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Structure–Sweetness Relationship in Thaumatin: Importance of Lysine Residues

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Abstract

To clarify the structural basis for the sweetness of thaumatin I, lysine-modified derivatives and carboxyl-group-modified derivatives were prepared by chemical modification followed by chromatographic purification. The sweetness of derivatives was evaluated by sensory analysis. Phosphopyridoxylation of lysine residues Lys78, Lys97, Lys106, Lys137 and Lys187 markedly reduced sweetness. The intensity of sweetness was returned to that of native thaumatin by dephosphorylation of these phosphopyridoxylated lysine residues except Lys106. Pyridoxamine modification of the carboxyl group of Asp21, Glu42, Asp60, Asp129 or Ala207 (C-terminal) did not markedly change sweetness. Analysis by far-UV circular dichroism spectroscopy indicated that the secondary structure of all derivatives remained unchanged, suggesting that the loss of sweetness was not a result of major disruption in protein structure. The five lysine residues, modification of which affected sweetness, are separate and spread over a broad surface region on one side of the thaumatin I molecule. These lysine residues exist in thaumatin, but not in non-sweet thaumatin-like proteins, suggesting that these lysine residues are required for sweetness. These lysine residues may play an important role in sweetness through a multipoint interaction with a putative thaumatin receptor.

Introduction

Thaumatin is a sweet-tasting protein isolated from the arils of *Thaumatococcus daniellii* Benth, a plant native to tropical West Africa (Van der Wel and Loeve, 1972). Naturally occurring thaumatin consists of six closely related proteins (I, II, III, a, b and c), all with a molecular mass of 22 kDa (207 amino acids) (Van der Wel and Loeve, 1972; Ledeboer *et al.*, 1984). Neither protein contains bound carbohydrate or unusual amino acids (Van der Wel and Loeve, 1972). The proteins have an isoelectric point of 12 (Van der Wel and Loeve, 1972). The sweet taste of thaumatins can be detected at threshold amounts 1600 times less than that of sucrose on a weight basis (equivalent to 10^5-fold less on a molar basis); the threshold values are near 10^{-4}% or 48 nM, making these proteins the most intensely sweet substances known (Van der Wel and Loeve, 1972; Higginbotham, 1979). The three-dimensional structure of thaumatin I has been determined at high resolution (De Vos *et al.*, 1985; Ogata *et al.*, 1992), revealing that the protein consists of three domains: (i) an 11 strand, flattened β-sandwich (1–53, 85–127 and 178–207, domain I); (ii) a small disulfide-rich region (54–84, domain III); and (iii) a large disulfide-rich region (128–177, domain II). A family of pathogenesis-related (PR) proteins has been referred to as thaumatin-like proteins, due to the remarkable homology between PR-5 proteins and thaumatin. Despite their structural relatedness to thaumatin, none of the PR-5 proteins has been reported to have a sweet taste (Dudler *et al.*, 1994; Peng *et al.*, 1997; Tattersall *et al.*, 1997).

Despite extensive studies in this field, the structural basis of thaumatin's sweetness is still uncertain. There is no obvious structural similarity between the three-dimensional structures of thaumatin I and another sweet protein, monellin (Ogata *et al.*, 1987; Somoza *et al.*, 1993). Chemical modification studies have suggested that thaumatin's basicity is not the dominating factor in determining the intensity of its sweetness (Van der Wel and Bel, 1976; Shamil and Beynon, 1990). These previous chemical modification studies did not identify the labeled amino acid residue, and so did not identify a structural determinant for sweetness. Slootstra *et al.* (Slootstra *et al.*, 1995) identified epitopes for monoclonal antibodies that reacted with both thaumatin and monellin, and suggested that two regions of thaumatin, amino acids 19–29 and 77–84, are the important determinants of sweet taste. A site-directed mutagenesis study has reported that mutation at Lys67, Lys137 or Tyr169 reduced sweetness by five-fold (Kim and Weickmann, 1994); the authors concluded that these residues contribute to determining sweet taste. Thus, the determinants of thaumatin's sweet taste remain controversial and obscure.

Two intensely sweet proteins, thaumatin and monellin, are both basic proteins (Van der Wel and Loeve, 1972; Morris *et al.*, 1973), suggesting that basicity could be important for

sweetness. Sequence analysis has revealed substantial homology between thaumatin and thaumatin-like proteins (Koiwa *et al.*, 1999), but there are differences in some amino acid residues; in particular, there is a characteristic pattern of lysine residues in thaumatin, which suggests that some lysine residues may play an important role in sweetness. Van der Wel has suggested in his review (Van der Wel, 1994) that an increase in the basicity of thaumatin, by amidation of its carboxyl groups, can increase its sweetness. This could suggest that positive charge on a thaumatin molecule could play some role in the increase in sweetness. Therefore, in this study the roles of positive charge and lysine residues in sweetness were investigated. Our approach was to prepare a variety of lysine-modified or carboxyl-group-modified thaumatin derivatives by chemical modification followed by chromatographic purification, and to examine the effects of the modifications on sweetness.

Materials and methods

Materials

Thaumatin I was purified from crude thaumatin powder (Sunsweet T; San-Ei Gen FFI, Osaka, Japan) by ion-exchange column chromatography followed by gel filtration column chromatography as described previously (Kaneko and Kitabatake, 1999). L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (type XIII), chymotrypsin (type II) and potato acid phosphatase were purchased from Sigma (St Louis, MO). Urea, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dithiothreitol and iodoacetamide were of specially prepared reagent grade; all other chemicals were of guaranteed grade from Nacalai Tesque Inc. (Kyoto, Japan).

Modification of lysine residues by pyridoxal 5′-phosphate and dephosphorylation

Thaumatin I was incubated with pyridoxal 5′-phosphate (PLP) at 25°C in 50 mM sodium phosphate (NaP_i) buffer, pH 7.0 (Lundblad and Noyes, 1984). The final concentrations of thaumatin I and PLP were 200 μM and 3 mM, respectively. After 15 min of reaction, a freshly prepared solution of sodium borohydride (30 mg/ml) was added to the reaction mixture to achieve a final concentration of 0.6 mg/ml. This concentration of sodium borohydride was sufficient to reduce the Schiff base completely without substantial loss of sweetness due to this reagent (addition of sodium borohydride without addition of PLP did not affect the sweetness of thaumatin I).

Phosphopyridoxylated thaumatin I was separated by ion-exchange column chromatography and extensively dialyzed against 5 mM NaP_i buffer, pH 5.0, at 4°C. The dialyzed sample (200 mg of protein in 50–60 ml) was applied on to a 1.1 × 5.0 cm column of SP Sephadex C-25 (Pharmacia, Uppsala, Sweden) previously equilibrated with 5 mM NaP_i buffer, pH 5.0. Elution was carried out using a stepwise gradient elution with buffers of increasing pH and ionic strength as follows: the flow-through was the first fraction; fractions 2–4 were eluted, respectively, with 100 ml of 5 mM NaP_i buffer, pH 7.0; 50 ml of the same buffer containing 50 mM NaCl; and 100 ml of 20 mM NaP_i buffer, pH 8.0, containing 100 mM NaCl. These fractions were dialyzed overnight against water at 4°C, lyophilized and stored at 4°C until use.

Mono-phosphopyridoxylated thaumatin I, in which one lysine residue was modified, was further purified using a hydroxyapatite column on fast protein liquid chromatography (FPLC) (Pharmacia). Phosphopyridoxylated thaumatin I was dissolved in 5 mM NaP_i buffer, pH 7.0, and applied to a hydroxyapatite column (TSKgel HA-1000, 7.5 × 75 mm; TOSOH, Tokyo, Japan) connected to an FPLC. The flow rate was 0.5 ml/min. Elution was monitored by a UV detector at 278 nm. Elution was performed with the following linear gradient of NaCl in 5 mM NaP_i buffer, pH 7.0: (i) 0 M from 0 to 15 min; (ii) 0–275 mM over 40 min; (iii) 275–500 mM over 5 min; and (iv) 500 mM for 10 min. Five peaks (*a–e*) were observed. Peak fractions were concentrated and desalted using a Centricon YM-10 centrifugal filter device (Millipore, Bedford, MA, USA) at 4°C.

These fractions were further purified by ion-exchange column chromatography on an FPLC equipped with a Cosmogel SP (7.5 × 75 mm; Nacalai Tesque) or a Cosmogel CM (7.5 × 75 mm; Nacalai Tesque) ion-exchange column. To purify fractions *a–d*, a Cosmogel SP ion-exchange column and two solvents [5 mM NaP_i buffer, pH 7.0 (solvent A) and 0.2 M NaP_i buffer, pH 8.0 (solvent B)] were used. The flow rate was 0.5 ml/min. Elution was monitored by a UV detector at 278 nm. The elution was performed with a linear gradient from 100% solvent A to 37.5% solvent B over 60 min, and then to 100% solvent B over 10 min. A Cosmogel CM-ion exchange column was used to purify fraction *e*. The elution was performed as described above, except for the gradient program, which was a linear gradient from 100% solvent A to 15% solvent B over 60 min, and then to 100% solvent B over 20 min. Peak fractions were pooled and desalted using a Centricon YM-10 centrifugal filter device.

5′-Phosphates on the PLP group of phosphopyridoxylated derivatives were eliminated using acid phosphatase (Sigma) (Bingham and Farrell, 1976). In brief, 1 mg of phosphopyridoxylated derivative was incubated with acid phosphatase (5 units) in 500 μl of 0.1 M sodium acetate buffer, pH 5.5, at 37°C for 24 h. The dephosphorylated derivative was separated by ion-exchange column chromatography using SP Sephadex C-25 resin. The sample was eluted with 0.2 M NaP_i buffer, pH 8.0. Samples were pooled and desalted using a Centricon YM-10 centrifugal filter device.

Modification of carboxyl groups

The carboxyl groups of thaumatin I were modified with

pyridoxamine (PM) using EDC as coupling agent (Carraway and Koshland, 1972). Thaumatin I (225 μM) was incubated for 2 h at 25°C with PM (0.2 M) and EDC (7.0 mM) in 5 mM NaP_i buffer, pH 5.0. The reaction mixture was extensively dialyzed at 4°C against 5 mM NaP_i buffer, pH 7.0. The dialyzed sample (200 mg of protein in 30–40 ml) was applied on to a SP Sephadex C-25 column (1.1 × 5.0 cm) equilibrated with 5 mM NaP_i buffer, pH 7.0. Elution was carried out using stepwise gradient elution with buffers of increasing the ionic strength as follows: the flow-through was the first fraction, and the second, third and fourth fractions were eluted respectively using 100 ml of 5 mM NaPi buffer, pH 7.0, containing 70 mM NaCl; 100 ml of the same buffer but containing 90 mM NaCl; and 50 ml of 5 mM NaPi buffer, pH 7.0, containing 400 mM NaCl, consecutively. The third fraction contained mono-PM-thaumatin I, whose one carboxyl group was modified with PM; this was dialyzed overnight against water at 4°C. The dialyzed sample was then lyophilized and stored at 4°C until use.

Mono-PM-thaumatin I was further purified by ion-exchange column chromatography on high performance liquid chromatography (HPLC) (LC-6A; Shimadzu, Kyoto, Japan) and then dissolved in 5 mM NaP_i buffer, pH 7.0, and applied to a Cosmogel SP (20 × 100 mm; Nacalai tesque) ion-exchange column connected to a HPLC. Elution was monitored by a UV detector at 278 nm. The flow rate was 3.0 ml/min. To perform ion-exchange column chromatography, the same two solvents (solvents A and B) were used and the elution was performed with a linear gradient: 100% solvent A for 5 min, 0–30% solvent B in Solvent A over 5 min, 30–50% over 50 min, then 50–100% over 10 min. Peak fractions were pooled, concentrated and desalted using a Centricon YM-10 centrifugal filter device at 4°C. They were then further purified by rechromatography using ion-exchange column chromatography, performed on HPLC apparatus equipped with a Cosmogel SP (7.5 × 75 mm; Nacalai Tesque) ion-exchange column. The flow rate was 0.5 ml/min. The elution was monitored by a UV detector at 278 nm. The same two solvents as used in the first chromatography were used and the elution was performed with a linear gradient from 100% solvent A to 25% solvent B over 15 min and to 35% solvent B over 60 min, and then to 100% solvent B in 70 min. Peak fractions were pooled and desalted using a Centricon YM-10 centrifugal filter device and were immediately used for further analysis.

Spectrophotometric quantification

The concentration of native thaumatin I and its derivatives were determined spectrophotometrically with a UV-160A spectrophotometer (Shimadzu) in 5 mM NaPi buffer, pH 7.0, using a molar extinction coefficient of ε_{278} of 17 000/M/cm (Van der Wel and Loeve, 1972). To obtain ε_{278}, a mass of 22 209 daltons (Iyenger *et al.*, 1979) was used. Chemical modification did not affect the ε_{278} value, as shown by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) procedure. Protein-bound PLP was determined at 325 nm using an ε_{325} of 4800/M/cm (Lundblad and Noyes, 1984; Green *et al.*, 1992).

Proteolytic digestion and peptide purification

Tryptic digestion was performed as follows. The lyophilized sample (final concentration 2 mg/ml) was dissolved in 8 M urea and 50 mM Tris–HCl buffer, pH 8.0. The sample was incubated with dithiothreitol (2 mM) for 15 min at 50°C and then carboxamidomethylated by the addition of freshly prepared 0.1 M aqueous iodoacetamide solution to a final concentration of 8 mM. The sample was incubated at 37°C for 20 min and was diluted five-fold with 50 mM Tris–HCl buffer, pH 8.0. TPCK-treated trypsin was added to a ratio of 1:7 (w/w) protease:substrate and the solution was incubated for 22 h at 37°C. Chymotryptic digestion was performed in the same manner as tryptic digestion, except that the ratio of protease:substrate was 1:2 (w/w).

The products of proteolytic digestion were applied on to a reversed-phase HPLC column (Cosmosil $5C_{18}$-AR-II: ODS, 4.6 × 150 mm; Nacalai tesque) connected to an HPLC apparatus (L-6200; Hitachi, Tokyo, Japan). The flow rate was 0.42 ml/min and elution was performed in 0.1% trifluoroacetic acid (TFA) with a linear gradient of 10–38% acetonitrile over 35 min, then to 90% acetonitrile in an additional 10 min. The eluent was monitored using an RF-530 fluorescence detector (Shimadzu): excitation, 327 nm; emission, 395 nm for detection of phosphopyridoxylated lysine (Lo *et al.*, 1992), or excitation, 315 nm; emission, 395 nm for pyridoxamine-labeled residues. The peaks were further purified by rechromatography using a reversed-phase HPLC column (Cosmosil $5C_{18}$-AR: ODS, 4.6 × 150 mm) and eluted in 0.1% TFA with a linear gradient of 10–50% methanol over 35 min, and then to 90% methanol in an additional 10 min. Flow rate and detection were the same as above.

Sensory analysis

The sweetness threshold of samples was evaluated by means of a triangle test for taste absolute threshold (Horio and Kawamura, 1998). Five healthy subjects, three males and two females, aged 25–48 years, participated in this trial. The samples were tasted in order of their concentration, starting with those of the lowest concentration. After each taste, subjects thoroughly rinsed their mouths with distilled water. The test solutions used were 20, 50, 100, 200 and 500 nM for native thaumatin I; 100, 200, 500, 1000, 2000, 5000 and 10 000 nM for the di- or tri-phosphopyridoxylated derivatives; 20, 50, 100, 200, 500 and 1000 nM for the mono-phosphopyridoxylated derivatives; 20, 50, 100, 200 and 500 nM of the pyridoxylated derivatives; and 20, 50, 100 and 200 nM of the PM-labeled derivatives. Protein was

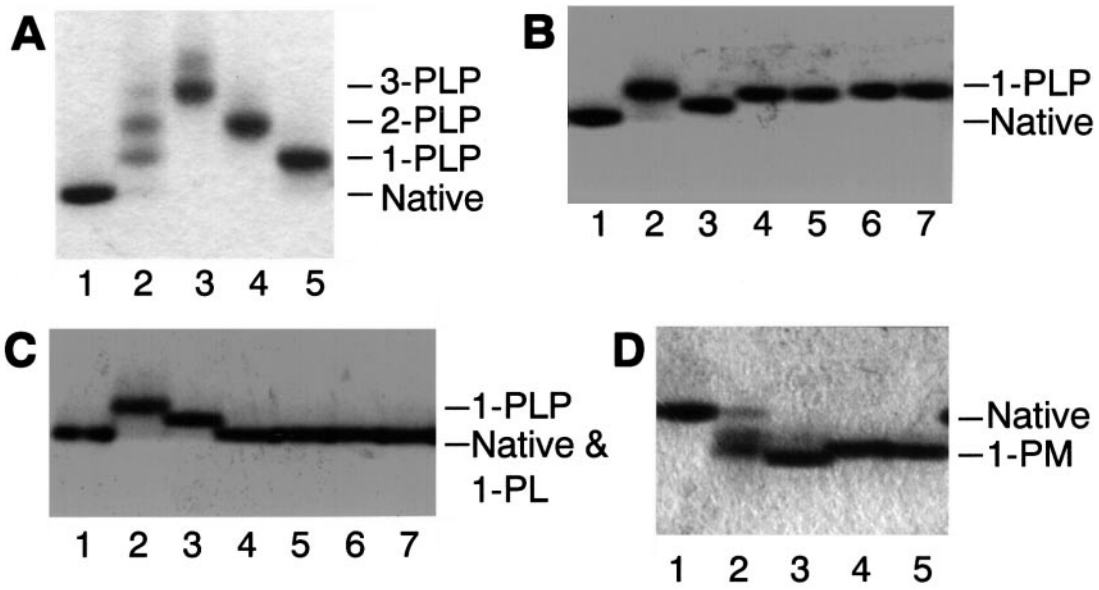

Figure 1 Native PAGE analysis of native thaumatin I and its derivatives. **(A)** Lane 1, native thaumatin I; lane 2, mixture of multiply phosphopyridoxylated derivatives before purification; lane 3, tri-phosphopyridoxylated (3-PLP) derivative; lane 4, di-phosphopyridoxylated (2-PLP) derivative; lane 5, mono-phosphopyridoxylated (1-PLP) derivative. **(B)** Lane 1, native thaumatin I; lane 2, mixture of phosphopyridoxylated derivatives before purification; lane 3, PLP-Lys106 derivative; lane 4, PLP-Lys78 derivative; lane 5, PLP-Lys187 derivative; lane 6, PLP-Lys137 derivative; lane 7, PLP-Lys97 derivative. **(C)** Lane 1, native thaumatin I; lane 2, mixture of phosphopyridoxylated derivatives before purification and without dephosphorylation; lane 3, PL-Lys106 derivative; lane 4, PL-Lys78 derivative; lane 5, PL-Lys187 derivative; lane 6, PL-Lys137 derivative; lane 7, PL-Lys97 derivative. **(D)** Lane 1, native thaumatin I; lane 2, mixture of mono-pyridoxamine-labeled derivatives before purification; lane 3, PM-Asp60 derivative; lane 4, PM-Asp21/Glu42/Asp129-derivative; lane 5, PM-Ala207 derivative. Five micrograms of protein was loaded in each lane and stained with Coomassie Brilliant Blue.

dissolved in 5 ml of 5 mM NaP_i buffer, pH 7.0, and kept at room temperature until testing. Relative sweetness (%) was calculated as 100 × (threshold of native thaumatin I)/(threshold of sample).

Other methods

Non-denaturing PAGE (native PAGE) was performed using a system for basic proteins (Reisfeld *et al.*, 1962). Five micrograms of protein sample was applied on to each lane of a 7.5% homogeneous native polyacrylamide gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. Circular dichroism (CD) spectra were obtained with a J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 25.0°C in 5 mM NaP_i buffer, pH 7.0. Far-UV CD spectra were recorded at a protein concentration of 4.5 μM with a 1 mm cell at wavelengths from 250 to 200 nm. The data were collected three times and are given as the average mean residue ellipticity. Matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometric (MALDI-TOF-MS) analyses were carried out using a Voyager RP BioSpectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MS, USA). Automated sequencing of peptides was performed in an Applied Biosystems 492 Protein Sequencer (PE Biosystems, Foster City, CA). Amino acid compositions were analyzed with an amino acid analyzer (L-8500A; Hitachi) after acid hydrolysis in the gas phase with 6 M hydrochloric acid containing 0.1% (v/v) phenol for 22 h at 110°C under vacuum.

Results

Importance of lysine residues

Preparation of mono-, di- and tri-phosphopyridoxylated derivatives

To prepare lysine-modified derivatives to examine the role of lysine residues in sweetness, thaumatin I was reacted with PLP. The reaction of thaumatin I with PLP generated many phosphopyridoxylated derivatives (Figure 1A, lane 2). Each of these derivatives was isolated by SP Sephadex C-25 ion-exchange column chromatography and the resulting samples were analyzed on a native polyacrylamide gel (Figure 1A, lanes 3–5). Judging from the electrophoretic mobility, the first, second and third fractions consisted mainly of tri-, di- and mono-phosphopyridoxylated derivatives, respectively. The number of modified residues was further confirmed by amino acid analysis (data not shown).

Sweetness of mono-, di- and tri-phosphopyridoxylated derivatives

The sweetness of native thaumatin I and its derivatives was evaluated. The resulting sweetness threshold values and the relative sweetnesses are listed in Table 1. Native thaumatin I showed intense sweetness at 500 nM (data not shown) and a

Table 1 Sweetness of thaumatin I derivatives determined by means of a triangle test for absolute taste threshold

Thaumatin I derivative	Sweetness threshold (nM)	Relative sweetness[a] (%)
Native	50	100
Mono-PLP	260 ± 120	22 ± 6
Di-PLP	1800 ± 400	3 ± 1
Tri-PLP	>10 000	<0.5
PLP-Lys78	140 ± 50	40 ± 12
PLP-Lys97	320 ± 140	19 ± 7
PLP-Lys106	260 ± 120	22 ± 6
PLP-Lys137	200	25
PLP-Lys187	260 ± 120	22 ± 6
PL-Lys78	50	100
PL-Lys97	50	100
PL-Lys106	260 ± 120	22 ± 6
PL-Lys137	50	100
PL-Lys187	50	100
PM-Asp60	50	100
PM-Asp21/Glu42/Asp129	44 ± 12	130 ± 60
PM-Ala207	50	100

Relative sweetness intensities of derivatives are expressed as a percentage of the sweetness (mean ± SD) of that of native thaumatin I.

[a]Relative sweetness (%) = 100 × (threshold of native thaumatin I)/(threshold of sample).

sweetness threshold of 50 nM. This latter value corresponds to that previously reported by Van der Wel and Loeve (Van der Wel and Loeve, 1972).

To examine the effect of phosphopyridoxylation of lysine residues on sweetness, the sweetness of the mono-, di- and tri-phosphopyridoxylated derivatives was investigated. As the number of the modified lysine residues increased, the sweetness decreased. Mono-, di- and tri-modification of lysine residue reduced sweetness by five-, 30- and >200-fold, respectively. These results suggest that lysine residues are involved in determining sweetness.

The position of functionally important lysine residues

Isolation of specifically phosphopyridoxylated derivatives

It is probable that the sites of modification of derivatives differ from each other, even in a mono-phosphopyridoxylated derivative obtained above. To examine whether a variety of derivatives exist, we subjected mono-phosphopyridoxylated derivative to hydroxyapatite FPLC. We observed five peaks, named *a–e* (Figure 2), indicating the existence of a variety of derivatives whose modified sites are different from each other. Each peak was further purified by rechromatography using ion-exchange FPLC. The purified derivatives were named phosphopyridoxylated derivatives *a–e*. Each derivative showed a single peak on analytical ion-exchange FPLC analysis with different retention times (data not shown) and gave a single sharp band on native PAGE (Figure 1B, lanes 3–7), suggesting the homogeneity

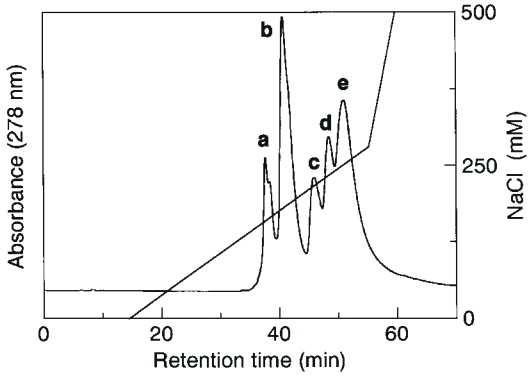

Figure 2 Purification of phosphopyridoxylated thaumatin I by hydroxyapatite FPLC. Five milligrams of phosphopyridoxylated thaumatin I, which binds one molecule of PLP per molecule, was applied to a TSK gel HA-1000 hydroxyapatite column connected to an FPLC apparatus. Elution was monitored by absorbance at 278 nm. The five major peaks denoted *a–e* were subjected to further purification. The shoulder beside peak *a* corresponds to native thaumatin I. Data are representative of five independent experiments.

of these derivatives. Incorporation of one PLP per mole of thaumatin I was further confirmed spectrophotometrically and by amino acid analysis (data not shown).

Next, we identified the residue that was modified in these mono-phosphopyridoxylated derivatives. Each derivative was reduced with dithiothreitol, carboxamidemethylated, digested with trypsin (derivatives *a* and *c-e*) or chymotrypsin (derivative *b*) and resolved by reversed-phase HPLC. The elution profiles of protease digests of derivatives *a–e* are shown in Figure 3A–E, respectively. Each profile showed one or two major peaks (Roman numerals) and some minor peaks. The eluted fractions corresponding to the major peaks were collected and subjected to additional reversed-phase HPLC. The fluorescent peptide was isolated and sequenced (Table 2, peaks A-I to E-I; X represents a modified residue). Table 2 also indicates the corresponding sequences in thaumatin I. The results show that a specific single lysine residue was modified in each derivative, revealing the homogeneity of all derivatives. Aspartic acid was observed at position 113 in this study, although other authors have reported asparagine at this position (Iyengar *et al.*, 1979). The incorrectness of the assignment by Iyengar *et al.* has been pointed out before (Lee *et al.*, 1988). The phosphopyridoxylated lysine residues in derivatives *a–e* were identified as Lys106, Lys78, Lys187, Lys137 and Lys97, respectively. Thus, these phosphopyridoxylated derivatives were named PLP-Lys106-thaumatin I, PLP-Lys78-thaumatin I, PLP-Lys187-thaumatin I, PLP-Lys137-thaumatin I and PLP-Lys97 thaumatin I, respectively.

Sweetness of specifically phosphopyridoxylated derivatives

To examine the effect of phosphopyridoxylation of each lysine residue on sweetness, the sweetness of specifically phosphopyridoxylated derivatives was investigated (Table 1). The sweetness intensities of the phosphopyridoxylated derivatives were reduced by 60–81%, almost similar to that

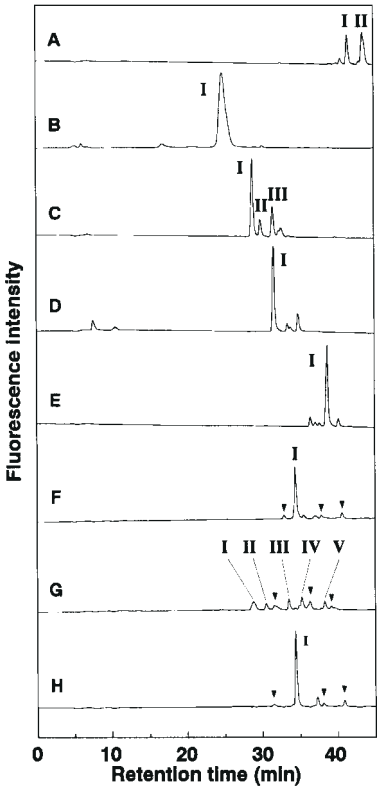

Figure 3 Reversed-phase HPLC profiles of proteolytic digests of thaumatin I derivatives. **(A–E)** Five phosphopyridoxylated-derivatives, *a–e*, were digested and subjected to reversed-phase HPLC as described in Materials and methods. Elution was detected by fluorescence (excitation, 327 nm; emission, 395 nm). **(F–H)** Three pyridoxamine-labeled derivatives, *a–c*, were digested and subjected to reversed-phase HPLC as described in Materials and methods. Elution was detected by fluorescence (excitation, 315 nm; emission, 395 nm). Peaks marked with arrows also appeared in control elution profiles of proteolytic digests of unmodified thaumatin I, and are therefore not attributed to modified species. Peaks detected by fluorescence, marked with Roman numerals, were expected to contain a modified residue and were purified by rechromatography using a reversed-phase HPLC column eluted with a methanol linear gradient as described in Materials and methods. Resulting peptides were sequenced.

of mono-phosphopyridoxylated thaumatin and significantly different from that of native thaumatin I. The extent of the decrease in sweetness of these derivatives was comparable to that of mutant thaumatin produced by Kim and Weickmann (Kim and Weickmann, 1994). Since the CD spectrum of these specifically phosphopyridoxylated derivatives was unchanged (see below), the decrease in sweetness caused by the incorporation of PLP could reflect the importance of these lysine residues for sweetness. There are two possible interpretations of this effect: (i) that lysine residues at these sites are necessary to exhibit sweetness fully; or (ii) that positive charges at these sites are necessary for sweetness, that is, replacement of positive charge (on a lysine residue) by a negative charge (on a phosphopyridoxylated lysine residue) should reduce the sweetness.

Table 2 Sequences of peptides containing a modified residue. The purified peptides containing a modified residue were sequenced.

Peak	Peptide (modified residue)	Cycles anal.	Sequence cycle
A-I	98–108 (Lys106)	12	DYIDISNIXGF
A-II	98–119 (Lys106)	23	DYIDISNIXGFNVPMNFSPTTR
B-I	76–80 (Lys78)	6	RCXRF
C-I	184–200 (Lys187)	18	VLNXPTTVTCPGSSNYR
C-II	184–200 (Lys187)	18	VLDXPTTVTCPGSSNYR
C-III	182–200 (Lys187)	20	SYVLDXPTTVTCPGSSNYR
D-I	126–139 (Lys137)	15	CAADIVGQCPAXLK
E-I	91–106 (Lys97)	17	SLNQYGXDYIDISNIK
F-I	54–62 (Asp60)	9	TDCYFDXSG
G-I	15–23 (Asp21)	9	AAASKGXAA
G-II	38–44 (Glu42)	7	TINVXPG
G-III	15–25 (Asp21)	11	AAASKGXAALD
G-IV	126–138 (Asp129)	14	CAAXIVGQCPAKL
G-V	38–43 (Glu42)	6	TINVXP
H-I	201–207 (Ala207)	8	VTFCPTA

X represents the modified amino acid residues, which cannot be detected by the method of sequencing used.

Role of lysine residues in sweetness

Preparation of specifically pyridoxylated derivatives

To clarify the possibilities above, the phosphate group of phosphopyridoxylated lysine residues was eliminated by phophatase to alter charge of this position from negative back to positive (see Discussion) and the effect on sweetness was examined. Elimination of the phosphate group was confirmed by a decrease in absorbance at 325 nm and by an increase in electrophoretic mobility on native PAGE (Figure 1C), relative to those of the phosphopyridoxylated derivatives. The dephosphorylated derivatives were named pyridoxylated (PL)-Lys106-thaumatin I, PL-Lys78-thaumatin I, PL-Lys187-thaumatin I, PL Lys137-thaumatin I, and PL-Lys97-thaumatin I, respectively, because a PL-lysine residue was formed from a PLP-lysine residue.

Sweetness of specifically pyridoxylated derivatives

The sweetness of the pyridoxylated derivatives is shown in Table 1. All of the pyridoxylated derivatives, except the PL-Lys106-thaumatin I derivatives, exhibited sweetness equivalent to that of native thaumatin I, i.e., the sweetness intensity of these derivatives was restored to that of native thaumatin by dephosphorylation of PLP-thaumatin.

The sweetness of PL-Lys78-, PL-Lys97-, PL-Lys137- and PL-Lys187-thaumatin I derivatives was equal to that of native thaumatin I. This indicates that the increase in sweetness was due to the positive charge on these lysine residues. Therefore, positive charges at Lys78, 97, 137 and 187 are important for sweetness and the distance between the α-carbon and the ε-amino group of these lysine residues may not be important for sweetness.

In contrast, the sweetness of PLP-Lys106-thaumatin I

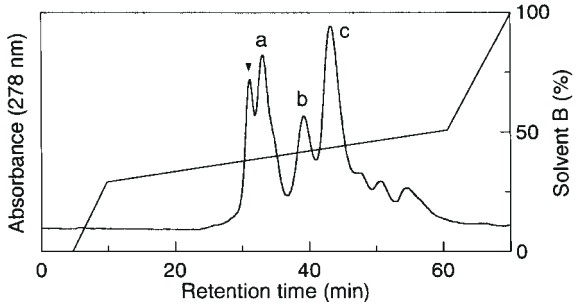

Figure 4 Purification of mono-PM-modified thaumatin I by ion-exchange HPLC. The carboxyl group-modified thaumatin I, which contains one molecule of PM per molecule, was applied to a Cosmogel SP ion-exchange column connected to an HPLC apparatus. Elution was monitored by absorbance at 278 nm. The three major peaks denoted *a–c* were subjected to further purification. The peak eluting before peak *a*, indicated by an arrow, corresponds to native thaumatin I. Data are representative of four independent experiments.

was not affected by dephosphorylation. The effect of dephosphorylation of PLP-Lys106 on sweetness was different from that of the other four residues. This may suggest that, at position 106, the side-chain structure is important for sweetness.

Effect on sweetness of elimination of a negative charge from the carboxyl group

Preparation of specific carboxyl-group-modified derivatives

In the experiments above, it was shown that the positive charges distributed on the thaumatin molecule are important for sweetness. The effect of elimination of negative charge from the thaumatin molecule was examined next. The carboxyl group of thaumatin was modified with pyridoxamine (PM). By this modification, a pyridoxal moiety was attached to a carboxyl group and then a negative charge was shielded. The reaction generated a variety of modified derivatives. To investigate if the effect on sweetness depended on the position of the modified residue, a mono-modified derivative was purified by SP Sephadex C-25 column chromatography followed by ion-exchange HPLC (Figure 4). The peak indicated by an arrow represents unmodified thaumatin I. Three other peaks, denoted *a–c*, were further purified by rechromatography using ion-exchange HPLC. The samples obtained were called PM-thaumatin *a–c*, respectively. Although PM-thaumatin *b* was heterogeneous (see below), we were unable to separate this fraction further. Incorporation of one mole of PM into the thaumatin I molecule was confirmed by MALDI-TOF-MS analysis (data not shown). Amino acid analysis and MALDI-TOF-MS analysis confirmed that there was no occurrence of side reactions, including modification of tyrosine residues (Carraway and Koshland, 1968) and chemical cross-linking (Carraway and Koshland, 1972).

To identify the modified residues, each derivative was reduced with dithiothreitol, carboxamidemethylated, digested with trypsin (derivatives *a* and *c*) or chymotrypsin (derivative *b*) and subjected to reversed-phase HPLC. The elution profiles are shown in Figure 3F–H. The profiles of tryptic digests of derivatives *a* and *c* showed one major peak (Roman numerals) and three minor peaks (arrows). The profile of chymotryptic digestion of derivative *b* showed five large peaks (Roman numerals) and three small peaks (arrows). The peaks marked with arrows also appeared in a control elution profiles (proteolytic digests of unmodified thaumatin I) and so were not attributed to any modified species. The eluted fractions corresponding to the peaks marked with Roman numerals were collected, and the fluorescent peptide was isolated by additional reverse-phase HPLC purification (data not shown). The amino acid sequences of the peptides in the isolated peaks are shown in Table 2 (peaks F-I to H-I; X represents a modified residue). The modification at Ala207 (C-terminal) in peptide H-I was further confirmed by a comparison of the protonated ion peaks observed at *m/z* 946.4 with the theoretical values of 946.1 for $(M + H^+)$, which was calculated from a peptide fragment from Val201 to Ala207 plus one molecule of PM. The results showed that Asp60 and Ala207 were specifically modified in derivatives *a* and *c*, respectively, indicating the homogeneity of these derivatives. In contrast, three modified residues, Asp21, Glu42, and Asp129, were found in derivative *b*. Together with the fact that one PM molecule was incorporated per one thaumatin I molecule, three kinds of mono-PM-thaumatin I were involved in the derivative *b*. It was possible to evaluate the relative proportions of each derivative by the fluorescence intensity of chymotryptic peptides: we found 44% of Asp21-modified derivative, 31% of Glu42-modified derivative, and 25% of Asp129-modified derivative. Thus, these derivatives *a–c* were denoted as PM-Asp60-thaumatin I, PM-Asp21/Glu42/Asp129-thaumatin I, and PM-Ala207-thaumatin I, respectively. Native PAGE analysis of these derivatives showed the increase in net charge of these molecules (Figure 1D).

Sweetness of specific carboxyl-group-modified derivatives

The effect on sweetness of PM modification of the carboxyl group was examined (Table 1). Although modification of the carboxyl group of Asp21, Glu42, Asp60, Asp129 or Ala207 (C-terminal) did not significantly affect the sweetness of thaumatin I, PM-Asp21/Glu42/Asp129-thaumatin I was a little sweeter. It is certain that elimination of negative charge or a decrease in the number of negative charges on the surface of thaumatin molecule does increase sweetness in specific conditions, but the details of how altering the surface charge of a thaumatin molecule affects the sweetness, including the specificity of the topological site, need to be clarified.

Structural properties of thaumatin I derivatives

To determine whether the chemical modifications affected the secondary structure of thaumatin I, far-UV CD spectra

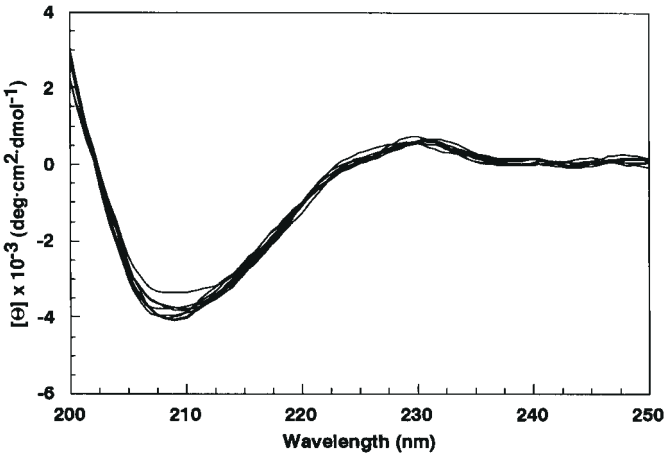

Figure 5 Far-UV CD spectra of thaumatin I derivatives. The far-UV CD spectra of native thaumatin I and derivatives were recorded in 5 mM NaP_i buffer, pH 7.0, as described in Materials and methods. The spectra of native thaumatin I and derivatives whose sweetness was affected are shown in this figure. The spectra from top to bottom at 225 nm are of native thaumatin I, PLP-Lys97-thaumatin I, PLP-Lys137-thaumatin I, PLP-Lys187-thaumatin I, PL-Lys106-thaumatin I, PLP-Lys106-thaumatin I, PLP-Lys78-thaumatin I and PM-Asp21/Glu42/Asp129-thaumatin I, respectively. Other derivatives gave similar spectra (not shown).

were measured. None of the modifications were associated with any detectable changes in the dichroic content (Figure 5), indicating that no major conformational change was induced by the modifications. These observations show that the loss of sweetness must have been due to local changes in structure, rather than being the result of major disruptions in protein structure.

Location of modified residues on the three-dimensional structure of thaumatin

The location of modified residues on the three-dimensional structure of thaumatin is shown in Figure 6. Lys106 is represented in red and Lys78, Lys97, Lys137 and Lys187 in blue. Five other positions, Asp21, Glu42, Asp60, Asp129 and Ala207 (C-terminal), are colored green. One side of the thaumatin molecule (shown at the front in Figure 6A and on the left in Figure 6B) contains all of the important lysine residues shown in this study. Asp60, Asp129 and Ala207 (C-terminal) are located on the opposite side. Lys78, Lys97, Lys137 and Lys187 are located in the flexible loop region, but Lys106 is not. A previous study suggested that the determinant of thaumatin's sweet taste was located in the flexible loop region (Kim and Weickmann, 1994), but our study does not support this suggestion.

Discussion

In this study, we investigated the role of positive charge and lysine residues in determining sweetness. Although thaumatin I contains 11 lysine residues (Iyengar *et al.*, 1979), only five specifically phosphopyridoxylated derivatives, modified on Lys78, Lys97, Lys106, Lys137 or Lys187, were isolated in this study. This indicates that these lysine residues are more reactive with PLP than are the other six lysine residues, which may indicate (Lundblad and Noyes, 1984) that these modified lysine residues are located on the surface of the molecule. Indeed, Kim and Weickmann (Kim and Weickmann, 1994) have reported that these five lysine residues are exposed more than the other six lysine residues. This could explain why these lysine residues play an important role in the perceived sweetness of thaumatin.

The electric charge of lysine residues was changed in this study. First, lysine residues were phosphopyridoxylated, which introduces a negative charge into the phosphate group on lysine residues. Second, the phosphate group was eliminated using phosphatase. The pyridoxal moiety contains one positively charged group (the NH group in the pyridine ring) and one negatively charged group (the OH group in phenol) (Fischer *et al.*, 1958). In addition, one positively charged group (NH of imino group) is thought to be involved in a pyridoxylated lysine residue, since an alkylamino group would be positively charged (Van der Wel, 1994). Thus, elimination of the phosphate group alters the charge at this position from negative back to positive. We examined how these changes in the electric charge of lysine residues affected sweetness.

Phosphopyridoxylation of Lys78, Lys97, Lys106, Lys137 or Lys187 reduced sweetness to ~20% and dephosphorylation of these phosphopyridoxylated lysine residues restored the intensity of sweetness to that of native thaumatin, except in the case of Lys106. This shows that the positive charges on these sites are important in determining sweetness. Although others have reported that the net charge of the molecule seems not to be the dominant factor determining the intensity of sweetness (Shamil and Beynon, 1990; Van der Wel, 1994), we have shown that the positive charges on Lys78, Lys97, Lys137 and Lys187 are required for sweetness.

The sweetness of PLP-Lys106-thaumatin I was not returned to that of native thaumatin by dephosphorylation of PLP-Lys106 , in contrast to the other four residues mentioned above. This may be because Lys106 itself plays a critical role in sweetness, or because an amino acid residue that is critical for sweetness is located near Lys106, or there may be other reasons. Although more extensive studies are needed to elucidate the exact role of Lys106 in determining sweetness, it seems that the role of Lys78, Lys97, Lys137 and Lys187 is, at least in part, to confer the positive charges required for sweetness.

We also investigated the role of carboxyl groups in determining sweetness. Van der Wel has suggested that an increase in the basicity of thaumatin, by amidation of its carboxyl groups, can increase its sweetness to some extent (Van der Wel, 1994). However, details have been obscure until now. Our study has shown that PM-Asp21/Glu42/Asp129-thaumatin I is sweeter than native thaumatin I, indicating that altering the charge of these sites from negative to neutral increases the sweetness. Elimination of

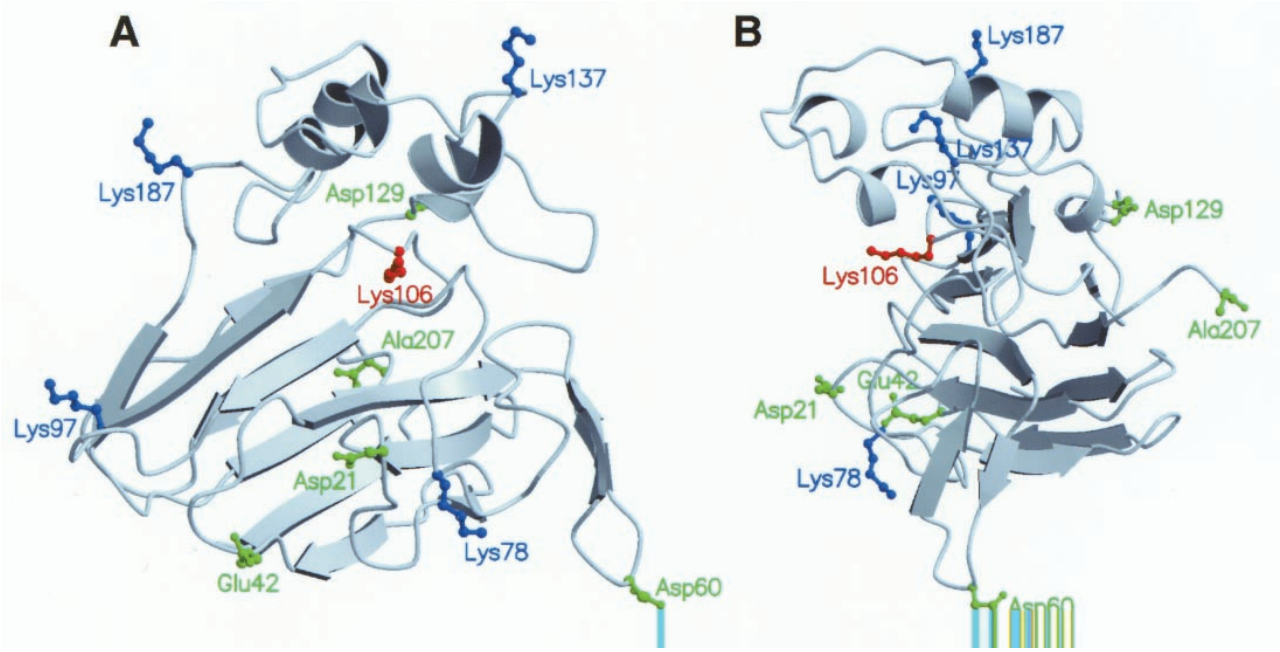

Figure 6 Location of modified residues on three-dimensional structure of thaumatin. Schematic representation of the structure of thaumatin in two different orientations (front view and side view). α-Helices are represented by belts, and helical ribbons and strands of β-sheets by arrows. The side chains of amino acid residues modified in this study are colored as follows: phosphopyridoxylation and pyridoxylation of residues colored red reduced sweetness; phosphopyridoxylation of residues colored blue reduced sweetness; modification with PM of residues colored green did not affect sweetness. These residues are shown as ball-and-stick models and labeled as Lys, Asp, Glu or Ala with numbers showing their positions in the primary sequence. The diagram was drawn using data for thaumatin (Ko *et al.*, 1994) (PDB #1THV) and the programs MOLSCRIPT (Kraulis, 1991) and Raster 3D (Merritt and Murphy, 1994).

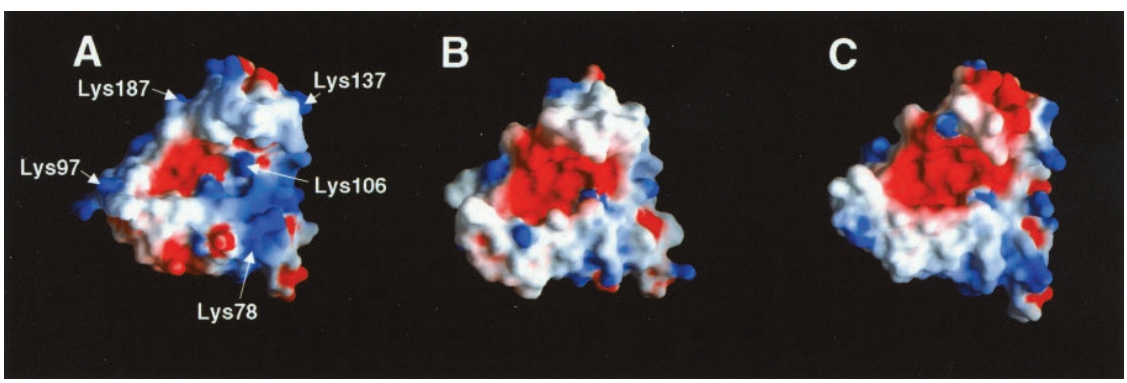

Figure 7 View of the surface topology of **(A)** thaumatin, **(B)** PR–5d and **(C)** zeamatin showing the molecular surface and surface electrostatic potential. The protein surface is colored according to the electrostatic potential, ranging from blue (most positive) through white (neutral) to red (most negative). Protein orientations are similar to that of thaumatin shown in Figure 6A. The lysine residues identified as important in this study are indicated by arrows. The figure was produced using GRASP (Nicholls and Honig, 1991).

negative charge from some region of the surface of thaumatin molecule could, therefore, increase the sweetness of thaumatin or reduce the threshold of sweetness, and non-specific positive charge(s) on the surface of thaumatin molecule could affect sweetness. It would be interesting to determine if altering the charge of a specific carboxyl group from negative to positive increased the sweetness. Although the present study showed that Asp60 and Ala207 (C-terminal) were not critical for sweetness, more extensive studies are needed to examine the roles of carboxyl group in determiming sweetness.

Of the five lysine residues we have shown to be important for sweetness, Lys106 is located in a cleft region in the three-dimensional structure of thaumatin, and Lys78, Lys97, Lys137 and Lys187 surround the cleft region. An X-ray crystallographic study of thaumatin and two thaumatin-like proteins, PR-5d and zeamatin, showed that the cleft region and the surrounding lysine residues in thaumatin are more basic than the corresponding region in PR-5d and zeamatin (Figure 7), although there were no large differences in the surface electrostatic potential of the opposite side among these three proteins. The corresponding positions are occu-

pied by acidic or neutral residues in six other thaumatin-like proteins, namely PR-5c, NP24, PR-5a, *Arabidopsis thaliana* thaumatin-like protein, PR-5K receptor kinase and *Oryza sativa* thaumatin-like protein (Koiwa *et al.*, 1999). These could be important features, because no other members of the family have been reported to be sweet (Dudler *et al.*, 1994; Koiwa *et al.*, 1997; Peng *et al.*, 1997; Tattersall *et al.*, 1997). Thus, the basicity of the cleft region and the four lysine residues surrounding the cleft region seem likely to be structurally required for sweetness.

The present results suggest a possible mechanism for the determination of sweetness in thaumatin. In this study, monophosphopyridoxylation of Lys78, Lys97, Lys106, Lys137 or Lys187 reduced sweetness significantly, but not completely. The results showed that these lysine residues contributed to sweetness almost equally, and increase in phosphopyridoxylation of lysine residue reduced the sweetness markedly. These findings suggest that sweetness could be determined by multipoint interaction of thaumatin with a putative receptor molecule. It is likely that the low threshold value of thaumatin, 50 nM, is accomplished by a high-affinity interaction between thaumatin and a putative receptor site. A number of high-affinity interactions occur through multipoint interaction, in which many amino acid side chains on a broad surface region are in contact with their counterparts, each one contributing partly to the total binding energy; examples include interactions between interleukin-3 and its receptor (Klein *et al.*, 1997), antigen and antibody (Amit *et al.*, 1986; Colman *et al.*, 1987), neurotoxin and its receptor (Kharrat *et al.*, 1989; Zilberberg *et al.*, 1997), cAMP dependent protein kinase and its peptide inhibitor (Knighton *et al.*, 1991), and collagen and integrin (Kamata *et al.* 1999). Thus, it is conceivable that the mechanism by which the sweetness of thaumatin is perceived also occurs through multipoint interaction of the molecule with a putative receptor molecule.

For other sweet proteins, monellin and brazzein, it has also been suggested that many residues spread over a broad surface region of the molecule are important in determining sweetness (Kohmura *et al.*, 1992; Somoza *et al.*, 1995; Assadi-Porter *et al.*, 2000). It has been suggested that two remote regions of the molecule determine sweetness. These findings are not inconsistent with a multipoint interaction model.

The five lysine residues, modification of which reduced sweetness, are all located on one side of the thaumatin molecule, i.e., the cleft-containing side, while the other residues studied, modification of which did not affect sweetness, are located mainly on the opposite side. These results suggest that the cleft-containing side of the thaumatin molecule is important for sweetness; this may, therefore, be the side of the molecule that interacts with a putative thaumatin receptor. This is not inconsistent with previous studies. Lys67 and Lys137, mutation of which reduces the sweetness of thaumatin (Kim and Weickmann, 1994), are located on the cleft-containing side. It is likely that many amino acid residues on the cleft-containing side of the molecule contribute, by some cumulative effect, to determining sweetness through multipoint interaction with the putative receptor molecule.

In this study, PLP, PL and PM were used as modifying agents. Since these compounds are natural coenzyme forms of pyridoxine (vitamin B_6), thaumatin derivatives modified with these compounds were considered safe for use in taste tests. The additional benefit of using these compounds is their fluorescence, which makes it easier to detect modified residues. Further studies of other chemical modifications or site-directed mutagenesis of residues on the cleft-containing side of the molecule may be useful for characterizing further the factors that determine the sweetness of thaumatin.

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