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# Effects of Chemical Modification of Lysine Residues on the Sweetness of Lysozyme

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## Abstract

Lysozyme is a sweet-tasting protein with a sweetness threshold value of around 7 μM. To clarify the effect of basicity at the side chain of lysine residues on the threshold values of sweetness, charge-specific chemical modifications such as guanidination, acetylation and phosphopyridoxylation of lysine residues were performed. Sensory analysis showed that the sweetness threshold value of lysozyme was not changed by guanidination, whereas it was increased markedly by acetylation and phosphopyridoxylation. To confirm the importance of the basicity in the lysine residues in detail, purification of acetylated (Ac-) and phosphopyridoxylated (PLP-) lysozymes using SP-ion exchange column chromatography was performed. The threshold values were not changed by modification with fewer than two residues (~7 μM), whereas the threshold values significantly increased to 15 and 34 μM when tetra-Ac and tri-PLP, respectively. Furthermore, sweetness was not detected at 30 μM (hexa-, penta-Ac and tetra-PLP). It should be noted that removal of the negative charges of the phosphate groups in the tri-PLP lysozyme by acid phosphatase resulted in the recovery of sweetness (6.4 μM), indicating that basicity at the position of the lysine residues is responsible for lysozyme sweetness and that strict charge complementarities might be required for interaction to its putative receptor.

**Key words:** basicity, chemical modification, lysine, lysozyme, sweet-tasting protein

## Introduction

Proteins are usually tasteless, with the exception of some proteins that elicit a sweet taste response on the human palate. These include thaumatin (van der Wel and Loeve, 1972), monellin (Morris and Cagan, 1972; van der Wel, 1972), brazzein (Ming and Hellekant, 1994), pentadin (van der Wel *et al.*, 1989), mabinlin (Liu *et al.*, 1993), curculin (Yamashita *et al.*, 1990) and lysozyme (Masuda *et al.*, 2001). Although most sweet-tasting proteins other than brazzein (p$I$ = 5.4) (Ming and Hellekant, 1994) have a high isoelectric point >7.0, no homology has been observed among these sweet-tasting proteins in amino acid sequences and in tertiary structures. A large number of studies have been performed to identify common features among sweet-tasting proteins. In particular, one of the most useful and fundamental methods for determination of residues involved in the elicitation of sweetness is the chemical modification of the side chains of specific residues (van der Wel and Bel, 1976; van der Wel, 1994; Kaneko and Kitabatake, 2001). Acetylation of thaumatin resulted in progressive diminution of sweetness intensity as the acetyl groups were introduced (van der Wel and Bel, 1976). Phosphopyridoxylation of thaumatin reduced its sweetness and five positively charged lysine residues (Lys78, Lys97, Lys106, Lys137 and Lys187), which are all located on one side of the thaumatin molecule, are important in the elicitation of sweetness (Kaneko and Kitabatake, 2001). Chemical modification studies and subsequent mutation studies of brazzein suggested that charge plays a significant role in interaction between brazzein and its receptor (Jin *et al.*, 2003a,b). In addition, chemical synthesis studies of monellin and mutation studies of single-chain monellin have suggested that charged residues are responsible for elicitation of sweetness (Kohmura *et al.*, 1992; Somoza *et al.*, 1995; Mizukoshi *et al.*, 1997; Sung *et al.*, 2001). Thus, the importance of charged residues on the surface of sweet-tasting proteins has been widely suggested. These indications are well in accord with the results of immunological investigation, suggesting that epitopes for monoclonal antibodies that recognized both thaumatin and monellin contain charged residues (Slootstra *et al.*, 1995).

Among sweet-tasting proteins, lysozyme is one of the most thoroughly characterized and its structure and function have been studied extensively (Blake *et al.*, 1967; Phillips, 1967; Imoto *et al.*, 1972; Jollès and Jollès, 1984; Smith *et al.*,

1993). Nevertheless, a detailed explanation of the sweetness of the lysozyme is still unknown. Chicken egg lysozyme consists of a single amino acid chain of 129 residues and has a molecular weight of 14 500. It is easily obtained and purified from egg white and has the possibility of being used as a sweetener. A previous study showed that the sweetness of lysozyme was independent of its enzymatic activity, as demonstrated by chemical modification of the carboxyl groups of catalytic residues (Masuda *et al.*, 2001). Furthermore, lysozyme from goose egg white, which is classified as a goose-type lysozyme and differs from chicken-type lysozyme in structure and molecular weight (20 500), elicited a similar sweet taste (Masuda *et al.*, 2001). Although most sweet-tasting proteins, including both types of lysozyme, are basic proteins, little is known about the relationship between sweetness and basicity. In particular, it is not clear whether a surface positive charge on a sweet-tasting protein is required for the elicitation of sweetness.

Thresholds are limits of sensory capacities and can be measured by a variety of psychophysical procedures based on the methods of limits. The three-alternative forced-choice (3-AFC) method is one of the standard methods for determining each person's threshold (Meilgaard *et al.*, 1999). To assess human threshold values of sweetness precisely, a large number of samples are required because small sample amounts are easily influenced by the dilution of saliva or the area of application of the taste stimulus.

In this study, we prepared a large amount of chemically modified lysozyme to evaluate its threshold values of sweetness as sensed by humans. To elucidate whether basicity is essential for the lysozyme taste stimulus, the role of the positive charge at the position of the lysine residues was investigated. We employed three types of modification: (i) guanidinated lysozyme (Gua-lysozyme), which converts lysine residues to homoarginine residues and gives the same number of positive charge; (ii) acetylated lysozyme (Ac-lysozyme), which reduces positive charges by blocking with a methyl group and decreases positive net charges on the molecular surface; and (iii) phosphopyridoxylated lysozyme (PLP-lysozyme), which introduces negatively charged phosphate groups into the side chain of the lysine residues of the lysozyme molecular surface. In the case of PLP-lysozyme, the negative charges of the phosphate groups can be removed by acid phosphatase so that it retains the positive charge as that of native lysozyme. This study describes the relationship between the properties of the side chain of the lysine residue and the threshold values of sweetness, which were evaluated by humans for sensory analysis.

## Materials and methods

### Materials

Lysozyme chloride from egg white was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL). *Micrococcus luteus* was purchased from Sigma Chemical Co. (St Louis, MO). Ethylene glycol chitin was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CM Toyopearl 650M was purchased from TOSOH Co. (Tokyo). *O*-Methylisourea was purchased from Wako Pure Chemical Industries, Ltd. Acid phosphatase (from potato, 6.7 units/mg solid) was from Sigma Chemical Co. Acetic anhydride, pyridoxal 5′-phosphate and trinitrobenzene sulfonic acid were obtained from Nacalai Tesque Inc. All other chemicals were of guaranteed reagent grade.

### Purification of lysozyme

Lysozyme chloride powder from egg white (3 g; Nacalai Tesque) was dissolved in 50 ml of distilled and deionized water (dd water), and the pH was adjusted to 9.5 with NaOH. Lysozyme was crystallized in the presence of sodium chloride at 4°C and was recrystallized five times (Alderton and Fevold, 1946).

### Protein concentration

The protein concentration of unmodified lysozyme was determined spectrophotometrically by using the absorbance at 280 nm ($E_{1\%}$ = 26.4) (Kumagai and Miura, 1989). For the chemically modified lysozyme, the protein concentration was measured by the BCA procedure using unmodified lysozyme as a standard.

### Chemical modification of lysine residues

#### *Guanidination*

Guanidination was performed as described elsewhere with slight modification (Cupo *et al.*, 1980). Briefly, purified lysozyme solution was dialyzed extensively against dd water. Eighteen milliliters of lysozyme solution (7.25 mg/ml) was mixed with 2.0 ml of 100 mM Tris–HCl buffer, pH 8.4, containing 1 M NaCl. To this was added 20 ml of *O*-methylisourea solution, prepared by dissolving 2.16 g of *O*-methylisourea in water. The pH was adjusted to 10.5 by the addition of 5 M NaOH. The reaction mixture was stirred for 4 days at 4°C. The reactant was precipitated by ammonium sulfate. After centrifugation, the precipitate was dissolved in water and dialyzed extensively against dd water at 4°C for sensory analysis and native polyacrylamide gel electrophoresis (PAGE). The number of modified lysine residues was measured by using trinitrobenzene sulfonic acid (TNBS) as described below.

#### *Acetylation*

Acetylation of lysozyme was performed as described (Yamasaki *et al.*, 1968). Briefly, 50 ml of lysozyme solution (30 mg/ml) was mixed with an equal volume of saturated sodium acetate solution. Then 5-, 60- and 300-fold molar excess per mol amino group of acetic anhydride was added with

vigorous stirring at 0°C. The reaction mixture was centrifuged and the supernatant obtained was dialyzed against dd water extensively at 4°C for sensory analysis and native PAGE analysis. The number of modified lysine residues was counted by using the TNBS method described below. Highly acetylated lysozyme was further purified by high-performance liquid chromatography (HPLC; L-6200, Hitachi, Ltd, Tokyo) with an SP-ion exchange HPLC column (COSMOGEL SP, 20 × 100 mm, Nacalai Tesque Inc.). The column was equilibrated with 50 mM sodium phosphate buffer, pH 5.8, and the protein was eluted with a linear gradient from pH 5.8 to pH 8.0. The purity of each fraction was confirmed by native PAGE. The purified samples were stored as ammonium sulfate precipitate until use.

*Phosphopyridoxylation*

Modification of lysine residues with pyridoxal 5′-phosphate was performed in 50 mM sodium phosphate buffer, pH 7.5 (Lundblad and Noyes, 1984). A 15- to 150-fold molar excess of pyridoxal 5′-phosphate was added to 25 ml of lysozyme solution (3 mg/ml). Reactions were carried out for 15 min at 25°C. Subsequently, a freshly prepared solution of sodium borohydride (30 mg/ml) was added to the reaction mixture to achieve a final concentration of 0.60 mg/ml. PLP-lysozyme was purified by HPLC with an SP-ion exchange HPLC column using two methods. For separation of highly modified PLP-lysozyme, the column was equilibrated with 50 mM sodium phosphate buffer, pH 5.0, and the protein was eluted with a pH linear gradient from pH 5.0 to pH 8.0. For separation of less modified PLP-lysozyme, protein was applied to the column previously equilibrated with 50 mM sodium phosphate buffer, pH 5.8, and then eluted with a pH linear gradient from pH 5.8 to pH 8.0. Subsequently, this solution was eluted with a linear salt gradient from 0 to 0.5 M NaCl in 50 mM sodium phosphate buffer, pH 8.0. The purity of each fraction was checked by native PAGE. The extent of phosphopyridoxylation of the lysine residues was determined spectrophotometrically from the absorbance at 325 nm by using an $\varepsilon_{325}$ of 4800 $M^{-1}cm^{-1}$ for protein-bound PLP (Lundblad and Noyes, 1984; Green *et al.*, 1992). Sensory analysis of each fraction was performed as described below.

### Removal of phosphate groups from PLP-lysozyme by acid phosphatase

5′-Phosphates on the PLP groups of tri-phosphopyridoxylated (3-PLP) lysozyme were enzymatically removed using acid phosphatase (Kaneko and Kitabatake, 2001). In brief, 3-PLP-lysozyme was incubated with acid phosphatase (30 units) in 0.1 M sodium acetate buffer, pH 5.5, for 4 days at 37°C. The dephosphorylated PLP-lysozyme was purified by a carboxymethyl ion exchange column equilibrated with 50 mM Tris–HCl buffer, pH 7.4. The protein was eluted with the same buffer containing 0.5 M NaCl, and the eluate was pooled and dialyzed against dd water for sensory analysis.

### Counting the number of modified lysine residues

One milliliter of protein solution (0.50 mg/ml) was incubated with 4 ml of 7 M urea in 0.1 M borate buffer, pH 8.0, and 1 ml of TNBS solution (3.00 mg/ml) in the dark for 3 h. The sample containing no protein served as a control. The number of free amino groups was calculated from the ratio of the absorbance at 346 nm for protein-bound TNBS ($\varepsilon_{346}$ = 12 500 $M^{-1}cm^{-1}$) to the absorbance at 280 nm for lysozyme ($\varepsilon_{280}$ = 39 000 $M^{-1}cm^{-1}$) (Yamasaki *et al.*, 1968).

### Sensory analysis of lysozyme

Three paper cups (A, B, C) were prepared, and 5 ml of the protein solution was put in one of the three cups. In the other two cups, the solvent (water) was placed. These three cups were given to the panel who was asked to indicate which cup had the taste-eliciting solution. Next, similar experiments were performed using the sample solution with higher protein concentration. Through this method, the threshold values for taste sensation were obtained for each protein sample. Sweetness intensity was evaluated on a scale from 0 to 5 using a scaling bar (Meilgaard *et al.*, 1999). 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 threshold value of sweetness is the concentration giving value 2. The threshold values were averaged and analyzed with one-way ANOVA (analysis of variance) and with the Kruskal–Wallis test on ranks for nonparametric data. A post-hoc test was performed by Fisher's LSD test. A $P < 0.05$ value was considered a significant difference in the statistical analysis (O'Mahony, 1986).

### Measurement of enzymatic activity of lysozyme

The enzymatic activity of lysozyme was determined by measuring the lysis of *M. luteus* suspension (lytic activity) or ethylene glycol chitin as a substrate (hydrolytic activity). Lytic activity was determined by measuring the clearing of turbidity of *M. luteus* suspension at 450 nm. Fifty microliters of lysozyme solution was added to 2.95 ml of the suspension in a cuvette at 25°C. 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 min was defined as 1 unit of enzymatic activity. The residual activity of lysozyme is represented as the percentage of that of native lysozyme. Hydrolytic activity toward ethylene glycol chitin was performed as follows (Imoto and Yagishita, 1971). A lysozyme solution (0.5 ml) in 0.1 M acetate buffer, pH 4.5, was mixed with 1 ml of 0.05% ethylene glycol chitin solution, and the mixture was incubated for 30 min at 40°C. After the reaction, 2 ml of color reagent (0.50 g potassium ferricyanide in 1 l of 0.5 M sodium carbonate) was added, and the mixture was

immediately boiled for 15 min in the dark. After cooling, an absorbance at 420 nm was measured.

### Polyacrylamide gel electrophoresis

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

### Determination of isoelectric points

The isoelectric points of the modified lysozyme variants were determined using a pH gradient electrophoresis system (Nihoneido Co., Tokyo). A linear sucrose gradient from 60 to 30% was loaded onto a 110 ml electrophoresis column in the presence of 1% ampholine (Amersham Bioscience AB, Uppsala, Sweden). After the protein sample containing 30% sucrose and 1% ampholine were loaded onto the top of the column, a linear sucrose gradient from 30 to 0% was loaded onto the same column. Electrophoresis was performed at a constant voltage of 800 V for 48 h at 1°C. After electrophoresis, samples were fractionated every 2 ml. The protein concentration of each fraction was measured by the absorbance at 280 nm, and the pH value of each fraction was measured on ice by using a pH meter (Horiba, Co. Ltd, Kyoto, Japan).

### Schematic representation of lysozyme

Molecular model of lysozyme was prepared by the MolFeat program using data for lysozyme (PDB193L) and expressed as a ribbon model and a space-filled model.

## Results

### Chemical modification of lysine residues

#### *Guanidination*

To examine whether the structural feature or the positive charge in lysine residues are essential for its sweetness, Gua-lysozyme that convert lysine residues to homoarginine residues was prepared. This modification did not change the number of positive charges. Six of seven amino groups from the ε-amino group of lysine residues and one α-amino group from N-terminal lysine residue have the potential to be modified with *O*-methylisourea. The extent of guanidination of lysozyme (5.64 μmol) was determined by the TNBS method. Measurement of absorbance at 346 nm showed that 36.5 μmol of the trinitrophenyl groups were introduced into 5.64 μmol of unmodified lysozyme, indicating that an average of 6.5 of the amino groups of a lysozyme molecule were modified by TNBS.

In the case of Gua-lysozyme, 4.1 μmol of the trinitrophenyl groups, that is, less than 1 amino group, was modified with TNBS, indicating that all six lysine residues including the N-terminal lysine residue were modified with *O*-methylisourea. Native PAGE showed that the mobility of Gua-lysozyme was almost the same as that of unmodified lysozyme, indicating that the surface positive charges of lysozyme were not changed by guanidination (Figure 1). Sensory analysis indicated that the threshold value of Gua-lysozyme was ~7.5 μM, which is almost identical to that of unmodified lysozyme (6.7 μM; see Figure 2). These results suggested that positive charges at the position of the side chain of the lysine residues are important for the elicitation of lysozyme sweetness.

#### *Acetylation*

The acetylation of lysozyme was performed in acetate buffer with a 5-, 60- and 300-fold molar excess of acetic anhydride per mol amino group. Native PAGE showed that the mobility of Ac-lysozyme decreased as the amount of acetic anhydride for treatment increased (Figure 3). This result shows that acetylation causes the reduction of the surface positive charges of a lysozyme molecule. TNBS analysis of these

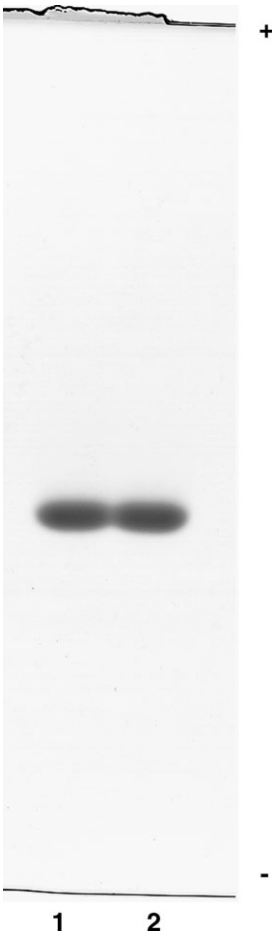


**Figure 1** Native PAGE pattern of Gua-lysozyme. Native PAGE for positively charged proteins was performed on 15% gel and stained with Coomassie brilliant blue. Lane 1, unmodified lysozyme (5 μg); lane 2, Gua-lysozyme (5 μg).

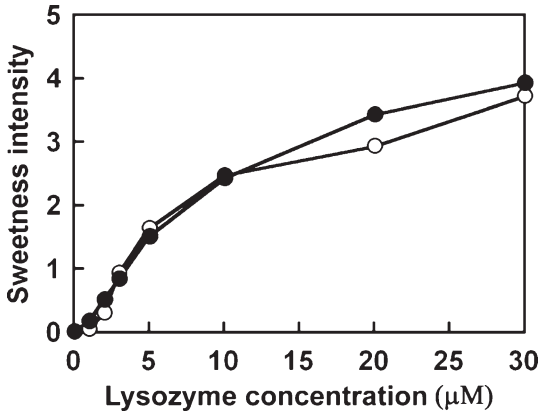


**Figure 2** Sweetness intensity of Gua-lysozyme. The sweetness intensity values of unmodified lysozyme (open circles) and Gua-lysozyme (closed circles) are plotted against lysozyme concentration. Intensity value: 0 = no taste; 1 = some taste stimulation; 2 = sweet taste; 3 = potently sweet taste. Intensity value 2 corresponds to the threshold of lysozyme sweetness.

Ac-lysozymes showed that 31.5, 22.4 and 18.0 μmol of trinitrophenyl groups were introduced into a lysozyme molecule (5.64 μmol) when treated with 5-, 60- and 300-fold molar excess of acetic anhydride, respectively. That is, 5.6, 4.0 and 3.2 amino groups were modified by TNBS on average, meaning that 1.4, 3.0 and 3.8 lysine residues of the lysozyme were acetylated, respectively. Sensory analysis showed that the threshold value of lysozyme sweetness increased as the number of acetylated residues increased (Figure 4), suggesting that positive charges at the position of the lysine residues would be involved in the lysozyme sweetness.

To clarify the effect of the threshold value of sweetness on acetylation in detail, further purification of crude Ac-lysozyme was performed using SP ion exchange column chromatography (Figure 5). Native PAGE suggested that each peak gave a single band and the mobility decreased in the order of peak A, B and C (Figure 6). TNBS analysis showed that 17.4, 10.1 and 5.00 μmol of the trinitrophenyl groups were introduced into the lysozyme of peaks A, B and C, respectively, indicating that 6.1, 5.2 and 3.9 amino groups was acetylated. Judging from above results, the Ac-lysozyme shown in peaks A, B and C of Figure 6 should correspond to hexa-, penta- and tetra-Ac-lysozyme (6-, 5- and 4-Ac-lysozyme), respectively. Sensory analysis of Ac-lysozyme corresponding to peaks A–C were performed and indicated that the thresholds of 4-, 5- and 6-Ac-lysozyme were 15, 38 and >50 μM, respectively (Figure 7, Table 1). This suggests that positive charges might play a significant role in the elicitation of lysozyme sweetness. Enzymatic activity, that is, the lytic activity of Ac-lysozyme against *M. luteus* was reduced by less than 10% of that of the unmodified one, indicating that positive charges should also be important for lytic activity against the cell wall (Table 1). However, the hydrolytic activity of 4-, 5- and 6-Ac lysozyme was 107, 94 and 89%, respectively, of that of unmodified lysozyme (Table 1). These results were


**Figure 3** Native PAGE pattern of Ac-lysozyme. Native PAGE was performed on 15% gel and stained with Coomassie brilliant blue. Lanes 1 and 5, unmodified lysozyme; lane 2, a Ac-lysozyme derivatives modified by a 5-fold excess of acetic anhydride; lane 3, by a 60-fold excess; lane 4, by a 300-fold excess.

in good agreement with the previous results (Yamasaki *et al.*, 1968), indicating that no conformational changes occurred in the catalytic and substrate binding site of lysozyme. The isoelectric points of 4-, 5- and 6-Ac-lysozyme were all above 10 (Table 1).

### *Phosphopyridoxylation*

Modification of lysine residues with pyridoxal 5′-phosphate was performed in the condition of 15- to 150-fold molar excess of pyridoxal 5′-phosphate to lysozyme. The number of phosphopyridoxylated-lysine residues was determined spectrophotometrically. PLP-lysozymes were fractionated by an SP-ion exchange column, as shown in Figure 8. Each peak was analyzed by native PAGE, indicating that the mobility of the band decreased as the number of phosphopyridoxylated residues increased (Figure 9). Sensory analysis showed

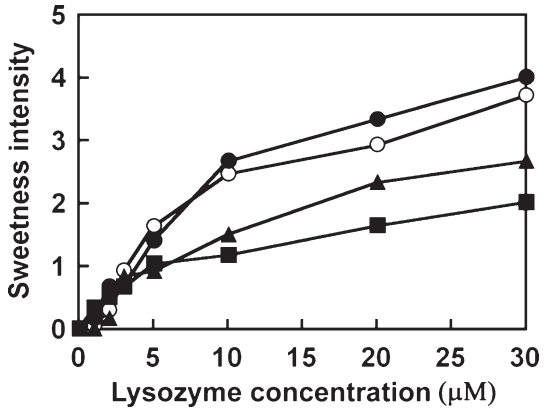


**Figure 4** Sweetness intensity of Ac-lysozyme derivatives. The values of sweetness intensity of unmodified lysozyme (open circles) and Ac-lysozyme derivatives (closed circles) correspond to lane 2 in Figure 3 and (close triangles) correspond to lane 3 in Figure 3; (close squares) correspond to lane 4 in Figure 3, and are plotted against lysozyme concentration. Evaluation of sweetness intensity was performed as in Figure 2.

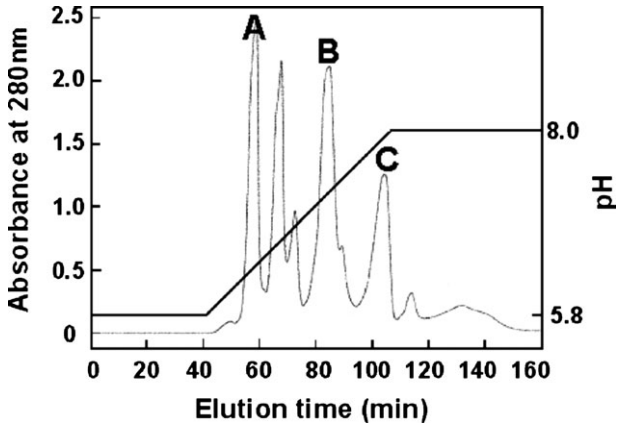


**Figure 5** Purification of Ac-lysozyme derivatives. Crude Ac-lysozyme (10 mg) was applied onto the SP-ion exchange HPLC column equilibrated with 50 mM sodium phosphate buffer (pH 5.8). Elution was performed with a linear pH gradient from pH 5.8 to pH 8.0 at a flow rate of 1.0 ml/min. The purified samples (peaks A–C) were precipitated by ammonium sulfate. The purified Ac-lysozyme was analyzed by native PAGE (Figure 6).

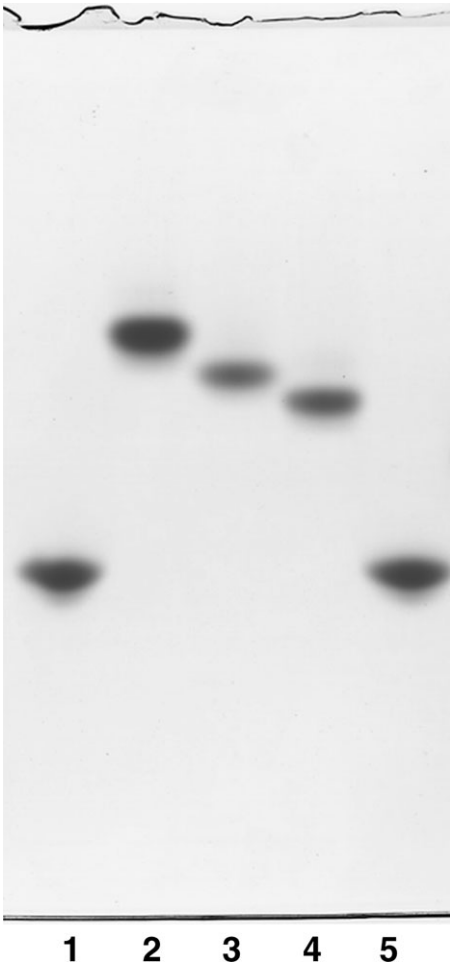


**Figure 6** Native PAGE pattern of purified Ac-lysozyme derivatives. Native PAGE was performed on 15% gel and stained with Coomassie brilliant blue. The extent of modification of Ac-lysozyme was determined by TNBS as described in the text. Lanes 1 and 5, unmodified lysozyme; lane 2, 6-Ac-lysozyme (peak A in Figure 5); lane 3, 5-Ac lysozyme (peak B in Figure 5); lane 4, 4-Ac lysozyme (peak C in Figure 5).

that the threshold value of sweetness of mono- and di-PLP-lysozyme (1- and 2-PLP-lysozyme) was 5.6 and 8.3 μM, respectively. These values were almost equivalent to that of unmodified lysozyme (6.7 μM). However, the threshold values of tri- and tetra-PLP-lysozyme (3- and 4-PLP-lysozyme) were 34 and >30 μM, respectively (Figure 10, Table 2). Furthermore, the sweetness of penta-phosphopyridoxylated lysozyme (5-PLP-lysozyme) was not observed at 50 μM. Isoelectric points (p*I*) of the 5-, 4- and 3-PLP-lysozymes were 6.5 7.3 and 8.7, respectively (Figure 11). These results indicated that surface charge of lysozyme was changed dramatically by phosphopyridoxylation. Enzymatic activity of 5-, 4- and 3-PLP-lysozyme against *M. luteus* was ~5% of that of unmodified lysozyme, and the hydrolytic activity of 5-, 4- and 3-PLP-lysozymes was 77, 88 and 88% of that of unmodified lysozyme, respectively (Table 2).

### Dephosphorylation of PLP-lysozyme

Dephosphorylation from PLP-lysozyme by acid phosphatase was examined by native PAGE. The mobility of the band increased with longer incubation with acid phosphatase, and the mobility of the sample treated for 96 h was almost identical to that of unmodified lysozyme (Figure 12). Dephosphopyridoxylated-3-PLP lysozyme was purified by CM Toyopearl 650M, and the sweetness and enzymatic activity of purified dephosphopyridoxylated-3-PLP lysozyme were investigated. The threshold value of dephosphorylated-PLP-lysozyme was 6.4 μM, which is almost the same as that of unmodified lysozyme, indicating that sweetness was recovered by the removal of the phosphate groups (Table 2). This result demonstrates

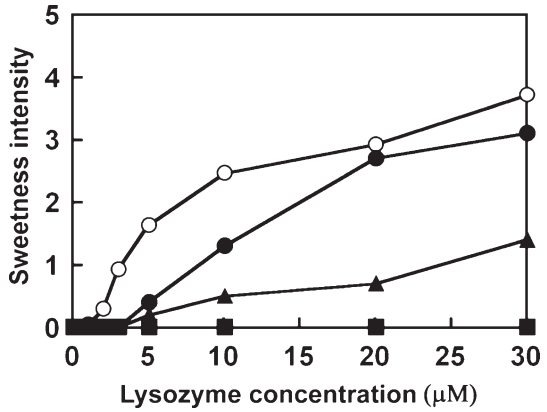


**Figure 7** Sweetness intensity of purified 4- to 6-Ac-lysozyme. The values of sweetness intensity of unmodified lysozyme (open circles) and 4-Ac-lysozyme (closed circles), 5-Ac-lysozyme (closed triangles) and 6-Ac-lysozyme (closed squares) are plotted against lysozyme concentration.

**Table 1** Properties of acetylated-lysozyme derivatives

| | Threshold of sweetness (μM) | pI | Hydrolytic activity[a] (%) | Lytic activity[a] (%) |
|---|---|---|---|---|
| Ac-6 residues | >50 | 10.0 | 89 | <5 |
| Ac-5 residues | 38 ± 13** | 10.8 | 94 | <5 |
| Ac-4 residues | 15 ± 6.3* | >11.0 | 107 | <5 |
| Unmodified | 6.7 ± 2.5 | 11.5 | 100 | 100 |

The threshold of sweetness is expressed as mean ± SD.
*$P < 0.05$, **$P < 0.01$.
[a]Lytic activity and hydrolytic activity were indicated as relative activity by taking the activity of unmodified lysozyme to be 100.

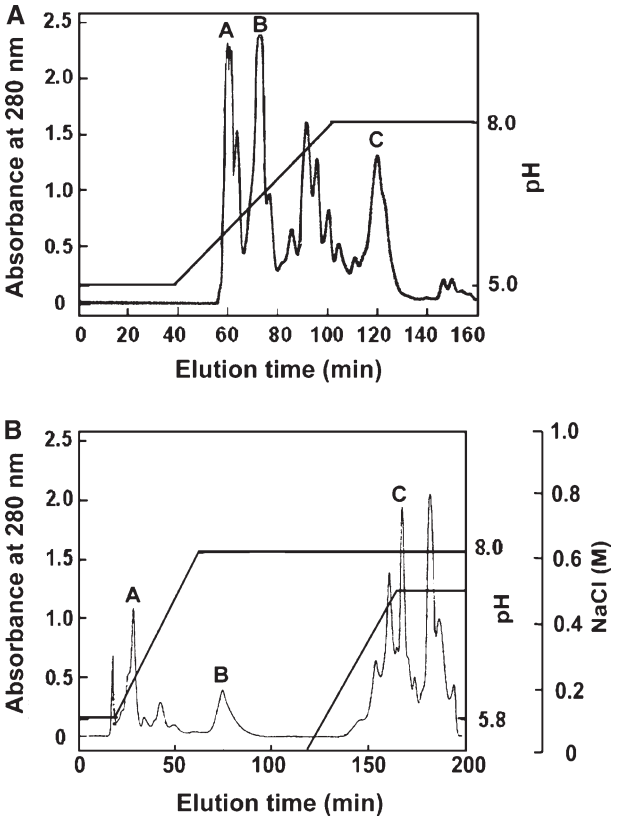


**Figure 8** Purification of PLP-lysozyme derivatives. **(A)** The highly modified PLP-lysozyme (10 mg) was applied onto the SP-ion exchange HPLC column equilibrated with 50 mM sodium phosphate buffer (pH 5.0). Elution was performed with a linear pH gradient from pH 5.0 to pH 8.0 in the same buffer at a flow rate of 1.0 ml/min. The fractions (peaks A–C) were collected and the number of modified lysine residues was determined spectrophotometrically. Each peak was analyzed by native PAGE (Figure 9). **(B)** The lower modified PLP-lysozyme (5 mg) was applied on the SP-ion exchange HPLC column equilibrated with 50 mM sodium phosphate buffer (pH 5.8). Elution was performed with a linear pH gradient from pH 5.8 to pH 8.0 in the same buffer at a flow rate of 1.0 ml/min. A subsequent salt gradient for 0–0.5 M NaCl was performed. The purified samples (peaks A–C) were analyzed by native PAGE (Figure 9).

that positive charges on the surface of a lysozyme molecule, rather than lysine residues, are important for the elicitation of sweetness by lysozyme. Enzymatic activity was also recovered to 98% of that of unmodified lysozyme.

## Discussion

The three-dimensional structure of the lysozyme molecule indicates that the molecule is made up of two domains. The first domain consists of the N- and C-terminal ends (residues 1–39 and 85–129) containing four helices. The second domain consists of a three-stranded anti-parallel β-pleated sheet. All six lysine residues (Lys1, Lys13, Lys33, Lys96, Lys97 and Lys116) are located on the molecule surface (Figure 13).

In the present study, we investigated the effects of the positive charge of lysine residues by chemical modification on the sweetness of lysozyme. Three types of charge-related modifications of the lysine residues were employed.

First, guanidination of the lysine residues was performed. The conversion of lysine to homoarginine residues by guanidination does not influence the surface net charge distribution of the lysozyme molecule, and the ε-amino group of the lysine residue is converted to the guanidino group containing a bulkier hydrophobic group. Our results are consistent with the previous results (Cupo *et al.*, 1980), that is, all six lysine residues were guanidinated. Sensory analysis of Gua-lysozyme showed that no significant change in the sweetness intensity was observed, suggesting that the guanidino groups as well as the ε-amino groups at the position of the lysine residues plays an important role in lysozyme sweetness.

Second, to clarify the role of the positive charge of the lysine residues on lysozyme sweetness, acetylation of the lysine residues was performed. Acetylation causes only a slight change in the stereochemical nature of the side chain of the lysine residue, and a decrease of the surface positive net charge of lysozyme. The reactivity of each lysine residue toward chemical

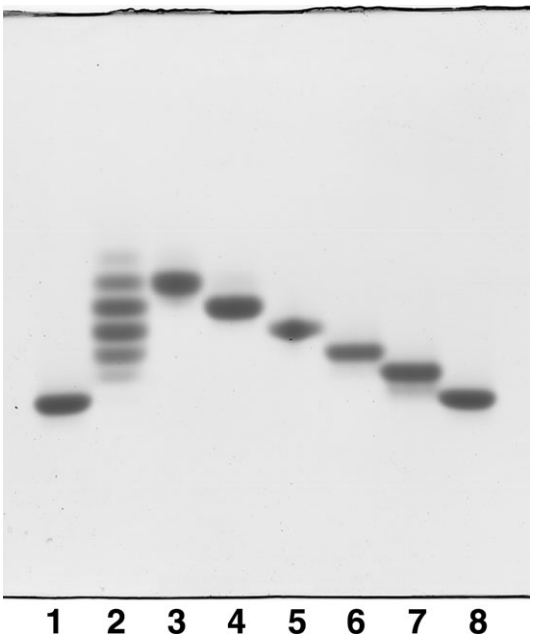


**Figure 9** Native PAGE pattern of purified PLP-lysozyme derivatives. Native PAGE was performed on 15% gel and stained with Coomassie brilliant blue. Lanes 1 and 8, unmodified lysozyme; lane 2, mixture of PLP lysozyme derivative; lane 3, 5-PLP-lysozyme, corresponding to peak A of Figure 8A; lane 4, 4-PLP-lysozyme, corresponding to peak B of Figure 8A; lane 5, 3-PLP-lysozyme, corresponding to peak C of Figure 8A or to peak A of Figure 8B; lane 6, 2-PLP-lysozyme, corresponding to peak B of Figure 8B; Lane 7, 1-PLP-lysozyme, corresponding to peak C of Figure 8B.

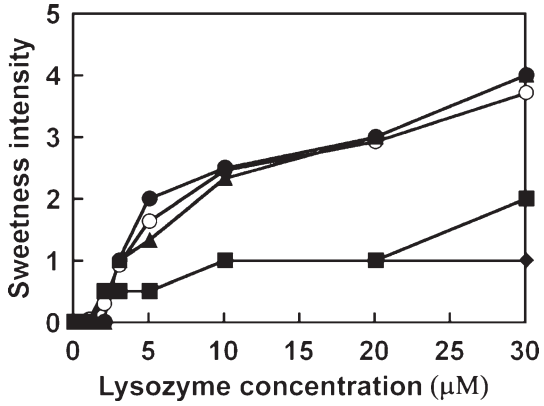


**Figure 10** Sweetness intensity of purified PLP lysozyme. The values of sweetness intensity of unmodified lysozyme (open circles) and 1-PLP-lysozyme (closed circles), 2-PLP-lysozyme (closed triangles), 3-PLP-lysozyme (closed squares) and 4-PLP-lysozyme derivative (closed diamonds) are plotted against lysozyme concentration.

**Table 2** Properties of PLP-lysozyme derivatives

| | Threshold of sweetness (μM) | p*I* | Hydrolytic activity[a] (%) | Lytic activity[a] (%) |
|---|---|---|---|---|
| PLP-5 residues | >50 | 6.5 | 77 | < 5 |
| PLP-4 residues | >30 | 7.3 | 88 | < 5 |
| PLP-3 residues | 34 ± 8.9** | 8.7 | 88 | 5.3 |
| PLP-2 residues | 8.3 ± 2.6 | 10.0 | 93 | 28 |
| PLP-1 residues | 5.6 ± 2.6 | 11.0 | 98 | 80 |
| Dephosphorylated PLP-3 residues | 6.4 ± 3.5 | >11 | 102 | 98 |
| Unmodified | 6.7 ± 2.5 | (11.5) | 100 | 100 |

The threshold of sweetness is expressed as mean ± SD.
$^{**}P < 0.01$.
[a]Lytic activity and hydrolytic activity are indicated as relative activity by taking the activity of unmodified lysozyme to be 100.

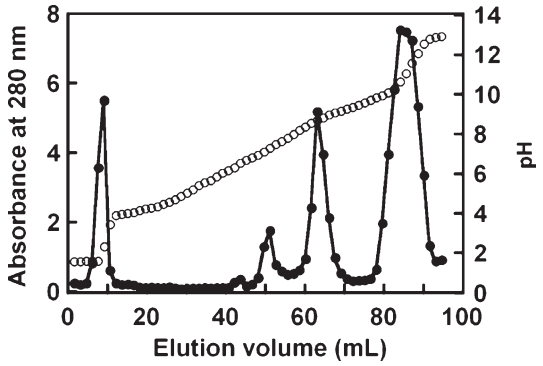


**Figure 11** Determination of isoelectric points of PLP lysozyme variants The isoelectric points of modified lysozyme variants were determined using a pH gradient electrophoresis system. Electrophoresis was performed in the presence of 1% ampholine at a constant voltage of 800 V for 48 h at a temperature of 1°C. After electrophoresis, the samples were fractionated in a volume of 2.0 ml. Absorbance at 280 nm was measured by a spectrophotometer; the pH value of each fraction was determined on ice by using a pH meter.

reagents is different because the reactivity of the six ε-amino groups is related to the surface accessibility of each ε-amino group (Lee and Richards, 1971). The lysine residues of lysozyme were acetylated in the order Lys97, Lys33, Lys1, Lys13 = Lys116, Lys96 (Suckau *et al.*, 1992). In the case of estrone glucuronide acylation and PEGylation, the lysine residues were modified in the order Lys33, Lys97, Lys116 (Smales *et al.*, 1999; Lee and Park, 2003). The reactive residues among the six lysine residues are Lys97 and Lys33, which correspond to the residues with the high accessibility values of 47.4 and 40.1%, respectively. Since acetylation and phosphopyridoxylation of less than two lysine residues has not influenced lysozyme sweetness, it seems that two lysine residues, Lys97 and Lys33, may not play a critical role in the elicitation of lysozyme sweetness. Since these two lysine residues are situated at opposite sides of the lysozyme molecule in the three-dimensional structure (Figure 13), it can be assumed that they would not simultaneously contact the same side of its putative receptor.


**Figure 12** Native PAGE pattern of dephosphopyridoxylated lysozyme derivatives 3-PLP-lysozyme (1.00 mg/ml) in 0.1 M sodium acetate buffer, pH 5.5, was mixed with acid phosphatase (30 units) and incubated for 4 days at 37°C. The extent of dephosphorylation of 3-PLP-lysozyme was monitored by native PAGE. Lanes 1 and 6, unmodified lysozyme; lane 2, 3-PLP-lysozyme derivative (without acid phosphatase); lane 3, dephosphorylated lysozyme (reacted for 48 h); lane 4, dephosphorylated lysozyme (reacted for 72 h); lane 5, purified dephosphorylated lysozyme (reacted for 96 h).

That 5- and 6- Ac-lysozymes do not exhibit sweetness means that positive charges at the different position of the lysine residues are involved in lysozyme sweetness. Enzymatic activity was measured to clarify whether the reduction of lysozyme sweetness can be attributed to the conformational changes induced by chemical modification. Hydrolytic activity of Ac-lysozyme against glycol chitin was almost the same as that of native lysozyme, which is not inconsistent with the previous report (Yamasaki *et al.*, 1968) (Table 1). These results suggest that no gross conformational changes in lysozyme would be induced by acetylation. Imoto *et al.* (1974, 1976a,b) also reported that alteration of the net charge in lysozyme by acetylation causes no gross conformational changes, which could not be detected by equilibrium methods using optical rotation and circular dichroism. In contrast to the above result, lysozyme sweetness and lytic activity against *M. luteus* decrease with an increase in the number of modified residues (Table 1). These results suggest that positive charges at the position of the lysine residues are important for lysozyme sweetness as well as for lytic activity.

These results were confirmed by phosphopyridoxylation experiments. Introduction of negative charges at the positions of the lysine residues led to a reduction of lysozyme sweetness as well as lytic activity. Furthermore, dephosphorylation of the negatively charged phosphate groups of 3-PLP-lysozyme resulted in recovery of sweetness and lytic activity. Taken together, positive charges at the positions of the lysine residues of the lysozyme molecule are important for the elicitation of sweetness, whereas the length or shapes of the side chain are not important.

It should be noted that the reduction of sweetness by phosphopyridoxylation was more effective than that by acetylation, suggesting that charge–charge interaction between lysozyme and its putative receptor would play a significant role in recognition of sweet-tasting protein. Strict charge complementarities might be required for interaction with its putative receptor. The cell surface of *M. luteus* is highly negatively charged, suggesting that electrostatic interaction might play a significant role in lytic activity (Yamasaki *et al.*, 1968; Kumagai *et al.*, 1992). Since most sweet-tasting proteins, including lysozyme, are basic proteins, it is conceivable that the charge–charge interaction would occur between sweet-tasting proteins and their putative receptors. Recently, a family of three G-protein-coupled receptors (T1Rs) selectively expressed in taste cells have been identified (Hoon *et al.*, 1999; Kitagawa *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Sainz *et al.*, 2001; Nelson *et al.*, 2001). A heteromeric—A heterodimeric receptor comprised of T1R2–T1R3, interacts with the sweet-tasting proteins monellin, thaumatin and brazzein, as well as with small sweet compounds (Li *et al.*, 2002; Jiang *et al.*, 2004). The mechanisms of interaction of sweet-tasting proteins with T1R2–T1R3 have been investigated by computer-aided docking simulation, which has suggested that most sweet-tasting proteins are centered on a large cavity of the T1R3 protomer. The electrostatic potentials of the large cavity of T1R3 are predominantly negative, and are a good complement to the positive charged surfaces of sweet-tasting proteins (Temussi, 2002; Spadaccini *et al.*, 2003; Tancredi *et al.*, 2004). Research on thaumatin suggested that five positively charged lysine residues are important for the elicitation of sweetness through multipoint interaction with its putative receptors (Kaneko and Kitabatake, 2001). Additionally, it has been suggested that the charge plays a significant role in the interaction between brazzein and its receptor through multipoint interaction in spite of its low isoelectric point of 5.4 (Jin *et al.*, 2003a).

As has been demonstrated with the sweet-tasting proteins, basicity at the position of the lysine residues plays a significant role in elicitation of lysozyme sweetness. Positive charges at different positions of the lysine residues should contribute to lysozyme sweetness through multipoint interaction that might occur with not only the cell surface of *M. luteus* but also with a putative receptor. Multipoint interaction among proteins is found in many biological

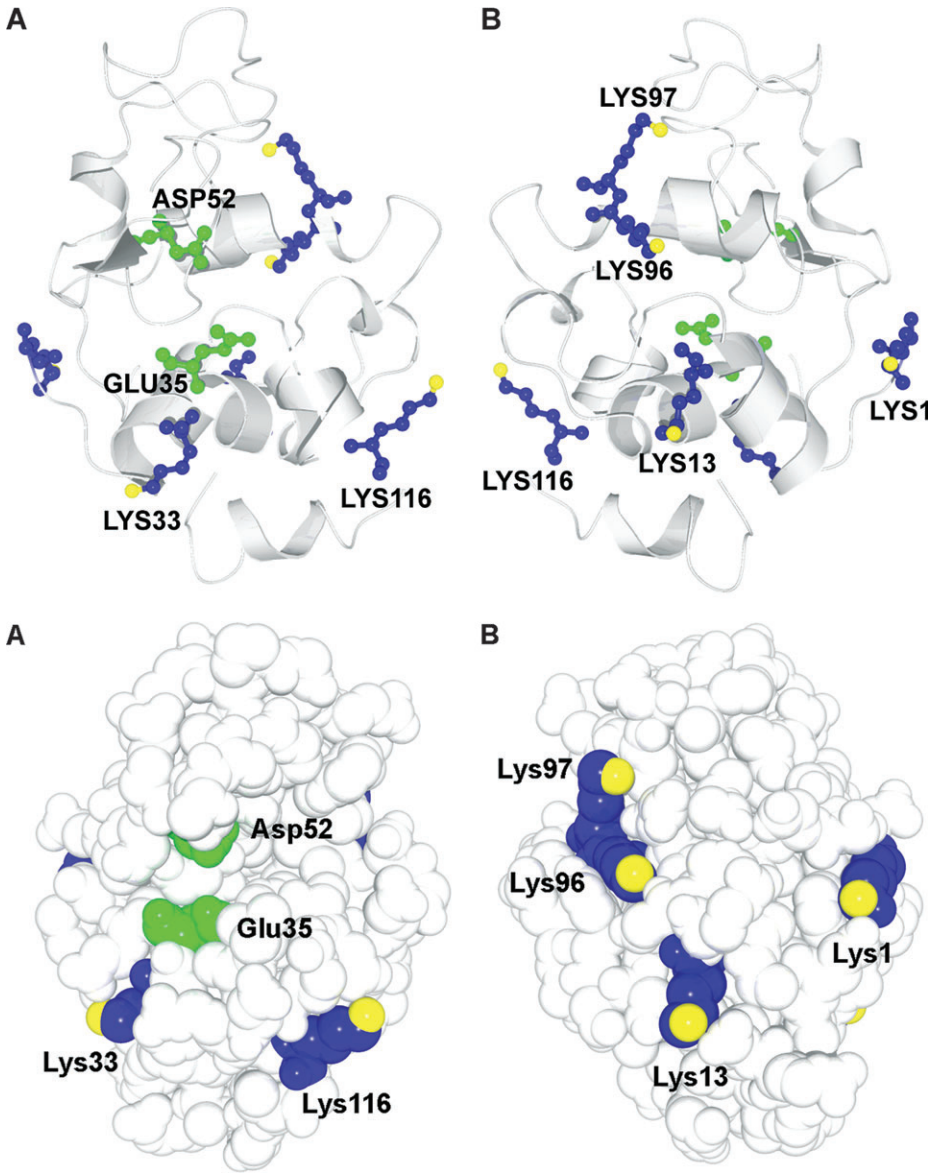


**Figure 13** Three-dimensional structure of hen egg lysozyme: schematic representation of the lysozyme in two different orientations. **(A)** The side of catalytic regions of lysozyme. **(B)** A different view of side A, rotated 180° about a vertical axis. The lysine residues are shown in blue; the ε-amino group of lysine residues is expressed in yellow. Catalytic residues Glu35 and Asp52 are indicated in green. The upper lane is expressed as a ribbon model; the lower lane is expressed as a space-filled model. The figure was drawn using data for lysozyme (PDB193L) and the program MolFeat.

phenomena. One typical example is antigen–antibody interaction, in which many amino acid residues—including charged residues and those at a broad surface region—participate with high affinity binding (Padlan, 1996). It appears that a low threshold of sweetness and a long-lasting aftertaste of sweet-tasting proteins compared with small molecular mass sweeteners such as sucrose and aspartame should be related to multipoint interaction with receptors. Although the mechanism of the interaction of sweet-tasting proteins with receptors has not been elucidated, strict charge-complementarities through multipoint interaction should be important between the surfaces of positively charged residues in sweet proteins and predominantly negatively charged counterparts, the putative receptors. To clarify the mechanism of interaction between receptors and sweet-tasting proteins, further investigations are needed using mutation analysis as well as chemical modification analysis. For these purposes, the charge-specific chemical modification approaches employed in this study would help in understanding the mechanism of the sweetness–structure relationship of sweet-tasting proteins, including lysozyme. It would also provide useful information for subsequent mutation analysis. Such an investigation is being conducted by genetic engineering to construct expression systems for recombinant

lysozyme (Masuda *et al.*, 2005) including site-directed mutagenesis.

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