Sweetness and Enzymatic Activity of Lysozyme

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Hen egg lysozyme elicits a sweet taste sensation for human beings. Effects of reduction of disulfide bonds, heat treatment, and chemical modification of hen egg lysozyme on both sweetness and hydrolytic activity were investigated. Both the sweetness and enzymatic activities were lost when the intradisulfide linkage in a lysozyme molecule was reduced and *S*-3-(trimethylated amino) propylated. The sweetness and enzymatic activity of lysozyme were lost on heating at 95 °C for 18 h. These facts suggest that tertiary structures of lysozyme are indispensable for eliciting a sweet taste as well as enzymatic activity. Although the modification of carboxyl residues in a lysozyme by glycine methylester or aminomethansulfonic acid resulted in the loss of enzymatic activity by blocking the catalytic residues, the sweetness was fully retained. These results indicate that the sweetness of lysozyme was independent of its enzymatic activity. The lysozyme purified from goose egg white similarly elicited a sweet taste, although goose (g-type) lysozyme is quite different from hen egg lysozyme (c-type) on the basis of structural, immunological, and enzymatic properties. These findings indicate that a specific protein property of lysozyme is required for sweetness elicitation and that the enzymatic activity and carbohydrates produced by enzymatic reaction are not related to the sweet taste.

Keywords: *Hen lysozyme; goose lysozyme; sweet protein; chemical modification; enzymatic activity*

INTRODUCTION

Some peptides and amino acids, as well as saccharides, polyols, and others, are known to elicit the sensation of sweetness (*1*). The ease of access to a putative receptor site on a taste cell is relevant to the elicitation of sweetness, and several models have been proposed to explain the perception of sweetness (*2*). In most cases, proteins are tasteless and flavorless, which seems to be because protein is too large a molecule to interact with specific putative receptor molecules on the taste cell. However, some proteins are known to elicit a sweet taste response on the human palate.

Six proteins, thaumatin (*3*), monellin (*4*, *5*), pentadin (*6*), mabinlin (*7*), curculin (*8*), and brazzein (*9*), were identified as eliciting a sweet taste. Among these, thaumatin and monellin, the most potently sweet, are derived from a plant in tropical West Africa and elicit sweet taste at a low concentration approximately 100 000-fold less than that of sucrose on a molar basis. Neither carbohydrates nor modified amino acids are contained in these proteins. Several investigations of structural and immunological properties of these two proteins have been performed; the three-dimensional structures of monellin and thaumatin were elucidated at high-resolution X-ray crystallography (*10*, *11*). In addition, antibodies cross-reacted with thaumatin and monellin were prepared to elucidate the consensus property in sweet proteins (*12*). However, no common features have been observed between these two proteins in amino acid sequences or in tertiary structures. Recently, it has been reported that the positive charges on the thaumatin molecule are essential to elicitation of a sweet taste (*13*). Researchers in the food and pharmaceutical industry have pointed out that hen egg white lysozyme also elicits a sweet taste, and the sweetness of c-type lysozymes has been reported (*14*).

Lysozyme is one of the most thoroughly characterized enzymes (*15*, *16*), and its structure and function have been extensively studied by X-ray crystallographic analysis (*17*, *18*), NMR investigation (*19*), and other methods. According to its amino acids sequence alignment and the specificity for the substrate, lysozyme is divided into three types: chicken-type (c-type), goose-type (g-type), and viral-type (v-type). Embden goose egg white lysozyme representative of the g-type lysozyme was reported by Dianoux and Jollès (*20*) and Canfield and McMurry (*21*). C-type and g-type lysozymes differ not only in molecular weight (*22*), but also in immunological properties (*23*), inhibition specificity, and enzymatic properties (*24*−*26*). Hen egg lysozyme, representative of the c-type lysozyme as designated by Prager et al. (*27*), consists of a single amino acid chain of 129 residues and has a molecular weight of 14 500. Many of the c-type lysozymes catalyze the hydrolysis of the β-1,4 glycosidic bonds between the C1 of *N*-acetyl muramic acids (MurNAc) and the C4 of *N*-acetyl glucosamines (GlcNAc) of the peptidoglycan in the bacterial cell walls and catalyze the hydrolysis of chitin, the homopolymer of GlcNAc. Three amino acid residues (Glu35, Asp52, and Asp101) are in the substrate binding site, and the side chains of Glu35 and Asp52 have been inferred as the essential groups for general acid−base catalysis of lysozyme (*18*).

A detailed explanation of the sweetness of the lysozyme is as yet unknown. It has not been clarified whether the sweetness of hen egg lysozyme is derived only from

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the protein molecule that is from its tertiary structure or from the carbohydrates produced by the lysozyme treatment. In addition, there is no information about g-type lysozymes, which also exhibit sweetness.

In the present work, we focused on whether the sweetness elicited by hen egg lysozyme was related to its enzymatic activity. First, we examined whether the four disulfide bonds that stabilize protein conformation are related to the sweetness of lysozyme and investigated the relationship between thermal denaturation and loss of sweetness. Chemical modification of carboxyl groups of lysozyme was employed to determine whether these residues influenced the sweetness. Finally, we investigated g-type, goose, and ostrich lysozymes.

MATERIALS AND METHODS

Materials. Hen egg lysozyme was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and further purification was performed by crystallization. Goose eggs were kindly donated by the Kyoto Municipal Zoo. Ostrich eggs were obtained from a country store (Nagano, Japan). Reduced and *S*-3-(trimethylated amino) propylated lysozyme was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *Micrococcus luteus* was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Ethyl-3-dimethylaminopropylcarbodiimide (EDC) and glycine methylester hydrochloride were obtained from Nacalai Tesque. Aminomethansulfonic acid was from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). CM Toyopearl 650M was purchased from Tosoh Co. (Tokyo, Japan). All other chemicals were of guaranteed reagent grade.

Purification of Lysozymes. Hen egg lysozyme was purified by crystallization five times at pH 9.5 in the presence of sodium chloride (*28*). The purification of goose and ostrich lysozymes was performed according to the method of Canfield and McMurry (*21*) and that of Jollès et al. (*29*), with slight modification. In brief, egg white (total 250 mL) was separated from egg yolk and diluted twice in distilled water, then stirred for 1 h at room temperature. Three times the volume (1500 mL) of saturated ammonium sulfate solution was added. The precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against 50 mM Tris-HCl, pH 7.4, at 4 °C for 2 days. After the removal of the precipitate by centrifugation (5000*g*), the supernatant was applied on the CM-Toyopearl 650M column (2.2 × 20 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and eluted with a linear gradient of 50 mM Tris-HCl containing 150 mM NaCl, and 50 mM Tris-HCl containing 500 mM NaCl at pH 7.4, using a low-pressure gradientor LPG-1000 (Tokyorikakikai, Co., Tokyo, Japan). The fractions containing lysozyme activity were combined and dialyzed against distilled water, then concentrated by using a Centriplus apparatus at 4 °C (Millipore Co., Bedford, MA).

Measurement of Enzymatic Activity of Lysozyme. Enzymatic activity of lysozyme was determined by measuring the clearing of turbidity of *M. luteus* suspension (substrate solution) at 450 nm. *M. luteus* suspension was prepared by mixing *M. luteus* (15 mg) with a 50 mM potassium phosphate buffer at pH 7.0 (100 mL) that had been sterilized by filtration. The mixture was suspended by gentle swirling and incubated at 25.0 °C for 2 h. Prior to reaction of the sample, the absorbance of this mixture was adjusted to 0.750 ± 0.050 at 450 nm (measured against air; cuvette path length = 1.0 cm) by dilution with the same buffer. Fifty microliters of lysozyme solution (0.47 μM) was added to 2.95 mL of the suspension in a cuvette at 25.0 °C and mixed. The decrease in absorbance was monitored by a Shimadzu UV−Vis spectrophotometer (UVmini-1240, Shimadzu Co., Kyoto, Japan). Concentration of lysozyme was determined spectrophotometrically from the absorbance at 280 nm ($E_{1\%}$ = 26.4, *30*). The residual activity of lysozyme is represented as the percentage of that of native lysozyme.

Sensory Analysis of the Sweetness of Lysozymes. The sweetness of each lysozyme derivative was evaluated as follows. Three subjects participated in a taste test. First, 3 mL of the lysozyme solution in a 2-fold dilution series (from 200 nM to 200 μM) was prepared. The lysozyme dilutions were tested in the order of increasing concentration. Ten-times and fifty-times higher concentrations of lysozyme solution (140 and 700 μM, respectively) were used for the sweetness assay.

SDS−Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed on a 13.5% gel according to the method of Laemmli (*31*).

Preparation of Lysozymes Heated at Various Temperatures. Recrystallized hen egg lysozyme was dissolved in water and dialyzed against 100 mM sodium acetate buffer, pH 4.0. Lysozyme in 100 mM sodium acetate buffer (protein concentration, 700 or 70 μM) was incubated in a dry thermo unit (model: TAH-1G, TAITEC, Co., Tokyo, Japan) at various temperatures (from 40 to 95 °C) for 25 h. Samples were periodically withdrawn and dialyzed extensively against distilled water. The concentration of the dialysate was adjusted to 700 or 140 μM for the sweetness assay.

Preparation of the Inactive Lysozyme. Chemical modification of the carboxyl groups in hen egg white lysozyme was carried out as follows, based on the methods reported by Hoare and Koshland (*32*), and Lin and Koshland (*33*). Lysozyme (700 μM) and aminomethansulfonic acid (250 mM) or glycine methyl ester (1.0 M) were mixed and dissolved in water at pH 4.75 and added to 100 mM 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) at 25 °C. The pH of the reaction mixture was maintained at 4.75 with 1 M HCl. The EDC addition was repeated every 1 h. Reaction mixtures were periodically withdrawn, and the reaction was quenched by the addition of 10× the volume of 1.0 M sodium acetate buffer, pH 4.75. These aliquots were dialyzed against distilled water and against 50 mM Tris-HCl buffer, pH 7.4. Dialysates were purified by CM Toyopearl columns as described above.

RESULTS AND DISCUSSION

Lysozyme is one of the most thoroughly characterized enzymes, and the sweetness of lysozyme seems to be widely known among food and pharmaceutical investigators. However, it is unclear why the hen egg lysozyme elicits sweetness, and whether its sweetness is derived from its protein structure or only from its primary structure or from the carbohydrate produced by hydrolysis of the cell walls through lysozyme treatment. For purposes of investigating this sweetness, we prepared various lysozyme samples.

Sweetness of Hen Egg Lysozyme for Human and Effects of Disulfide Reduction and *S*-3-(Trimethylated Amino) Propylation on Sweetness. Taste stimulation by hen egg lysozyme was detected at 10 μM, and sweetness was perceived at 20 μM (Figure 1). Above 30 μM, strong sweetness stimulation was maintained. The sweetness of *S*-3-(trimethylated amino) propylated lysozyme was not detected up to 50 μM (Figure 1). Enzymatic activity of *S*-3-(trimethylated amino) propylated lysozyme was not detected below 50 μM. These findings indicate that a specific conformation of lysozyme, which is maintained by intramolecular disulfide linkage, is required for the hen egg lysozyme to taste sweet. The taste of *S*-3-(trimethylated amino) propylated lysozyme had a potent astringency and aftertaste.

Heat Treatment of Lysozyme. Sweetness of lysozyme was not remarkably changed by heating at 70 °C for 25 h or 80 °C for 8 h (Table 1), whereas no sweetness was perceived from the lysozyme heated at 95 °C for 18 h. Lysozyme incubated for 25 h at 95 °C had a potent astringency and aftertaste. Enzymatic activity was not altered by heating below 60 °C, increased on heating at 80 °C for the first 2 h, and decreased at prolonged heating at 80 °C. The residual activity after 25 h heating

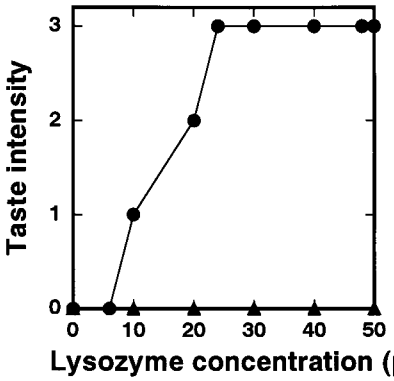

Figure 1. Sweetness intensity of reduced and *S*-3-(trimethylated amino) propylated lysozyme and native lysozyme. The sweetness intensity of native (closed circle) and reduced and *S*-3-(trimethylated amino) propylated lysozyme (closed triangle) are plotted according to increases of concentration. Intensity values: 0 = no taste; 1 = taste stimulation; 2 = sweet taste; and 3 = potently sweet taste.

Table 1. Sweetness and Enzymatic Activity of Heat-Treated Lysozymes

	sweetness[a]		enzymatic activity[b]
	70 μM	700 μM	(10^3 units/mg)
40 °C, 25 h	+	+	57
50 °C, 25 h	+	+	57
60 °C, 25 h	+	+	63
70 °C, 25 h	+	+	35
80 °C, 8 h	+	+	31
95 °C, 6 h	+	+	19
95 °C, 18 h	−	−	0

[a] Sweetness of the sample solution (70 and 700 μM) was elucidated by the sensory method shown in the text; +, sweet taste; −, no sweet taste. [b] Enzymatic activity indicates units per 1 mg of protein. One unit is defined as the decrease in absorbance at 450 nm of 0.001/min at 25 °C, pH 7.0. The activity of native lysozyme is 50 000 units/mg.

at 80 °C was approximately 8% of that of native lysozyme. Heating at 95 °C also showed that enzymatic activity rapidly decreased (Figure 2). The loss of enzymatic activity of lysozyme seems to be induced by chemical reactions including deamidation of Asn and/or hydrolysis of the Asp−X peptide bonds (*34*, *35*), suggesting the necessity of a specific protein conformation for sweetness elicitation as well as for enzymatic activity. In addition, reduced and *S*-3-(trimethylated amino) propylated lysozyme and 25 h-heated lysozyme exhibited a potent astringency.

Chemical Modification of Lysozyme. To clarify whether the regions of protein molecule eliciting of sweet taste is the same as that of enzymatic activity, carboxyl groups of lysozymes, which appeared to include Glu35, Asp52, and Asp101, were modified with glycine methyl ester and aminomethan sulfonic acid (*32*, *33*). Sweetness and enzymatic activity were plotted against reaction time (Figure 3). No difference was observed in sweetness between native and chemically modified lysozyme. However, the enzymatic activity of the lysozyme modified with glycine methyl ester was less than 5% of that of native lysozyme after 5 h reaction. The modification with aminomethansulfonic acid gave almost the same results as those of glycine methyl ester. These results clearly indicate that sweetness is not related to enzymatic activity and that these two functions are entirely independent. The sweetness of hen egg lysozyme seems to require a specific conformation that differs from that required for enzymatic activity.

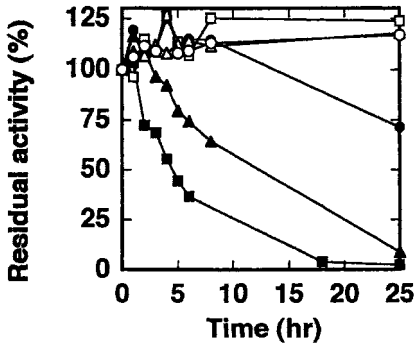

Figure 2. Residual activity of hen egg lysozyme at various temperatures. Lysozyme (700 or 70 μM) in 100 mM acetate buffer at pH 4.0 was heated at various temperatures: 40 °C (○), 50 °C (△), 60 °C (□), 70 °C (●), 80 °C (▲), and 95 °C (■). Enzymatic activity of lysozyme was measured by the clearing of turbidity of *M. luteus* at 450 nm. The residual activity of lysozyme is represented as a percentage of that of native lysozyme.

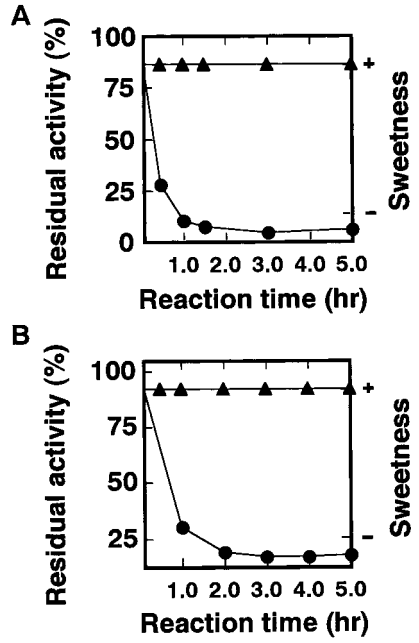

Figure 3. Sweetness and residual activity of chemically modified lysozymes. Lysozyme (700 μM) is chemically modified with (A) glycine methyl ester (1.0 M) and (B) aminomethansulfonic acid (0.25 M) in the presence of EDC, at pH 4.75. Closed circles indicate residual activity, and triangles indicate sweetness.

Sweetness of g-Type Lysozyme. The lysozyme from hen egg white belongs to the c-type lysozymes, and those from goose and ostrich egg white belong to the g-type lysozymes. Goose egg lysozyme consists of a single amino acid chain of 185 residues and has a molecular weight of 20 500. Ostrich lysozyme has a slight sequence homology to hen lysozyme (*29*), although the N-terminal portion of g-type lysozyme contains four half-cystine residues that are not observed in hen lysozyme (*36*). Lysozymes from hen, goose, and ostrich were purified from respective eggs, and their purity was confirmed by SDS−PAGE (Figure 4). The molecular weight of hen, goose, and ostrich lysozymes was approximately 14 500, 20 000, and 20 000, respectively. Although both types of lysozymes are basic proteins and exhibit muramidase activity, there are significant differences between these types of lysozymes not only in size, amino acid sequence, and tertiary structure, but

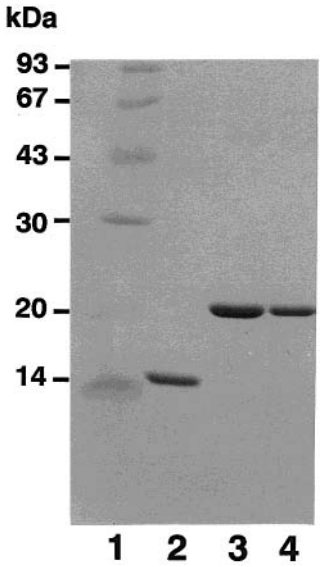

Figure 4. SDS−PAGE analysis of hen and goose egg lysozymes. SDS−PAGE was performed on a 13.5% gel and stained with Coomassie Brilliant Blue. Lane 1, MW marker; lane 2, hen egg lysozyme; lane 3, goose egg lysozyme; lane 4, ostrich egg lysozyme.

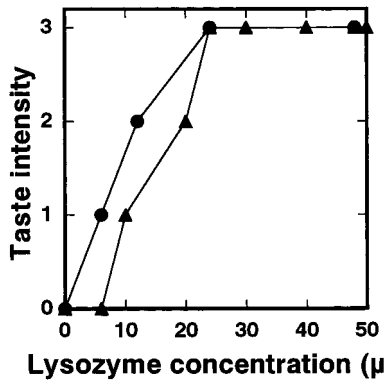

Figure 5. Sweetness intensity of goose egg lysozyme and hen egg lysozyme. The sweetness intensity of goose lysozyme (closed circle) and hen lysozyme (closed triangle) are plotted according to increases in concentration. Intensity values are the same as those used in Figure 1.

also in enzymatic specificity and inhibition properties. The lowest concentration for the perception of sweetness of g-type lysozyme, that is, the threshold, was around 6 μM, which was slightly lower than that of hen egg lysozyme (10 μM) (Figure 5). The sweetness of the g-type lysozyme is clearer and has less aftertaste than that of hen lysozyme.

As for sweet protein, several proteins including thaumatin were known. Although the basicity of the protein molecule seemed to be important for sweetness, some acidic sweet proteins have been found (*9*). Recently, the importance of lysine residues for elicitation of sweetness by thaumatin was emphasized by modification of lysine residues on one side of the thaumatin molecule (*13*). On the basis of the above consideration, we suggest that the elicitation of sweetness by both types of lysozymes derive from the tertiary structure, which is required for maintenance of the localization of the surface positive charge of the protein molecule. Further investigation is needed to clarify the details of the sweetness of lysozymes, that is, to elucidate and distinguish among similarly sweet proteins. Such studies, including a recombinant lysozyme and mutagenesis approach, are in progress.

Egg white lysozyme is an acceptable food protein, and its application as a food preservative and as a therapeutic agent has been expanding. Given that lysozyme is stable at an acid pH, it has potential application as a sweetener in the food industry. However, some fundamental studies on the use of lysozyme in foods are also needed.

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LITERATURE CITED

(1) Janusz, J. M. In Progress in sweetners; Grenby, T. H., Ed.; Elsevier Science Publishers LTD: England, 1989; pp 1−46.
(2) Eggers, S. C.; Acree, T. E.; Shallenberger, R. S. Sweetness chemoreception theory and sweetness transduction. *Food Chem.* **2000**, 68, 45−49.
(3) Van der Wel, H.; Loeve, K. Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus danielli* Benth. *Eur. J. Biochem.* **1972**, *31*, 221−225.
(4) Van der Wel, H. Isolation and characterization of the sweet principle for *Dioscoreophyllum cumminsii* (Stapf) Diels. *FEBS Lett.* **1972**, *21*, 88−90.
(5) Morris, J. A.; Cagan, R. H. Purification of monellin, the sweet principle of *Dioscoreophyllum cumminsii*. *Biochim. Biophys. Acta* **1972**, *261*, 114−122.
(6) Van der Wel, H.; Larson, G.; Hladik, A.; Hladik, C. M.; Hellekant, G..; Glaser, D. Isolation and characterization of pentadin, the sweet principle of *Pentadiplandra brazzeana* Baillon. *Chem. Senses* **1989**, *264*, 6655−6659.
(7) Liu, X.; Hu, Z.; Maeda, S.; Aiuchi, T.; Nakaya, K.; Kurihara, Y. Purification, complete amino acid sequence and structure characterization of the heat stable sweet protein, mabinlin II. *Eur. J. Biochem.* **1993**, *211*, 281−287.
(8) Yamashita, H.; Theeraship, A.; Nakaya, T.; Nakamura, Y.; Kurihara, Y. Purification and complete amino acid sequence of a new type of sweet protein with taste-modifying activity, curculin. *J. Biol. Chem.* **1990**, *265*, 15770−15775.
(9) Ming, D.; Hellekant, G. Brazzein, a new high-potency thermostable sweet protein from *Pentadiplandra brazzeana* B. *FEBS Lett.* **1994**, *355*, 106−108.
(10) Ogata, C. M.; Hatada, M.; Tomlinson, G.; Shin, W.-C.; Kim, S.-H. Crystal structure of the intensely sweet protein monellin. *Nature* **1987**, *328*, 739−742.
(11) Ogata, C. M.; Gordon, P. F.; de Vos, A. M; Kim, S.-H. Crystal structure of a sweet tasting protein thaumatin I, at 1.65 Å resolution. *J. Mol. Biol.* **1992**, *228*, 893−908.
(12) Slootstra, J. W.; De Geus, P.; Haas, H.; Verrips, C. T.; Meloen, R. H. Possible active site of the sweet-tasting protein thaumatin. *Chem. Senses* **1995**, *20*, 535−543.
(13) Kaneko, R.; Kitabatake, N. Structure sweetness relationship study of sweet protein thaumatin: Importance of lysine residues, *Chem. Senses* **2001**, *26*, 167−177.
(14) Maehashi, K.; Udaka, S. Sweetness of lysozyme. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 605−606.
(15) Jollès, P.; Jollès, J. Review of progress in lysozyme research. *Mol. Cell. Biochem.* **1984**, *53*, 165−189.
(16) Imoto, T.; Johnson, L. N.; North, A. C. T.; Phillips, D. C.; Rupley, J. A. In Boyer, P. D. (ed.), *The Enzymes*, Vol. 7, Academic Press: New York, 1972, pp 665−868.

(17) Blake, C. C. F.; Johnson, L. N.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, V. R. Crystallographic studies of the activity of hen egg-white lysozyme, *Proc. R. London Ser. B. Biol. Sci.* **1967**, *167*, 378−388.
(18) Phillips, D. C. The hen egg white lysozyme molecule, *Proc. Nat. Acad. Sci. U.S.A.* **1967**, *57*, 484−495.
(19) Smith, L. J.; Sutcliffe, M. J.; Redfield, C.; Dobson, C. M. Structure of hen lysozyme in solution. *J. Mol. Biol.* **1993**, *229*, 930−994.
(20) Dianoux, A,-C..; Jollès, P. Study of a lysozyme poor in cystine and tryptophan: the lysozyme of goose egg white. *Biochim. Biochem. Acta,* **1967**, *133*, 472−479.
(21) Canfield, R. E.; McMurry, S. Purification and characterization of a lysozyme from goose egg white. *Biochem. Biophys. Res. Commun.* **1967**, *26*, 38−42.
(22) Simpson, R. J.; Morgan, F. J. Complete amino acid sequence of Embden goose (*Anser anser*) egg-white lysozyme. *Biochim. Biophys. Acta* **1983**, *744*, 349−351.
(23) Arnheim, N.; Hindenburg, A.; Begg, G. S.; Morgan, F. J. Multiple genes for lysozyme in birds. Studies on black swan egg white lysozymes. *J. Biol. Chem.* **1973**, *248*, 8036−8042.
(24) Canfield, R. E.; Kammerman, S.; Sobel, J. H.; Morgan, F. J. Primary structure of lysozymes from man and goose. *Nature, New Biol.* **1971**, *232*, 16−17.
(25) Jollès, P.; Saint-Blancard, J.; Charlemagne, D.; Dianoux, A.-C.; Jollès, J.; Le Baron, J. Comparative behavior of six different lysozymes in the presence of an inhibitor. *Biochim. Biophys. Acta* **1968**, *151*, 532−534.
(26) Arnheim, N.; Steller, R. Multiple genes for lysozyme in birds. *Arch. Biochem. Biophys.* **1970**, *141*, 656−661.
(27) Prager, E. M.; Wilson, A. C.; Arnheim, N. Widespread distribution of lysozyme g in egg white of birds. *J. Biol. Chem.* **1974**, *249*, 7295−7297.
(28) Alderton, G.; Fevold, H. L. Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. *J. Biol. Chem.* **1946**, *164*, 1−5.
(29) Jollès, J.; Périn J.-P.; Jollès, P. The ostrich (*Struthio camelus*) egg-white lysozyme. *Mol. Cell. Biochem.* **1977**, *17*, 39−44.
(30) Kumagai, I.; Miura, K. Enhanced bacteriolytic activity of hen egg-white lysozyme due to conversion of Trp62 to other aromatic amino acid residues, *J. Biochem.* **1989**, *105*, 946−948.
(31) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **1970**, *227*, 680−685.
(32) Hoare, D. G.; Koshland, D. E., Jr. A procedure for the selective modification of carboxyl groups in proteins, *J. Am. Chem. Soc.* **1966**, *88*, 2057−2058.
(33) Lin, T.-Y.; Koshland, D. E., Jr. Carboxyl group modification and the activity of lysozyme, *J. Biol. Chem.* **1969**, *244*, 505−508.
(34) Ahern, T. J.; Klibanov, A. M. The mechanisms of irreversible enzyme inactivation at 100 °C. *Science* **1985**, *228*, 1280−1284.
(35) Tomizawa, H.; Yamada, H.; Imoto, T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 °C. *Biochemistry* **1994**, *33*., 13032−13037.
(36) Schoentgen, F.; Jollès, J.; Jollès, P. Complete amino acid sequence of ostrich (*Struthio camelus*) egg-white lysozyme, a goose type lysozyme. *Eur. J. Biochem.* **1982**, *123*, 489−497.

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