromatography as described in 'Experimental procedures'. Aliquots of fractions were subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gels. Protein bands were detected by staining with Coomassie blue (A), transferred to polyvinylidene difluoride (PVDF) with brazzein detected by immunoblotting with anti-brazzein antibody (B) or measured by enzyme-linked immunosorbent assay (ELISA) (C). The positions of standard proteins of the indicated molecular weights ($\times 10^{-3}$) are given on the left of the gel and blot. Lane 1, phosphate-buffered saline (PBS) seed extract; lane 2, flow through (unbound) fraction; lane 3, PBS wash; lane 4, third PBS wash; lanes 5–9, fractions eluted with low-pH buffer.

Constructs 2 and 5 employed an embryo-preferred promoter to drive expression of type 2 or type 3 brazzein proteins, respectively, and thus the brazzein protein is predicted to accumulate to the highest level in the embryo. In order to assess brazzein accumulation in seed tissues, whole grain from lines derived from constructs 2 and 5 was generated, and each grain pool was fractionated using a dry-milling procedure to isolate the germ component of the seed. Brazzein measured in fractions from the milling of construct 5 grain is shown in Figure 2C. Brazzein was concentrated fivefold in the germ fraction as a function of fraction dry weight when compared with whole grain. Similar results were also observed on fractionation of grain from construct 2 lines (data not shown). Thus, standard dry milling effectively enriched the brazzein content using grain from lines in which expression was targeted to the embryo.

Characterization of brazzein protein expressed in maize

The highest brazzein-expressing lines employed the use of a plant signal sequence fused to the amino-terminal end of brazzein to direct newly expressed protein to the plant cell wall. Generally, the signal sequence is removed during transport of the protein to the cell wall (Hood *et al*., 1997). Therefore, in order to assess whether proper removal of the signal sequence occurred in the transgenic maize lines, brazzein proteins were purified from construct 2 and construct 5 lines. Immunoaffinity chromatography using anti-brazzein antibody covalently coupled to Sepharose was employed to purify either type 2 or type 3 brazzein from construct 2 and construct 5 lines, respectively. Aliquots of samples taken during chromatography were analysed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie. Results for the isolation of brazzein from construct 5 grain are shown in Figure 3. The major 6.5-kDa band detected by Coomassie staining in fractions eluted by low pH (Figure 3A, lanes 7–9) was also detected by immunoblotting using anti-brazzein antibody (Figure 3B, lanes 7–9), and the presence of the band correlated with the detection of brazzein by ELISA (Figure 3C). Immunoaffinity chromatography of an extract derived from the grain of a

Table 1 N-terminal amino acid sequence analysis of brazzein purified from maize seed of construct 2 and 5 lines

Sequence origin	First 10 amino acids*
Type 2 brazzein	qdkckkvyen†
Construct 2 brazzein	sqdkxkkvye
Type 3 brazzein	dkckkvyeny
Construct 5 brazzein	dkxkkvyeny

*'x' indicates cycle in which assignment of amino acid could not be made.
†Denotes predicted coding sequence for brazzein without conversion of amino-terminal glutamine to pyroglutamate.

construct 2 line yielded similar results (supplementary figure, see later). Purified, maize-derived brazzein proteins from both construct 2 and 5 lines were subjected to Edman degradation to obtain the N-terminal amino acid sequence (Table 1). The amino-terminal sequences of natural type 2 and type 3 forms of brazzein are shown for reference. Unassigned amino acids within the analysed sequences correspond to the locations of cysteine residues in the brazzein open reading frame known to form intramolecular disulphide bridges in the brazzein protein (Kohmura *et al*., 1996). The results indicate that brazzein in construct 5 lines is processed accurately to produce a natural type 3 amino-terminus. However, brazzein purified from a construct 2 line possesses an additional amino acid at the amino-terminal end, a serine. This amino acid corresponds to the last amino acid in the signal sequence employed in construct 2, and thus these results suggest processing of the fusion protein one amino acid removed from glutamine.

One potential application of brazzein is as a refined food additive. Therefore, a purification method was developed in order to generate sufficient quantities of purified type 3 brazzein protein for sensory evaluation in order to test whether purified material retains high intrinsic sweetness. An outline of the protocol used to enrich the brazzein protein is shown in Figure 4A. Fractions generated during a representative purification run were assayed for the presence of brazzein protein, and the results are summarized in Table 2. Analyses of fractions by SDS-PAGE were also performed, and the results are shown in Figure 4B. Brazzein was enriched to approximately 70% of soluble protein by this protocol, and yielded a single band by SDS-PAGE. This protocol was repeated several times to generate a sufficient amount of purified type 3 brazzein for sensory testing.

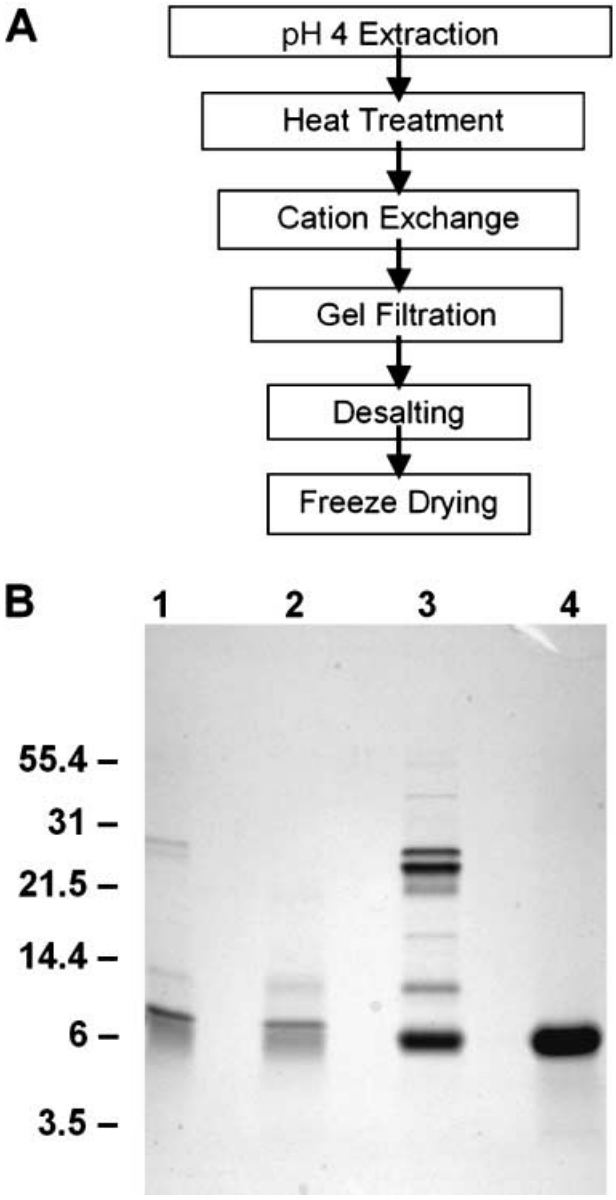

Figure 4 Enrichment of type 3 brazzein for taste testing. (A) Flow chart of the multistep purification scheme for the isolation of brazzein from maize seed. Pooled seed from construct 5 lines was used for the purification of type 3 brazzein as per the procedure outlined in 'Experimental procedures'. Aliquots of pooled fractions obtained from various steps were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 18% gel. (B) Coomassie blue-stained gel. The positions of standard proteins of the indicated molecular weights ($\times 10^{-3}$) are given on the left. Lane 1, crude extract of type 3 brazzein maize seed (5 μg); lane 2, heated/filtered extract (5 μg); lane 3, brazzein peak from cation exchange chromatography (5 μg); lane 4, type 3 brazzein peak from size exclusion chromatography (5 μg).

Sensory analysis of corn brazzein

As potential applications for corn-expressed brazzein may require the use of either a purified protein or a less purified fraction derived from corn seed, it was useful to determine the sweetness intensity of brazzein both within a corn fraction and as a purified protein. In order to determine whether brazzein protein within germ fractions possessed intrinsic

Table 2 Purification of brazzein protein from seed derived from a construct 5 maize line*

Purification step	Total protein† (mg)	Brazzein‡ (mg)	Purity (% TSP)	Fold enrichment	Yield (%)
Crude extract	1148.5	7.5	0.65	1	100
Heated extract	697.1	7.1	1.02	1.6	95
SP Sepharose peak	22	4.9	19.7	30.1	65
Superdex 30 peak	6.1	4.3	70.7	108.1	58

TSP, total soluble protein.

*Purification of type 3 brazzein from 80 g corn flour derived from a construct 5 line.

†Total protein in fractions was determined using the BCA Assay (Pierce).

‡Brazzein content of fractions was determined by analysis using a brazzein-specific enzyme-linked immunosorbent assay (ELISA).

Table 3 Relative sweetness intensity of corn-expressed brazzein in maize germ flour and as type 3 brazzein protein purified from maize

Source material*	Brazzein type	Concentration of brazzein in taste sample (p.p.m.)	Measured sweetness (SEV) (%)†	Brazzein sweetness intensity‡
Germ flour	Type 2	20	1.8	900
Germ flour	Type 3	10	2.0	1700
Purified protein	Type 3	40	4.8	1200
Purified protein	Type 3	80	7.5	940

*Germ flour containing either type 2 or type 3 brazzein was derived from seed of construct 2 and construct 5 lines, respectively.

†Sucrose equivalence value (SEV) corresponds to the intensity of sweetness relative to sucrose solutions (w/v %).

‡Relative sweetness intensity compared with sucrose by weight of brazzein component.

sweetness, germ flour was subjected to sensory evaluation. Control germ flour and germ flour from construct 2 and 5 lines (0.04% and 0.02% of germ flour dry weight corresponding to brazzein, respectively) were used to generate extracts that were evaluated for sweetness intensity relative to sucrose solution reference standards using standard testing methods. Extracts from germ flour containing either type 2 or type 3 brazzein were tasted and scored relative to the sweetness intensity of sucrose reference standards prepared at 1.0%, 2.0%, 3.0%, 4.0% and 5.0%; they were found to possess sweetness comparable with solutions of 1.8% and 2.0% sucrose, respectively (Table 3). This corresponds to sweetness equivalence of approximately 40% and 35% sucrose for germ flour containing 0.02% type 3 and 0.04% type 2 brazzein, respectively. Based on the brazzein content measured for the germ flour fractions, these results indicate that type 3 brazzein flour is the sweetest per unit brazzein, delivering a sweetness intensity of approximately 1700 times that of sucrose on a per weight basis at 2.0% sucrose, compared with 900 times that of sucrose for corn type 2 brazzein. No sweetness was detected under the conditions of this analysis in control germ flour. These results clearly demonstrate that brazzein expressed in maize possesses a high intrinsic sweetness intensity, which survives seed harvesting, storage, fractionation, de-fatting and milling procedures.

The evaluation of the sweetness intensity of purified type 3 brazzein from corn is also shown in Table 3. Two concentrations of brazzein mixed into a citrate buffer system were evaluated in comparison with a panel of sucrose standards generated by dissolving sucrose in the same citrate buffer. The results indicate that purified, type 3 corn brazzein possesses a sweetness intensity of 940–1200 times that of sucrose at the relative sucrose intensities tested. This is in the range reported for type 3 brazzein expressed in a bacterial expression system (Assadi-Porter *et al.*, 2000b). Thus, recombinant brazzein, whether within a corn fraction or purified from corn seed, retains its naturally high sweetness intensity.

Germ flour product application tests

In order to examine the suitability of brazzein corn germ for potential product applications, the germ flour from grain derived from a construct 2 line was used in product application tests. Germ flour was used to formulate recipes for Sports Protein Bars and Reduced Carbohydrate Muffins. Resultant product test samples were examined by a taste panel for sweetness intensity and taste qualities relative to products formulated using germ flour from non-transgenic, control corn. The brazzein-containing bar was described as 'sweeter' than the product prepared using control germ and

'exhibited a smoother, more sugar-like taste profile'. The mouth-feel was also described as 'slightly warmer and fuller' than control material. Similar results were described for the Reduced Carbohydrate Muffin, with the brazzein product being described as 'sweeter and fuller in flavour'. In these evaluations, it was estimated that brazzein flour contributed a sweetness of 25%–30% that of sucrose on a per weight basis, demonstrating that the sweetness of brazzein in germ flour survived the conditions used for the preparation of two potential products.

Discussion

Plants are increasingly being employed as biofactories for the commercial production of a number of valuable proteins (reviewed in Hood and Jilka, 1999; Streatfield and Howard, 2003; Horn *et al*., 2004). Biopharming has been used in the development of oral vaccines, enzymes for industrial applications and pharmaceutical proteins. Production in plant expression systems provides a number of advantages over other expression systems: (i) plants have been shown to accurately express a wide variety of heterologous proteins which undergo complex post-translational processing; (ii) production can be scaled up rapidly to inexpensively produce very large quantities of product; (iii) plants do not harbour human pathogens, thus minimizing the fear of harmful contaminants; and (iv) the expression of proteins in seeds provides a natural site for the long-term storage of proteins. The expression of transgenic proteins in edible crops has the particular advantage of producing materials that can be directly consumed, a characteristic that has been exploited for the purpose of oral vaccine development (Streatfield and Howard, 2003). Maize is a particularly suitable vehicle for the commercial production of consumable proteins, in that it is a widely grown, efficient and dependable crop that serves as a major source for basic foods, and it is already supported by a highly efficient agribusiness infrastructure for grain transportation, storage and processing. Furthermore, the use of this plant expression system has already led to the commercial production of several proteins (Hood *et al*., 1999; Woodard *et al*., 2003).

We report here the use of a maize expression system for the economical production of the intensely sweet protein, brazzein, for both low- and high-intensity sweetener markets. The high expression of brazzein protein in corn seed embryo resulted in a germ fraction with an intrinsic sweetness of up to 40% of the value of sucrose by weight. This is particularly striking considering that the seed pool used for the generation of the germ fraction tested possessed an expression level of only approximately 50 μg type 3 brazzein per gram seed. Brazzein expression levels in single seeds of up to 4% TSP have already been obtained, which corresponds to approximately 400 μg brazzein per gram seed. This suggests that seed from the highest expressing maize lines possesses germ containing a sweetness intensity greater than that of sucrose on a per weight basis. Furthermore, with the capacity to increase brazzein expression levels through continued backcrossing of lines into elite inbred germplasm and the generation of homozygous hybrid parent seed for production, it is not unreasonable to project a potential for unfractionated whole grain to exceed the sweetness intensity of sucrose. This strategy to achieve high-level recombinant protein expression without transgene silencing through the selection of lines maintaining both high-level expression over multiple generations and herbicide resistance has been successfully employed (Hood *et al*., 2003). Furthermore, *Agrobacterium*-mediated transformation limits the copy number of gene insertions, thereby aiding in gene stability even in the homozygous state (Muller *et al*., 1987; Last and Gray, 1990; Frary and Hamilton, 2001; Choi *et al*., 2003). Thus, together, these approaches should enable a further increase in the already high-level brazzein protein expression as generational breeding continues.

The fact that brazzein protein expressed in maize possesses the same sweet phenotype as the natural product is critical for recombinant protein production. In the case of thaumatin, several approaches using other expression systems yielded recombinant protein that, for unknown reasons, was not sweet (Edens *et al*., 1982; Edens and van der Wel, 1985; Lee *et al*., 1988). The demonstration that brazzein-containing germ flour can be used directly in potential product applications without further enrichment to supply sweetness and improved product flavour shows the potential of this unpurified material as a low-intensity sweetener. Using a germ fraction for product sweetening not only provides a low-calorie alternative to sucrose, but also gives the intrinsic bulking properties necessary to replace the volume lost on removal of sugar, yielding a combined sweetener and bulking agent. As type 3 brazzein purified from transgenic maize seed possesses a sweetness intensity 1200 times that of sucrose on a per weight basis, material enriched in this fashion could potentially serve as a high-intensity sweetener to further extend the range of product applications of corn-expressed brazzein. The high stability of brazzein protein is an advantageous characteristic useful to the food industry, and makes corn-produced brazzein suitable for a broad range of market applications, with two such applications demonstrated here. It is also possible that brazzein from corn could be used to enrich a high-fructose corn syrup fraction to generate a formulation with increased sweetness.

Maize lines expressing type 3 brazzein hold the greatest potential for commercial application. First, type 3 brazzein-containing lines possess the highest expression levels observed in maize. Second, maize-expressed type 3 brazzein is correctly processed, producing a protein bearing the amino-terminal end of the sweetest natural form of brazzein. Third, the high intrinsic sweetness intensity of corn-expressed type 3 brazzein is retained in germ flour and in purified protein, where the sweetness intensity approximates that obtained in type 3 brazzein produced in a recombinant bacterial system (Assadi-Porter *et al*., 2000b). Maize holds the added advantage of the cost-efficient protein production of type 3 brazzein packaged in a naturally edible product. In this report, germ flour from a line designed to express type 2 brazzein was used for application testing. The transformation and identification of lines designed to express type 2 brazzein preceded the development of type 3 lines, and thus at the time of initial application testing sufficient quantities of type 3 material were unavailable. However, as type 3 brazzein within germ flour has been shown to retain high-intensity sweetness, it is reasonable to expect a similar performance for type 3 brazzein in germ flour application tests. Continued development of the most promising lines expressing type 3 brazzein will enable further application testing to evaluate the potential of this material.

Interestingly, inaccurate processing of the type 2 brazzein expression product generated a variant form of brazzein without an N-terminal glutamine, which did not undergo conversion to pyroglutamate to generate type 1 brazzein. Although this variant retained sweetness, despite the fact that the N-terminus is known to play a role in sweetness determination (Assadi-Porter *et al*., 2000b), its sweetness intensity was not improved over type 3 brazzein. Higher sweetness intensity variant forms of brazzein have been engineered with an intrinsic sweetness approximately twice that observed for the type 3 form (Assadi-Porter *et al*., 2000b), and the use of a maize system to express these variant forms could potentially generate even sweeter varieties of maize. However, type 3 brazzein is the highest intensity natural form of the protein, and thus type 3-containing lines still hold the advantage of gaining regulatory approval and acceptance as a natural alternative to synthetic sweeteners.

Others have reported the engineering of sweet protein expression into transgenic plants, but the employment of such systems for commercial protein production has yet to become a reality. The economic feasibility of a recombinant plant system for protein production requires high-level expression of the heterologous protein in a commercially viable crop species. Witty (1990) obtained transgenic *Solanum tuberosum* that produced recombinant thaumatin with a sweet phenotype, but the product yield was low. Penarrubia *et al*. (1992) reported the expression of monellin, the sweet protein from the red berries of the West African plant, *Dioscoreophyllum cumminsii* Diels, in transgenic tomato and lettuce, but the expression of only 23.9 μg monellin/g wet weight was achieved, compared with up to 400 μg/g seed reported here. Attempts have been made to express mabinlin II, a sweet-tasting protein derived from the fruit of the Chinese plant, *Capparis masaikai* Levl, in transgenic *S. tuberosum* tubers (Xiong and Sun, 1996), but explicit results have yet to be reported. Even if the expression limitations are overcome in these systems, the feasibilities of lettuce, tomato or potato as commercially viable crops for recombinant protein production have yet to be established. Thus, the high-level brazzein protein expression reported here in a plant system already proven as viable for the commercial production of recombinant protein may enable the eventual commercial production of sweet protein in transgenic plants.

The commercialization of products containing recombinant brazzein will require regulatory approval. Recent reports of the identification of major food allergens for pathogen-related thaumatin-like proteins (PR-5 family of plant allergens) have underscored the need to evaluate the allergenicity of products of biotechnology destined for the food supply (reviewed in Hoffman-Sommerguber, 2002). Interestingly, despite the substantial homology of thaumatin to known thaumitin-like allergens, thaumatin has received approval from several regulatory bodies following thorough toxicological and safety testing. Comparison of brazzein with peptides in the Farrp allergen database reveals no substantial homology to known allergens (not ≥ 35% identity over the 53-amino acid length of type 3 brazzein). Furthermore, the indigenous population of Africa has safely used the natural source of brazzein to sweeten foods for many years, suggesting that brazzein protein does not pose any unusual risk to health. However, the relatively high stability of brazzein protein, a benefit to utility in applications in which high-temperature processing is required, suggests that the evaluation of food allergen potential will be an important factor for the regulatory approval of brazzein.

Experimental procedures

Construction of plasmids

Starting with the published amino acid sequence (Ming and Hellenkant, 1994), the sequences for type 2 and type 3 brazzein were back-translated using the Backtranslate program

of the Wisconsin GCG Package against a codon table tabulated for highly expressed maize (corn) genes. The resulting DNA sequences were scanned for the presence of undesirable sequences, e.g. polyadenylation signals, 5′ and 3′ consensus splice sites, other mRNA destabilizing sequences and undesirable endonuclease restriction enzyme sites. The DNA sequences were modified to eliminate these sites by choosing alternative codons. The resulting sequences were then constructed using a series of synthesized overlapping complementary oligonucleotides and the polymerase chain reaction (PCR) to amplify the resulting synthetic sequence. Convenient restriction sites were also engineered into the 5′ and 3′ ends of the optimized gene to facilitate cloning. These sequences were subcloned into a maize expression cassette within a transformation vector that included right and left border sequences of an *Agrobacterium tumefaciens* Ti plasmid and the maize-optimized *pat* gene of *Streptomyces viridochromogenes* (White *et al*., 1992) expressed from the cauliflower mosaic virus (CaMV) 35S promoter, conferring resistance to the herbicide bialaphos, as per the strategy reported by Hood *et al*. (2003). Eight different constructs were generated. For constructs 1–3 and 5–8, a maize codon-optimized version of the barley alpha-amylase signal sequence (BAASS; Rogers, 1985) was incorporated to provide a cell secretion signal at the N-terminus. The brazzein-containing open reading frames were subcloned into vectors downstream of a constitutive promoter (a maize polyubiquitin-like promoter; Hood *et al*., 2003), an embryo-preferred promoter (a maize globulin-1 promoter; Belanger and Kriz, 1991) or an endosperm-preferred promoter (maize 22-kDa alpha-zein gene cluster, GenBank accession no. AF090447, nucleotides 1–867). For constructs 7 and 8, the 5′ UTRs of TEV (GenBank accession no. M15239, nucleotides 2–143) and MDMV (GenBank accession no. AJ001691, nucleotides 1–138) were constructed using a series of synthesized overlapping complementary oligonucleotides and the PCR to amplify the resulting synthetic sequences prior to subcloning 3′ of the promoter sequence and 5′ of BAASS. Construct 6 is essentially the same as construct 5 except for the placement of restriction sites to accommodate subsequent subcloning for the creation of constructs 7 and 8. Figure 1 contains a list of the expression constructs that were generated and used for the initiation of corn transformation.

Agrobacterium-mediated maize transformation

Plant expression constructs were introduced into maize using an *A. tumefaciens*-mediated approach described in Streatfield *et al*. (2001). Ears from either the maize line Hi II, or a hybrid generated by crossing Hi II with an elite inbred, were harvested and used for transformation. The resulting transgenic plant tissues were selected for growth in the presence of 5 μM bialaphos (PhytoTechnology Laboratories, Overland Park, KS) to ensure that stable integration of genetic material had occurred. A series of 10–20 plants was grown from each independent transformation event. Plants were pollinated with elite hybrid germplasm, and the first-generation (T_1) seed was harvested and analysed for recombinant protein expression by ELISA for quantification of the brazzein protein. Transgenic lines expressing high levels of recombinant protein were backcrossed with commercial maize lines. Pollen from transgenic lines was also used to pollinate commercial lines in order to bulk up transgenic seed rapidly.

Preparation of soluble protein extracts from corn seed

Individual dry seeds were pulverized using a mortar and pestle, and pools of seed (25–50) were ground in a coffee grinder. Pulverized materials were then shaken vigorously in a tube with a steel ball bearing with 500 μL phosphate-buffered saline containing 0.05% Tween® 20 (PBST) per either single seed or 0.1-g pooled seed sample. Soluble protein was recovered in the supernatant by centrifugation of the homogenized tissue. TSP concentrations in plant extracts were determined using an assay for protein–dye binding (Bradford, 1976).

Quantification of recombinant brazzein in transgenic corn

A sandwich-ELISA was used to quantify brazzein levels in plant extracts and chromatography fractions. Briefly, plates were coated with rabbit anti-brazzein antibody in 0.05 M carbonate/bicarbonate buffer at pH 9.6 by incubating for several hours at 4 °C. The wells were then blocked by incubating with blocking buffer [PBST containing 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO)] at 37 °C for 1 h. Soluble protein extracts were added to the wells and incubated at 4 °C overnight. Biotinylated rabbit anti-brazzein antibody, diluted in blocking buffer, was added to the wells and incubated at 37 °C for 1 h. Streptavidin–alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA) in blocking buffer was then added to the wells and incubated at 37 °C for 1 h. The ELISA plates were washed four times with PBST between each step. Finally, *para*-nitrophenylphosphate (Sigma) was added to each well, followed by incubation at 37 °C for 30 min, and the absorbance at 405 nm was recorded. A dilution series of recombinant

yeast-derived type 1 brazzein protein standard and a non-transgenic negative corn control sample were included in the assay for reference.

Amino-terminal sequence analysis of brazzein isoforms

Whole corn seed from type 2 and type 3 corn lines was separately ground and extracted with phosphate-buffered saline (PBS) at a v/w ratio of 4.5 : 1 (PBS to sample) by rotating the sample for 30 min at 4 °C, and the supernatant was clarified by centrifugation at 20 000 ***g*** for 15 min. Protein A Sepharose-purified rabbit anti-brazzein antibody (5 mg) was coupled to a 2-mL Aminolink Plus column (Pierce, Rockford, IL) as per the manufacturer's instructions. Brazzein-containing extracts were applied by gravity to the anti-brazzein column (2 mL) pre-equilibrated in PBS. The column was washed with PBS (8 mL), followed by 3 × concentrated PBS (11 mL), and bound protein was eluted with low-pH buffer (20 mM glycine, pH 2.5, 150 mM NaCl) and collected as 1-mL fractions directly into tubes containing 1 M MOPS (3-(N-Morpholino)propanesulphonic acid), pH 8 (81 μL), to neutralize the samples. Fractions containing brazzein were pooled and concentrated via ultracentrifugation on centricon-3 membrane filtration units (Billerica, MA). Brazzein samples (1 μg each for corn type 2 and corn type 3) were electrophoresed on 10–20% SDS-PAGE gels, and then transferred to polyvinylidene difluoride (PVDF) membranes. Bands were visualized by staining with GelCode Blue (Pierce), excised from PVDF and subjected to automated Edman degradation by the method of Matsudaira (1987) using a Hewlett-Packard G1000A automated sequencer at the Texas A&M Protein Chemistry Laboratory.

Purification of recombinant type 3 brazzein from transgenic corn for sensory analysis

Corn meal from a line programmed to express type 3 brazzein was extracted with buffer A (20 mM sodium acetate, pH 4.0), containing 30 mM NaCl at a ratio of 5 : 1 v/w, by stirring at 4 °C for 1 h. The supernatant was clarified by centrifugation, heated to 80 °C for 30 min, and then clarified a second time by filtration through a 0.2-μm filter. The supernatant was further fractionated by cation exchange chromatography by direct loading on to SP-Sepharose Fast Flow (Pharmacia) equilibrated in buffer A. Bound protein was eluted with a linear gradient to 500 mM NaCl generated over 15 column volumes, and fractions were examined for brazzein content by ELISA. Brazzein-containing fractions were pooled and concentrated via ultrafiltration on 3-kDa membranes. Retentates typically contained > 95% of the brazzein from this concentration step, and were subsequently applied to a 307-mL Superdex 30 column (Amersham Biosciences, Upsala, Sweden) equilibrated in buffer A containing 300 mM NaCl. Brazzein eluted in a single peak at the same elution volume position (6.5 kDa) as brazzein purified from yeast. This protocol was repeated multiple times to generate sufficient material for sensory evaluation. Purified brazzein was concentrated via ultrafiltration on 3-kDa membranes, desalted on a G-25 column (Amersham Biosciences) equilibrated in water, and then freeze dried. Fifty per cent of the lyophilized sample weight was estimated to be brazzein content based on ELISA values and sample mass, with residual moisture contributing approximately 20% of the sample weight.

Sweetness intensity testing

For the evaluation of the sweetness intensity of corn germ flour, sucrose equivalence values were determined by a panel of tasters using samples prepared as a 5% solution of the corn germ flour suspended in water. Type 2 and type 3 brazzein corn germ flour samples, containing brazzein protein at 0.04% and 0.02% of dry weight, respectively, were compared with a non-transgenic control germ flour sample. Germ flour samples were suspended using a high shear mixer and the extract was filtered prior to tasting. For the assessment of extract sweetness intensity, solutions were tasted and scored relative to the sweetness intensity of sucrose reference standards prepared at 1.0%, 2.0%, 3.0%, 4.0% and 5.0% in water. For the evaluation of the sweetness intensity of type 3 brazzein purified from corn, brazzein solution test samples were prepared in a citric/citrate model soft drink buffer. A lyophilized type 3 brazzein protein sample, estimated to contain brazzein at 50% of dry weight, was dissolved at 80 p.p.m. and 160 p.p.m. to generate brazzein concentrations of 40 p.p.m. and 80 p.p.m., respectively. Bench tasting was carried out to determine concentrations approximately equal to 4% and 8% sucrose. Sucrose solutions ranging from 3% to 9% concentration at steps of 0.5%, prepared in a citric/citrate buffer, were used as reference standards to enable the scoring of sample sweetness intensity. The results of the quantification of sweetness intensities are presented in Table 3.

For the evaluation of brazzein corn germ flour in application tests, products were formulated using control corn germ flour and type 2 brazzein-containing corn germ flour from a construct 2 line as follows. (i) For Sports Protein Bar application testing, soya protein isolate (26 g) was blended with de-fatted corn germ (12.63 g), fructo-oligosaccharide syrup (46.14 g), inulin (7.43 g), glycerol (1.86 g), sunflower oil (5.57 g)

and emulsifier (0.37 g) to produce a bar with a high protein content. Preparation was conducted in a planetary mixer; the liquid components of the formulation were heated to 60 °C, mixed with the dry powders and then sheeted and cut prior to taste testing. (ii) For Reduced Carbohydrate Muffin application testing, polydextrose (19.35 g), de-fatted corn germ (20.43 g), whey protein concentrate (3.6 g), water (13.5 g), whole egg (18 g), shortening (13.5 g), baking soda (1.35 g), salt (0.27 g), dried blueberries (6.64 g) and flaked wheat (3.36 g) were blended and baked in a rotary oven at 160 °C for 35 min. Products were evaluated by a small panel of tasters for appearance, texture, flavour and aftertaste. Particular attention was paid to the effects on sweetness and flavour derived from incorporating brazzein germ flour into the formulation.

Fractionation of grain

Grain was fractionated into component parts using standard milling practices, essentially as described previously (Lamphear *et al*., 2002).

Electrophoresis

SDS-PAGE was performed at 100–150 V for 90–150 min using a Novex XCell II minigel electrophoresis system. Protein bands were visualized by staining with Coomassie using GelCode Blue reagent (Pierce) or immunoblotting. For immunoblotting, unstained proteins were transferred to PVDF membranes at 30–35 V for 60 min using a Novex XCell II minigel electrophoresis system. Blots were incubated at room temperature in blocking solution (Tris-buffered saline, pH 8.0, containing 0.05% Tween 20 and 5% non-fat dried milk) for 1 h, and then with rabbit anti-brazzein antiserum diluted 1 : 10 000 in blocking solution overnight, followed by incubation for 1 h with donkey anti-rabbit peroxidase conjugate (Jackson Immunoresearch Laboratories) diluted 1 : 10 000 in blocking solution. Immunoreactive bands were detected using ECL reagent (Amersham Biosciences, Upsala, Sweden) as per the manufacturer's recommendations.

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Supplementary material

The authors have provided the following supplementary figure, which can be downloaded from http://www.blackwellpublishing.com/products/journals/suppmat/PBI/PBI105/PBI105sm.htm: **Figure S1** Isolation of brazzein protein from maize seed bearing construct 2 by immunoaffinity chromatography. An extract was prepared from seed of a type 2 construct, and subjected to immunoaffinity chromatography as described in 'Experimental procedures'. Aliquots of fractions were subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gels. Protein bands were detected by staining with Coomassie blue (A), and the brazzein content was measured by enzyme-linked immunosorbent assay (ELISA) (B). The positions of standard proteins of the indicated molecular weights ($\times 10^{-3}$) are shown on the left of the gel. Lane 1, phosphate-buffered saline (PBS) seed extract; lane 2, flow through (unbound) fraction; lane 3, PBS wash; lane 4, third PBS wash; lanes 5–9, fractions eluted with low-pH buffer.

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