Chem. Senses 30: 667–681, 2005

doi:10.1093/chemse/bji060
Advance Access publication September 14, 2005

Structure–Sweetness Relationship in Egg White Lysozyme: Role of Lysine and Arginine Residues on the Elicitation of Lysozyme Sweetness

Tetsuya Masuda, Nobuyuki Ide and Naofumi Kitabatake

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Correspondence to be sent to: Naofumi Kitabatake, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan. e-mail: kitabatake@kais.kyoto-u.ac.jp

Abstract

Lysozyme is one of the sweet-tasting proteins. To clarify the structure–sweetness relationship and the basicity–sweetness relationship in lysozyme, we have generated lysozyme mutants with *Pichia* systems. Alanine substitution of lysine residues demonstrated that two out of six lysine residues, Lys13 and Lys96, are required for lysozyme sweetness, while the remaining four lysine residues do not play a significant role in the perception of sweetness. Arginine substitution of lysine residues revealed that the basicity, but not the shape, of the side chain plays a significant role in sweetness. Single alanine substitutions of arginine residues showed that three arginine residues, Arg14, Arg21, and Arg73, play significant roles in lysozyme sweetness, whereas Arg45, Arg68, Arg125 and chemical modification by 1,2-cyclohexanedione did not affect sweetness. From investigation of the charge-specific mutations, we found that the basicity of a broad surface region formed by five positively charged residues, Lys13, Lys96, Arg14, Arg21, and Arg73, is required for lysozyme sweetness. Differences in the threshold values among sweet-tasting proteins might be caused by the broadness and/or the density of charged residues on the protein surface.

Key words: basicity, lysozyme, mutation, *Pichia pastoris*, structure–function of sweet protein, sweet-tasting proteins

Introduction

The sense of taste provides humans with valuable information for distinguishing the property or quality of food. Although most substances that elicit taste are low–molecular weight compounds, five proteins, that is, thaumatin (van der Wel and Loeve, 1972), monellin (Morris and Cagan, 1972; van der Wel, 1972), mabinlin (Liu *et al.*, 1993), curculin (Yamashita *et al.*, 1990), and brazzein (Ming and Hellekant, 1994), were identified as sweet-tasting proteins and miraculin (Kurihara and Beidler, 1968), curculin, and neoculin (Shirasuka *et al.*, 2004; Suzuki *et al.*, 2004) were known as taste-modifying proteins. Although the three-dimensional structures of some of the sweet-tasting proteins, thaumatin (Ogata *et al.*, 1992; Ko *et al.*, 1994), monellin (Somoza *et al.*, 1993; Lee *et al.*, 1999; Spadaccini *et al.*, 2001), and brazzein (Caldwell *et al.*, 1998), are known, no common feature has been identified in the tertiary structure or in the amino acid sequence. Most sweet proteins, thaumatin and monellin in particular, are basic proteins and have high isoelectric points. An exception is a small sweet-tasting protein, brazzein, which has an isoelectric point of 5.4 (Ming and Hellekant, 1994). To identify the sweetness determinants for sweet-tasting proteins, studies involving systematic chemical modification and mutational analysis would provide valuable information about the mechanism for the elicitation of sweetness in proteins as well as for the interaction between sweet-tasting proteins and its putative receptors.

Besides the five sweet-tasting proteins, hen egg white lysozyme, representative of the chicken-type lysozyme, also elicits a sweet taste (Masuda *et al.*, 2001). Lysozyme can be obtained from egg white in large quantities by a simple purification method and can be used as a sweetener. Lysozyme is one of the most thoroughly characterized proteins, consists of a single amino acid chain of 129 residues, and has a molecular mass of 14.5 kDa. In its three-dimensional structure, lysozyme is composed of two domains. Six subsites make up the active site for the lysis of cell walls, and Glu35 and Asp52 are identified as catalytic residues (Blake *et al.*, 1967; Phillips, 1967; Imoto *et al.*, 1972; P. Jollès and J. Jollès, 1984; Smith *et al.*, 1993) (Figure 1). Thus, intensive research on the structure–enzyme activity of lysozyme has been performed and reported, whereas a detailed explanation of the sweetness of lysozyme is yet unknown. We have previously demonstrated that the sweetness of lysozyme was independent of its enzymatic activity, as shown by the chemical modification of the carboxyl groups of the catalytic residues (Masuda *et al.*, 2001). Furthermore, lysozyme from goose egg white, which is

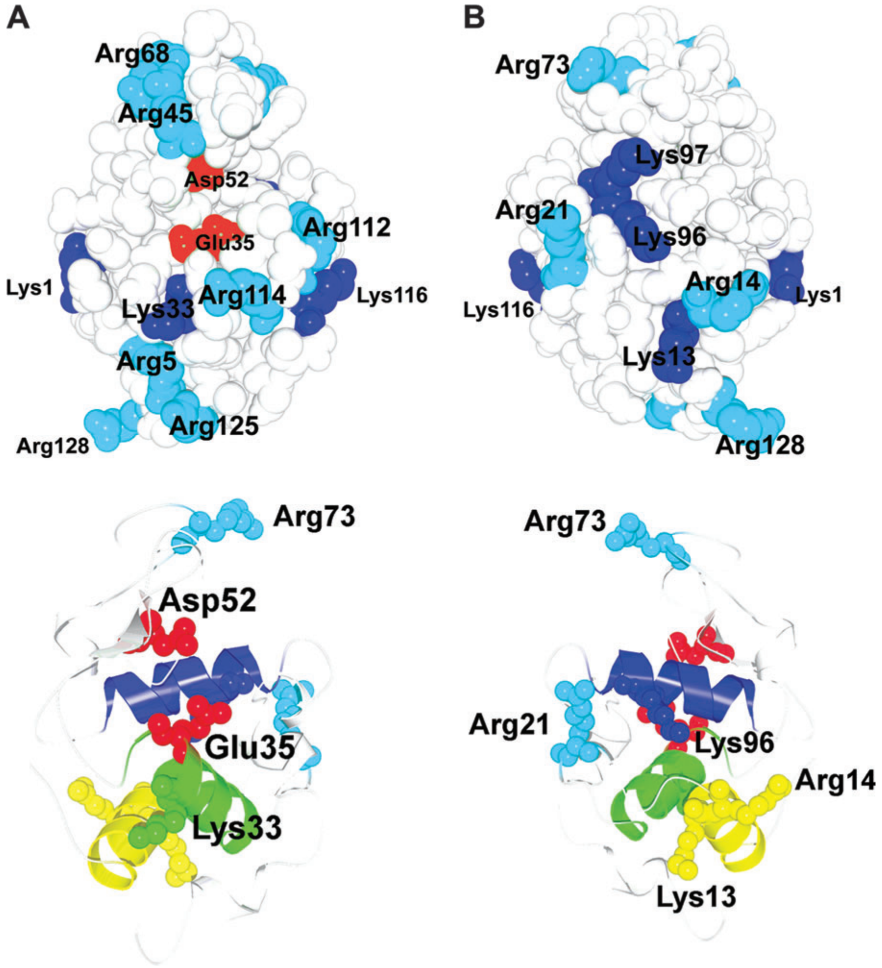

Figure 1 Three-dimensional structure of hen egg white lysozyme. Schematic representation of lysozyme in two different orientations. **(A)** Side of catalytic regions of lysozyme. **(B)** Different view of side A, rotated 180° about a vertical axis. The figure was drawn using data for lysozyme (PDB: 193L) and expressed as a space-filled model (upper) and a ribbon model (lower). The lysine residues are indicated in blue and the arginine residues are indicated in cyan. Catalytic residues, Glu35 and Asp52, are indicated in red.

classified as goose-type lysozyme and differs from chicken-type lysozyme in structure and molecular mass (20.5 kDa), similarly elicited a sweet taste (Masuda *et al.*, 2001). Although most sweet-tasting proteins including both types of lysozymes are basic proteins, it is unclear whether the surface positive charge of sweet-tasting proteins is required for elicitation of sweetness.

To clarify the importance of the positively charged regions on the protein surface, we have previously performed charge-specific chemical modification of lysine residues (Masuda *et al.*, 2005b). The results demonstrated that the threshold (T) values were not changed by modification with fewer than two residues, whereas the T values significantly increased with tetra-acetylation and tri-phosphopyridoxylation, indicating that basicity at the side chain of the lysine residues plays a significant role in the elicitation of the sweetness of lysozyme. However, in the previous study, it has not been completely determined which of the lysine residues were chemically modified. There are two possibilities for the effects of lysine residues. One is that the specific lysine residue is involved in sweetness and the T value would increase when its specific residue was modified. The other is that no specific lysine residues are involved in sweetness and the basicity of the protein is important for sweetness. If the latter were the important factor, the other positively charged residue would be involved in the elicitation of the sweetness of lysozyme. Lysozyme contains 6 lysine residues and 11 arginine residues. Thus, lysozyme consists of about twice the number of positively charged arginine residues as lysine residues. It is unclear whether the basicity of the arginine residues plays a significant role in the elicitation of sweetness, and the role of basicity in the arginine residue in eliciting sweetness in lysozyme should be clarified. In efforts to ascertain them, investigations using site-directed mutagenesis in combination with human sensory analysis have provided valuable information for elucidating the structure–sweetness relationship as well as the basicity–sweetness relationship in lysozyme.

Here, we prepared lysozyme mutants by *Pichia* systems to evaluate the T values of sweetness as sensed by humans. To reveal the critical positively charged residues for eliciting lysozyme sweetness and to investigate the basicity–sweetness relationship in lysozyme, the substitution of lysine residues with alanine or arginine residues and the substitution of arginine residues with alanine residues were performed. From this investigation, we identified the consensus properties among the sweet-tasting proteins based on multipoint interaction observed in protein–protein interaction.

Materials and methods

Materials, strains, and plasmids

Escherichia coli strain Top 10F (*recA*, *endA1*, *HsdR*), *Pichia pastoris* strain X-33 (wild type), and pPIC6α expression vector, which contains both *Saccharomyces cerevisiae* pre–pro α-mating factor secretion signal and blasticidin resistance gene (*bsd*), were obtained from Invitrogen (Groningen, The Netherlands). XL_1-Blue supercompetent cells and *PfuTurbo* DNA polymerase were obtained from Stratagene (La Jolla, CA). Restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA). Yeast extract, bacto peptone, bacto tryptone, bacto agar, yeast nitrogen base (YNB) without amino acids, and casamino acid were obtained from Difco Laboratories (Detroit, MI). CM-Toyopearl 650M was obtained from Tosoh Co. (Tokyo, Japan). *Micrococcus luteus* was purchased from Sigma (St Louis, MO). Blasticidin hydrochloride was obtained from Kaken Pharmaceutical Co., Ltd (Tokyo, Japan). All other chemicals were of guaranteed reagent grade for biochemical use.

Medium

Escherichia coli cells were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) or low-salt LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Low-salt LB medium was used for selection of transformants by blasticidin. *Pichia pastoris* was grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or a buffered minimal glycerol (BMG) consisting of 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol. Induction of recombinant protein was performed with a buffered methanol medium (BMM) that was identical to BMG except that it contained 1% casamino acids and 0.5% methanol instead of glycerol.

Site-directed mutagenesis

The PCR2.1-TOPO vector carrying a mature lysozyme gene (named TOPO-LYS) was constructed as described previously and used as the template for mutagenesis (Masuda *et al.*, 2005a). Polymerase chain reaction (PCR) was performed by using the high fidelity of *PfuTurbo* DNA polymerase with two synthetic oligonucleotide primers containing the desired mutation. Double- or triple-site mutants were generated in the same way using the mutated lysozyme genes as templates. After the PCR product was digested with *Dpn*I, the amplified nicked vector incorporating the desired mutations was transformed to XL_1-Blue supercompetent cells. The mutations were confirmed by DNA sequencing using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK).

Construction of lysozyme expression vector and transformants

The plasmid DNA of lysozyme containing the desired mutation was digested by *Xho*I and *Not*I. The resulting fragment (approximately 0.4 kb) was gel purified using a QIAquick Gel Extraction Kit (QIAGEN, GmbH, Hilden, Germany) and ligated into the yeast shuttle vector pPIC6α that had also been digested with the same enzymes. The ligated plasmid was transformed into XL_1-Blue supercompetent cells. DNA sequencing was performed to confirm that the mutated fragment was correctly cloned in the frame of the signal sequence of pPIC6α without insertion or deletion. The pPIC6α vector carrying the desired mutations was linearized by *Pme*I and transformed into the *Pichia* X-33 strain by electroporation (Electroporator 2510, Eppendorf, Hinz GmbH, Hamburg, Germany). Multicopy transformants were selected on the basis of blasticidin resistance as described previously (Masuda *et al.*, 2005a).

Expression of lysozyme mutants

Small-scale expression of lysozyme mutants was performed as follows. Blasticidin-resistant transformants were grown in 200 ml of BMG medium at 28°C to an A_{600} of 2–6. Cells were collected by centrifugation (1500 × *g*) and resuspended in BMM to an A_{600} of 1 and cultivated for 5 days with the addition of methanol at 24-h intervals. After 5 days, the supernatants were collected by centrifugation (4000 × *g*) and the expressed lysozyme in the supernatant was determined by lytic activity as described later. Large-scale expression of lysozyme mutants was achieved in a 3-l fermenter. The temperature was maintained at 28°C with a heating unit (Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and a refrigerated circulating bath (RTE9, Thermo NESLAB, Newington, NH). The pH was adjusted to 5.0 by the addition of 25% ammonium hydroxide. The concentration of dissolved oxygen was maintained at above 20% by use of an oxygen supply unit (MOS-25, Tokyo Rikakikai Co. Ltd). Detailed conditions were described previously (Masuda *et al.*, 2005a). Purification of the lysozyme mutants was performed using cation exchange chromatography, as described subsequently.

Purification of lysozyme mutants

Purification of the lysozyme mutants was performed by cation exchange chromatography as described previously (Masuda *et al.*, 2005a). In brief, the supernatant was diluted fivefold with distilled, deionized water and directly applied on a CM-Toyopearl 650M column (2.2 × 20 cm) previously equilibrated with 50 mM Tris–HCl buffer (pH 7.4). The bound lysozyme was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer using a low-pressure gradientor LPG-1000 (Tokyo Rikakikai Co. Ltd). Protein purity was identified by native polyacrylamide gel electrophoresis (PAGE) analysis as described subsequently. The protein concentration was determined spectrophotometrically from the absorbance at 280 nm ($E_{1\%}$ = 26.4, Kumagai and Miura, 1989) and further quantified by a densitometer using 0.5 μg of lysozyme as a standard (Atto Co., Tokyo, Japan). The purified lysozyme fractions were combined and stored at 4°C as a precipitate with ammonium sulfate until use.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS)–PAGE was performed in 13.5% gel according to the method of Laemmli (1970). Native PAGE was performed using a system of a 15% homogeneous native polyacrylamide gel for the basic protein (Reisfeld *et al.*, 1962). The gels were stained with Coomassie Brilliant Blue R-250.

N-Terminal sequence analysis

N-Terminal sequence analysis was performed in a gas-phase sequencer (Procise 490, PE Applied Biosystems) using the Edman degradation method. Purified lysozyme was directly spotted on the polyvinylidene difluoride (PVDF) membrane and inserted in the analysis cuvette.

Measurement of lytic activity of lysozyme

Enzymatic activity of lysozyme was determined by measuring the clearing of the turbidity of a suspension of *M. luteus* (substrate solution) at 450 nm (Masuda *et al.*, 2001). The decrease in absorbance was monitored by a Shimadzu UV–VIS spectrophotometer (UVmini-1240, Shimadzu Co., Kyoto, Japan). A decrease in absorbance of 0.001 per minute was defined as 1 unit of enzymatic activity.

Fluorescence measurement

Fluorescence spectra of recombinant and mutant lysozymes were recorded in 10 mM sodium acetate buffer, pH 5.5, on a fluorescence spectrophotometer (F-3000; Hitachi, Ltd, Tokyo, Japan) with an excitation wavelength of 280 nm; the emission wavelength ranged from 300 to 450 nm. The excitation and the emission band passes were set at 5 nm. The protein concentration was adjusted to 2.5 μM.

Modification of arginine residues by 1,2-cyclohexanedione

Modification of arginine residues was performed as described elsewhere (Patthy and Smith, 1975a,b; Suckau *et al.*, 1992). 1,2-Cyclohexanedione (CHD) was added to lysozyme solution (140 μM) in 0.2 M sodium borate buffer, pH 9.0. Reactions were allowed to continue for 120 min at 37°C and were then terminated by dilution with 5% acetic acid. Subsequently, samples were dialyzed against 1% acetic acid and 50 mM acetate buffer, pH 4.0. These crude CHD–lysozymes were further purified by CM ion exchange column chromatography. The column was equilibrated with 50 mM sodium acetate buffer (pH 4.0), and the protein was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer.

Sensory analysis of lysozyme variants

The sweetness threshold of the samples was evaluated by means of a triangle test for taste threshold (Kaneko and Kitabatake, 2001; Masuda *et al.*, 2005b). Six subjects (ages 22–52) participated. Three paper cups, one containing 5 ml of protein solution and the others containing 5 ml of distilled water, were given to the panel, who was asked to indicate which cup had the taste-eliciting solution. The sample solutions were provided in the order of concentration from the lowest (1 μM) to the highest (30 μM) level of sweetness. Sweetness intensity was evaluated on a scale from 0 to 5 using a scaling bar. The value 0 means no taste detected from the test solution; the value 1 means that the sample solution elicited some taste stimulation. The value 2 represents the concentration at which the panel member detected sweetness from the sample solution. That is, the T value of sweetness is the concentration giving the value 2. The T values were averaged and analyzed with one-way analysis of variance or with the Kruskal–Wallis test on ranks for nonparametric data, as described previously (Masuda *et al.*, 2005b). A *post hoc* test was performed by Fisher's least significant differences test. A $P < 0.05$ value was considered as a significant difference in the statistical analysis.

Schematic representation of sweet-tasting proteins

Molecular model of sweet-tasting proteins was prepared by the MolFeat program using Protein Data Bank (PDB) data for lysozyme (PDB: 193L), thaumatin (PDB: 1THW), monellin (PDB: 4MON), and brazzein (PDB: 1BRZ).

Results

Construction of lysozyme expression vector

To avoid undesired mutations, mutation was performed on the TOPO-LYS vector. After confirmation of the introduction of the desired mutation, the vector was digested by *Xho*I and *Not*I, and the resulting fragments were cloned back to the yeast shuttle vector pPIC6α (Figure 2).

Production and properties of the lysozyme variants

Expression of recombinant lysozyme was performed by using a flask or a fermenter. Recombinant lysozyme in culture supernatant was purified by CM ion exchange chromatography and approximately 400 mg/l of recombinant lysozyme

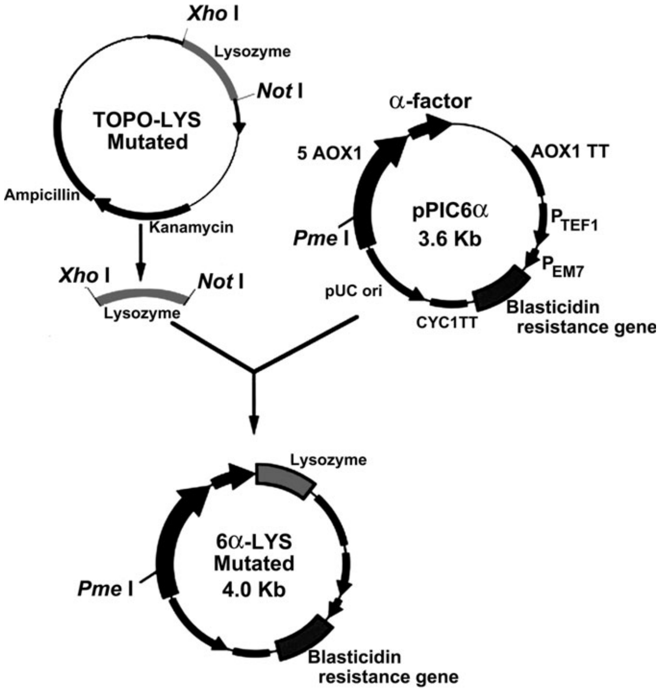

Figure 2 Ligation of the lysozyme gene with yeast shuttle vector pPIC6α. The lysozyme gene predigested with *Xho*I and *Not*I was ligated into the *Xho*I and *Not*I sites of yeast shuttle vector pPIC6α.

was obtained. The recombinant lysozyme was purified to homogeneity as evidenced by SDS-PAGE and native PAGE, giving a single band with a similar molecular size and a similar charge property to that of egg white lysozyme. The N-terminal sequence of the recombinant lysozyme was investigated by a gas-phase sequencer (Procise 490, PE Applied Biosystems) and was identical to that of egg white lysozyme, indicating correct processing at the *Kex*2 cleavage site. The T value of sweetness of recombinant lysozyme was around 7.0 μM, which is almost the same value as that of egg white lysozyme. In addition, enzymatic activity against *M. luteus* was also the same as that of egg white lysozyme. These results mean that the *Pichia* system was able to generate enough recombinant lysozyme with correct conformation to perform sensory analysis, which requires a large sample volume. In addition to recombinant lysozyme, mutants of lysozyme were produced by the *Pichia* system and purified as previously described (Masuda *et al.*, 2005a). The purity of the mutant lysozyme derivatives obtained after purification was checked by native PAGE, and a single band was obtained. The mobility of the band decreased if Lys or Arg was substituted with Ala, but it did not change if Lys was substituted with Arg (Figure 3), indicating that charge-specific mutation could be introduced into a lysozyme molecule. Fluorescence spectra of recombinant and mutant lysozymes in buffer are shown in Figure 4. The fluorescence spectrum of each mutant was similar to that of recombinant lysozyme and was different from that of recombinant lysozyme in 6 M guanidine hydrochloride. These results suggest that most of the mutations did not lead to an alteration in the microenvironment of tryptophan residues and did not induce gross conformational changes of the lysozyme molecule. From these results, we also assumed that all the mutants had a similar structure to that of recombinant lysozyme and that the effect of mutation on the sweetness of lysozyme might be induced by a minor local structural change involving the replacement of a side chain of an amino acid residue.

Basicity at Lys13 and Lys96 is required for lysozyme sweetness

Six lysine residues (Lys1, Lys13, Lys33, Lys96, Lys97, and Lys116) occur in a lysozyme molecule. The side chains of Lys1, Lys13, and Lys97 project over the surface of the lysozyme molecule, and some, but not all, of the side chains of Lys33, Lys96, and Lys116 are buried (Imoto *et al.*, 1972). The T values of the sweetness of the lysozyme mutants (Lys to Ala) are summarized in Table 1. The single mutation of Lys96Ala causes a significant increase in the threshold, from 6.7 to 18.3 μM, that is, the sweetness was reduced to about one-third of that of recombinant lysozyme. This means that Lys96 is involved in the elicitation of the sweetness of lysozyme. A previous chemical modification study suggested that acetylation and phosphopyridoxylation of less than two lysine residues resulted in no significant effects of sweetness of lysozyme (Masuda *et al.*, 2005b). The results in this study clearly showed that a specific lysine residue involved in sweetness. Since the T value of a double mutant of continuous Lys96Ala-Lys97Ala was 16.7 μM, no significant difference in the T values was observed between the single mutant Lys96Ala and the double mutant Lys96Ala-Lys97Ala. These results showed that Lys96 plays a significant role in the elicitation of lysozyme sweetness, whereas Lys97 does not contribute to the elicitation of lysozyme sweetness. This implication was further confirmed by the result that the T value of double mutant Lys33Ala-Lys97Ala is 5.8 μM and almost the same as that of the recombinant, indicating that neither Lys33 nor Lys97 is responsible for the elicitation of the sweetness of lysozyme. These two lysine residues are exposed to the surface of the lysozyme molecule and are believed to the most reactive residues in chemical modifications (Suckau *et al.*, 1992). This result means that two lysine residues, Lys33 and Lys97, might be chemically modified and modification at these residues did not influence the sweetness of lysozyme. The effects of mutation of the other lysine residues, Lys1, Lys13, and Lys116 were also investigated. The T value of a single mutation of N-terminal lysine residue to alanine residues, Lys1Ala, was similar to that of the recombinant. The T value of the double mutant of Lys116Ala-Arg125Ala was also similar to that of the recombinant. These findings indicate that neither Lys1 nor Lys116 is involved in the elicitation of the sweetness of lysozyme. In contrast, the T value of Lys13Ala was significantly

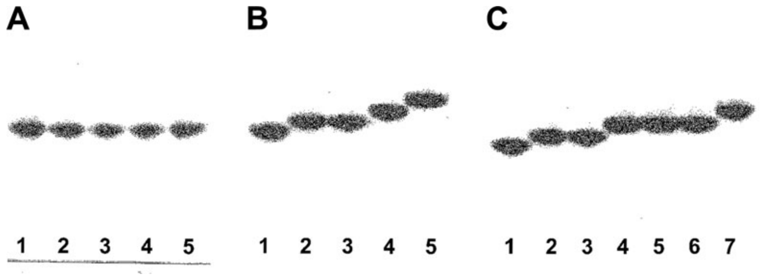

Figure 3 Native PAGE analysis of recombinant lysozyme variants. Native PAGE was performed on 15% gel and stained with Coomassie Brilliant Blue. **(A)** Lys to Arg mutants: lane 1, egg white lysozyme; lane 2, Lys1Arg; lane 3, Lys13Arg; lane 4, Lys33Arg; lane 5, Lys96Arg. **(B)** Lys to Ala mutants: lane 1, egg white lysozyme; lane 2, Lys1Ala; lane 3, Lys13Ala; lane 4, Lys1Ala-Lys13Ala; lane 5, Lys1Ala-Lys13Ala-Lys33Ala. **(C)** Arg to Ala mutants: lane 1, egg white lysozyme; lane 2, Arg14Ala; lane 3, Arg21Ala; lane 4, Arg14Ala-Arg21Ala; lane 5, Arg14Ala-Arg73Ala; lane 6, Arg21Ala-Arg73Ala; lane 7, Arg14Ala-Arg21Ala-Arg73Ala.

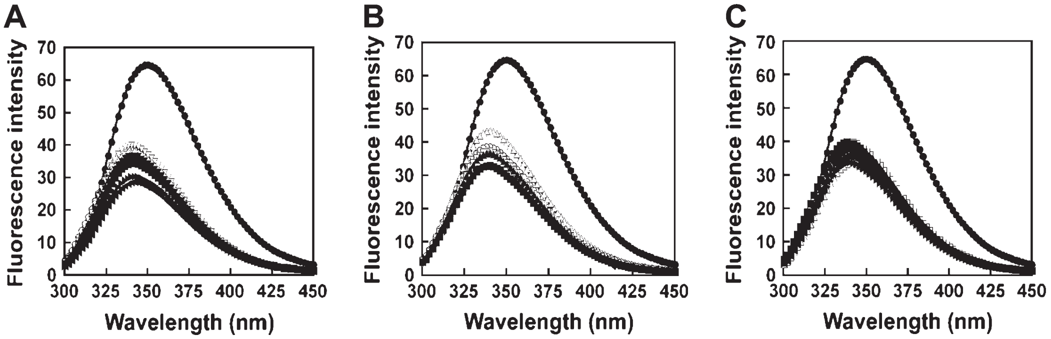

Figure 4 Fluorescence spectra of mutant lysozymes. Fluorescence spectra of mutant lysozymes were recorded in 10 mM sodium acetate buffer, pH 5.5, on a fluorescence spectrophotometer with an excitation wavelength of 280 nm and an emission wavelength ranging from 300 to 450 nm. **(A)** Lys to Arg mutants. **(B)** Lys to Ala mutants. **(C)** Arg to Ala mutants. Recombinant lysozyme in 10 mM sodium acetate buffer, pH 5.5 (○), and recombinant lysozyme in 6 M guanidine hydrochloride (●).

increased to 16.0 μM, indicating that Lys13 plays an important role in the elicitation of the sweetness of lysozyme. The T value of double mutant Lys1Ala-Lys13Ala was similar to that of Lys13Ala. The T value of triple mutant Lys1Ala-Lys13Ala-Lys33Ala was 15.0 μM, which is also similar to that of Lys13Ala. These results suggest that neither Lys1 nor Lys33 is responsible for lysozyme sweetness and that only Lys13 is involved in the elicitation of lysozyme sweetness. Although the expression of Lys13Ala-Lys96Ala was attempted to clarify the effects of mutation of two critical lysine residues, double mutant Lys13Ala-Lys96Ala could not be obtained by *Pichia* systems.

Basicity of guanidino groups as well as ε-amino groups is significant for lysozyme sweetness

To clarify whether the structural features or the positive charges in lysine residues, that is, the length and/or shapes of the side chain are essential for its sweetness, mutation from lysine residue to arginine residue was performed. Since mutation Lys13Ala or Lys96Ala causes significant reduction of lysozyme sweetness, the effects of the mutations of Lys13Arg and Lys96Arg on the T values were investigated. The results indicated that the T values as well as the enzymatic activity of the mutants of Lys96Arg and Lys13Arg were not different from that of recombinant lysozyme. These results indicate that the basicity of the guanidino groups of arginine residues as well as the ε-amino groups of lysine residues in these regions is also available for the elicitation of sweetness. As expected, mutation from lysine residue to arginine residue at the lysine residues that are not involved in sweetness resulted in no significant difference in the T values or enzymatic activity (Table 2). Since the p*K*a value of the arginine residue is slightly higher than that of the lysine residue, the basicity of the protein surface would be increased. To clarify these effects, di-, tri-, tetra-, and hexa-mutants (Lys to Arg) were generated. The results showed that no significant change of T values of sweetness was observed even if all lysine residues are substituted to arginine residues (Table 2).

Lysozyme sweetness is independent of its enzymatic activity

The enzymatic activities of lysozyme mutants (Lys to Ala) ranged from 48% to 97% of that of egg white lysozyme (Table 1). The enzymatic activities decreased as the number of mutated lysine residues increased. In particular, the enzymatic activity of Lys96Ala-Lys97Ala and Lys1Ala-Lys13Ala-Lys33Ala was 49% and 48% of that of egg white lysozyme,

Table 1 T values of sweetness and enzymatic activity of lysozyme mutants (Lys to Ala)

	T value[a]	Increase in T value[b]	Enzymatic activity[c]	Relative sweetness[d]
Recombinant	6.7 ± 2.6	—	102	750
K96A	18.3 ± 6.2**	11.6	97	270
K96AK97A	16.7 ± 5.7**	10.0	49	300
K33AK97A	5.8 ± 1.9	−0.9	93	860
K1A	9.2 ± 1.9	2.5	82	540
K116AR125A	7.5 ± 2.5	0.8	71	670
K13A	16.0 ± 8.0**	9.3	92	310
K1AK13A	14.7 ± 5.8**	8.0	62	340
K1AK13AK33A	15.0 ± 6.3**	8.3	48	330
Native	7.0 ± 3.1	0.3	100	710

[a] T value of sweetness is observed with sensory analysis and expressed as mean ± SD.
[b] Increase in T value is the difference of T value from recombinant lysozyme (6.7 μM).
[c] Lytic activity is determined using *Micrococcus luteus* as a substrate and is indicated as relative activity by taking the activity of native lysozyme to be 100.
[d] Relative sweetness was determined by (T value of sucrose 5 mM)/(T value of sample).
$^{**}P < 0.01$.

respectively, suggesting that the positive charges of the side chains of the lysine residues influence lytic activity through electrostatic interaction. We have previously shown that acetylation and phosphopyridoxylation of lysozyme resulted in progressive diminution of sweetness intensity as well as enzymatic activity against *M. luteus* when the acetylated or phosphopyridoxylated groups were introduced into lysine residues (Masuda *et al.*, 2005b). Although the positive charges of the lysine residues influence the lytic activity of lysozyme, mutations Lys13Ala and Lys96Ala affected the sweetness of lysozyme but no significant decrease in enzymatic activity was observed in comparison to that of egg white lysozyme. These results show that the lytic activity of lysozyme is independent of lysozyme sweetness. Of the six lysine residues, two specific lysine residues, Lys13 and Lys96, play a significant role in lysozyme sweetness, whereas these two lysine residues do not contribute to lytic activity. In the three-dimensional structure, these two lysine residues are located at the opposite sides of the catalytic cleft of the lysozyme molecule (Figure 1). It is noted that the sweetness T value of tetra-acetylated lysozyme is 15.0 μM, which is similar to that of single mutation, Lys13Ala or Lys96Ala. It is assumed that the acetylated lysine residues of tetra-acetylated lysozyme would include one of the critical sweetness determinant residues of Lys13 or Lys96 and that the positive charges at the Lys13 and Lys96 positions of the lysozyme molecule are important for the elicitation of lysozyme sweetness.

Table 2 T values of sweetness and enzymatic activity of lysozyme mutants (Lys to Arg)

	T value[a]	Increase in T value[b]	Enzymatic activity[c]	Relative sweetness[d]
Recombinant	6.7 ± 2.6	—	102	750
K1R	9.3 ± 2.6	2.6	107	540
K13R	8.3 ± 2.4	1.6	102	600
K96R	5.8 ± 1.9	−0.9	102	860
K116R	7.5 ± 3.5	0.8	105	670
K33R	8.3 ± 2.4	1.6	98	600
K96RK97R	5.3 ± 1.2	−1.4	98	940
K33RK96RK97R	8.3 ± 2.4	1.6	95	600
K96RK97RK116R	8.3 ± 2.4	1.6	100	600
K1RK13RK96RK97R	7.5 ± 3.5	0.8	95	670
K1RK13RK33RK96RK97RK116R	7.0 ± 4.3	0.3	95	710
Native	7.0 ± 3.1	0.3	100	710

[a] T value of sweetness is observed with sensory analysis and expressed as mean ± SD.
[b] Increase in T value is the difference of T value from recombinant lysozyme (6.7 μM).
[c] Lytic activity is determined using *Micrococcus luteus* as a substrate and is indicated as relative activity by taking the activity of native lysozyme to be 100.
[d] Relative sweetness was determined by (T value of sucrose 5 mM)/(T value of sample).

Arg14, Arg21, and Arg73 located on the same side of a lysozyme molecule as that of Lys13 and Lys96 also play a significant role in lysozyme sweetness

Chemical modification and mutation studies of lysine residues in lysozyme showed that the basicity at two positively charged lysine residues, Lys13 and Lys96, is involved in lysozyme sweetness. To consider the effects of other positively charged residues, we attempted to substitute arginine residues with alanine residues. Although two lysine residues, Lys13 and Lys96, which are responsible for lysozyme sweetness, are situated at opposite sides of the catalytic cleft on the lysozyme molecule, three arginine residues, Arg14, Arg21, and Arg73, are located on the same side formed by two critical lysine residues (Figure 1). Single mutations Arg14Ala and Arg21Ala raised the T values of lysozyme sweetness slightly to 14.2 and 13.3 μM, respectively, with significance $P < 0.05$ (Table 3). However, the mutation of Arg73Ala led to a significant increase in the T value to 18.3 μM ($P < 0.01$) (Table 3). These results indicated that three Arg residues, Arg14, Arg21, and Arg73, contribute to lysozyme sweetness and that Arg73 is the most significant of the three. The enzymatic activity of Arg14Ala was slightly lower than that of Arg21Ala and Arg73Ala. These results suggest that the

Table 3 T values of sweetness and enzymatic activity of lysozyme mutants

	T value[a]	Increase in T value[b]	Enzymatic activity[c]	Relative sweetness[d]
Recombinant	6.7 ± 2.6	—	102	750
R14A	14.2 ± 6.1*	7.5	80	350
R21A	13.3 ± 4.7*	6.6	98	380
R73A	18.3 ± 3.7**	11.6	98	270
R45A	13.0 ± 6.0	6.3	80	380
R68A	6.7 ± 2.4	0.0	95	750
R45AR68A	14.0 ± 4.9*	7.3	80	360
K116AR125A	7.5 ± 2.5	0.8	71	670
Native	7.0 ± 3.1	0.3	100	710

[a] T value of sweetness is observed with sensory analysis and expressed as mean ± SD.

[b] Increase in T value is the difference of T value from recombinant lysozyme (6.7 μM).

[c] Lytic activity is determined using *Micrococcus luteus* as a substrate and is indicated as relative activity by taking the activity of native lysozyme to be 100.

[d] Relative sweetness was determined by (T value of sucrose 5 mM)/(T value of sample).

$*P < 0.05$; $**P < 0.01$.

Figure 5 Native PAGE analysis of CHD–lysozymes. Native PAGE was performed on 15% gel and stained with Coomassie Brilliant Blue. Lane 1, egg white lysozyme; lane 2, CHD–lysozyme with three modified Arg residues; lane 3, CHD–lysozyme with two modified Arg residues

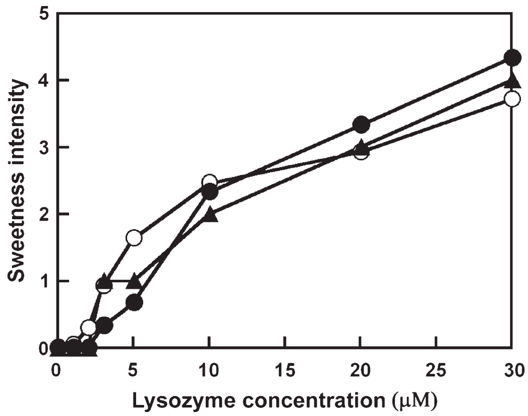

Figure 6 T values of sweetness of CHD–lysozymes. Sweetness intensity of CHD–lysozymes plotted against lysozyme concentration. Egg white lysozyme (○), CHD–lysozyme with two modified Arg residues (●), and CHD–lysozyme with three modified Arg residues (▲).

mutation of Arg14Ala would cause subtle conformational changes of the lysozyme molecule (Table 3).

Arginine residues located on a side different from that related to the elicitation of lysozyme sweetness might not play a significant role in lysozyme sweetness

The effects of other arginine residues located on a side different from that related to the elicitation of lysozyme sweetness were investigated. First, two arginine residues, Arg45 and Arg68, were investigated. These two residues are located in a different loop and form a ridge on the surface of the lysozyme molecule (Figure 1). Single mutation of Arg68Ala did not influence the T value of sweetness. Mutation of Arg45Ala slightly increased the T value with no significance (Table 3). The double mutant of Arg45Ala-Arg68Ala gave a T value similar to that of Arg45Ala, indicating that these two arginine residues play a minor role in lysozyme sweetness (Table 3). Chemical modification of arginine residues by CHD resulted in a mixture of lysozyme molecules of which two or three arginine residues were modified (Figure 5). Further purification was performed by a CM ion exchange column. Sensory analysis indicated that these CHD-modified lysozymes exhibited the same sweetness as that of unmodified lysozyme (Figure 6). It was reported that modification of the arginine residues by CHD occurred selectively in Arg5 and Arg125 (Suckau *et al.*, 1992). Arg5 and Arg125 are each located on different sides of the five critical sweetness determinants (Figure 1), indicating that these residues are not involved in lysozyme sweetness. It was partly confirmed by the results of the previous mutation, indicating that the T value of Lys116Ala-Arg125Ala is no different from that of recombinant lysozyme (Table 3).

Effects of double and triple mutations on lysozyme sweetness

The effects of double and triple mutation of lysine and/or arginine residues on lysozyme sweetness were investigated. The T value of Arg21Ala-Arg73Ala is 25.0 μM, which is higher than that of recombinant lysozyme (6.7 μM) by 18.3 μM (increase in T value; Table 4). This value is almost the additive sum of the effect of each residue. Because the T value of Arg21Ala and Arg73Ala is 13.3 and 18.3 μM, respectively, the increase in the T value from that of recombinant lysozyme is 6.6 and 11.6 μM, respectively. Therefore, the calculated T value of Arg21Ala-Arg73Ala is 24.9 μM (24.9 = 6.7 + 6.6 + 11.6), which is quite close to the observed value of 25.0 μM. It is strongly suggested that both Arg21 and Arg73 are directly involved in the elicitation of lysozyme sweetness and that each residue contributes to sweetness independently. The T value of Arg14Ala-Arg21Ala increased to 18.0 μM, which is slightly higher than that of Arg14Ala (14.2 μM) or Arg21Ala (13.3 μM). The calculated T value of Arg14Ala-Arg21Ala corresponds to 20.8 μM (20.8 = 6.7 + 7.5 + 6.6), which is slightly higher than the observed T value by 2.8 μM (Table 4).

In contrast, the T value of the double mutant Arg14Ala-Arg73Ala is 15.0 μM and is similar to that of Arg14Ala and Arg73Ala whose T value is 14.2 and 18.3 μM, respectively (Table 4). The calculated T value of Arg14Ala-Arg73Ala is 25.8 μM (25.8 = 6.7 + 7.5 + 11.6), which is significantly higher than the observed T value by 10.8 μM. These results indicate that the introduction of further mutation did not induce an increase in the T value, and effects for preventing the loss of sweetness were observed. It is assumed that the mutation of Arg14Ala might cause subtle conformational changes of the protein molecule and, with the combination of the mutation of Arg73Ala, might enhance the positive charge distribution on the surface of the protein molecule. Since Arg14 is located adjacent to Lys13, the distributions of positive charges around Lys13 and Arg14 might be affected. These rearrangements of positive charges could conveniently prevent a reduction in the sweetness of lysozyme. However, such effects have not been detected in relation to the triple mutation of Arg14Ala-Arg21Ala-Arg73Ala whose T value is more than 30 μM (Figure 7, Table 4).

Effects of two adjacent charged residues on lysozyme sweetness

To confirm the effects of the positive charges around Lys13 and Arg14, two triple mutants, Lys1Ala-Lys13Ala-Arg14Ala and Lys1Ala-Lys13Ala-Arg21Ala, were prepared. The T value of Lys1Ala-Lys13Ala-Arg14Ala was 20.8 μM (Table 4). The calculated T value of Lys1Ala-Lys13Ala-Arg14Ala was 26.0 μM, which is slightly higher than the observed T value by 5.2 μM (20.8 μM). These results indicate that the deletion of both positively charged residues, Lys13 and Arg14, resulted in an increase of the T value, that is, a reduction of sweetness. Since a significant difference in the T value was observed between Arg21Ala and Lys1Ala-Lys13Ala-Arg14Ala ($P = 0.0282$), it is likely that continuous positively charged residues Lys13 and Arg14 play a significant role in lysozyme sweetness.

In contrast, the T value of Lys1Ala-Lys13Ala-Arg21Ala was 15.0 μM (Table 4). The calculated T value of Lys1Ala-Lys13Ala-Arg21Ala was 25.1 μM, which is significantly higher than the observed T value by 10.1 μM (25.1 μM). This was similar to the result obtained by double mutation Arg14Ala-Arg73Ala.

Taking these findings together, five positively charged residues located at the opposite sides of the catalytic cleft of a lysozyme molecule are essential for lysozyme sweetness. Of the five charged residues, three charged residues, Arg73, Lys13, and Lys96, are particularly important for the elicitation of lysozyme sweetness; Arg14 and Arg21 might assist three charged residues.

Table 4 T values of sweetness and enzymatic activity of double and triple mutants of lysozyme

	T value[a]	Increase in T value[b]	Calculated T value[c]	Enzymatic activity[d]
Recombinant	6.7 ± 2.6	—	—	102
R21AR73A	25.0 ± 5.0**	18.3	24.9 (6.7 + 6.6 + 11.6)	80
R14AR21A	18.0 ± 4.0**	11.3	20.8 (6.7 + 7.5 + 6.6)	69
R14AR73A	15.0 ± 5.0**	8.3	25.8 (6.7 + 7.5 + 11.6)	78
R14AR21AR73A	>30	>23.3	32.4 (6.7 + 7.5 + 6.6 + 11.6)	48
R45AR68AR73A	26.7 ± 11.1**	20.0	24.6 (6.7 + 0 + 6.3 + 11.6)	53
K1AK13AR14A	20.8 ± 1.9**	14.1	26.0 (6.7 + 2.5 + 9.3 + 7.5)	66
K1AK13AR21A	15.0 ± 5.0**	8.3	25.1 (6.7 + 2.5 + 9.3 + 6.6)	65
Native	7.0 ± 3.1	0.3	—	100

[a] T value of sweetness is observed with sensory analysis and expressed as mean ± SD.
[b] Increase in T value is the difference of T value from recombinant lysozyme (6.7 μM).
[c] Calculated T value corresponds to the additive value of each single mutant.
[d] Lytic activity is determined using *Micrococcus luteus* as a substrate and is indicated as relative activity by taking the activity of native lysozyme to be 100.
**$P < 0.01$.

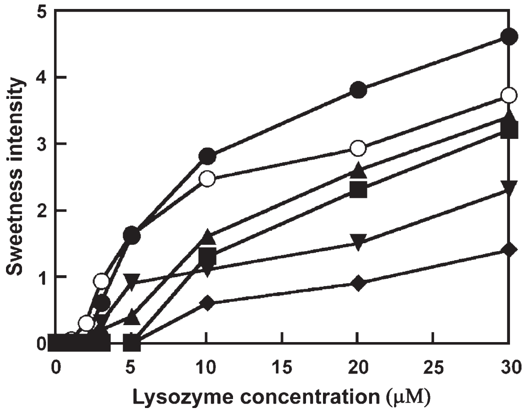

Figure 7 T values of sweetness of Arg to Ala mutants. The sweetness intensities of lysozyme derivatives are plotted against lysozyme concentration. Egg white lysozyme (○), recombinant lysozyme (●), Arg14Ala-Arg73Ala (▲), Arg14Ala-Arg21Ala (■), Arg21Ala-Arg73Ala (▼), and Arg14Ala-Arg21Ala-Arg73Ala (◆). Intensity value: 0 = no taste, 1 = taste stimulation, 2 = sweet taste, and 3 = potently sweet taste. Intensity value 2 corresponds to the T value of lysozyme sweetness.

Discussion

The critical residues for lysozyme sweetness are gathered together on a given surface area of a lysozyme molecule

Previous studies of the chemical modification of lysozyme have demonstrated that acetylation and phosphopyridoxylation of less than two lysine residues have no influence on lysozyme sweetness. Since the reactive residues among the six lysine residues are considered to be Lys97 and Lys33, mutation of Lys33Ala-Lys97Ala was performed. The T value of Lys33Ala-Lys97Ala was almost the same as that of recombinant lysozyme, indicating that these two residues are not involved in the elicitation of sweetness. However, mutation of Lys96Ala-Lys97Ala as well as Lys1Ala-Lys13Ala resulted in an increase of the T value compared to that of recombinant lysozyme. In addition, a single mutation showed that the basicity of the side chains of two lysine residues, that is, Lys13 and Lys96, plays a significant role in lysozyme sweetness. These results demonstrated that the basicity of a specific residue is involved in sweetness and that the T value increases when the specific residue is modified and changed. In the three-dimensional structure of lysozyme, Lys13 is located at the end of a helix A and Lys96 is in a helix C (Imoto *et al.*, 1972). The distance of the side chain of the ε-amino group between Lys13 and Lys96 is about 13 Å (Figure 8A). These two lysine residues occur on the same side of the molecule, which is located at the opposite sides of the catalytic cleft referred to as the R region. Although Lys96 and Lys97 are sequentially adjacent, the distance between these residues is only 9 Å, and both residues are located in the same R region, only Lys96 is involved in lysozyme sweetness. These results indicate that a strictly defined distribution of positive charges on the surface of the lysozyme molecule is needed to produce lysozyme sweetness. Lys1 and Lys116, lysine residues that are not required for sweetness, are located at the edge of the R region and are far from Lys13 and Lys96, ranging from 17 to 26 Å. Lys33 is located on the same side of the catalytic cleft referred to as the F region and opposite that of the R region and does not participate in lysozyme sweetness. These results demonstrate that only two out of the six lysine residues, that is, Lys13 and Lys96, are located on the same side and are required for the elicitation of lysozyme sweetness.

To confirm whether the lysine residue itself is important for the elicitation of lysozyme sweetness or if only basicity is required, mutation of lysine residues to arginine residues was performed. The T values of Lys13Arg and Lys96Arg are similar to that of recombinant lysozyme, showing that the ε-amino groups at the positions of Lys13 and Lys96 are replaceable by the guanidino groups without alteration of the T value. Although mutation from lysine to arginine residue causes conversion from the ε-amino group to the formation of a bulkier hydrophobic group of guanidino groups, the net surface charge distribution of the lysozyme molecule is maintained. This means that the positive charges at the positions of Lys13 and Lys96 of the lysozyme molecule are important for the elicitation of sweetness, whereas the size, shape, and length of the side chains of the residues are not strictly critical for sweetness. Although the p*K*a value of the side chain of the arginine residue is about 12.5 and is slightly higher than that of the lysine residue at 10.5, mutation from lysine to arginine residue would increase the protein basicity. However, the T values of mono-, di-, tri-, tetra-, and hexa-Lys to Arg mutants were almost identical to that of recombinant lysozyme. These results indicate that the sweetness of lysozyme could not simply be raised by the increase of the basicity from the lysine residue to the arginine residue. Taken together, it is assumed that the basicity formed by these two specific lysine residues, Lys13 and Lys96, which are separated but are on the same side of the molecule, would play a significant role in the elicitation of sweetness.

Since the importance of basicity at Lys13 and Lys96 for lysozyme sweetness has been clarified, the effects of arginine residues on lysozyme sweetness have been investigated. The alanine substitution of the arginine residues revealed that Arg14, Arg21, and Arg73 are important for lysozyme sweetness. In the three-dimensional structures of the lysozyme molecule, Arg14, Arg21, and Arg73 are located on the same side of the lysozyme molecule, referred to as the R region (Figure 1). The distances between the three arginine residues range from 17 to 29 Å (Figure 8A). The distances between each of the three critical arginine residues and each critical lysine residue range from about 11 to 34 Å. Thus, the regions formed by these five positively charged residues would gather in a given area on the surface of the protein and spread about 250 Å^2. These results suggest that positively charged arginine residues as well as lysine residues on the R regions play a significant role in lysozyme sweetness. It should be noted that

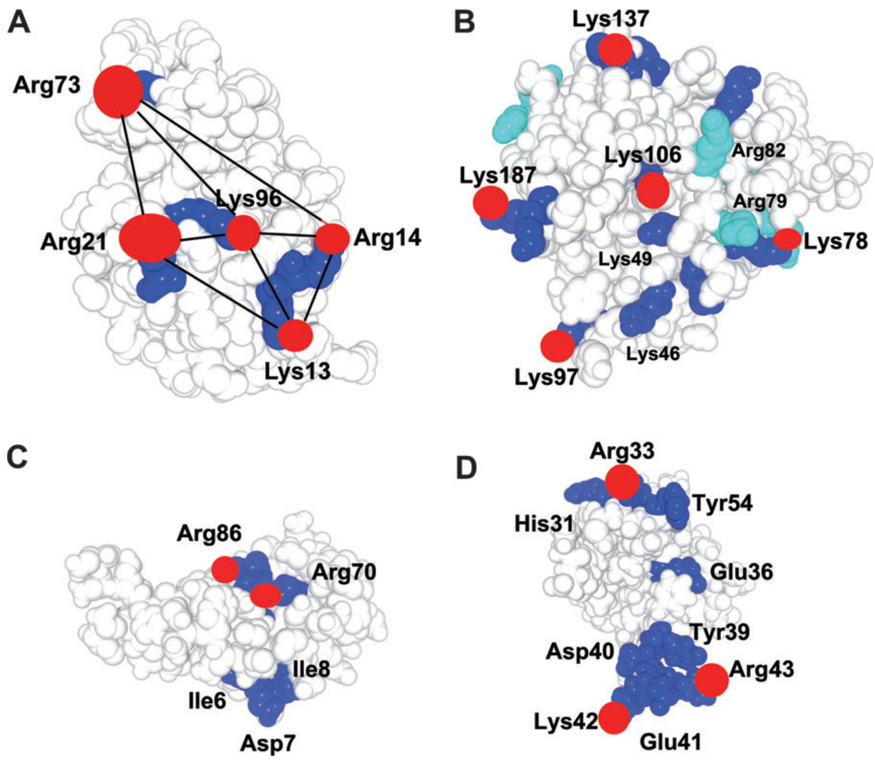

Figure 8 Sweetness determinants of sweet-tasting proteins. The figure was drawn using data for lysozyme (PDB: 193L) **(A)**, thaumatin (PDB: 1THW) **(B)**, monellin (PDB: 4MON) **(C)**, and brazzein (PDB: 1BRZ) **(D)** and expressed as a space-filled model. The critical residues for sweetness are indicated in blue, and the positively charged side chains are indicated in red.

the T values of two mutants, Arg14Ala and Arg21Ala, were significantly higher than the T value of recombinant lysozyme with significance $P < 0.05$. In addition, mutation of Arg73 to alanine residue led to a significant increase in the T value to 18.3 μM ($P < 0.01$), indicating that Arg73 is the critical residue for sweetness and that the contributions for the elicitation of sweetness of Arg14, Arg21, and Arg73 are not the same. It should also be noted that Lys13, Arg73, and Lys96 are more important than are Arg14 and Arg21 in this regard. It is likely that three critical residues, Arg73, Lys96, and Lys13, line up in a straight line on the R region of the surface of the lysozyme molecule (Figure 8A), while Arg14 and Arg21, which are located to the side of the straight line, might assist these three residues in the elicitation of sweetness. The regions formed by the five positively charged residues also contain negatively charged residues, Asp18, Asp87, and Asp101. We have previously performed chemical modification of Glu and Asp residues with glycine methyl ester and aminomethansulfonic acid and investigated the sweetness and enzymatic activity of lysozyme (Masuda *et al.*, 2001). Through these modifications, the enzymatic activity (lytic activity) of lysozyme was lost because catalytic Glu35, Asp52, and Asp101 were modified. It has been reported that 10 of 11 carboxyl residues in lysozyme are modified by carbodiimide reactions (Hoare and Koshland, 1966; Lin and Koshland, 1969). Since chemically modified lysozymes elicit a sweet taste in a manner similar to unmodified lysozyme, carboxyl residues including catalytic residues would not play a significant role in lysozyme sweetness.

T values of double mutant did not result from additive values from each single mutant

A large number of studies have been performed to identify the critical regions of sweet-tasting proteins (Kohmura *et al.*, 1992; Somoza *et al.*, 1995; Assadi-Porter *et al.*, 2000; Kaneko and Kitabatake, 2001; Jin *et al.*, 2003), while little has been studied regarding the effects of double and triple mutations of critical residues on sweetness. The double mutant, Arg21Ala-Arg73Ala, gave a T value of 25.0 μM, which is almost the same as the calculated T value (24.9 μM). The T values of double mutant Arg14Ala-Arg21Ala (18.0 μM) and triple mutant Lys1Ala-Lys13Ala-Arg14Ala (20.8 μM) were slightly lower than the calculated T values (20.8 and 26.0 μM) by 2.8 and 5.2 μM, respectively. In contrast, the T values of double mutant Arg14Ala-Arg73Ala (15.0 μM) and triple mutant Lys1Ala-Lys13Ala-Arg21Ala (15.0 μM) were significantly lower than the calculated T values (25.8 and 25.1 μM) by 10.8 and 10.1 μM, respectively. As expected from the calculated T value of each single mutant, mutations of the pairs of residues located on a relatively vertical line of the lysozyme molecule shown in Figure 8A, that is, Arg21-Arg73 or Lys13-Arg14, resulted in a reduction of sweetness. Conversely, mutations of the pairs of residues located on a relatively horizontal line in Figure 8A, that is, Arg14-Arg73 or Lys13-Arg21, resulted in a T value that was lower than the calculated T value. These results suggest that both Arg14 and Arg73 contribute to elicitation of sweetness, but when one of them was changed to an alanine residue, the effect of another residue might be changed. Since the enzymatic activity of Arg14Ala is 80% less than that of native lysozyme, mutation might cause subtle conformational changes of lysozyme around Lys13, which is adjacent to Arg14. It has been reported that Lys13 forms a salt bridge with Leu129 in three-dimensional structures (Imoto *et al.*, 1972), suggesting that the disruption of the salt bridge might induce a new positive charge distribution on the protein surface. This could occur as follows. When a lysozyme molecule interacts with a putative receptor on the taste cell, both Arg14 and Arg73 might interact weakly with a receptor. While one of them changes to an alanine residue, interaction of another residue, for example, Lys13, with a receptor might also change somewhat. Contribution of each Arg14 and Arg73 to sweetness would not necessarily be equal, and the increase of the T value by the mutations was not simply the result of additive T values from each single mutant. As is the case with the Arg14Ala-Arg73Ala mutation, the Lys1Ala-Lys13Ala-Arg21Ala mutation might induce the rearrangement of a positively charged environment on the surface of the protein molecule to prevent the loss of sweetness.

Considering these results, it could be demonstrated that basicity of the five charged residues is required for lysozyme sweetness and that combinations of basicity from Arg21-Arg73 and Lys13-Arg14 are particularly important for maintaining lysozyme sweetness. Since the distance of the side chains between Arg21 and Arg73 is approximately 16 Å, which is closer than that between Arg21 and Lys13 or Arg73 and Arg14, it is assumed that the basicity of both Arg21 and Arg73 might be incorporated with the elicitation of sweetness. Similarly, with Arg21-Arg73, the basicity from Lys13 and Arg14 is also important for maintaining lysozyme sweetness. It is assumed that the basicity of Lys13, Lys96, and Arg73 might directly influence the sweetness of lysozyme. The basicity of Arg14 and Arg21 might indirectly support the basicity around Lys13 or Arg73 to help prevent a reduction of sweetness.

Elicitation of sweetness of sweet-tasting proteins differed from that of small sweeteners

The T value of lysozyme sweetness is around 7 μM, which is about 700 times lesser than that of sucrose. Although the consensus AH-B-γ entity has been observed in small sweeteners (Shallenberger and Acree, 1967; Kier, 1972; Temussi *et al.*, 1978), no common features have been found among sweet-tasting proteins. Tancredi *et al.* (2004) have searched for the glucophores (sweet finger) that are observed in low–molecular mass sweeteners on the surface of sweet-tasting proteins and found possible mimicking parts of sweet-tasting proteins. They synthesized cyclic peptides consisting of these parts by a solid-phase technique. None of them elicited a sweet taste, suggesting that the mechanism of the elicitation of sweetness by proteins differs from that of low–molecular mass sweeteners. Chemical modification studies and mutation studies of thaumatin showed that Lys46, Lys67, Lys78, Lys97, Lys106, Lys137, and Lys187 are involved in sweetness (Kim and Weickmann, 1994; Kaneko and Kitabatake, 2001). These residues are separated in a range from 20 to 40 Å (Figure 8B). Site-directed mutagenesis of single-chain monellin and solid-phase peptide synthesis studies of monellin suggested that the surface patch formed by Ile6, Asp7, Ile8, and Gly9 as well as another surface patch formed by Arg86, Arg70, and Asp72 participate in the elicitation of sweetness (Ariyoshi and Kohmura, 1994; Somoza *et al.*, 1995). These two regions are separated in a range of approximately 11–25 Å (Figure 8C). A site-directed mutagenesis analysis of brazzein has suggested that residues 29–33, 36, and 39–43 as well as the C-terminal regions are responsible for the elicitation of sweetness (Jin *et al.*, 2003). The critical residues are separated by distances of 8–19 Å from each other (Figure 8D). Besides the three sweet-tasting proteins, the critical regions for the sweetness of lysozyme are separated as shown in Figure 8A and are gathered in a given area on the surface of the protein. As indicated earlier, charged residues at the surface of sweet-tasting proteins could play an important role in the elicitation of sweetness. It is likely that the mechanisms of the elicitation of sweetness might be different from those of low-mass sweeteners such as AH-B entity. A low T value of sweetness and a long-lasting aftertaste of sweet-tasting proteins compared to those of low–molecular mass sweeteners would be due to the large number of charged residues involved in eliciting sweetness.

The broadness and density of charged residues on a protein surface could play a significant role in the elicitation of sweetness of sweet-tasting proteins

The T value of lysozyme sweetness is around 7 μM, which is about 200 times greater than that of thaumatin and monellin. At first, to ascertain the differences of T values among sweet-tasting proteins, we investigated the sweetness determinants and charged environment on the surface of sweet-tasting proteins. The distances separating each of the critical five residues in thaumatin are in a range of 20–40 Å. In contrast, the distance of each critical residue of lysozyme ranges from 11 to 34 Å, and the sweetness determinants of thaumatin are broader than those of lysozyme (Figure 8). In addition, thaumatin includes other positively charged arginine residues and aromatic tyrosine residues in this region. These residues are not found in other thaumatin-like proteins (pathogenesis-related [PR]-5 and zeamatin), which are remarkably homologous with thaumatin in their tertiary structure but do not elicit a sweet taste (Dudler *et al.*, 1994; Koiwa *et al.*, 1999). It is likely that the specific sweet-tasting property of thaumatin might be derived from a broad surface area and a specific structural property such as a cleft and might have the potential to form a stable conformation to induce a potent sweetness in comparison to lysozyme. The sweetness determinants of single-chain monellin are broad and separated (Ariyoshi and Kohmura, 1994; Somoza *et al.*, 1995). Besides charged residues, N-terminal regions, Ile6, Ile8, and Gly9, are involved in sweetness, suggesting that a specific conformation might be required to elicit sweetness as well as to form a stable conformation. In brazzein, the critical residues for sweetness are separated by distances of 8–19 Å (Jin *et al.*, 2003). It should be noted that the mutation of negatively charged residues Asp29 and Glu41 to positively charged residues (Lys) resulted in an increase of sweetness, suggesting that charge plays a significant role in sweetness. Although brazzein is not a basic protein (pI = 5.4), it is a small protein and large numbers of charged residues including negatively charged residues are involved in sweetness. It is likely that the density of the charged environment on the protein surface might be higher than that of lysozyme, giving it the potential to interact with its putative receptors. As demonstrated earlier, positively charged residues on the surface of sweet-tasting proteins could play an important role in the elicitation of sweetness. The differences in T values might be caused by differences in the broadness of the areas formed by the charged residues and the densities of the charged residues.

Recently, a family of three G-protein–coupled receptors (T1Rs) selectively expressed in taste cells has been identified (Hoon *et al.*, 1999; Kitagawa *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001). The receptor composed of T1R2 and T1R3 functions as sweet receptor, which interacts with low–molecular mass sweeteners as well as sweet-tasting proteins thaumatin, monellin, and brazzein (Nelson *et al.*, 2001; Li *et al.*, 2002; Zhang *et al.*, 2003; Zhao *et al.*, 2003; Jiang *et al.*, 2004). It has been suggested that the cysteine-rich region of T1R3 determines responses to sweet-tasting proteins monellin and brazzein (Jiang *et al.*, 2004). Since most experiments were conducted at high protein concentrations (1000 times greater than its T value), it seems that other residues in the receptor molecules might be involved in the interaction with sweet-tasting proteins or that other signal transduction mechanisms are involved in the elicitation of the sweetness of proteins. It has been reported that the elicitation of sweetness was evoked in the absence of T1R3 (Damak *et al.*, 2003). From this, it might be postulated that other receptors and/or pathways besides the T1R2–T1R3 systems are involved in the interaction of sweet-tasting proteins. The mechanisms of interaction of sweet-tasting proteins with T1R2–T1R3 were investigated by computer-aided docking simulation, which suggested that the electrostatic potentials of a large cavity of T1R3 are predominantly negative and in good complement to the positively charged surfaces of sweet-tasting proteins (Temussi, 2002). Although the mechanism of the interaction of lysozyme with putative receptors has not been elucidated, positively charged residues on the protein surface might have the potential to elicit the sweet taste of lysozyme. Mutation analysis of the lysozyme molecule in this study provides a novel strategy for developing the perception of tastants as well as the mechanism of signal transduction.

In summary, the basicity of a broad surface region formed by five positively charged residues, Lys13, Lys96, Arg14, Arg21, and Arg73, are required for lysozyme sweetness. Particularly, the basicity formed by the combinations of Arg21 and Arg73 and of Lys13 and Arg14 is important for maintaining lysozyme sweetness.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (T.M. and N.K.).

References

Ariyoshi, Y. and **Kohmura, M.** (1994) *Solid-phase synthesis and structure-activity relationships of analogs of the sweet protein monellin.* J. Soc. Synth. Org. Chem. Jpn., 52, 359–369.

Assadi-Porter, F.M., Aceti, D., Cheng, H. and **Markley, J.L.** (2000) *Sweetness determinant sites of brazzein, a small, heat-stable, sweet-tasting protein.* Arch. Biochem. Biophys., 376, 252–258.

Blake, C.C.F., Johnson, L.N., Mair, G.A., North, A.C.T., Phillips, D.C. and **Sarma, V.R.** (1967) *Crystallographic studies of the activity of hen egg-white lysozyme.* Proc. R. Lond. Ser. B Biol. Sci., 167, 378–388.

Caldwell, J.E., Abildgaard, F., Dzakula, Z., Ming, D., Hellekant, G. and **Markley, J.L.** (1998) *Solution structure of the thermostable sweet-tasting protein brazzein.* Nat. Struct. Biol., 5, 427–431.

Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, Z., Varadarajan, V., Zou, S., Jiang, P., Ninomiya, Y. and **Margolskee, R.F.** (2003) *Detection of sweet and umami taste in the absence of taste receptor T1r3.* Science, 301, 850–853.

Dudler, R., Mauch, F. and **Reimmann, C.** (1994) *Thaumatin-like proteins.* In Witty, M. and Higginbotham, J.D. (eds), Thaumatin. CRC Press, Boca Raton, FL, pp. 193–199.

Hoare, D.G. and **Koshland, D.E. Jr** (1966) *A procedure for the selective modification of carboxyl groups in proteins.* J. Am. Chem. Soc., 88, 2057–2058.

Hoon, M.A., Adler, E., Lindemeier, L., Battey, J.F., Ryba, N.J.P. and **Zuker, C.S.** (1999) *Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity.* Cell, 96, 541–551.

Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. and **Rupley, J.A.** (1972) *Vertebrate lysozymes.* In Boyer, P.D. (ed.), The Enzymes, vol. 7. Academic Press, New York, pp. 665–868.

Jiang, P., Ji, Q., Liu, Z., Snyder, L.A., Benard, L.M.J., Margolskee, R.F. and **Max, M.** (2004) *The cystein-rich region of T1R3 determines responses to intensely sweet proteins.* J. Biol. Chem., 279, 45068–45075.

Jin, Z., Danilova, V., Assadi-Porter, F.M., Aceti, D.J., Markley, J.L. and **Hellekant, G.** (2003) *Critical regions for the sweetness of brazzein.* FEBS Lett., 544, 33–37.

Jollès, P. and **Jollès, J.** (1984) *Review of progress in lysozyme research.* Mol. Cell. Biochem., 53, 165–189.

Kaneko, R. and **Kitabatake, N.** (2001) *Structure-sweetness relationship in thaumatin: importance of lysine residues.* Chem. Senses, 26, 167–177.

Kier, L.B. (1972) *A molecular theory of sweet taste.* J. Pharm. Sci., 61, 1394–1397.

Kim, S.H. and **Weickmann, J.L.** (1994) *Crystal structure of thaumatin I and its correlation to biochemical and mutational studies.* In Witty, M. and Higginbotham, J.D. (eds), Thaumatin. CRC Press, Boca Raton, FL, pp. 135–149.

Kitagawa, M., Kusakabe, Y., Miura, H., Ninomiya, Y. and **Hino, A** (2001) *Molecular genetic identification of a candidate receptor gene for sweet taste.* Biochem. Biophys. Res. Commun., 283, 236–242.

Ko, T.P., Day, J., Greenwood, A., and **McPherson, A.** (1994) *Structures of three crystal forms of the sweet protein thaumatin.* Acta Crystallogr. D, 50, 813–825.

Kohmura, M., Nio, N. and **Ariyoshi, Y.** (1992) *Highly probable active site of the sweet protein monellin.* Biosci. Biotechnol. Biochem., 56, 1937–1942.

Koiwa, H., Kato, H., Nakatsu, T., Oda, J., Yamada, Y. and **Sato, Y.** (1999) *Crystal structure of tobacco PR-5d protein at 1.8 Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins.* J. Mol. Biol., 286, 1137–1145.

Kumagai, I. and **Miura, K.** (1989) *Enhanced bacteriolytic activity of hen egg-white lysozyme due to conversion of Trp62 to other aromatic amino acid residues.* J. Biochem., 105, 946–948.

Kurihara, K and **Beidler, L.M.** (1968) *Taste-modifying protein from miracle fruits.* Science, 161, 1241–1243.

Laemmli, U.K. (1970) *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 227, 680–685.

Lee, S.Y., Lee, J.H., Chang, H.J., Cho, J.M., Jung, J.W. and **Lee, W.** (1999) *Solution structure of a sweet protein single-chain monellin determined by nuclear magnetic resonance and dynamical simulated annealing calculations.* Biochemistry, 38, 2340–2346.

Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M. and **Adler, E.** (2002) *Human receptors for sweet and umami taste.* Proc. Natl Acad. Sci. USA, 99, 4692–4696.

Lin, T.Y. and **Koshland, D.E. Jr** (1969) *Carboxyl group modification and the activity of lysozyme.* J. Biol. Chem., 244, 505–508.

Liu, X., Hu, Z., Maeda, S., Aiuchi, T., Nakaya, K. and **Kurihara, Y.** (1993) *Purification, complete amino acid sequence and structure characterization of the heat stable sweet protein, mabinlin II.* Eur. J. Biochem., 211, 281–287.

Masuda, T., Ide, N. and **Kitabatake, N.** (2005b) *Effects of chemical modification of lysine residues on the sweetness of lysozyme.* Chem. Senses, 30, 253–264.

Masuda, T., Ueno, Y. and **Kitabatake, N.** (2001) *Sweetness and enzymatic activity of lysozyme.* J. Agric. Food Chem., 49, 4937–4941.

Masuda, T., Ueno, Y. and **Kitabatake, N.** (2005a) *High yield secretion of the sweet-tasting protein lysozyme from the yeast Pichia pastoris.* Protein Expr. Purif., 39, 35–42.

Max, M., Shanker, Y.G., Huang, L., Rong, M., Liu, Z., Campagne, F., Weinstein, H., Damak, S. and **Margolskee, R.F.** (2001) *Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac.* Nat. Genet., 28, 58–63.

Ming, D. and **Hellekant, G.** (1994) *Brazzein, a new high-potency thermostable sweet protein from Pentadiplandra brazzeana B.* FEBS Lett., 355, 106–108.

Montmayeur, J.P., Liberles, S.D., Matsunami, H. and **Buck, L.B.** (2001) *A candidate taste receptor gene near a sweet taste locus.* Nat. Neurosci., 4, 492–498.

Morris, J.A. and **Cagan, R.H.** (1972) *Purification of monellin, the sweet principle of Dioscoreophyllum cumminsii.* Biochem. Biophys. Acta, 261, 114–122.

Nelson, G., Hoon, A., Chandrashekar, J., Zhang, Y., Ryba, N.J.P. and **Zuker, C.S.** (2001) *Mammalian sweet taste receptors.* Cell, 106, 381–390.

Ogata, C.M., Gordon, P.F., de Vos, A.M. and **Kim, S.H.** (1992) *Crystal structure of a sweet tasting protein thaumatin I, at 1.65 Å resolution.* J. Mol. Biol., 228, 893–908.

Patthy, L. and **Smith, E.L.** (1975a) *Reversible modification of arginine residues. Application to sequence studies by restriction of tryptic hydrolysis to lysine residues.* J. Biol. Chem., 250, 557–564.

Patthy, L. and **Smith, E.L.** (1975b) *Identification of functional arginine residues in ribonuclease A and lysozyme.* J. Biol. Chem., 250, 565–569.

Phillips, D.C. (1967) *The hen egg white lysozyme molecule.* Proc. Natl Acad. Sci. USA, 57, 484–495.

Reisfeld, R.A., Lewis, U.J. and **Williams, D.E.** (1962) *Disk electrophoresis of basic proteins and peptides on polyacrylamide gels.* Nature, 195, 281–283.

Sainz, E., Korley, J.N., Battey, J.F. and **Sullivan, S.L.** (2001) *Identification of a novel member of the T1R family of putative taste receptors.* J. Neurochem., 77, 896–903.

Shallenberger, R.S. and **Acree, T.** (1967) *Molecular theory of sweet taste.* Nature, 216, 480–482.

Shirasuka, Y., Nakajima, K., Asakura, T., Yamashita, H., Yamamoto, A., Hata, S., Nagata, S., Abo, M., Sorimachi, H. and **Abe, K.** (2004) *Neoculin as a new taste-modifying protein occurring in the fruit of Curculigo latifolia.* Biosci. Biotechnol. Biochem., 68, 1403–1407.

Smith, L.J., Sutcliffe, M.J., Redfield, C. and **Dobson, C.M.** (1993) *Structure of hen lysozyme in solution.* J. Mol. Biol., 229, 930–994.

Somoza, J.R., Cho, J.M. and **Kim, S.H.** (1995) *The taste-active regions of monellin, a potently sweet protein.* Chem. Senses, 20, 61–68.

Somoza, J.R., Jiang, F., Tong, F., Kang, S.H., Cho, J.M. and **Kim, S.H.** (1993) *Two crystal structures of a potently sweet protein. Natural monellin at 2.75 resolution Å and single-chain monellin at 1.7 Å resolution.* J. Mol. Biol., 234, 390–404.

Spadaccini, R., Crescenzi, O., Tancredi, T., De Casamassimi, N., Saviano, G., Scognamiglio, R., Di Donato, A. and **Temussi, P.A.** (2001) *Solution structure of a sweet protein: NMR study of MNEI, a single chain monellin.* J. Mol. Biol., 305, 505–514.

Suckau, D., Mak, M. and **Przybylski, M.** (1992) *Protein surface topology-probing by selective chemical modification and mass spectrometric peptide mapping.* Proc. Natl Acad. Sci. USA, 89, 5630–5634.

Suzuki, M., Kurimoto, E., Nirasawa, S., Masuda, Y., Hori, K., Kurihara, Y., Shimba, N., Kawai, M., Suzuki, E. and **Kato, K.** (2004) *Recombinant curculin heterodimer exhibits taste-modifying and sweet-tasting activities.* FEBS Lett., 573, 135–138.

Tancredi, T., Pastore, A., Salvadori, S., Esposito, V. and **Temussi, P.A.** (2004) *Interaction of sweet proteins with their receptor.* Eur. J. Biochem., 271, 2231–2240.

Temussi, P.A. (2002) *Why are sweet proteins sweet? Interaction of brazzein, monellin and thaumatin with the T1R2-T1R3 receptor.* FEBS Lett., 526, 1–4.

Temussi, P.A., Lelj, F. and **Tancredi, T.** (1978) *Three-dimensional mapping of the sweet taste receptor site.* J. Med. Chem., 21, 1154–1158.

van der Wel, H. (1972) *Isolation and characterization of the sweet principle for Dioscoreophyllum cumminsii (Stapf) Diels.* FEBS Lett., 21, 88–90.

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

Yamashita, H., Theeraship, A., Nakaya, T., Nakamura, Y. and **Kurihara, Y.** (1990) *Purification and complete amino acid sequence of a new type of sweet protein with taste-modifying activity, curculin.* J. Biol. Chem., 265, 15770–15775.

Zhang, Y., Hoon, M.A., Chandrashekar, J., Mueller, K.L., Cook, B., Wu, D., Zuker, C.S. and **Ryba, N.J.P.** (2003) *Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways.* Cell, 112, 293–301.

Zhao, G.Q., Zhang, Y., Hoon, M.A., Chandrashekar, J., Erlenbach, I., Ryba, N.J.P. and **Zuker, C.S.** (2003) *The receptors for mammalian sweet and umami taste.* Cell, 115, 255–266.

Accepted August 12, 2005