Contents lists available at ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

Expression of functional plant sweet protein thaumatin II in the milk of transgenic mice

Rui Lu[a,1], Xiaoming Li[b,1], Yancui Wang[a,*], Le Jin[a,*]

[a] Jiangsu Food and Pharmaceutical Science College, Huaian, Jiangsu, China
[b] Jiangsu Agri-Animal Husbandry Vocational College, Taizhou, Jiangsu, China

ARTICLE INFO

Article history:
Received 9 August 2020
Received in revised form 1 November 2020
Accepted 11 November 2020
Available online 27 November 2020

Keywords:
Thaumatin
Expression
Sweetness
Transgenic mice
Mammary gland bioreactor

ABSTRACT

Thaumatin is a kind of natural sweet protein which has the characteristics of high sweetness, low calorie, safe and non-toxic, etc. And it has high commercial value in food processing, medicine and other fields.

In this study, we employed a sequence that encode thaumatin II, along with goat beta-casein 5′ and 3′ regulatory elements, to construct a mammary gland-specific expression vector. Transgenic mice were generated using pronuclear microinjection, and the expression of thaumatin and its biological activities were assayed in the milk.

PCR and southern blot analysis confirmed that the mice harbor *thaumatin II* transgenes. Thaumatin II retaining their sweetness was expressed in the mammary glands of transgenic mice, as determined by ELISA, western blot and sweetness intensity test.

This research describes an initial step in the production of plant protein thaumatin-sweetened milk from large animals such as cow and goat.

1. Introduction

There is currently a worldwide trend to reduce sugar consumption, since high intake of sugar can instigate risk factors such as diabetes (Kahn and Sievenpiper, 2014), cardiovascular diseases (Johnson et al., 2009) and obesity (Collaboration, N.C.D. Risk Factor, 2016). This trend is currently met by using artificial non-nutritive sweeteners. However, these artificial sweeteners have also been shown to have the same adverse health effects as sugar (Suez et al., 2014; Pearlman et al., 2017; Anand and Winkelmayer, 2012; Azad et al., 2016; Drouin-Chartier et al., 2019; Hodge et al., 2018; Mullie and Clarys, 2017), and their metabolites may cause environmental pollution (Gatidou et al., 2020). Sweet proteins are not associated with adverse health effects, and thaumatin is one of the most studied and most promising alternatives to sugars and artificial sweeteners (Joseph et al., 2019; Yamamoto et al., 2020; Kant, 2005). The thaumatin was first found from the fruit arils of a tropically grown plant called *Thaumatococcus daniellii* (Benth) belonging to the family Marantaceae in tropical West Africa, and it has high water solubility, heat and acidic resistance, and taste properties like those of carbohydrate sweeteners (van der Wel and Loeve, 1972; Breiteneder, 2004; Kaneko and Kitabatake, 2001a). Thaumatin has gained attention because 1) it was reported to be nearly 100,000 times sweeter than sucrose on a molar basis and about 3000 times sweeter on a weight basis, and the threshold value of sweetness of thaumatin is about 50 nM (van der Wel and Loeve, 1972); 2) its safety has been confirmed by the relevant studies and since 1984, and it has been approved for use in the EU (food additive E957)(Mortensen, 2006; Zemanek and Wasserman, 1995); 3) it has been used in the food industry as a sweetener and flavor enhancer, and in pharmaceutics as a component of special low-calorie diets (Faus, 2000).

There are at least five intensely sweet forms: two main ones- thaumatin I and II, and three minor ones- thaumatin

* Corresponding authors.
E-mail addresses: 20191002@jsfpc.edu.cn (Y. Wang), 20191014@jsfpc.edu.cn (L. Jin).
[1] These authors made equal contribution to this work.
https://doi.org/10.1016/j.fbp.2020.11.006
0960-3085/

a, b and c (Iyengar et al., 1979; Edens et al., 1982). All of these forms have a molecular mass of about 22 kDa and have an isoelectric point of 12 (van der Wel and Loeve, 1972). To date, thaumatin II is the best-studied variant of thaumatin, because it was characterized by high stability and maintains sweetness even after heating to 80 °C at pH 2.0 for 4 h (Kaneko and Kitabatake, 2001b). These properties of thaumatin II make it an attractive ingredient for the food industry, and the major applications are seen as an additive in dairy products, chewing gum, ice cream, soft drinks, etc (Joseph et al., 2019). The local people in West Africa have consumed the arrowroot fruit for a long time, and there is no report of harm to human health (van der Wel and Loeve, 1972; Joseph et al., 2019).

The natural source of thaumatin was extracted routinely in Africa and sold for years, marketed by Tate & Lyle (UK) under the brand name Talin. But the *Thaumatococcus daniellii* (Benth) has extremely high requirements for the growth environment, and researchers from all over the world have introduced it without success (Kiełkiewicz et al., 2012). Because of the difficulties and limitations of obtaining natural source of thaumatin, numerous attempts have been performed to produce recombinant thaumatin in different genetically engineered microorganisms and transgenic plants (Edens et al., 1982; Masuda et al., 2010; Schestibratov and Dolgov, 2005; Bartoszewski et al., 2003). Compared with other bioreactor systems for the production of recombinant proteins, transgenic animal mammary gland-specific bioreactors are attractive platforms because their ability to produce complex, biologically active proteins in an efficient and economic manner is superior to those of bacteria, mammalian cells, transgenic plants, and insects (Houdebine, 2018; Wang et al., 2013; Lu et al., 2018). To the best of our knowledge, there are no reports of using transgenic animal mammary gland bioreactors to express thaumatin.

The overall objective of this study was to evaluate the feasibility of producing thaumatin II in the milk of transgenic animals. Thaumatin II transgenic mice were generated that possess a mammary gland-specific thaumatin II gene, the expression and sweetness of thaumatin II was confirmed. Our results showed that recombinant thaumatin II protein can be secreted in the milk of transgenic mice and maintain sweetness.

2. Materials and methods

2.1. Ethics statement

All animal procedures and study designs were conducted in accordance with the guide for the care and use of laboratory animals (Ministry of Science and Technology of the People's Republic of China). Animal surgical procedures were performed under anesthesia, and all efforts were made to minimize animal pain, suffering, and distress. The ICR mice were housed at 25 ± 1 °C on a 12-h light/ dark cycle with free access to food and tap water.

2.2. Construction of the thaumatin expression vector

The *thaumatin II* cDNA was artificially synthesized by Sangon Biotech (Shanghai) Co., Ltd harboring *Xho* I sites at the 5′ and 3′ termini. The *thaumatin II* (J01209.1) mature peptide coding sequence was fused with a Kozak translation initiation sequence and a goat β-lactoglobulin signal peptide coding region. Codon optimization of the *thaumatin II* gene was performed using the gene project software (Sangon Biotech, China) for expression in mammary gland epithelial cells. The synthesized gene was cloned into a pBC1 milk expression vector (Invitrogen Life Technologies, US), which harbors a goat beta-casein promoter. The resulting vector was designated as pBC1-thaumatin II (Fig. 1).

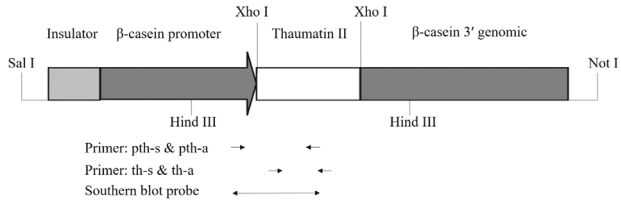

Fig. 1 – Schematic of pBC1-thaumatin II. Insulator: chicken β-globin insulator (2X); β-casein promoter: goat β-casein promoter; Thaumatin II: optimized Thaumatin II coding region; β-casein 3′ genomic: β-casein 3′ genomic fragment; loci of primers and Southern blot probe in the identification of transgenic mice were also illustrated.

2.3. Generation of transgenic mice

The transgenic mice were generated by pronuclear microinjection. A 16.4-kb transgene fragment, obtained from the pBC1-thaumatin II plasmid with *Not* I and *Sal* I double-digestion, was purified using a QIAquick gel extraction kit (Qiagen, Germany) and diluted to 5 ng/μL in TE buffer, as described elsewhere (Chen et al., 2008).

2.4. Detection of exogenous gene insertions in the mice genome

One pair of pBC1-thaumatin II specific primers were designed to identify the transgenic mice. The primers were: pth-s: 5′-TTGACAAGTAATACGCTG-3′; pth-a: 5′-TTACAAGAATAGGGAAGG-3′. The amplified products were 776 bp in size. Sequencing analysis was performed by Sangon Biotech (Shanghai) Co., Ltd.

Genomic DNA extracted from the tail of transgenic and wild-type (WT) mice and plasmids pBC1-thaumatin II as positive controls were digested overnight by the restriction enzyme *Hind* III. The digoxigenin-labeled probe was amplified using the primers pth-s and pth-a. After agarose gel electrophoresis for 4 h, the DNA was transferred onto a nylon membrane (Roche, Switzerland) for blotting. The nylon membrane was hybridized with probes for 18 h and incubated with an antibody (biotin-labeled mouse anti-digoxin) for 0.5 h. The expected size of the positive bands was about 5.2 kb. The reagents used for Southern blot analysis were purchased from Boster Co., Ltd (Wuhan, Hubei, China).

2.5. Detection of transcription of recombinant thaumatin in the mammary gland tissues

Total RNA was isolated using Trizol (Tiangen, China) from the mammary gland, liver, heart, spleen, lung, kidney, pancreas and uterus of transgenic mice. Primers th-s: 5′-TACCACCCTGGCCGAGTT-3′ and th-a: 5′-CAGAAGGTCACCCTGTAGTTGC-3′ were used to verify the presence of the thaumatin II gene. The mouse housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control with

primers GA1 (5′-GGTGAAGGTCGGTGTGAACG-3′) and GA2 (5′-CTCGCTCCTGGAAGAGGTG-3′).

2.6. Detection of recombinant thaumatin in the milk

The mice milk was collected as previous reported (DePeters and Hovey, 2009; Yan et al., 2013). A mixture of thaumatin I and thaumatin II proteins (T7638, Sigma-Aldrich, USA) was used as positive control. Samples was boiled at 100°C for 10 min in SDS-PAGE loading buffer and then separated on 12% polyacrylamide Tris–glycine gels. Separated proteins were electrophoretically transferred to PVDF membranes (0.45 μm, Pall, USA). The membranes were blocked with 5% FBS/TBST overnight at 4°C and then incubated with a polyclonal antibody raised in rabbits against thaumatin protein (1:2000 dilution in 5% FBS/TBST) at room temperature for 1.5 h, followed by three washes with TBST. The membranes were incubated with HRP-conjugated goat anti rabbit IgG (1:3000 dilution in 5% FBS/TBST; Biosharp Life Science, China) at room temperature for 1 h, followed by three washes with TBST. Immunodetection was conducted using ECL as substrate (Millipore Corporation, Billerica, MA, US) according to the manufacturer's instructions.

Thaumatin II expression levels were measured by enzyme-linked immunosorbent assay (ELISA). The whey samples were diluted to 1:100 with PBS for ELISA. Rabbit polyclonal antibody to thaumatin (1:2000 dilution in 5% FBS/ TBST) was used as primary antibody. HRP-conjugated goat anti-rabbit IgG (1:3000 dilution in 5% FBS/ PBS; Biosharp Life Science, China) were used as secondary antibody in a TMB substrate kit assay (Sangon, Biotech, China). Absorbance was measured using a microplate reader (Rayto, China) at 450 nm. Standard curves were constructed using the thaumatin standard (T7638, Sigma-Aldrich, USA), and the thaumatin concentrations in the samples were calculated using the regression equations of the standard curves, which were based on their OD values.

2.7. Sweetness intensity testing

The boiled milk was subjected to a double-blind taste. The participants included 16 volunteers, including 8 females and 8 males (aged 18–50 years), and none of the them exhibited any irregularities in their sense of taste. They were randomly selected and fully disclosed of the purpose and their roles of the study.

The tasting protocol is designed according to the standard for Measuring Sensations of Taste (Yan et al., 2013; Green et al., 1996). Briefly, 0%, 0.6%, 2%, 4%, 6%, and 10% (w/v) sucrose solutions were prepared as positive controls, and the milk of wild-type (WT) mice as the negative control. 50 μL 2-fold diluted mouse milk was dropped onto the anterior part of the tongue. The subject's mouth was rinsed three times by drinking water after each test. The order of presentation of the samples was randomized and the taste test for each sample was repeated three times. The score was based on the feedback of the categories "not sweet" (0), "uncertain whether sweetness was tasted" (0.5), "faintly sweet" (1.0), "sweet" (2.0), "very sweet" (3.0) and "extremely sweet" (4.0). The average score for 2% and 4% sucrose was established at 1.0 or 2.0, respectively. The sweetness scores were first evaluated with repeated measurement ANOVA (analysis of variance) followed by one-way ANOVA of the scores for different variants. P values <0.05 were considered significant.

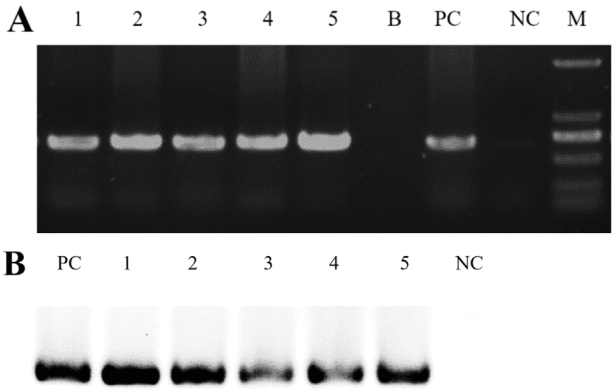

Fig. 2 – Identification of the thaumatin II gene in transgenic mice. A PCR detection of the thaumatin II gene in transgenic mice. NC: genomic DNA from wild-type mouse; Lanes 1-5: Genomic DNA of 5 founder transgenic mice; B: water; M: DL2000 DNA marker; PC: pBC1-Thaumatin II plasmid mixed with genomic DNA from wild-type mouse as positive control. B Identification of thaumatin II gene in transgenic mice by Southern blot analysis. PC: pBC1-Thaumatin II plasmid digested with Hind III as positive control; 1-5: 5 founder transgenic mice genomic DNA digested with Hind III; NC: wild-type mouse genomic DNA digested with Hind III as negative control.

3. Results

3.1. Construction of the Thaumatin expression vectors

The chimeric cDNA fragments were inserted into pBC1 vector to generate recombinant plasmid pBC1- Thaumatin II. The size of the recombinant plasmid construct was confirmed by restriction analysis. The result of sequencing indicated that the coding region of the *thaumatin II* DNA fragment was fused in-frame to upstream of the vector pBC1.

3.2. Generation of transgenic mice

Transgenic mice were produced by pronuclear microinjection. 62 fertilized eggs were microinjected, of which 53 were transferred into 6 surrogate mice recipients. A total of 46 kids were born by the surrogate mice recipients.

3.3. Transgene integration in the genome of transgenic mice

The integration of the *thaumatin II* gene into the genome of the transgenic mice was confirmed by PCR and Southern blot analysis.

Primers pth-s and pth-a were used for PCR amplification and the digoxigenin-labeled amplicon was used as probe for Southern blot analysis. Of the 40 pups born, 5 (1 male and 4 female) transgenic mice were identified by PCR analysis (Fig. 2A) and were further verified by Southern blot analysis (Fig. 2B).

3.4. Thaumatin II mRNA transcription in mammary glands and other tissues

We verified tissue-specific expression of thaumatin II in transgenic and wild type (WT) mice at five weeks of age by reverse transcription PCR (RT-PCR). As shown in Fig. 3, the thaumatin II

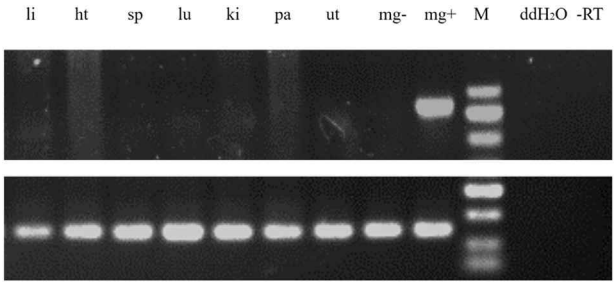

Fig. 3 – RT-PCR analysis of transgenic mouse from various tissues to determine the tissue specificity of thaumatin II in transgenic mice. The tissues analyzed were from liver (li), heart (ht), spleen (sp), lungs (lu), kidneys (ki), pancreas (pa), uterus (ut), normal mammary glands (mg-), lactating mammary gland (mg+); M: DNA Marker; ddH$_2$O: double distilled water; -RT: no M-MuLV Reverse Transcriptase in the reaction to rule out genomic DNA contamination. The bottom panel is the result of the RT-PCR analysis of the mouse GAPDH gene.

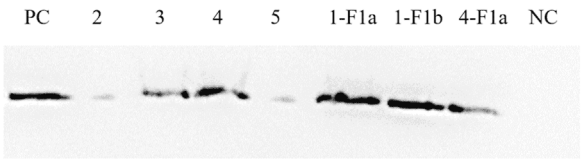

Fig. 4 – Western blot analysis of expression of thaumatin II in the milk of transgenic mice. PC: 2 μg mixture of thaumatin I and thaumatin II proteins (T7638, Sigma-Aldrich, USA) as positive control; 2, 3, 4, 5: 10 μL whey sample of the female founder transgenic mice; 1-F1a, 1-F1b, 4-F1a: 10 μL whey sample of the female F1 generation transgenic mice; NC: 10 μL whey sample from a wild-type mouse as negative control.

transcripts were found in lactating mammary glands, but not in other tested tissues (liver, heart, spleen, lung, kidney, pancreas and uterus) of the transgenic mice. Mouse GAPDH was used as the control for the reverse-transcribed RNA. Distilled deionized water, non-transgenic mammary tissue and samples without reverse transcription were used as the negative controls. This finding demonstrates the high tissue-specificity of *thaumatin II* gene transcription, with no leaky expression.

3.5. *Detection of thaumatin II in the milk of transgenic mice*

Thaumatin II was detected in the milk of the 4 female founder transgenic mice and 3 female progeny, whereas no corresponding signal was detected in the no-transgene milk. The sizes of thaumatin II protein was approximately 22 kDa, (Fig. 4), which indicated that the goat β- lactoglobulin signaling peptide was correctly cleavaged in the mice mammary gland epithelial cell.

The expression levels of thaumatin II were determined using an ELISA (Table. 1). The levels of thaumatin II in the milk of the 4 different female founder mice were as follows: line 2, 1.97 mg/L; line 3, 7.38 mg/L; line 4, 16.72 mg/L; and line 5, 1.69 mg/L. The thaumatin II expression levels in the milk from 3 different F1 progeny were as follows: 1-F1a, 19.57 mg/L; 1-F1b, 21.98 mg/L; 4-F1a, 15.17 mg/L. These result show that the thaumatin II gene is specifically expressed in the mammary gland and that the transgene acquired a germline transmission.

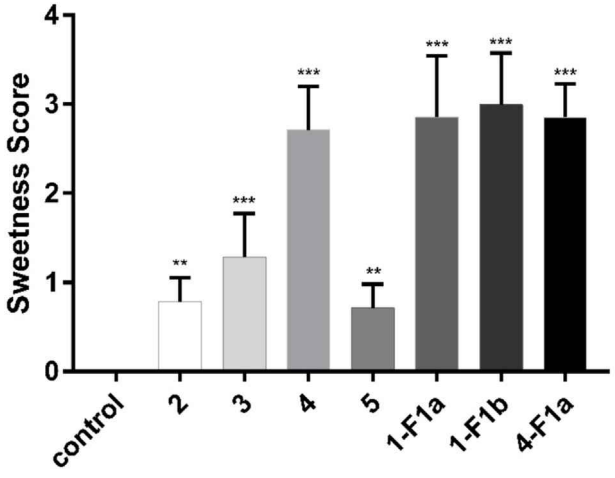

Fig. 5 – Sweetness test of recombinant thaumatin II. Sweetness was scored from 1 to 4 as follows: (0) not sweet, (0.5) uncertain if sweetness was tasted, (1.0) faintly sweet, (2.0) sweet, (3.0) very sweet and (4.0) extremely sweet. Results of psychophysical experiments with thaumatin II are shown; data were averaged for the 16 volunteers. Error bars represent SD. Column patterns indicate different levels of sweetness compared with the control (non-transgenic milk); the others are the sweetness of milk from the different transgenic mice. The results are shown as means ± S.D. ***P < 0.001 compared with control.

3.6. *Sweetness analysis of thaumatin II in transgenic mouse milk*

To determine whether the thaumatin II in transgenic mice milk retained its sweetness, a sensory evaluation of the milk was performed and all of the samples were diluted 2 times with double distilled water. Milk from three different WT mice was used as the negative control. To test the thermal stability of thaumatin II in the milk of transgenic mice, the milk was heated at 100 °C for 5 min. The sweetness of the 2-fold diluted milk from mouse line 1 and line 4 (including their F_1 offspring) were comparable to that of 4.0% sucrose solution (Fig. 5) and is significantly differed from that of the control milk ($P < 0.05$). The milk from mouse lines 3 was less sweet (less than 2.0% sucrose solution). The sweetness of the 2-fold diluted milk from mouse line 2 and 5 were comparable with that of a 0.6% sucrose solution. The sweetness and the concentrations of the thaumatin II in the milk had positive correlation.

4. Discussion

In this study, we constructed a vector for the mammary gland-specific expression of thaumatin II and generated transgenic mice carrying the vector. The ELISA, Western blotting, and

Table 1 – The expression levels of thaumatin II in the milk of transgenic mice.

Mouse	Line 2	Line 3	Line 4	Line 5	1-F1a	1-F1b	4-F1a
Concentration (mg/L)	1.97	7.38	16.72	1.69	19.57	21.98	15.17

sweetness intensity testing showed that bioactive thaumatin II was efficiently expressed in the milk of transgenic mice. These results demonstrated that transgenic animals could be used as bioreactors in the production of thaumatin II protein, thereby constituting an initial step for the production of plant protein thaumatin-sweetened milk from large animals such as dairy cow and goat.

The various expression levels of thaumatin II protein in different mouse lines might be due to 1) the random integration of the transgene, which leads to positional effects on its expression (Dobie et al., 1996; Robertson et al., 1995; Haruyama et al., 2009); 2) the different copy numbers of the thaumatin II transgenes (Kong et al., 2009). The thaumatin II concentrations in the milk of our transgenic mice were not as high as other proteins previously reported (Lu et al., 2019). Although the concentrations do not meet the requirements of mass production of pure thaumatin II, the expression enabled the milk to have a high level of sweetness. The sweetness rating score of the 2 times diluted milk from mouse line 1 (with 20.8 mg/L thaumatin II protein) is comparable to that of 4% sucrose solution. This implies that our recombinant thaumatin II is about 3000 times sweeter than sucrose by weight, which is consistent with natural thaumatin II. These results indicate plant protein thaumatin II could be efficiently expressed and well post-translational modified in the mammary gland of animals.

Cow and goat milk has high content of some amino acids (especially sulfuric ones), saturated short chained fatty acids and minerals (mainly K, Ca, Mg, Fe, Cu and Mn) (Farrell et al., 2004; Litwinczuk et al., 2015). So their milk is a common food or food additive worldwide, and milk is also a healthy food for the diabetics. We believe that thaumatin II-sweetened milk can be used as a food (additive) without prior purification, a procedure similar to pharmaceutical proteins expressed in mammary glands, such as tissue plasminogen activator (Lu et al., 2019) and human C1 inhibitor (van Veen et al., 2012). Therefore, the processing cost and production time are greatly reduced, which greatly facilitates market development. And besides, our experimental results show that boiling does not cause thaumatin II to lose its sweetness, so thaumatin II-sweetened milk can still maintain its sweetness during manufacturing. We believe that thaumatin II-sweetened milk is a safe and healthy food for everyone, and especially for the diabetics.

5. Conclusion

The present study has verified that mammary gland-specific expression vector of thaumatin II functional gene can be expressed in transgenic mice milk, while the recombinant protein retains its biological activity. Recombinant thaumatin expressed in the mammary glands has rarely been reported, and our studies provide an initial step to produce plant protein thaumatin-sweetened milk from large animals such as cow and goat for healthcare applications in future investigations.

Author contribution

R.L., L.J. and Y.C.W. conceived and designed the experiments. R.L. and X.M.L. acquired the data. R.L., L.J. and Y.C.W. contributed materials/analysis tools. R.L. wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

Thanks to the resources and personnel Jiangsu Food and Pharmaceutical Science College for their assistance in this research.

References

Anand, S., Winkelmayer, W.C., 2012. 'Causal or casual?-The association between consumption of artificially sweetened carbonated beverages and vascular disease'. J. Gen. Intern. Med. 27, 1100–1101.

Azad, M.B., Sharma, A.K., de Souza, R.J., Dolinsky, V.W., Becker, A.B., Mandhane, P.J., Turvey, S.E., Subbarao, P., Lefebvre, D.L., Sears, M.R., Investigators Canadian Healthy Infant Longitudinal Development Study, 2016. 'Association between artificially sweetened beverage consumption during pregnancy and infant body mass index'. JAMA Pediatr. 170, 662–670.

Bartoszewski, G., Niedziela, A., Szwacka, M., Niemirowiczlogzczytt, K., 2003. 'MOdification of tomato taste in transgenic plants carrying a thaumatin gene from Thaumatococcus daniellii Benth'. Plant Breed. 122, 347–351.

Breiteneder, H., 2004. 'Thaumatin-like proteins – a new family of pollen and fruit allergens'. Allergy 59, 479–481.

Chen, H.L., Huang, J.Y., Chu, T.W., Tsai, T.C., Hung, C.M., Lin, C.C., Liu, F.C., Wang, L.C., Chen, Y.J., Lin, M.F., Chen, C.M., 2008. 'Expression of VP1 protein in the milk of transgenic mice: a potential oral vaccine protects against enterovirus 71 infection'. Vaccine 26, 2882–2889.

Collaboration, N.C.D. Risk Factor, 2016. 'Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants'. Lancet 387, 1377–1396.

DePeters, E.J., Hovey, R.C., 2009. 'MEthods for collecting milk from mice'. J. Mammary Gland Biol. Neoplasia 14, 397–400.

Dobie, Kenneth W., Muriel, Lee, Fantes, Judith A., 1996. 'Variegated Transgene Expression in Mouse Mammary Gland Is Determined by the Transgene Integration Locus'.

Drouin-Chartier, J.P., Zheng, Y., Li, Y., Malik, V., Pan, A., Bhupathiraju, S.N., Tobias, D.K., Manson, J.E., Willett, W.C., Hu, F.B., 2019. 'Changes in Consumption of Sugary Beverages and Artificially Sweetened Beverages and Subsequent Risk of Type 2 Diabetes: Results From Three Large Prospective U.S. Cohorts of Women and Men'. Diabetes Care 42, 2181–2189.

Edens, L., Heslinga, L., Klok, R., Ledeboer, A.M., Maat, J., Toonen, M.Y., Visser, C., Verrips, C.T., 1982. 'Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in Escherichia coli'. Gene 18, 1–12.

Farrell Jr., H.M., Jimenez-Flores, R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K.F., Swaisgood, H.E., 2004. 'NOmenclature of the proteins of cows' milk–sixth revision'. J. Dairy Sci. 87, 1641–1674.

Faus, I., 2000. 'ReCent developments in the characterization and biotechnological production of sweet-tasting proteins'. Appl. Microbiol. Biotechnol. 53, 145–151.

Gatidou, G., Vazaiou, N., Thomaidis, N.S., Stasinakis, A.S., 2020. 'Biodegradability assessment of food additives using OECD 301F respirometric test'. Chemosphere 241, 125071.

Green, Barry G., Dalton, Pamela, Cowart, Beverly, Shaffer, Greg, Rankin, Krystyna, Higgins, Jennifer, 1996. 'Evaluating the' Labeled magnitude scale' for measuring sensations of taste and smell'. Chem. Senses 21, 323–334.

Haruyama, N., Cho, A., Kulkarni, A.B., 2009. 'Overview: engineering transgenic constructs and mice. Curr. Protoc. Cell Biol., Chapter 19: Unit 19 10.

Hodge, A.M., Bassett, J.K., Milne, R.L., English, D.R., Giles, G.G., 2018. 'Consumption of sugar-sweetened and artificially sweetened soft drinks and risk of obesity-related cancers'. Public Health Nutr. 21, 1618–1626.

Houdebine, L.M., 2018. 'Production of pharmaceutical proteins by transgenic animals'. Rev Sci Tech 37, 131–139.

Iyengar, R.B., Smits, P., van der Ouderaa, F., van der Wel, H., van Brouwershaven, J., Ravestein, P., Richters, G., van Wassenaar, P.D., 1979. 'The complete amino-acid sequence of the sweet protein thaumatin I'. Eur. J. Biochem. 96, 193–204.

Johnson, R.K., Appel, L.J., Brands, M., Howard, B.V., Lefevre, M., Lustig, R.H., Sacks, F., Steffen, L.M., Wylie-Rosett, J., 2009. Physical Activity American Heart Association Nutrition Committee of the Council on Nutrition, Metabolism, Epidemiology the Council on, and Prevention. 2009.' Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association'. Circulation 120, 1011–1020.

Joseph, J.A., Akkermans, S., Nimmegeers, P., Van Impe, J.F.M., 2019. 'Bioproduction of the recombinant sweet protein thaumatin: current state of the art and perspectives'. Front. Microbiol. 10, 695.

Kahn, R., Sievenpiper, J.L., 2014. 'DIetary sugar and body weight: have we reached a crisis in the epidemic of obesity and diabetes?: we have, but the pox on sugar is overwrought and overworked'. Diabetes Care 37, 957–962.

Kaneko, R., Kitabatake, N., 2001a. 'STructure-sweetness relationship in thaumatin: importance of lysine residues'. Chem. Senses 26, 167–177.

Kaneko, R., Kitabatake, N., 2001b. 'Sweetness of sweet protein thaumatin is more thermoresistant under acid conditions than under neutral or alkaline conditions'. Biosci. Biotechnol. Biochem. 65, 409–413.

Kant, R., 2005. 'SWeet proteins–potential replacement for artificial low calorie sweeteners'. Nutr. J. 4, 5.

Kiełkiewicz, Małgorzata, Gajc-Wolska, Janina, Ślusarz, Sylwia, Szwacka, Maria, 2012. 'Growth, development and yield of transgenic 35S-thaumatin II-expressing cucumber plants – open field evaluation'. Sci. Hortic.

Kong, Qingran, Wu, Meiling, Yanjun, Huan, Li, Zhang, Liu, Haiyan, Gerelchimeg, Bou, Luo, Yibo, Mu, Yanshuang, Liu, Zhonghua, Bridger Joanna, Mary, 2009. 'Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs'. PLoS One 4, e6679.

Litwinczuk, Zygmunt, Pastuszka, Robert, Barlowska, Joanna, 2015. 'NUtritional value and health-promoting properties of goat milk'. Med. Weter.

Lu, R., Zhang, T., Wu, D., He, Z., Jiang, L., Zhou, M., Cheng, Y., 2018. 'Production of functional human CuZn-SOD and EC-SOD in bitransgenic cloned goat milk'. Transgenic Res. 27, 343–354.

Lu, Rui, Zhang, Ting, Song, Shaozheng, Zhou, Minya, Cheng, Yong, 2019. 'Accurately cleavable goat beta-lactoglobulin signal peptide efficiently guided translation of a recombinant human plasminogen activator in transgenic rabbit mammary gland'. Biosci. Rep., BSR20190596.

Masuda, T., Tamaki, S., Kaneko, R., Wada, R., Fujita, Y., Mehta, A., Kitabatake, N., 2010. 'Cloning, expression and characterization of recombinant sweet-protein thaumatin II using the methylotrophic yeast Pichia pastoris'. Biotechnol. Bioeng. 85, 761–769.

Mortensen, Alicja, 2006. 'SWeeteners permitted in the European Union: safety aspects'. Scand. J. Food Nutr. 50, 104–116.

Mullie, P., Clarys, P., 2017. 'Consumption of artificially sweetened beverages during pregnancy is associated with a twofold higher risk of infant being overweight at 1 year'. Evid. Nurs. 20, 11.

Pearlman, M., Obert, J., Casey, L., 2017. 'The association between artificial sweeteners and obesity'. Curr. Gastroenterol. Rep. 19, 64.

Robertson, G., Garrick, D., Wu, W., Kearns, M., Martin, D., Whitelaw, E., 1995. 'Position-dependent variegation of globin transgene expression in mice'. Proc Natl Acad Sci U S A 92, 5371–5375.

Schestibratov, K.A., Dolgov, S.V., 2005. 'Transgenic strawberry plants expressing a thaumatin II gene demonstrate enhanced resistance to Botrytis cinerea'. Sci. Hortic. 106, 0–189.

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C.A., Maza, O., Israeli, D., Zmora, N., Gilad, S., Weinberger, A., Kuperman, Y., Harmelin, A., Kolodkin-Gal, I., Shapiro, H., Halpern, Z., Segal, E., Elinav, E., 2014. 'Artificial sweeteners induce glucose intolerance by altering the gut microbiota'. Nature 514, 181–186.

van der Wel, H., Loeve, K., 1972. 'Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from Thaumatococcus daniellii Benth'. Eur. J. Biochem. 31, 221–225.

van Veen, H.A., Koiter, J., Vogelezang, C.J., van Wessel, N., van Dam, T., Velterop, I., van Houdt, K., Kupers, L., Horbach, D., Salaheddine, M., Nuijens, J.H., Mannesse, M.L., 2012. 'Characterization of recombinant human C1 inhibitor secreted in milk of transgenic rabbits'. J. Biotechnol. 162, 319–326.

Wang, Yanli, Zhao, Sihai, Bai, Liang, Fan, Jianglin, Enqi, Liu, 2013. 'Expression systems and species used for transgenic animal bioreactors'. Biomed Res. Int. 2013, 580463.

Yamamoto, M., Terada, Y., Motoyama, T., Saito, T., Ito, K., 2020. 'SWeet proteins lysozyme and thaumatin are protein-type agonists for the calcium-sensing receptor'. Biochem. Biophys. Res. Commun. 521, 227–231.

Yan, S., Song, H., Pang, D., Zou, Q., Li, L., Yan, Q., Fan, N., Zhao, X., Yu, H., Li, Z., Wang, H., Gao, F., Ouyang, H., Lai, L., 2013. 'Expression of plant sweet protein brazzein in the milk of transgenic mice'. PLoS One 8, e76769.

Zemanek, E.C., Wasserman, B.P., 1995. 'Issues and advances in the use of transgenic organisms for the production of thaumatin, the intensely sweet protein from Thaumatococcus danielli'. Crit. Rev. Food Sci. Nutr. 35, 455–466.