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Antioxidant, anti-inflammatory, and anti-allergic activities of the sweet-tasting protein brazzein

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ABSTRACT

Sweet-tasting proteins may be useful as low-calorie sugar substitutes in foods, beverages, and medicines. Brazzein is an attractive sweetener because of its high sweetness, sugar-like taste, and good stability at high temperature and wide pH ranges. To investigate the bioactivities of brazzein, the antibacterial, antifungal, antioxidant, anti-inflammatory, and anti-allergic activities were determined *in vitro*. Brazzein showed no antibacterial and antifungal activities, although it showed approximately 45% or greater similarity to defensin, which has antimicrobial effects, and drosomycin, which is used as an antifungal agent. However, brazzein exhibited strong antioxidant effects, showing ABTS radical scavenging activity (IC_{50} = 12.55 μM) and DPPH activity (IC_{50} > 30 μM). Brazzein also showed anti-inflammatory activity and anti-allergic activity in a β-hexosaminidase assay (IC_{50} > 15 μM) and cyclooxygenase-2 inhibition assay (IC_{50} = 12.62 μM), respectively. These results suggest that brazzein has antioxidant, anti-inflammatory, and anti-allergic activities and considerable potential as a functional sweetener.

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1. Introduction

Low-calorie sugar substitutes are currently in high demand because the over-consumption of sugar and artificial sweeteners has a variety of side effects such as diabetes and obesity (Kant, 2005). Sweet-tasting proteins show potential as a low-calorie sugar substitutes for use in foods, beverages, and medicines. Brazzein is a sweet-tasting protein isolated from the West African fruit *Pentadiplandra brazzeana* Baillon. It is composed of a single chain of 54 amino acid residues and has a molecular weight of 6.5 kDa, which is smaller than that of other sweet proteins such as thaumatin and monellin. Brazzein has been reported to be approximately 500–2000-fold sweeter than sucrose (Assadi-Porter, Aceti, & Markley, 2000). In addition, up to approximately 80% of naturally extracted brazzein is present in the major form with 54 amino acid residues, while the remaining 20% exists as the minor form and lacks pyro-glutamate at the N-terminus. The minor form is approximately 2-fold sweeter than the major form (Assadi-Porter et al., 2010). Furthermore, brazzein exhibits high water solubility as well as high pH durability and thermostability because of its four disulfide bonds (Ming & Hellekant, 1994). Thus, brazzein shows potential as a sugar substitute.

Numerous proteins and peptides have been reported to show beneficial effects in living organisms, such as antimicrobial, antioxidant, anti-hypertensive, and anti-inflammatory effects (Espitia et al., 2012; Yu, Yin, Zhao, Liu, & Chen, 2012). However, the bioactivities of the sweet-tasting protein brazzein remain unknown.

In the present study, to investigate the bioactivity of brazzein in addition to its sweetness, we utilized the Basic Local Alignment Search Tool (BLAST) program and Vector Alignment Search Tool (VAST) program from the U.S. National Center for Biotechnology Information (NCBI) to compare the primary and higher-order structure of different proteins or peptides. Moreover, *in vitro* assays were performed to verify the results obtained using BLAST and VAST. Furthermore, the antioxidant, anti-inflammatory, and anti-allergic activities of brazzein were investigated. This is the first study to comprehensively examine the biological activities of brazzein.

2. Materials and Methods

2.1. Chemicals and reagents

CM-Sepharose resin was supplied by GE Healthcare (Little Chalfont, UK) and Coomassie Brilliant Blue R-250 for protein staining was obtained from Fluka (St. Louis, MO, USA). Polypeptide SDS-PAGE molecular weight standards were purchased from

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Bio-Rad (Hercules, CA, USA). 2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), L-ascorbic acid, celecoxib, anti-DNP IgE, DNP-BSA, Griess reagents, ketotifen fumarate, lipopolysaccharides, N^G-methyl-L-arginine acetate salt (L-NMMA), *p*-nitrophenyl *N*-acetyl-β-D-glucosamine, *p*-nitrophenyl α-D-glucopyranoside, and norfloxacin were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMSO and MTT formazan were supplied by Duchefa Biochemie (Amsterdam, The Netherlands). All other chemicals and reagents used were commercially available and of the highest grade.

2.2. Similarity analysis of primary and tertiary structures of proteins and docking simulation

The sequence of brazzein was retrieved from NCBI (http://www.ncbi.nlm.nih.gov). Similarities of the primary and tertiary structures of proteins were analyzed using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and VAST+ (https://www.ncbi.nlm.nih.gov/Structure/vastplus/vastplus.cgi) programs, respectively.

To investigate the interaction between human cyclooxygenase-2 (COX-2) and brazzein, docking simulation was performed. Crystal structure of des-pE1M-brazzein (PDB ID: 2LY5) and human COX-2 (PDB ID: 5IKG) were obtained from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). Docking simulation was carried out using the Global Range Molecular Matching (GRAMM-X) program (http://vakser.compbio.ku.edu/resources/gramm/grammx/) provided by the Center for Computational Biology in University of Kansas (Lawrence, KS, USA). The results of docking simulation were analyzed using DIMPLOT from the European Bioinformatics Institute (Cambridgeshire, UK) and 'SEQMOL' (http://biochemlabsolutions.com).

2.3. Preparation of brazzein

The *Kluyveromyces lactis* strain GG799 and expression vector pKLAC2 containing the α-mating factor signal sequence were utilized to produce brazzein (New England Biolabs, Ipswich, MA, USA). *K. lactis* is the strain certified by the Food and Drug Administration (FDA) as 'Generally Recognized as Safe (GRAS)'. 'GRAS' is a status label assigned by the FDA when a substance is not known to be hazardous to health and thus has been approved for use in foods. Expression vector construction, transformation, and expression for recombinant brazzein (minor form, des-pE1M-brazzein) production in *K. lactis* were described previously (Jo, Noh, & Kong, 2013; Yun, Kong, Chung, Kim, & Kong, 2016). Expressed recombinant brazzein was purified by CM-Sepharose chromatography as described previously (Jo et al., 2013). The fractions containing purified brazzein were dialyzed against distilled water and freeze-dried. The purity of recombinant brazzein was confirmed by reversed-phase HPLC column chromatography and SDS-polyacrylamide gel electrophoresis as described previously (Jo et al., 2013). The molecular weight marker was polypeptide SDS-PAGE molecular weight standards, while Coomassie Blue R-250 was used for protein staining.

2.4. In vitro assays for determining the bioactivity of brazzein

2.4.1. Antibacterial activity

The agar dilution method and broth dilution method (Andrews, 2001; Wiegand, Hilpert, & Hancock, 2008) were applied to assess the antibacterial effects of brazzein. The strains except for *Streptococcus pyogenes* were analyzed by a disk diffusion method using Mueller Hinton Agar (Difco, Detroit, MI, USA), while *S. pyogenes* was evaluated by the broth dilution method using Mueller Hinton II broth (Difco). The concentrations of the sample and a positive control, norfloxacin, were 0.01–20 μM. All experiments were performed in triplicate and the strains for the antibacterial assay are shown in Table 1. *Escherichia coli* CCARM 0010 and *Streptococcus pneumoniae* CCARM 0031 were used as controls. Because the cultivation of these strains is difficult, cultivation was conducted by the Korean National Research Resource Bank (Seoul, Korea).

2.4.2. Antifungal activity

To determine the antifungal activity of brazzein, the disc diffusion method was applied (Carson, Cookson, Farrelly, & Riley, 1994). Fungi were evenly spread onto potato dextrose agar (Difco), and then sterile paper discs with 150 μM of brazzein were placed on the media. The zone of inhibition was measured after incubating the dishes at 37 °C for 24 h. All experiments were performed in triplicate. The strains for the antifungal assay are shown in Table S1 and all strains were purchased from the Korean National Research Resource Bank.

2.4.3. Antioxidant capacity

The ABTS free radical scavenging assay was conducted as described by Arnao, Cano, and Acosta (2001). Absorbance was measured at 734 nm using an ELISA reader. The negative control was ABTS in potassium sulfate solution and l-ascorbic acid was used as the positive control. The final concentration of brazzein used in the DPPH and ABTS assay was 15–30 μM. All experiments were conducted in triplicate and the data are presented as the means ± standard deviation (SD).

The DPPH assay was conducted as described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications. The prepared DPPH solution (1 mM) and brazzein were reacted in a ratio of 1:1 and the mixing solution was incubated for 30 min to obtain reliable results. Absorbance was measured at 517 nm using an ELISA reader (SPECTRAmax 190PC, Molecular Devices, Eugene, OR, USA). The negative control was DPPH solution in ethanol and the positive control was l-ascorbic acid. All experiments were conducted in triplicate and the data are presented as the means ± SD.

2.4.4. Anti-inflammatory activity and cytotoxicity

The inhibitory effects on nitric oxide (NO) synthesized from murine macrophage RAW 264.7 cells were evaluated as described by Moro et al. (2012). NO production was determined as the accumulation of nitrite in the culture supernatant using the Griess reagent and absorbance was measured at 540 nm. Brazzein was treated at the concentrations of 1–15 μM and L-NMMA was used as a positive control. Furthermore, an MTT colorimetric assay was carried out to determine the cytotoxicity of brazzein (Moro et al., 2012). All experiments were conducted in triplicate and the data are presented as the means ± SD.

2.4.5. Anti-allergic activity

The inhibitory effects on the release of β-hexosaminidase from rat basophilic leukemia (RBL-2H3) cells (Korean Cell Line Bank) were evaluated as described previously by Matsuda, Tewtrakul, Morikawa, Nakamura, and Yoshikawa (2004). Ketotifen fumarate was used as a positive control and absorbance was measured at 405 nm using an ELISA reader. The final concentration of sample and ketotifen fumarate was 1–15 μM. The inhibitory effects of brazzein towards COX-2 (EC 1.14.99.1) were determined in a COX-2 inhibition assay with a COX-2 (human) Inhibitor Screening Assay Kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. The positive control was celecoxib and the sample (3–25 μM) was diluted with distilled water. All experiments were performed in triplicate and the data are presented as the means ± SD.

Table 1
Strains used for antibacterial activity assay and minimal inhibitory concentrations of brazzein and norfloxacin towards the strains.

Microorganisms			MIC (μM)	
Genus	Species	CCARM No.	Brazzein	Norfloxacin
Staphylococcus	*aureus*	0201	>20	15.7
Staphylococcus	*aureus*	0204	>20	62.6
Staphylococcus	*aureus*	0205	>20	31.3
Streptococcus	*pyogenes*	0206	>20	501.1
Streptococcus	*pyogenes*	0207	>20	62.6
Streptococcus	*pyogenes*	0208	>20	501.1
Pseudomonas	*aeruginosa*	0219	>20	31.3
Pseudomonas	*aeruginosa*	0223	>20	31.3
Pseudomonas	*aeruginosa*	0224	>20	31.3
Pseudomonas	*aeruginosa*	0225	>20	3.8
Escherichia	*coli*	0230	>20	<1.9
Escherichia	*coli*	0235	>20	3.8
Escherichia	*coli*	0236	>20	<1.9
Escherichia	*coli*	0237	>20	31.3
Escherichia	*coli*	0238	>20	7.8
Salmonella	*typhimurium*	0240	>20	<1.9
Hafnia	*alvei*	0013	>20	<1.9
Vibrio	*parahaemolyticus*	7001	>20	<1.9
Vibrio	*parahaemolyticus*	7002	>20	<1.9
Vibrio	*parahaemolyticus*	7003	>20	<1.9
Enterobacter	*cloacae*	0252	>20	3.8
Enterobacter	*cloacae*	0253	>20	<1.9
Streptococcus	*mutans*	0079	>20	250.5
Bacillus	*cereus*	0002	>20	31.3
Bacillus	*cereus*	0120	>20	7.8
Bacillus	*cereus*	0136	>20	7.8
Bacillus	*subtilis*	0003	>20	3.8
Klebsiella	*pneumoniae*	0015	>20	<1.9
Staphylococcus	*aureus*	0202	>20	15.7
Staphylococcus	*aureus*	0203	>20	62.6
Bacillus	*mycoides*	0216	>20	7.8
Bacillus	*megaterium*	0218	>20	7.8
Streptococcus	*aronson B*	0209	>20	501.1
Streptococcus	*faecium D*	0210	>20	<1.9
Salmonella	*enteritidis*	8038	>20	3.8
Salmonella	*enteritidis*	8039	>20	3.8
Salmonella	*typhimurium*	8009	>20	3.8
Salmonella	*typhimurium*	8229	>20	15.7
Klebsiella	*oxytoca*	0248	>20	<1.9
Klebsiella	*aerogenes*	0249	>20	3.8

3. Results and discussion

3.1. Expression and purification of brazzein

Recombinant brazzein was produced using the yeast *K. lactis GG799*, which is 'Generally recognized as safe (GRAS)'. For maximum secretion yield of recombinant brazzein, *K. lactis* cells containing the brazzein gene were grown in YPGal medium (pH 5.0) with 2% injection of the inoculum for 96 h at 30 °C. Recombinant brazzein was efficiently produced in the active form in the medium at an amount approximately 90% of the total secretory proteins. Secreted recombinant brazzein was applied to a CM-Sepharose column that had been equilibrated with 50 mM sodium acetate at pH 4.0 and was eluted with 400 mM NaCl. Purified recombinant brazzein was obtained approximately 90–100 mg/L and its purity was confirmed by SDS-PAGE and HPLC. Purified recombinant brazzein appeared as a single band on SDS-PAGE, with an apparent M_r of 6500 Da (Fig. S1). The elution time for the folded recombinant brazzein was 9 ± 0.5 min, as determined by reversed phase-HPLC (results not shown). The purified recombinant brazzein was used for subsequent bioactivity experiments.

3.2. Similarity analysis of primary and tertiary structures of brazzein

Understanding the similarity between the primary and tertiary structures of proteins will provide insight into their biochemical functions and evolution. Generally, BLAST is utilized to investigate the similarity of primary structures (amino acid sequences) between peptides and/or proteins. The alignment results of similar sequences determined using the BLAST program are shown in Fig. 1A. The primary structure of brazzein showed approximately 42–45% similarity with the antibacterial peptide defensin and a trypsin inhibitor.

Generally, proteins function depends on the tertiary structures rather than the primary structures of proteins. Moreover, tertiary structures of proteins are more evolutionarily conserved than primary structures. VAST was used to analyze the similarity of tertiary structures between brazzein and other proteins. Based on the results obtained using the VAST program, the tertiary structure of brazzein was very similar to those of antimicrobial protein 1, drosomycin used as an antifungal agent, scorpion alpha-toxin OD1, neurotoxin, and plant defensin (Fig. 1B). The tertiary structure of brazzein showed approximately 0.72–1.40 Å RMSD values against these structures.

3.3. Bioactivity analysis of brazzein

The sweet-tasting protein brazzein shares several common features with antimicrobial proteins that contribute to their antimicrobial actions, as follows: typically small sizes less than 10 kDa and isoelectric points ranging from pH 2 to 7 (Yeaman & Yount, 2003). Furthermore, brazzein has approximately 34% similarity

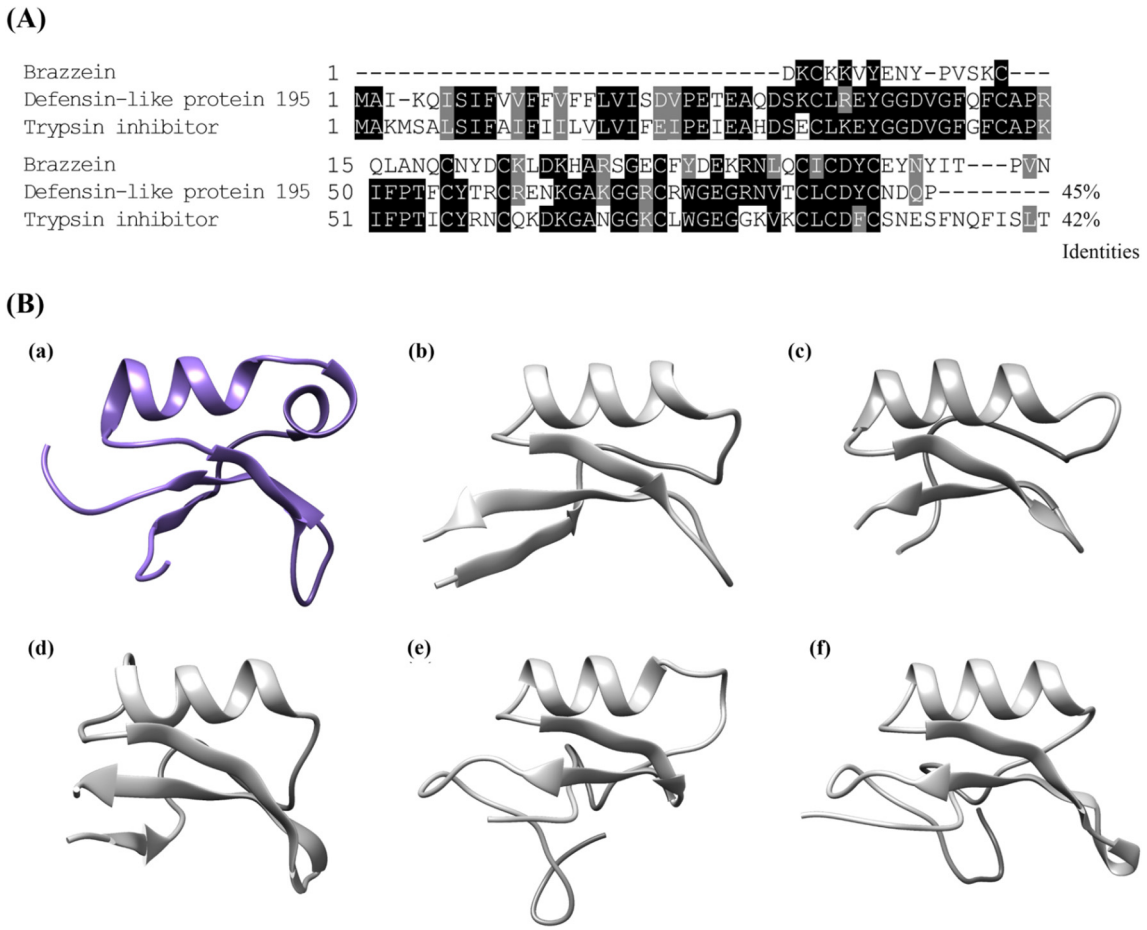

Fig. 1. Similar proteins with the primary and tertiary structures of brazzein determined using the BLAST and VAST programs. (A) Multiple alignment of brazzein (2LY5_A), defensin-like protein 195 [Arabidopsis thaliana] (XP_018437331.1) and putative trypsin inhibitor 4 [Arabidopsis thaliana] (CAG15211.1) using the T-coffee and BOXSHADE 3.21 programs. Identical residues are shaded in black and similar residues are shown in gray. (B) Tertiary structure of proteins with high structural similarity with brazzein. (a) Brazzein; (b) Aesculus hippocastanum antimicrobial protein 1; (c) Drosomycin; (d) Scorpion alpha-toxin OD1; (e) Tityus serrulatus neurotoxin; (f) Petunia hybrida defensin 1 PhD1.

with an antibacterial peptide, Ah-AMP-1 (PDB 1BK8), which exhibits an antibacterial effect (Yount & Yeaman, 2004). In this study, the antibacterial activity of brazzein was measured against the strains related to the tooth decay and food poisoning (Table 1). Unexpectedly, brazzein showed no antibacterial activity at concentrations of 0.01–20 μM at pH 5.5. A similar result was observed in a previous study by Yount and Yeaman (2004); the previous study suggested that brazzein have minimal antibacterial effects against *Bacillus subtilis*, *Staphylococcus aureus*, and *E. coli* at pH 5.5. Interestingly, antimicrobial peptides exhibited pH-specific activities. At pH 7.5, brazzein showed antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*, and *Candida albicans* (Yount & Yeaman, 2004). Wadhwani et al. (2009) also suggested that the solvent and pH of antimicrobial peptide samples varied in their antibacterial effects, although the samples and strains were the same.

Most antifungal peptides have α-helixes, β-sheets, and disulfide bonds in common (Giangaspero, Sandri, & Tossi, 2001). Brazzein also contains these common structures of antifungal peptides (Fig. 1B). Determination of the three-dimensional structures of brazzein revealed that brazzein contains one short α-helix (residues 21–29) and three antiparallel β-sheets (strand I, residues 5–7; strand II, residues 44–50; strand III, residues 34–39) (Caldwell et al., 1998; Nagata et al., 2013). Brazzein also contains four disulfide bonds (Cys4-Cys52, Cys16-Cys37, Cys22-Cys47, and Cys26-Cys49), resulting in a structure with good stability. Moreover, the three-dimensional structure of brazzein shows high similarity to those of antimicrobial protein 1, drosomycin known as a key component of antifungal defense in *Drosophila melanogaster* (Zhang & Zhu, 2009), scorpion toxin, neurotoxin, and plant defensin, although their primary structures exhibit low similarity (Fig. 1B). In this study, the antifungal activity of brazzein was measured against strains (*Neurospora crassa*, *Geotrichum candidum*, *Fusarium oxysporum* and *Aspergillus fumigatus*) that were inhibited by drosomycin, and against strains (*Aspergillus niger*, *Aspergillus oryzae*, *Fusarium sporotrichioides*, *Penicillium rugulosum and Trichoderma reesei*) related to food poisoning and spoilage. In contrast to the VAST results, brazzein showed no antifungal activity at 150 μM against all fungi tested (Table S1). Previous studies reported that antifungal peptides contain specific amino acids such as proline, glycine, arginine, histidine, and tryptophan (Bulet, Stocklin, & Menin, 2004; Zasloff, 2002). However, brazzein contained few of these amino acids (one proline, one glycine, two arginines, one histidine, and no tryptophan) (Fig. S2A). Thus, both the three-dimensional structure of the protein and composition of amino acids related to inhibition mechanism may be necessary for the antifungal activity of this protein.

Free radicals and reactive oxygen species are highly reactive molecules generated by normal cellular processes, environmental stresses, and UV irradiation. Excessive radicals in the human body damage nucleic acids, carbohydrates, proteins, and lipids, which can lead to inflammation, aging, and several diseases such as cancer and cardiac diseases (Lovo, Patil, Phatak, & Chandra, 2010).

There are various methods for determining the capacity of antioxidants. The ABTS and DPPH free radical scavenging assays were applied in this study. Fig. 2 shows that 30 μM brazzein exhibited antioxidant capacity in the ABTS assay (82.3 ± 1.7%) and DPPH assay (35.7 ± 2.6%). In particular, the antioxidant capacity of brazzein in the ABTS assay was greater than that of L-ascorbic acid, which is the most commonly used antioxidant.

According to previous studies, amino acids in protein can interact with free radicals, resulting in antioxidant activity (Østdal, Davies, & Andersen, 2002). Specific amino acids including those with a sulfhydryl group (methionine and cysteine) or aromatic ring (tryptophan, tyrosine, and phenylalanine) contain a hydrogen atom that can interact with free radicals (Elias, McClements, & Decker, 2005). Histidine with an imidazole-ring structure and lysine residues were also reported to show antioxidant activity (Elias et al., 2005; Wang & Mejia, 2005). Brazzein has many residues reported to have antioxidant activity (eight cysteine, seven lysine, six tyrosine, one phenylalanine, and one histidine residue), accounting for approximately 43% of the total number of amino acids (Fig. S2A). Furthermore, the surface accessibility of the amino acids constituting brazzein was investigated using 'NetSurfP-1.1' (Peterson, Petersen, Anderson, Nielsen, & Lundegaard, 2009). The results showed that two of the six tyrosine, one histidine, and all lysine residues were exposed on the surface of brazzein (Fig. S2B), indicating that these residues play an important role in antioxidant capacity. The eight-cysteine residues in brazzein did not affect the radical scavenging activity of brazzein, as these residues formed four disulfide bonds. Compared to the ABTS assay, the DPPH assay showed that brazzein had weaker radical scavenging activity. The ABTS assay is based on the generation of the ABTS cation radical, which is suitable for both hydrophilic and hydrophobic antioxidants, whereas the DPPH assay uses a radical dissolved in organic solvent and is thus applicable to hydrophobic systems (Floegel, Kim, Chung, Koo, & Chun, 2011). Based on this information, the antioxidant capacity of the sweet protein brazzein was better in the ABTS assay than in the DPPH assay. Taken together, we suggest that brazzein has a high content of amino acids with antioxidant ability and exerts considerable antioxidant activity.

Macrophages or monocytes promote the secretion of pro-inflammatory cytokines such as interleukin (IL) family proteins and nitric oxide (NO) by stimulating lipopolysaccharides (Sharma, Al-Omran, & Parvathy, 2007). NO is produced from L-arginine by NO synthase, and overproduction of NO induces an acute inflammatory reaction in humans. To assess the anti-inflammatory effects of brazzein, NO production in murine macrophage RAW 264.7 cells was measured. Prior to the NO assay, the cytotoxicity of brazzein was observed by the MTT colorimetric assay. The MTT assay showed that 1–15 μM brazzein had no cytotoxic effect on RAW 264.7 cells, as the cell viability was approximately 100% at all concentrations (Fig. 3A). Moreover, the anti-inflammatory activity of brazzein was determined in an NO assay. The results shown in Fig. 3B indicate that NO production from RAW 264.7 cells was not significantly decreased by 15 μM brazzein (11.83 ± 2.27%) and its IC_{50} value exceeded 15 μM. In contrast, L-NMMA, known as an NO synthase inhibitor, reduced NO by approximately 35% at the same concentration with an IC_{50} value of 39.83 μM. These results suggest that brazzein is not cytotoxic, but shows some anti-inflammatory activity. During inflammation, reactive nitrogen species such as a nitrous oxide, nitrogen dioxide, peroxynitrite, and NO are produced with ROS (Nakagawa & Yokozawa, 2002). Among these, NO is an unstable free radical; when excessively produced, it functions as a toxic radical, damaging cells and tissues. As described above, brazzein contains numerous residues reported to have radical scavenging capacity, accounting for approximately 43% of the total number of amino acids. Taken together, we suggest that the anti-inflammatory effect

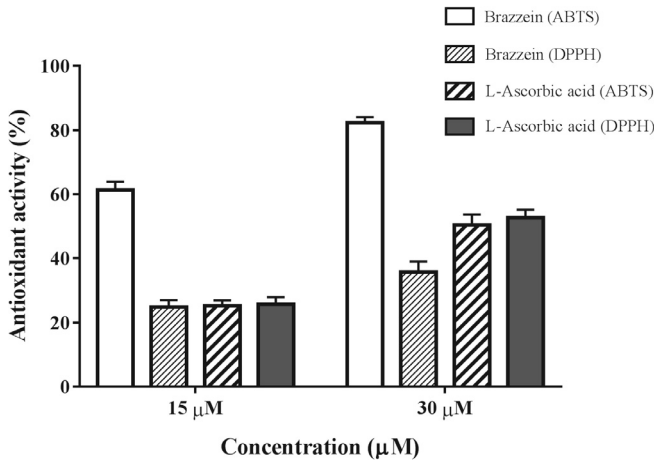

Fig. 2. DPPH and ABTS radical-scavenging activity of brazzein. L-Ascorbic acid was used as a positive control.

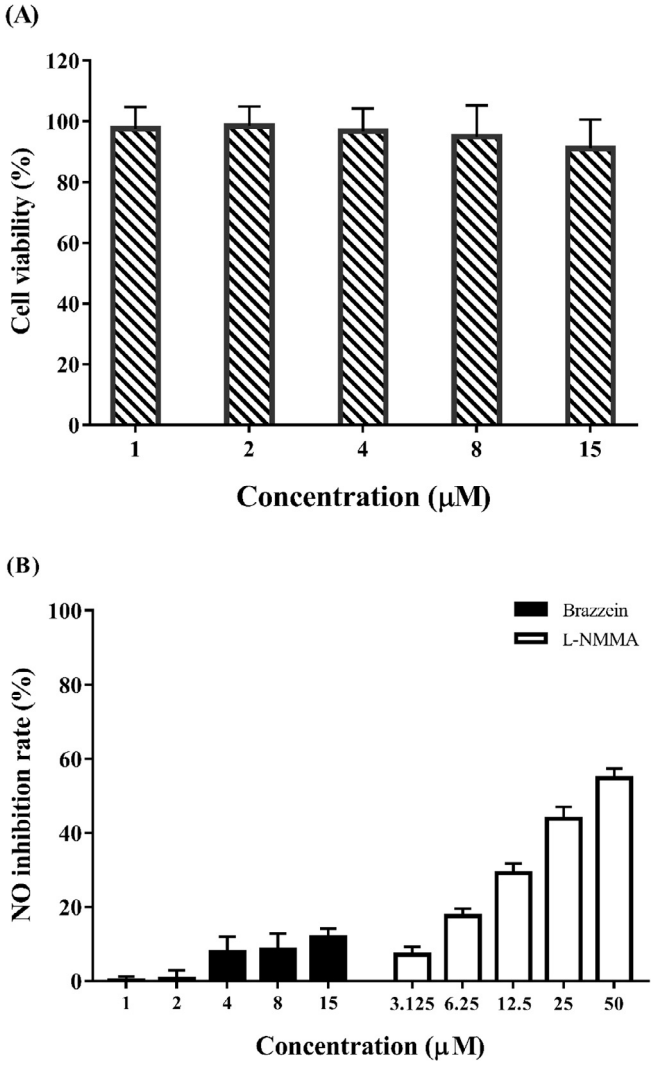

Fig. 3. (A) Cytotoxic effect of brazzein and (B) anti-inflammatory activity of brazzein and L-NMMA (control) against murine macrophage RAW 264.7 cells.

Table 2
Anti-allergic activity of brazzein by β-hexosaminidase release inhibition assay and COX-2 inhibition assay.

β-Hexosaminidase assay		COX-2 inhibition assay	
Sample	IC_{50} (μM)	Sample	IC_{50} (μM)
Brazzein	>15.00	Brazzein	12.62 ± 2.69
Ketotifen fumarate	79.83 ± 2.27	Celecoxib	0.026 ± 0.001
Quercetin[a]	5.70	Rofecoxib[b]	0.52
–	–	Ibuprofen[b]	180.00
–	–	Mefenamic acid[b]	7.00

[a] Value for quercetin was taken from Matsuda et al. (2004).
[b] Values for rofecoxib, ibuprofen, and mefenamic acid were taken from Duggan et al. (2011).

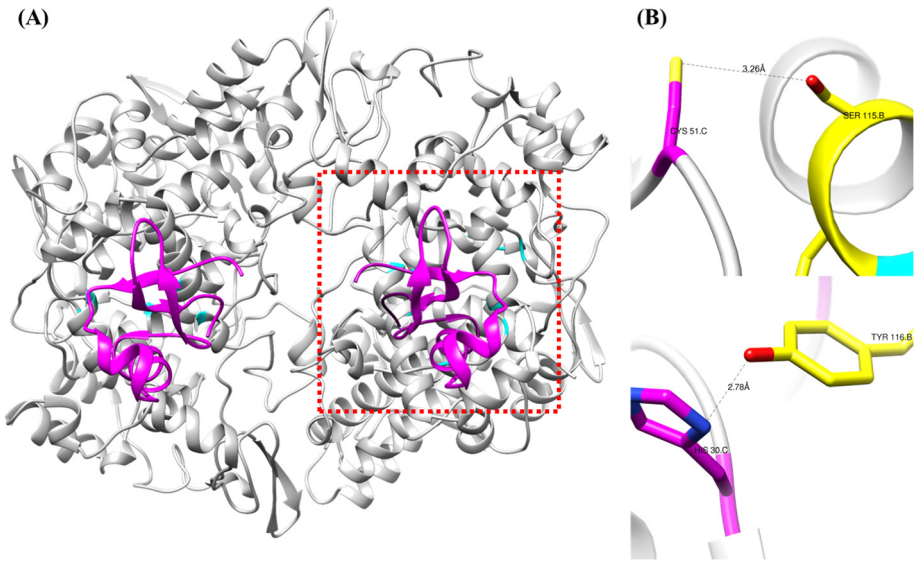

Fig. 4. Molecular docking of brazzein and human cyclooxygenase-2 (COX-2). (A) Structure model of human COX-2 homodimer in complex with brazzein. Active sites of human COX-2 are colored in cyan and brazzein is colored in magenta. (B) Enlargement of the interaction zone (hydrogen bonding near the active site of human COX-2 and brazzein) surrounded by red dots in panel (A). Hydrogen bonds form between Cys51 of brazzein (magenta) and Ser115 of COX-2 (yellow). Hydrogen bonds also form between His30 of brazzein and Tyr116 of COX-2. Hydrogen bonds are depicted as black dotted lines with distances.

of brazzein is be related to its radical scavenging effects based on its amino acid composition rather than its cytotoxicity on cells.

There are several ways to induce allergic reactions (Coombs & Gell, 1968). Among them, type I hypersensitivity is closely related to histamine. When mast cell degranulation occurs, histamine is secreted with β-hexosaminidase, which is used as an indicator of histamine release. Histamine leads to an allergic reaction, telangiectasia, enhancement of capillary permeability, and increase in heart rate (Marshall, 2004; Mekori & Metcalfe, 2000). In addition, prostaglandin functions as mediator of pathogenic mechanisms, including the acute inflammatory response. The biosynthesis of prostaglandin derived from arachidonic acid depends on COX-2, which is not expressed under normal conditions in most cells, but is elevated dramatically during inflammation (Ricciotti & FitzGerald, 2011). Based on the results of the β-hexosaminidase assay, 15 μM brazzein inhibited approximately 27% of β-hexosaminidase release from RBL-2H3 cells and its IC_{50} value exceeded the testable concentration (Table 2). Brazzein also showed inhibitory potency for COX-2 activity with an IC_{50} value of 12.62 μM. The potency of brazzein was lower than those of the COX-2 inhibitors rofecoxib and celecoxib. However, the potency was significantly higher than that of ibuprofen and similar to that of mefenamic acid. Based on these results, brazzein inhibited not only the secretion of histamine from mast cells but also the synthesis of prostaglandin by suppressing COX-2.

To determine the inhibition mechanism of brazzein towards human COX-2, twenty docking models were obtained by docking simulation using GRAMM-X. Dissociation constants and the Gibbs free energy of each model were calculated by SEQMOL. DIMPLOT was used to estimate the interactions between amino acids of human COX-2 and brazzein. Among the results, we selected the complex model showing the strongest binding force (K_d = 8.54-E−08) (Fig. 4A). Human COX-2 consists of a membrane-associated heme-containing homodimer and each subunit has its own active site (Carvalho et al., 2015). Docking simulation revealed that the His30 and Cys51 residues of brazzein form hydrogen bonds with the Ser115 and Tyr116 residues of the human COX-2 subunit and are located near the active site of human COX-2 (Fig. 4B). Therefore, brazzein appears to inhibit COX-2 activity by binding closely to the active site of COX-2.

4. Conclusion

By similarity analysis of the primary and tertiary structures between brazzein and proteins, the antibacterial and antifungal activities of brazzein were predicted. However, brazzein did not show antibacterial and antifungal activities *in vitro*. Notably, brazzein exhibited considerable antioxidant activity in the ABTS and DPPH assays as well as an anti-allergic effect in the

β-hexosaminidase assay and COX-2 inhibition assay. Moreover, brazzein also shown an anti-inflammatory effect. Based on these results, brazzein can be used as a functional sweetener with various bioactivities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.06.084.

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