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# Sweet Plant Proteins and Their Recombinant Production

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**Abstract:** There is a growing interest and increasing awareness of consumers towards natural food products, therefore there is a shift in food industry to produce foods with natural ingredients. On the other hand, high amount of sweetener use in food industry is another health concern. An interesting group of natural sweeteners are sweet proteins, which have hundreds/thousands times higher sweetness than sucrose. Sweet proteins have high sweetness but low calorie values and are of interest as they can be used as healthy alternatives to natural or artificial sweeteners. Known plant sweet proteins are produced by tropical plants and this limits the amount of protein that can be obtained. In order to increase the amount of protein, many studies have been carried out on the recombinant production of plant sweet proteins using different expression systems. In this article, sources, types, physicochemical and structural properties of sweet plant proteins and studies on their recombinant production are reviewed with insights to future studies.



# Tatlı Bitkisel Proteinler ve Rekombinant Üretimleri

**Anahtar Kelimeler**
Doğal tatlandırıcılar, Rekombinant üretim, Tatlı bitkisel proteinler

**Öz:** Tüketicilerin doğal gıda ürünlerine karşı olan ilgisi ve artan bilinçleri nedeniyle, gıda endüstrisi doğal içeriklere sahip gıdalar üretmeye yönelmiştir. Öte yandan gıda endüstrisinde yüksek miktarda tatlandırıcı kullanımı da bir diğer sağlık sorunudur. Tatlı proteinler, sakkarozdan yüzlerce/binlerce kat daha fazla tatlılığa sahip ilgi çekici doğal tatlandırıcılardır. Tatlı proteinler, yüksek tatlılığa ancak düşük kalori değerlerine sahiptir ve doğal veya yapay tatlandırıcılara sağlıklı alternatifler olarak kullanım potansiyelleri yüksektir. Bilinen bitki tatlı proteinleri tropik bitkiler tarafından üretilir ve bu, elde edilebilecek protein miktarını sınırlar. Protein miktarını arttırmak için farklı ekspresyon sistemleri kullanılarak bitkisel tatlı proteinlerin rekombinant üretimi üzerine birçok çalışma yapılmıştır. Bu makalede, tatlı bitki proteinlerinin kaynakları, türleri, fizikokimyasal ve yapısal özellikleri ve rekombinant üretimi ile ilgili çalışmalar derlenmiş ve yeni yapılabilecek çalışmalar üzerinde durulmuştur.

## 1. INTRODUCTION

Nowadays, the importance of nutrition and a balanced diet for protection of public health is increasing. In recent years, there has been a growing interest in reducing the sugar content in food products by both consumers and producers with the increase in health problems caused by high amounts of sugar consumption. Attempts to reduce the sugar content in food products led the food industry to use artificial sweeteners however, there are still discussions about the negative health effects of those; including toxic and carcinogenic effects of aspartame and acesulfame K [1], [2]. Although some synthetic sweeteners derived from cyclamic acid and cyclamate are still allowed in USA, but not in EU due to consumer demands. Market research results in developed countries show that many consumers prefer

natural foods [3]. Following these, studies have focused on finding alternatives obtained from natural sources such as sweeteners from plants [4] and sweet plant proteins, which were discovered many years ago have gained importance again. These sweet plant proteins with naturally sweet or taste-modifying properties are seen as natural and healthy alternatives to existing synthetic low-calorie sweeteners.

To date, six sweet plant proteins have been identified all from tropical plants; brazzein, curculin (neoculin), mabinlin, miraculin, monellin and thaumatin [5]. The main advantage of these proteins over carbohydrate based sweeteners is that, they have extremely high sweetness index with an insignificant amount of calories. Several research studies showed that sweet plant proteins do not have allergic or toxic effects [6].In this article, the physicochemical and structural properties of sweet plant proteins, their interactions with the human taste receptor, their current production strategies with an emphasis on recombinant production are reviewed.

### 1.1. Sweet Plant Proteins

All known sweet plant proteins have been discovered from tropical plants (Figure 1); mabinlin from China, curculin from Malaysia, and other sweet plant proteins are isolated from fruits of plants growing in the rainforests of Africa [5]. The comparison of amino acid sequences of these proteins show that there is no considerable sequence similarity [7]; these proteins have different number of amino acid sequences with almost no homology. The three-dimensional atomic structures of all sweet plant proteins were solved by X -ray crystallography or NMR and it was revealed that there are also no structural similarities among sweet plant proteins. In addition the level of sweetness of sweet plant proteins are also different from each other (Table 1).

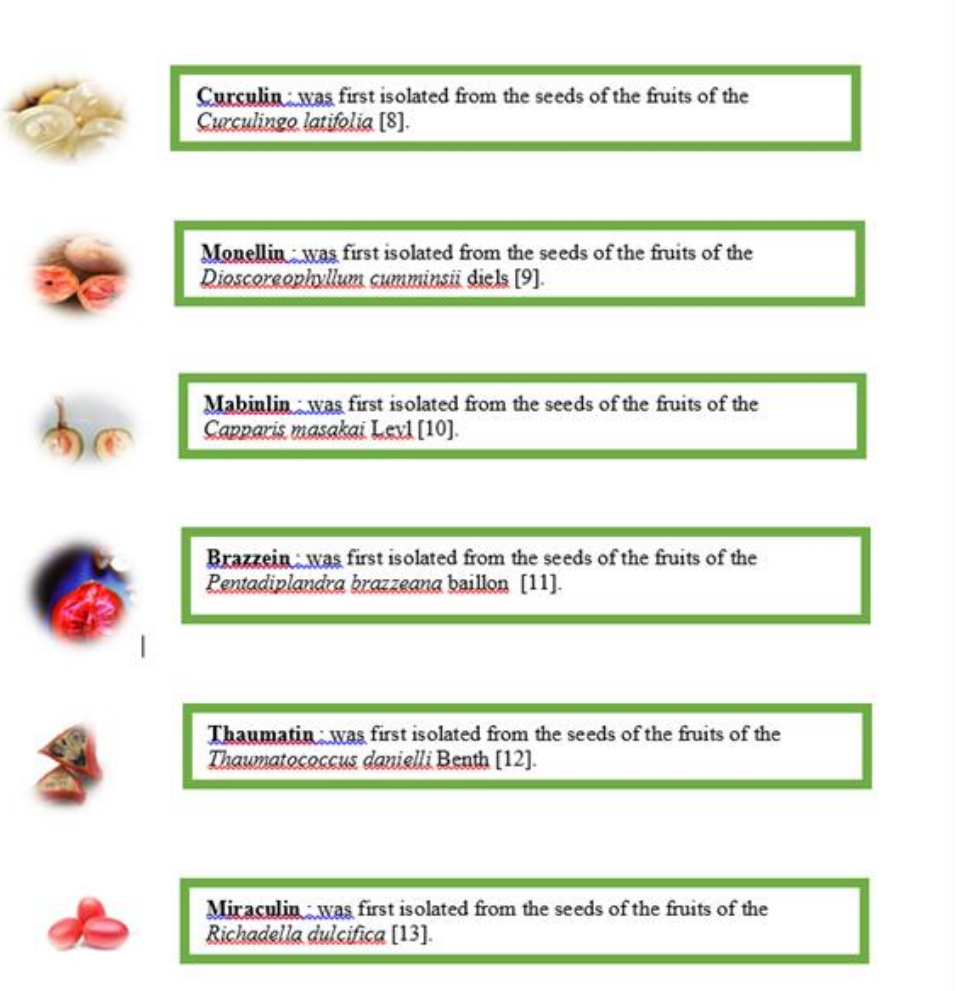


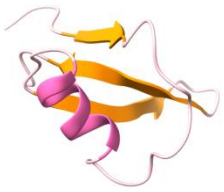
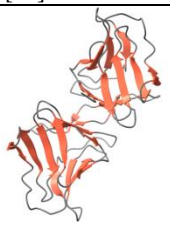
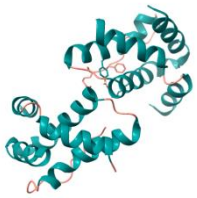
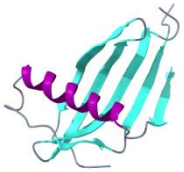
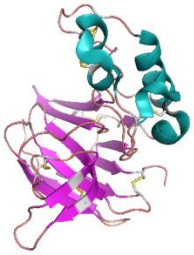

**Figure 1.** Plant sources of sweet proteins and initial isolation studies[8]–[13]

### 1.1. 1. Thaumatin

The sweet protein thaumatin, obtained from the *Thaumatococcus danielli Benth* fruit is the first approved and commercialized sweet plant protein. Thaumatin is metabolized similarly to other proteins, this protein is suitable for use by patients with diabetes due to its very low caloric value. Several studies showed that thaumatin does not have any toxic, allergic or other harmful effects in the amounts used as sweetener [14].

Thaumatin has been approved for use as a food additive (E957) by European Union since 1984 [21]. The use of thaumatin for modification and enhancing of flavors has

**Table 1.** Structural features of sweet proteins

| Protein | Source | Comparison of sucrose with sweetness on a molar basis [15] | Molecular weight (kDa) and amino acid content | Three-dimensional structure and PDB code |
|---|---|---|---|---|
| **Brazzein** | *Pentadiplvera brazzeana* | 17,000 | 6.5 kDa<br>54 aa |  <br>2BRZ[16] |
| **Curculin** | *Curculigo latifolia* | - | 24,9 kDa<br>114 aa |  <br>2DPF[17] |
| **Mabinlin II** | *Capparis masaikai* | ~400 | 12.4 kDa<br>105 aa |  <br>2DS2[18] |
| **Monellin** | *Dioscoreophyllum cumminsii* | 100,000 | 10.7 kDa<br>94 aa |  <br>3MON[19] |
| **Thaumatin** | *Thaumatococcus daniellii* | 100,000 | 22.2 kDa<br>207 aa |  <br>3WOU [20] |



also been approved by the Food and Drug Administration (FDA) (FEMA GRAS Number 3732) and is marketed in the USA under the trade name Talin. Thaumatin is used as a sweetener and flavor enhancer in candies, chewing gums, soft drinks, dairy products and ice cream in the food industry in Europe and Japan [7], [21]. Furthermore, thaumatin is used as a sweetener in toothpastes and to suppress the bitter taste of some drugs and food supplements. Although thaumatin has a very high sweetness level, its taste is different and more dominant than sucrose and fructose, so it leaves an undesirable sweetness effect in the mouth. For this reason, it is usually used in combination with other sweeteners [14]. Thaumatin has two isoforms; I and II, and commercial thaumatin extracted from the plant consists of a mixture of these two isoforms [14]. Thaumatin isoforms differ only at four amino acid positions, and their three-dimensional structures are almost identical. There are 16 cysteine amino acids in the thaumatin amino acid sequence, resulting in eight intramolecular disulfide bridges. The high number of disulfide bridges give thaumatin high thermal stability [22]. Thaumatin has been shown to be resistant to pasteurization, cooking, and other similar high-temperature processes when pH is 5.5 and lower [23].

However, at neutral pH and above, thaumatin loses its stability and sweetness [14], due to its basic nature with an isoelectric point of around 7.5.

### 1.1. 2. Brazzein

Brazzein is obtained from fruits of *Pentadiplandra brazzeana Baillo* and is the smallest of sweet plant proteins. Brazzein has a high degree of heat stability due to presence of four disulfide bridges in its structure [11], [24]. Brazzein has high potential in different food applications as it is water-soluble and retains its sweetness in a wide pH range [25]. Brazzein is ready to be commercialized as a sugar replacer and awaits approval from FDA [3].

### 1.1. 3. Monellin

Monellin, obtained from the fruit of *Dioscoreophyllum cumminsii diels*, consists of two polypeptide chains. These polypeptide chains fold into two different domains, one completely helical and one formed by beta sheets (Table 1). These two domains are held together by noncovalent bonds, therefore the structure of monellin is highly unstable. Monellin denatures at temperatures above 50°C, and loses its sweetness completely[15].

### 1.1. 4. Mabinlin

The sweet plant protein mabinlin, obtained from the fruit *of Capparis masakai Levl*, consists of two polypeptides, similar to monellin. However, unlike monellin, the mabinlin structure has completely alpha helix folding. In addition, strong disulfide bonds hold the two chains together, and therefore mabinlin has high thermal stability. There are four types of mabinlin isoforms in nature; I, II, III and IV. Among them, the sweetness of mabinlin II has been shown to be around 400 times higher than sucrose [26]. It has been reported in studies that the sweetness of mabinlin-III and -IV did not change after 1 hour at 80 °C, and mabinlin-II did not change even after 48 hours of incubation at the boiling point [2], [11]. Mabinlin does not yet have legal status with the European Food Safety or FDA and is not commercially used as a sweetener in food production**.**

### 1.1.5. Curculin and Miraculin

Curculin, isolated from the fruit of *Curculingo latifolia*, is not directly sweet, but at acid pH values have the ability to transform sour flavors into sweet ones [5]. Curculin's sweetness is almost equal to sucrose on a molar basis and is not durable to temperatures above 50°C [27]. There are two isoforms of curculin, and these two isoforms come together to form a heterodimeric structure, called neoculin. While neoculin has very little sweetness at neutral pH values, its sweetness effect increases at acidic pH values and becomes a sweet protein. Therefore, neoculin has important potential use, especially in fruit juices and nectars with low pH and soft drinks.

Miraculin, a sweet plant protein derived from the fruit of *Richadella dulcifica*, is a taste modifying protein, similar to curculin, that can change the sour taste to sweet taste in acidic pH environments. The structure of the miraculin protein is not yet resolved [28].

## 1.2. Interactions of Sweet Proteins with The Taste Receptor T1R2/T1R3

Humans can detect five basic tastes: sweet, salty, umami, bitter (bitter) and sour. Taste processing takes place first at the level of taste receptor cells. There are four types of taste receptor cells, including the immature Type IV. Mature cells Type I, Type II, Type III; detect salty, sweet-umami-bitter taste and sour taste; respectively[29]. Class C G protein receptors (GPCR) in type II taste cells initiate the molecular pathway that enables the detection of sweet, umami and bitter taste. Sweet taste receptors have been shown to exist in heterodimeric form and the receptor has been identified as T1R2 / T1R3 [30].

When the T1R2/T1R3 receptor binds to the sugar molecule, taste is perceived with the initiation of signal transduction within the cell, and sweet plant proteins create the same effect when they bind to the receptor [5]. In studies examining the three-dimensional structures of known sweet proteins, it has been observed that all of them, except mabinlin, contain beta sheets (Table 1). However, a common "sweet" structural region could not be identified in [15]. For this reason, studies are still ongoing to understand how exactly sweet proteins bind to the receptor in order to mimic sugar molecules at the molecular level [5].



## 1.3. Recombinant Sweet Plant Proteins Production Studies

The importance of using sweet plants proteins instead of carbohydrate or artificial sweeteners in designing healthy foods is clear. However, due to the fact that these proteins are produced by tropical plants, access to these plants is limited, and the amount of protein obtained from the plant depends on the plant quality[14]. Therefore alternative methods have been investigated and attempts have been made to produce sweet plant proteins using various transgenic plants. Recombinant protein production levels have been observed to be quite low from genetically modified plants [15]. Although studies on recombinant production with transgenic plants or animals have increased in recent years [31]–[34], these approaches are still controversial in terms of economics, sustainability and ethical terms. Numerous studies have been conducted on recombinant production of sweet plant proteins from microorganisms [14].

**Table 2.** Sweet plant proteins produced recombinantly from different expression systems

| Protein | Microorganism | Protein production efficiency | Reference |
|---|---|---|---|
| Brazzein | *Picha pastoris* | 44-345 mg/L | [36] |
| Brazzein | *Kluyveromyces lactis* | 238.9 g/L | [37] |
| Brazzein | *Kluyveromyces lactis* | 170 mg/L | [38] |
| Brazzein | *Bacillus licheniformis* | 5mg/L | [39] |
| Brazzein | *Kluyveromyces lactis* | 107 mg/L | [40] |
| Brazzein | *Escherichia coliBL21(DE3)*<br>*Escherichia coliSHuffle T7* | 1.8 - 2.3 mg/L<br>7.2 - 8.4 mg/L | [41] |
| Brazzein | *Kluyveromyces lactis* | 30-100 mg/L | [42] |
| Brazzein | *Lactococcus lactis* | 1,20-1,65- mg/L | [43]–[45] |
| Brazzein | *Lactobacillus spp* | N.D | [46] |
| Brazzein | *Pichia pastoris* | 90 mg/L | [47] |
| Mabinlin II | *Lactococcus lactis* | 32,5-59,1 mg/L | [48] |
| Monellin | *Escherichia coli* | 500 mg/L | [49] |
| Monellin | *Pichia pastoris* | 262~271 mg/L | [50] |
| Monellin | *Lactococus lactis* | 0,40 mg /L | [51] |
| Monellin | *Pichia pastoris* | 150 mg/L | [52] |
| Monellin | *Saccharomyces cerevisiae* | 675 mg/L | [53]. |
| Monellin | *Saccharomyces cerevisiae* | 410 mg/L | [54]. |
| Thaumatin | *Picha pastoris* | 5,6 mg/L | [55] |
| Thaumatin | *Pichia pastoris* | 0.129- 0.399 mg/L | [56] |
| Thaumatin | *Aspergillus awamori* | 5-25 mg/L | [57] |
| Thaumatin | *Picha pastoris* | 100 mg/L | [58] |
| Thaumatin | *Escherichia coli* | 40 mg/L | [59] |

Although progress has been made in the production and purification of thaumatin, problems have been encountered in adequate production of other proteins in bacteria, yeast and mold cells [35]. Almost 50 years have passed since the discovery of these proteins, yet optimization studies for recombinant production are still ongoing.

The selection of the expression system in the production of recombinant proteins is very important in terms of protein quality, functionality, productivity and yield. Studies have been carried out on the recombinant production of sweet plant proteins using different microorganisms and expression systems and these are summarized in Table 2.

In studies using *Escherichia coli*, one of the organisms most used in recombinant protein production, it has been observed that sweet plant proteins are generally produced in small amounts, similarly, the efficiency of protein production with *Lactococcus* species is low. Higher recombinant protein amounts are achieved using yeast expression systems.

A great deal of research has been done on the recombinant production and engineering of sweet plant proteins. Among these proteins, thaumatin is the most studied protein, whose sweetness and heat stability have been improved by different methods [2], [14]. In most of the studies conducted before 2000 on recombinant thaumatin [60]–[64]protein yield was reported to be low. In a study conducted in 2000, protein production efficiency was increased by optimizing the gene encoding thaumatin II according to *E.coli* codon usage.

In this study, the produced recombinant thaumatin protein was indistinguishable from natural thaumatin in terms of biochemical, spectroscopic and organoleptic properties [59].Very successful results have been obtained from *Pichia pastoris* expression system for the recombinant production of thaumatin. It has been shown that protein production efficiency is increased by cloning with the extracellular secretion signal naturally found in thaumatin protein sequence and transferring three gene copies instead of one [58]. In another study, protein disulfide isomerase enzyme was also cloned together with thaumatin and it was shown that the production efficiency of recombinant thaumatin increased in the presence of this chaperone [55].

Due to the difficulties and limitations of obtaining brazzein protein from its natural source, numerous attempts have also been made to produce brazzein from microorganisms. Studies on brazzein expression in *E. coli* showed that recombinant brazzein is localized in the insoluble fraction and requires denaturing conditions for purification [7], [65]. In subsequent studies, recombinant brazzein gene was synthesized by optimizing *Bacillus subtilis* codon preference, and recombinant protein was successfully produced from *E. coli* and *Bacillus licheniformis* cells with this synthetic gene, and both recombinant proteins were shown to have sweetness properties. A purification procedure was established for recombinant brazzein produced by *B. licheniformis*, and approximately 5 mg/L brazzein of high purity was obtained [39]. In another, in order to optimize the expression of brazzein protein from *E. coli*, codon-optimized gene was cloned into two different strains and results showed that the strain used had an effect on protein yield [41]. Use of different lactic acid bacteria were also investigated for expression of recombinant

brazzein, but low amounts of recombinant protein were produced[43], [46], [66].

Studies using *Pichia pastoris* show that recombinant brazzein is obtained in active form with a yield of approximately 30−90 mg/L [67]. In a study examining the extracellular secretion of recombinant brazzein from *P. pastoris*, brazzein was cloned with seven different signal peptides and three of these tested signal peptides increased the protein production efficiency. These signal peptides have been reported to be natural signal peptides of chicken lysozyme, *Aspergillus niger* alpha-amylase and Saccharomyces cerevisiae alpha-mating factor proteins [36]. In the study with *Kluyveromyces lactis*, which is used as a different yeast expression system, 107 mg/L of recombinant brazzein was obtained [40]. In another study using *K. lactis*, the effect of co-expression of chaperones involved in the formation of disulfide bonds with brazzein was examined and recombinant brazzein was produced in the range of 30-100 mg / L [42].

In another study performed on the *K. lactis* expression system, conditions such as pH, temperature, expression time, concentration of the inducer and carbon source, and induction time were optimized to increase brazzein production, and 1:2 (w/w) glucose: galactose induction at 25°C was resulted in an 2.5 fold increase in protein production [38]. Therefore, *K. lactis* will be a suitable expression system for recombinant brazzein by optimizing the pH, temperature, ratio of carbon source and nitrogen source, time of induction, as well as inducer, yeast extract and glycerol concentration [40]. Among the sweet plant proteins, the least studied protein is mabinlin. Mabinlin has less sweetness than other proteins and therefore has attracted less attention. Mabinlin structure consists of two independent polypeptides, and chain B was shown to be adequate for the sweetness of mabinlin [68]. The recombinant mabinlin II chain B produced from *E.coli* expression system has a sweet taste; however, it has been reported that the protein can only be purified from the inclusion body by the denaturation/renaturation method. In experiments using *L. lactis* expression system, recombinant mabinlin was produced and secreted out of the cell, albeit in very low amounts. This study is the first step towards the production of mabinlin II from the food grade *L. lactis* system[48].

Different studies have been carried out using *E.coli*, *L.lactis*, *P.pastoris* and *S.cerevisiae* expression systems for the production of recombinant monellin. In a study with *L.lactis*, it was shown that the use of codon-optimized gene produced a low amount of monellin [51]. In studies with *S. cerevisiae*, another microorganism with a safe expression system for use in foods, 410-675 mg/L recombinant monellin could be produced with low yields [53], [54]. The tendency of monellin to denature at high temperatures limits the use of recombinant protein in food applications [7]. To overcome this situation, amino acid mutations were made in monellin sequence and 150 mg/L protein could be produced using the *P. pastoris* expression system [52]. In another study using *P. pastoris*, the effect of cell density on monellin production was examined and highest protein levels; 270 mg/L; were obtained with lower cell density fermentations [50].

## 2. CONCLUSION

Sweet proteins have been the focus of attention of food industry for many years. However, they have not been widely used due to their limited availability. The successful transformation of these proteins for recombinant production will increase their use in food industry as a low calorie replacer for sweeteners and sugars. Apart from the six sweet plant proteins that have been studied so far, the identification of other similar plant proteins is one of the current research topics. By using bioinformatic tools and databases, the identification of new proteins that are similar to sweet proteins both in terms of primary sequence and tertiary structure will accelerate and enlarge the studies in this field. In addition, studies on increasing the sweetness level and recombinant production yields of these proteins with protein engineering approaches are still ongoing. In addition to all these, determining the physicochemical, functional, textural and sensory effects of sweet proteins on the food matrix to which they are added are important research topics in terms of creating new food formulations. It is predicted that in the coming years, sweet proteins will be produced on large scale and used more widely in the food industry as natural sweeteners.



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