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Single step purification of 2S albumin from *Theobroma cacao*

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ABSTRACT

Diabetes is prevalence in Malaysia affecting 21 % of her adults. The occurrence of this metabolic disease is linked to the excessive intake of high calorie sugar. Although non-sugar sweeteners are available, artificial sweeteners pose adverse side effects to human health while natural sweeteners such as sweet proteins suffer low heat stability thus limiting their utilization in food processing. However, sweet protein mabinlin from the plant mabinlang (Capparis masaikai Levl.) is a promising sugar substitute due to its excellence high heat stability and intense sweetness. Nevertheless, its mass production is hampered by the scarcity of the plant. Recently, 2s albumin in cacao (*Theobroma cacao*) showed high similarity to mabinlin and has more potential to be produced industrially because cacao are grown by many countries including Malaysia. However, there is no report regarding cocoa mabinlin characteristics, to date. In this study, we had purify 2s albumin, a protein that is correspond to mabinlin in cacao via Ion Exchange Chromatography (IEX). All peaks were pooled and collected, and then run through SDS- PAGE to confirm their purity. Peak 5 eluted from Q HP column in IEX gave a single protein band at 21 kDa after staining with silver nitrate solution. This study hopefully will lead to development of alternative low calorie sweetener from local resources of Malaysia, thus impact on reducing high sugar consumption related diseases, especially diabetes.

1. Introduction

In recent years, the prevalence of the diseases related to the consumption of sugars, such as obesity, diabetes, hyperglycemia, and caries, has increased dramatically. In Malaysia, the number of diabetes cases in 2014 had exceeded 3 million cases, which is the highest among ASEAN countries. The high sugar intake is believed to closely relate to this issue. In this respect, the high number diabetes case is acceptable since Malaysia is the 8th highest sugar consumers in the world. By the 1990s Malaysians were consuming an average of 24 teaspoons of sugars per day now the figure has jumped to 26 teaspoons. The aforementioned facts suggest reduction of sugar consumption in daily life. Yet, due to culture, sugar free diet is not possible, or else consumption of sweeteners is sometimes unavoidable for food or beverages. Therefore, the best way in addressing this issue is by sugar replacement with low-calorie sweeteners. Despite artificial sweeteners (aspartame, saccharin, neotame, sucralose, and acesulfame potassium) have higher sweetness and lower calorie compared to sugar (Schiffman, 2012), yet these compounds are often linked to some diseases due to their side effects, including psychological problems, mental disorder, bladder cancer and heart failure (Masuda and Kitabatake, 2006).

Some proteins have been known to elicit the sensation of sweetness (Gnanavel and Muthukumar, 2011; Rega et al., 2015; Masuda and Kitabatake, 2006). These proteins, so-called sweet tasting proteins, include thaumatin, monellin, mabinlin, brazzein, egg lysozyme, and neoculin (previously considered as curculin) (Picone and Temussi, 2012) are considered to be promising as low calorie sweeteners for sugar replacement (Masuda and Kitabatake, 2006). Monellin and thaumatin are considerably exhibiting higher sweetness compared to other sweet-tasting proteins, but these proteins are sensitive against heat treatment. This limits application of these proteins in higher temperature, including hot beverages.

Despite no common structural features among sweet tasting proteins, however, it is believed that

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the proteins elicit their sweet taste, in human and other mammals, by interacting with sweet taste receptor (Nelson et al., 2001; Margolskee, 2002; Li et al., 2002; Matano et al., 2015). The sweet receptor consists of a heterodimer of two sequence-related subunits that belong to the class C Gprotein- coupled receptor (GPCR) family: Taste type 1 Receptor 2 (T1R2) and Taste type 1 Receptor 3 (T1R3). On the basis of their sequence similarities to mGluR1, mGluR3, mGluR7, and rhodopsin (Kunishima et al., 2000; Muto et al., 2007; Palczewski et al., 2000) each subunit has been proposed to consist of three main structural domains: the Venus flytrap module (VFTM) containing lobes 1 and 2, which can be in an "open" or a "closed" conformation; the cysteine-rich domain (CRD), which was named for nine conserved cysteine residues in the mGluR subgroup of family C; and the hepta-helical transmembrane domain (TMD) (Pin et al., 2004).

Among all of the sweet tasting proteins, only mabinlin is stored in seeds, and the rest are accumulated in fruits; all of them show lower similarity in amino acid sequence. In term of heat stability, mabinlin is known to be a heat-stable sweet tasting protein (Liu et al., 1993). Mabinlin is firstly found in mature seeds of Capparis masaikai Lvl., which grows in a subtropical region of Yunnan province of China (Liu et al., 1993). At least four isoforms, namely, mabinlin I-1, mabinlin II, mabinlin III, and mabinlin IV, were found in the fruits (Nirasawa et al., 1994; Gu et al., 2015). Among them, mabinlin II is heat-stable and its sweetness is not lost after 48 h of incubation at 80 °C. The amino acid sequence of mabinlin II was determined and found to be a heterodimer composed of the A chain of 33 amino acid residues and the B chain of 72 amino acid residues (Liu et al., 1993).

The heat stability of mabinlin II has been shown due to the presence of disulfide bonds in its structure. A-chain and B-chain of mabinlin II contain two and six cysteine residues, respectively. The formation of two interchain disulfide bridges at Cys(A5)-Cys(B21) and Cys(A18)-Cys(B10), and two intrachain disulfide bridges at Cys(B11)-Cys(B59) and Cys(B23)-Cys(B67) were reported already (Nirasawa et al., 1994). Cleavage of the disulfide bridges destabilizes this protein and result in in complete loss of the sweet activity of mabinlin II (Nirasawa et al., 1994).

Recently, Niemenak et al. (2015) identified a mabinlin-like protein in cacao (*Theobroma cacao*) using proteomic approach. We believed it is a cacao 2S albumin protein, since it was reported having high similarity and identity with mabinlin from mabinlang (Kochhar et al., 2001). This protein is highly expressed in zygotic embryos, which might be due to its physiological function as energy storage. Given the cacao industry in Malaysia grew to become the third major commodity crop, mabinlin-like protein from cacao is promising to be further developed as sugar replacement. Currently, cacao is used mainly in chocolate based products, which been monopoly by giant company like Nestle. This reduced the marketability of products from small scale plantation and makes them difficult to compete in larger marketplace. Upon successful finding of sweet protein from cacao perhaps will give them the opportunity to divert the usage of their cacao. However, further development of this protein from cacao is limited by the lack of study on its sweetness, thermal stability and the mechanism by which it elicit sweetness response and, if any, thermal stability.

So far, there is report on whether this protein belongs to mabinlin II variant or the others. According to Moreno and Clemente (2008), most of the 2S albumins are considered major allergens in walnut, Brazil nut, oilseed rape, castor bean, and mustard seed. They are water-soluble seed storage proteins present in dicotyledonous plants, including legumes. They share a characteristic four helix, four disulfide bridges structure (Monsalve et al., 2004) with cereal α-amylase/trypsin inhibitors and nonspecific lipid transfer proteins (nsLTP) and are typically heterodimers, composed of a large and small subunit joined by one or two disulfide bridges. These two subunits result from post-translational processing of the precursor protein at several sites, close to the N-terminus, internally, and at the C-terminus (D'Hond et al., 1993). Besides, several 2S albumins show antimicrobial and antifungal properties (Agizzio et al., 2003).

Hence, we conducted this study in order to understand the 2S albumin from cacao, whether or not it having these kinds of properties, and exhibit sweetness as its homologue from mabinlang.

Furthermore, the molecular mechanism of sweetness is due to interaction of sweet tasting proteins and sweet receptors T1R2/T1R3 that are predicted to be unique for each sweet tasting protein. However, the mechanism by which mabinlin interacts to T1R2/T1R3 remains to be further studied. Post-translational modifications (PTM) are also predicted to play important role in the sweetness. Yet, the presence and type of PTM of cacao mabinlin need to be comprehensively studied.

The crucial step before characterize the properties of cacao mabinlin is purification of the protein. Various methods were commonly used to obtain purified protein including precipitation, gel size exclusion and ion exchange chromatography. However, further development of this protein from cacao is limited by the lack of study on its sweetness, thermal stability and the mechanism by which it elicit sweetness response and, if any, thermal stability.

In this study, we reported a single purification step via Ion Exchange Chromatography (IEX) of 2S albumin, a protein that is corresponding to mabinlin in cacao. Our objective is to obtain a novel sweet protein from cacao as a promising sugar replacer.

2. Methodology

The pods of T. cacao were collected from "Pusat Penyelidikan Bioteknologi, Lembaga Koko Malaysia,

Sabah". The pulp of 200 g seeds was removed completely before ground with 20 mM phosphate buffer, pH 7.8 (containing 100 mM NaCl and 1 mM DTT) with a ratio of 1:10 seeds to buffer at 4°C. The mixture then filtered with 4 layer of cheese cloth, thrice. After 3 times ultracentrifugation (35,000 rpm at 4° C, 30 minutes), the supernatant was concentrated by freeze dry and kept in -80°C.

2.1. Purification of 2S Albumin

The dried cacao powders were re-suspended in 50 mL of glycine buffer (50 mM, pH 9) before dialysis for 16 hours. The solution was subsequently passed through a sterile 0.22 µm filter and stored in 4° C. Chromatographic steps were performed at room temperature using Akta Prime Purification System. The filtered cacao extract was applied to a Q HP column (5 mL) equilibrated with 50 mM glycine/ NaOH, pH 9 at flow rate 1 mL / min. The proteins adsorbed on the column were eluted with a linear gradient of NaCl (0 – 1.0 M). Fractions for each peaks were pooled and collected. Pure 25µg of 2s albumin was obtained from 200 g of cacao seeds.

2.2. SDS- PAGE electrophoresis

Protein samples were denatured with two times SDS sample buffer (1M Tris HCl pH 6.8, 10 % (w/v) SDS, 80 % glycerol, 0.5 M EDTA, 1 % BPB, 2-mercaptoethanol) coupled with boiled at 95°C for 5 minutes. Protein separation was made on 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) based on Laemmli method (1970) method. The separation gel (30 % acrylamide solution, 1.5 M Tris HCl (pH 8.8), 10 % SDS solution, deionized water, 10 % APS (w/v), TEMED) were prepared first and left approximately 45 minutes before stacking gel (30 % acrylamide solution, 0.5 M Tris HCl (pH 6.8), 10% SDS solution, deionized water, 10 % APS (w/v), TEMED) were added and left for another 45 minutes. Then, 20 µL protein samples for each peak were load in the well and the gels were run at 150 V for 1 hour.

2.3. Gel visualization

Proteins had been visualized using Silver Nitrate Staining kit protocol. The gels were placed in the 250 mL fixing solution (100 mL ethanol (95%), 25 mL acetic acid glacial, MiliQ) for minimum 30 minutes with gentle shaking. Then, the gels were sensitized with 250 mL sodium thiosulfate solution (75 mL ethanol (95 %), 1.25 mL Glutardialdehyde (25% w/v), 10 mL sodium thiosulphate (5 % w/v), 1 packet of sodium acetate (17g) and mili Q) for another 30 minutes. The sensitizer solution was discarded and the gels were rinsed with miliQ for 20 seconds, thrice. 250 mL silver nitrate solution (25 mL silver nitrate (2.5 % w/v), 0.1 mL formaldehyde (37 % w/v) and miliQ) was added and the gels were gently agitated for 20 minutes. The container was covered with aluminum foils to protect from bright light. The silver nitrate solution was then discarded and the gels were rinse with miliQ for 20 seconds thrice before 250 mL developing solution (1 packet sodium carbonate (6.25g), 0.05 mL formaldehyde (37 %) and miliQ) was added. The gels were developed until protein spots were clearly visible. As soon as an adequate degree of staining had been achieved, the developer solution was discarded and the gels were rinsed with miliQ and then immersed with 250 mL stop solution (1 packet EDTA-Na_2●$2H_2O$ (3.65 g) and miliQ) for 10 minutes. Lastly, the gels were washed with miliQ before proceed with image digitalization by using the "Image Lab Bio-rad" software.

3. Results and discussion

Although most of 2S albumins have been identified as major allergens in many allergenic nuts and seeds (Pastorello et al., 2002), there is no report regarding its properties in cacao. As suggested by Liu et al. (1993) mabinlin is a storage protein that having sweetness activity. This was supported by their finding of the mabinlin homologues in the seeds of mabinlang.

In this study, we focus on purify 2S albumin from cacao since it was reported to have high similarity (55 %) and identity (43%) with mabinlin (Kochhar et al., 2001). According to Niemenak et al. (2015), isoelectric point (pI) for cacao mabinlin is 5.9. Thus, Q HP column was use in this study due to its strong anion binding capacity, which remains charged and have high loading capacities over broad pH ranges, as claimed by the manufacturer. The elution pattern is shown in Fig. 1.

After the purification process, Q HP column yielded several peaks, which we believe represented major seed proteins such as vicilin, since it was up-regulated in cacao zygotic embryos during maturation (Niemenak et al., 2015). Besides, cacao seeds are known to contain two prominent storage proteins (Voigt et al., 1994): an albumin and a globulin fraction (Kratzer et al., 2009).

Voigt et al. (1994) also suggested proteins constitute 10-15 % dry weight of cocoa seeds, the second most abundant constituent after cocoa fat. They had employing SDS-PAGE showed four predominant proteins represent 95 % (w/w) suggested that the total seed protein content is composed of 52 and 43 % of albumin and globulin fractions, respectively (Voigt et al., 1994).

When comparing many purification of 2S albumin from various plants, most of them involved at least two steps of purification processes. For example 2S albumin from peanut (Arachis hypogaea L.), size exclusion chromatography coupled with ion exchange chromatography was used (Duan et al., 2013). While in hazelnut, combination of size exclusion and RP- HPLC chromatography were used (Garino et al., 2010).

However in this study, we used only single step purification via ion exchange chromatography, after

omitting ammonium sulfate precipitation. This is to maintain native state of the proteins as well as to expedite the purification process. Plus, risk of protein denaturation also reduced. Ion exchange chromatography method provides the concomitant purification of the target protein and the small particle size (34µm) of the column allows fast adsorption and desorption even at high sample loadings and flow rates. Besides, the column can be reused, which decreases the cost of materials. Hence, it is the most suitable method for fast protein purification.

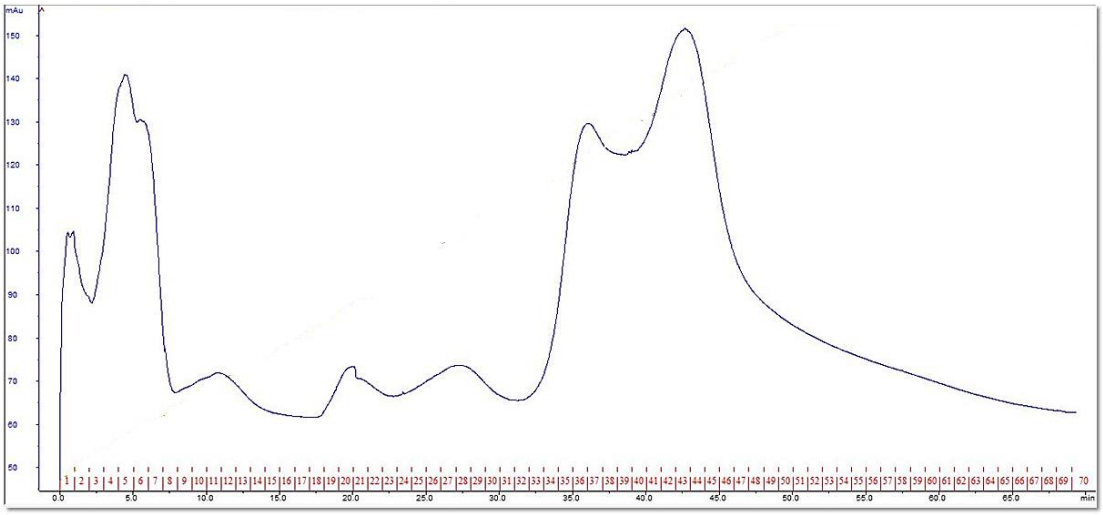

Fig. 1: Protein elution profile on Ion Exchange Chromatography (IEX) in Q HP column. 5 g sample was re-suspended in 50 mM glycine/ NaOH, pH 9.0 and eluted with linear gradient of NaCl (0- 1.0 M)

The purity of the proteins eluted was confirmed via SDS-PAGE, stained with silver staining protocol. This is to utilize the protein binding properties of silver ions, which are then reduced to silver metal using a developing solution, creating a visible image. The primary benefit of silver staining is high sensitivity, as it is able to detect less than 1 ng of protein (Weiss et al., 2009), making it extremely useful for applications involving low protein levels.

From the results, we can see multiple protein bands present before purification (Fig. 2) were removed after undergo ion exchange chromatography (Fig. 3). A single protein band was obtained in lane (5), showed a highly purified protein. However, three protein bands were obtained in lane (6), most probably due to concentrated mixture of cacao proteins that having same pI. In lane (6) and (7), the band not entirely pure since there are very faint bands appeared, might be some proteins trapped during purification process. Whereas for lane (F.T) until lane (4), no band were present, may be because no or very small concentration of protein was eluted.

Based on the molecular weight of the protein bands, we suggest that protein at 47 kDa, 31 kDa and 15 kDa are most probably subunits of vicilin-like globulin in cacao (Niemenak et al., 2015). Whereas protein at 21 kDa was concluded to be 2S albumin, a protein that is correspond to mabinlin in cacao referring to Kochhar et al. (2000).

In addition, electrophoretic patterns of total cocoa proteins showed that there are a number of other highly expressed proteins in cocoa seed (Lerceteau et al., 1999). It is quite possible that one or more of these other relatively abundant cocoa seed play vital role in cacao flavor, as well as other function which remains to be identified.

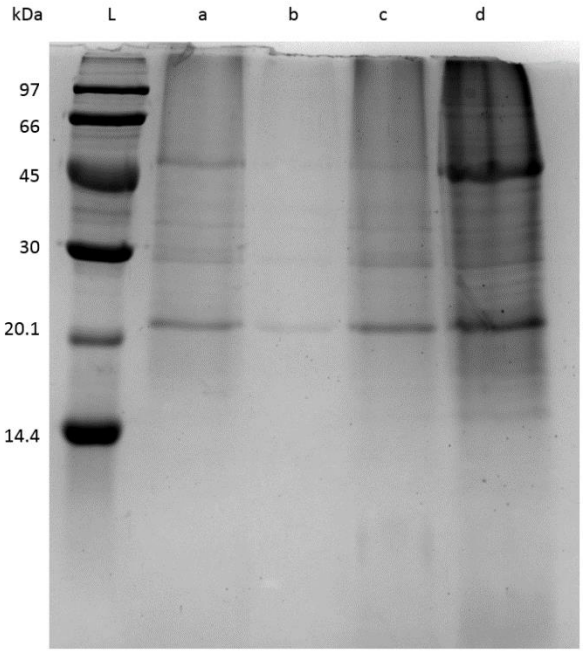

Fig. 2: SDS-PAGE showing protein bands after seed extraction with phosphate buffer, pH 7.8 containing 100mM NaCl and 1mM DTT. Lane (L) indicates protein marker; (a) supernatant; lane (b) diluted supernatant; (c) diluted pellet and (d) pellet

In later paper, we will describe in details on structures and activities of cacao 2S albumin obtained in this study including sweetness, thermal stability and post- translational involved.

4. Conclusion

Single successive chromatography steps, anion exchange resulted in apparent homogeneity of the

protein preparation as judged by SDS-PAGE followed by Silver Nitrate staining protocol. It can be concluded that a single native cacao 2S albumin, of molecular weight 21 kDa has been produced and purified in this study.

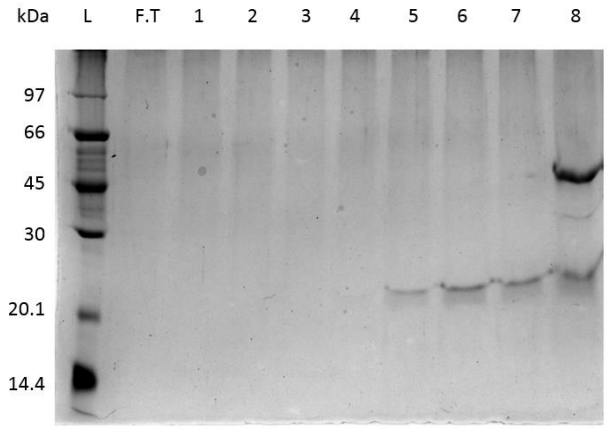

Fig. 3: SDS-PAGE showing purity of eluted protein from IEX: Lane (L) indicate protein marker; lane (F.T) flow through; lane (1) peak 1; lane (2) peak 2; lane (3) peak 3; lane (4) peak 4; lane (5) peak 5; lane (6) peak 6, lane (7) peak 7 and lane (8) peak 8. Band in red box indicate 2S albumin protein that is correspond to mabinlin in cacao

References

Agizzio AP, Carvalho AO, Suzanna de Fátima FR, Machado OL, Alves EW, Okorokov LA, Samarão SS, Bloch C, Prates MV, and Gomes VM (2003). A 2S albumin-homologous protein from passion fruit seeds inhibits the fungal growth and acidification of the medium by Fusarium oxysporum. Archives of Biochemistry and Biophysics, 416(2): 188-195.

D'Hondt K, Van Damme J, Van Den Bossche C, Leejeerajumnean S, De Rycke R, Derksen J, Vandekerckhove J, and Krebbers E (1993). Studies of the role of the propeptides of the Arabidopsis thaliana 2S albumin. Plant Physiology, 102(2): 425-433.

Garino C, Zuidmeer L, Marsh L, Lovegrove A, Morati M, Versteeg S, Schilte P, Shewry P, Arlorio M, and van Ree R (2010). Isolation, cloning, and characterization of the 2S albumin: A new allergen from hazelnut. Molecular Nutrition and Food Research, 54(9): 1257-1265.

Gnanavel M and Muthukumar SP (2011). Identification of novel sweet protein for nutritional applications. Bioinformation, 7(3): 112-114.

Gu W, Xia Q, Yao J, Fu S, Guo J, and Hu X (2015). Recombinant expressions of sweet plant protein mabinlin II in Escherichia coli and food-grade Lactococcus lactis. World Journal of Microbiology and Biotechnology, 31(4): 557-567.

Kochhar S, Gartenmann K, and Juillerat MA (2000). Primary structure of the abundant seed albumin of Theobroma cacao by mass spectrometry. Journal of Agricultural and Food Chemistry, 48(11): 5593-5599.

Kochhar S, Gartenmann K, Guilloteau M, and McCarthy J (2001). Isolation and characterization of 2S cocoa seed albumin storage polypeptide and the corresponding cDNA. Journal of Agricultural and Food Chemistry, 49(9): 4470-4477.

Kratzer U, Frank R, Kalbacher H, Biehl B, Wöstemeyer J, and Voigt J (2009). Subunit structure of the vicilin-like globular storage protein of cocoa seeds and the origin of cocoa- and chocolate-specific aroma precursors. Food Chemistry, 113: 903 -913.

Kunishima JN, Shimada Y, Tsuji Y, Sato Y, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, and Morikawa K (2000). Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature, 407(6807): 971-977.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Lerceteau E, Rogers J, Pétiard V, and Crouzillat D (1999). Evolution of cacao bean proteins during fermentation: a study by two-dimensional electrophoresis. Journal of the Science of Food and Agriculture, 79(4): 619-625.

Li X, Staszewski L, Xu H, Durick K, Zoller M, and Adler E (2002). Human receptors for sweet and umami taste. Proceedings of the National Academy of Sciences, 99(7): 4692-4696.

Liu X, Hu Z, Maeda S, Aiuchi T, Nakaya K, and Kurihara Y (1993). Purification, complete amino acid sequence and structural characterization of the heat-stable sweet protein, mabinlin II. European Journal of Biochemistry, 211(1-2): 281-287.

Margolskee RF (2002). Molecular mechanisms of bitter and sweet taste transduction. Journal of Biological Chemistry, 277(1): 1-4.

Masuda T and Kitabatake N (2006). Developments in biotechnological production of sweet proteins. Journal of Bioscience and Bioengineering, 102(5): 375-389.

Matano M, Nakajima K, Kashiwagi Y, Udaka S, and Maehashi (2015). Sweetness characterization of recombinant human lysozyme. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 188: 8-14.

Monsalve RI, Villalba M, Rico M, Shewry PR, and Rodríguez R (2004). Plant Food Allergens. Blackwell Publishing Ltd, Hoboken, USA.

Moreno FJ and Clemente A (2008). 2S albumin storage proteins: what makes them food allergens?. The Open Biochemistry Journal, 2(1): 16-28.

Muto T, Tsuchiya D, Morikawa K, and Jingami H (2007). Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. Proceedings of the National Academy of Sciences, 104(10): 3759–3764.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Rhyba NJ, and Zuker CS (2001). Mammalian sweet taste receptors. Cell, 106(3): 381-390.

Niemenak N, Kaiser E, Maximova SN, Laremore T, and Guiltinan MJ (2015). Proteome analysis during pod, zygotic and somatic embryo maturation of Theobroma cacao. Journal of Plant Physiology, 180: 49-60.

Nirasawa S, Nishino T, Katahira M, Uesugi S, Hu Z, and Kurihara Y (1994). Structures of heat-stable and unstable homologues of the sweet protein mabinlin. The FEBS Journal, 223(3): 989-995.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, and Yamamoto M (2000). Crystal structure of rhodopsin: AG protein-coupled receptor. Science, 289(5480):739-745.

Picone D and Temussi PA (2012). Dissimilar sweet proteins from plants: Oddities or normal components?. Plant Science, 195: 135-142.

Pin JP, Kniazeff J, Goudet C, Bessis AS, Liu J, Galvez T, Acher F, Ronard P, and Prezeu L (2004). The activation mechanism of class-CG-protein coupled receptors. Biology of the Cell, 96(5): 335-342.

Rega MF, Di Monaco R, Leone S, Donnarumma F, Spadaccini R, Cavella S, and Picone D (2015). Design of sweet protein based sweeteners: hints from structure–function relationships. Food Chemistry, 173: 1179-86.

Schiffman SS (2012). Rationale for further medical and health research on high-potency sweeteners. Chemical Senses, 37(8): 671-679.