Enhanced Antimicrobial Action of Lysozyme against Gram-Negative and Gram-Positive Bacteria Due to Modification with Perillaldehyde

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Hen egg white lysozyme was covalently modified to various degrees with perillaldehyde, a major phenolic aldehyde in the steam distillate of the green leaves of Japanese shiso [*Perilla frutescens* (Labiatae)], via Schiff base formation followed by reduction with sodium borohydride. Free amino group determinations revealed three lysozyme derivatives, designated LPa_1, LPa_2 and LPa_4, which contain 0.5, 2.4, and 3.9 residues of perillaldehyde, respectively. These modifications had no dramatic effect on the solubility of the lysozyme derivatives. The lytic activity of the modified lysozymes against *Micrococcus lysodeikticus* was slightly decreased as the degree of modification increased, but even LPa_4 retained 73% of the activity of the unmodified lysozyme. Fluorescence spectra revealed some conformational changes associated with derivatization of lysozyme with perillaldehyde. Lysozyme modified with four perillaldehyde residues exerted a markedly enhanced antimicrobial activity against both Gram-negative bacteria (*Escherichia coli* K12) and Gram-positive bacteria (*Staphylococcus aureus*) compared to the activity of perillaldehyde or lysozyme alone or even their mixtures when examined at the same mole basis. The present approach strongly suggests that the perillaldehyde–lysozyme conjugate can be of value as a novel bactericidal agent in formulated drug or food systems. This approach also, suggests that the catalytic function of lysozyme may be utilized in the development of new therapies or food preservatives by conjugating the molecule with antibacterial compounds which cannot be used alone as food ingredients.

Keywords: *Antimicrobial action; lysozyme; perillaldehyde; protein modification; fluorescence spectra*

INTRODUCTION

Drug discovery has relied on the systematic screening of natural products and synthetic chemicals. One natural product commonly used as a food preservative, particularly in Japan, is the edible green leaves of *Perilla frutescens* (Labiatae). It has long been implicated in traditional Chinese herb medicines (Duke, 1988). The major volatile component in the leaves was found to be perillaldehyde (Kang et al., 1992). Perillaldehyde has moderate antimicrobial activity against a broad spectrum of microbes. However, the involvement of perillaldehyde in food or drug applications seems to be hampered because of the strong odor and poor water solvation of the chemical and the high dose required to achieve favorable antibacterial activity.

It has recently been reported that lysozyme, when modified with fatty acids or a hydrophobic peptide, can be converted into a potent bactericidal agent against *Escherichi coli* K12 (Ibrahim et al., 1991, 1993, 1994). These results argued that the bacteriolytic activity of lysozyme can be utilized to deliver a membrane binding domain to the inner membrane of the bacteria. This proposal was based on the fact that lysozyme has a high tendency to interact with lipopolysaccharides (LPS) (Ohno and Morrison, 1989) and subsequently distort the normal packing between phosphate groups of phospholipids and LPS in the outer membrane (a permeability barrier) of Gram-negative bacteria. Despite this action of lysozyme on the integrity and the permeability of the outer membrane, it has little effect on the viability of the bacteria. In view of this action of lysozyme on the permeability barrier of Gram-negative bacteria, we postulated that lysozyme may facilitate the delivery of hydrophobic antibiotics to the killing site of bacteria (the inner membrane). Here, our strategy of developing a potent bactericidal lysozyme against both Gram-negative and Gram-positive bacteria was based on coupling perillaldehyde, a naturally occurring phenolic aldehyde, to lysozyme to utilize the catalytic function and polycationic properties of lysozyme to facilitate the localization and concentration of the antimicrobial perillaldehyde in the killing site of bacteria. Regardless of the benefits success would have for industrial applications, achievement of this goal would provide evidence that lysozyme can be utilized to deliver hydrophobic antibiotics, which are known to be excluded by the outer membrane, to the Gram-negative bacteria. This paper reports that lysozyme derivatives incorporating two or four preillaldehyde residues are very active in killing both *Escherichia coli* K12 (a Gram-negative bacterium) and *Staphylococcus aureus* (a Gram-positive bacterium).

MATERIALS AND METHODS

Lysozyme purified from fresh hen egg white and recrystallized five times was a product of Taiyo Kagaku Co., Mie, Japan. The microbial substrate of lysozyme, *Micrococcus lysodeikticus* cells, was purchased from Sigma Chemical Co. (St. Louis, MO). Perillaldehyde (molecular mass 150.22 Da), extracted from the green leaves of *P. frutescens* (Labiatae) was obtained from Tokyo Kasei Co. (Tokyo, Japan). As test microorganisms *E. coli* K12 (IFO 3301) and *S. aureus* (IFO 14462) were purchased from Institute for Fermentation, Osaka (IFO), Japan. Unless otherwise stated, all reagents used in this study were of reagent grade.

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0021-8561/94/1442-1813$04.50/0

Modification of Lysozyme. Perillaldehyde was covalently attached to the ϵ-amino group of lysyl residues of lysozyme through Schiff base formation followed by reduction with sodium borohydride ($NaBH_4$) using the method of Wang and Tu (1969). To obtain lysozyme with various degrees of modification, perillaldehyde/lysozyme mole ratios of 3, 7, and 50 were used. The mole content of lysine residues in lysozyme was considered to be seven residues (six side chains of lysine plus the amino terminus). Two milliliters of hexane containing an appropriate amount of perillaldehyde (31.2, 73.8, and 520 μM final concentration in the reaction mixture) was gradually added to 10 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 150 mg of lysozyme (10.4 μM). An equal volume of sodium borohydride, 1 mg/mL, in ice-cold 0.3 M potassium phosphate buffer, pH 7.0, containing 2.5 M NaCl, was added to the reaction mixture. The mixture was then allowed to stand for 30 min, with gentle agitation at 0 °C. The addition of $NaBH_4$ was repeated twice at 30-min intervals. Lysozyme was precipitated by adding 2 volumes of saturated sodium sulfate (Na_2SO_4). The precipitate was collected by centrifugation (26000*g* for 30 min, at 20 °C), and resuspended in 30 mL of distilled water. Thereafter, this solution was dialyzed exhaustively for three days against daily exchanges of distilled water in the cold. The lysozyme derivatives were then lyophilized and used for the following tests. A control sample, without addition of perillaldehyde in hexane, was taken through all steps of preparation.

The protein concentration of lysozyme derivatives was quantitated using the modified Lowry method of Miller (1959).

The degrees of modification were determined by measuring the free amino groups of control and modified lysozyme, using trinitrobenzenesulfonic acid, (TNBS) reagent. A 100-μL portion of lysozyme solution (0.1% in 50 mM potassium phosphate buffer, pH 7.4) was incubated with 1 mL of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS at 37 °C for 2 h. Then 1 mL of 10% SDS and 1 mL of 1 N HCl were added before the absorbance at 344 nm was measured. Modified lysine residues were calculated from the difference between lysine content in unmodified and modified lysozymes, considering the mole content of free amino groups in lysozyme molecule is seven.

Protein solubility was assessed by measuring protein concentration after the solution of lysozyme or its derivatives (0.1% protein in 50 mM sodium phosphate buffer, pH 7.0) was passed through a membrane filter (0.45 μM) according to the method of Miller (1959). Solubility was expressed as percent of protein concentration in the filtrate of modified lysozyme with respect to that of control sample.

Lysozyme Activity. The lytic action of lysozyme derivatives against *M. lysodeikticus* cells was determined according to turbidometric methods based on the decrease in turbidity of a cell suspension following the addition of lysozyme derivatives. The lysis of cells in 50 mM potassium phosphate buffer, pH 6.2, was monitored at four different protein concentrations. A 100-μL aliquot of solutions of the lysozyme derivatives (final concentrations of 0.11, 0.21, 0.43, or 0.85 μg/mL) was added into 1.9 mL of *M. lysodeikticus* cell suspension (final concentration of 190 μg/mL dry weight in the same buffer). This lysis mixture gave initial absorbance of 0.75–0.8 at the onset of the experiment. The decrease in absorbance at 600 nm (25 °C) was monitored using a Hitachi U-2000 recording spectrophotometer.

Antimicrobial Activity. The antimicrobial assay of lysozyme derivatives employed *E. coli* K12 3301 (as a representative microorganism of Gram-negative bacteria) and *S. aureus* IFO14462 (as a Gram-positive bacteria). In certain experiments, a 1.0 mL of diluted, midlogarithmic phase cultures (2×10^5 cells/mL in 10 mM potassium phosphate buffer, pH 7.0) was mixed with a 1.0-mL solution of the lysozyme derivatives (200 μg/mL protein in the same buffer). The antimicrobial action of mixtures of lysozyme and perillaldehyde was also examined. Perillaldehyde was dissolved in an appropriate amount of dimethylformamide (DMF) and mixed with lysozyme solution to give final volume of 1 mL. Each assay, thus, contained 0.2% DMF, which did not affect the growth of the microorganisms tested. The final protein concentration was 100 μg/mL of bacteria suspension containing 10^5 cells. Bacteria were incubated with lysozyme derivatives for 1 h at 37 °C. A 100-μL portion of dilutions was plated out onto two separate MacConkey (for *E. coli* assay) or brain heart infusion (BHI) (for *St. aureus* assay) agar plates. Colony forming units (CFU) were counted after incubation at 35 °C overnight. Percentage survival was represented with respect to control lysozyme.

Unless otherwise stated, all experiments of the present study were performed in triplicate.

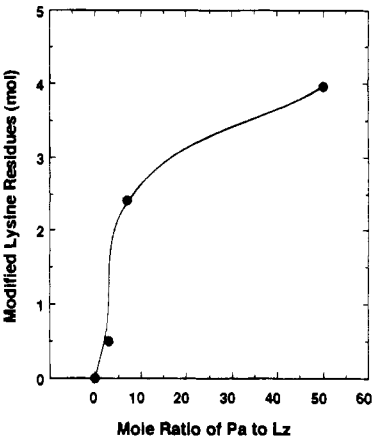

Figure 1. Number of modified lysine residues of lysozyme as a function of increasing mole ratio of perillaldehyde (Pa) to lysozyme (Lz) in the reaction mixture.

RESULTS

Degree of Modification. To achieve a stable amide bond between perillaldehyde and the ϵ-amino groups of lysyl residues, lysozyme was incubated with different mole ratios of the phenolic aldehyde, perillaldehyde, under reducing conditions. The extent of modified lysine residues as a function of increasing mole ratio of perillaldehyde is shown in Figure 1. As shown, incubation of 3, 7, or 50 mole excess of perillaldehyde to lysozyme produced lysozyme derivatives with 0.5, 2.4, or 3.9 mol of modified lysine residues, respectively. Although the perillaldehyde was present in a 7-fold molar excess over each ϵ-amino group on the lysozyme, the maximum degree of modification did not exceed 4 mol/mol (in the case of 50 mole ratio of Pa to Lz). It seems most likely, therefore, that only two lysine residues are readily reactive while the other residues may be buried in the interior of the molecule or might be implicated in salt bridges that are known to maintain the compact folded structure of lysozyme (Bashford and Karplus, 1990). Lysozyme derivatives incorporating 0.5, 2.4, and 3.9 residues of perillaldehyde were designated LPa_1, LPa_2, and LPa_4, respectively.

Solubility of Modified Lysozyme. The effects of this modification on the solubility and solution turbidity of lysozyme are shown in Figure 2. As can be seen, this modification had minimal effect on the protein solubility, and 92% of the solubility was retained even with the maximum degree of modification obtained in this study (LPa_4). Although a progressive increase in turbidity occurred with increasing degrees of lysozyme modification, the changes were very small.

Fluorescence Spectra of Modified Lysozyme. Figure 3 shows the fluorometric emission spectra of lysozyme modified to various degrees with perillaldehyde. When the protein was excited at 355 nm, a production of fluorescence was observed which exhibited emission maxima at 360 nm (Figure 3A). A progressive

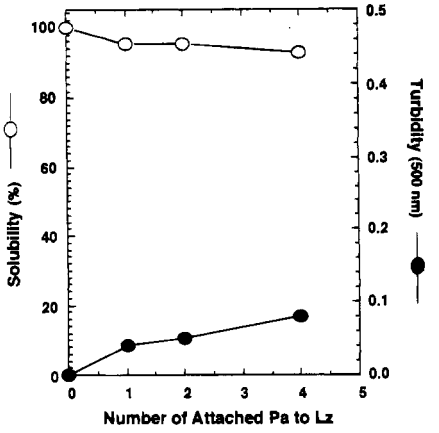

Figure 2. Protein solubility and solution turbidity of lysozyme derivatives. Values of solubility of modified lysozyme types are represented as the ratio to unmodified control: (○) solubility; (●) solution turbidity at 500 nm.

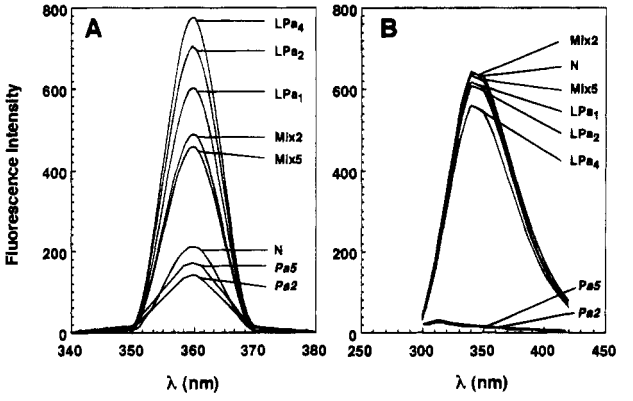

Figure 3. Fluorescence emission spectra of lysozyme modified to various degrees with perillaldehyde recorded upon excitation at 355 (A) or 280 nm (B). N indicates native lysozyme. LPa_1, LPa_2, and LPa_4 indicate lysozyme derivative incorporating 0.5, 2.4, and 3.9 mol of perillaldehyde, respectively. Mix2 and Mix5 indicate mixtures of perillaldehyde and lysozyme at mole ratios of 2 and 5, respectively. Pa2 and Pa5 indicate the spectrum of perillaldehyde alone at mole concentrations similar to that in Mix2 and Mix5, respectively.

increase of fluorescence intensity emerged with an increase in the degree of modification. It should be pointed out that a similar behavior was obtained with a mixture between perillaldehyde and lysozyme at mole ratios of 2 and 5 or even with perillaldehyde alone. However, the maximum fluorescence intensities of LPa_2 and LPa_4 were much greater than that of mixtures of perillaldehyde and lysozyme at approximately similar mole ratios (Mix2 and Mix5), suggesting that covalent attachment of perillaldehyde to lysozyme caused irreversible conformational changes in the lysozyme molecule, leading to changes in the environment of the aromatic side chain chromophores (tryptophan and tyrosyl residues). On the contrary, an increase in the number of attached perillaldehyde residues resulted in a remarkable decrease in the fluorescence intensity of lysozyme when excited at 280 nm (Figure 3B). The fluorescence quenching that accompanied the covalent attachment of perillaldehyde to lysozyme was not observed when they were only mixed, even when the mole ratio was similar to that in their adduct. Increase in the emission intensity of lysozyme excited at 355 nm reflects the exposure of the attached perillaldehyde residues to the exterior of the protein. Fluorescence quenching at excitation of 280 nm indicates conformational changes accompanying such modification of

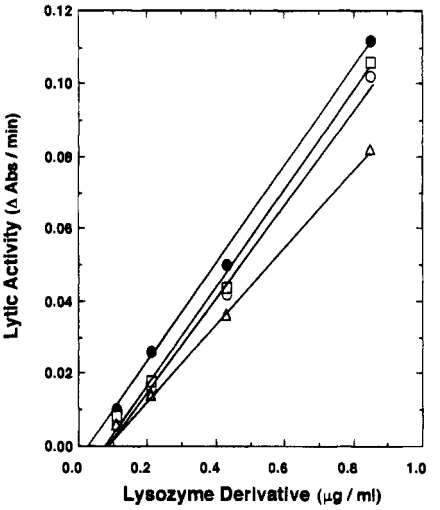

Figure 4. Lytic activities of lysozyme derivatives as a function of protein concentration: (●) native; (□) LPa_1; (○) LPa_2; (△) LPa_4.

lysozyme. These conformational changes did not occur when perillaldehyde and lysozyme were mixed without being conjugated. These results, however, demonstrate that the environment around the chromophoric residues of modified lyozyme is different from that of the unmodified one.

Lytic Activity of Modified Lysozyme. Lytic activities of lysozyme derivatives are shown in Figure 4. The enzymatic activities are expressed as the initial rate of decrease in the turbidity of a *M. lysodeikticus* suspension assessed at different protein concentrations. Although it is clear that modification with perillaldehyde resulted in a consistent decrease in the lytic activity of lysozyme, lysozyme derivative incorporating four perillaldehyde residues retained 73% activity with respect to unmodified lysozyme. Indeed, the results suggest that considerable bacteriolytic activity as well as water solubility of lysozyme can be preserved even with a degree of modification up to four residues per molecule of lysozyme. It has been reported that interaction of an aldehyde such as hexanal leads to extensive polymerization of lysozyme with significant damage in its amino acid residues and subsequent loss of the enzymatic activity (Tashiro et al., 1985; Matoba et al., 1984). In accordance with the literature data, our perillaldehyde–lysozyme conjugate appears to be a promising agent as its enzymatic activity is restored to a considerable extent and also because the undesirable polymerization did not occur.

Antimicrobial Activity of Modified Lysozyme with Perillaldehyde. Antibacterial actions of lysozyme derivatives were assessed by routinely employing *E. coli* K12 IFO 3301 (as a Gram-negative bacteria) and *S. aureus* IFO 14462 (as a Gram-positive bacteria). Figure 5 shows the antimicrobial activity of lysozyme covalently modified to various degrees with perillaldehyde and mixtures of lysozyme and perillaldehyde at mole ratios of 2 and 5 against *E. coli* K12. As shown, modified lysozyme types exerted significant bactericidal activity and the antimicrobial activity, was greatly intensified with an increase in the degree of modification. These antimicrobial effects were not observed with the mixture of lysozyme and perillaldehyde at mole ratios approximately similar to those in their conjugates. It should be pointed out that perillaldehyde alone had a very weak antimicrobial action against *E. coli* K12 when tested at a 5-fold molar excess over the concentration

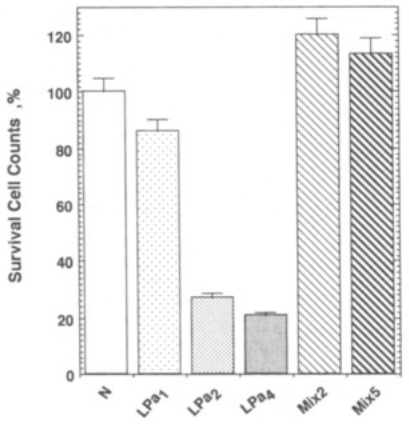

Figure 5. Antimicrobial activities of lysozyme derivatives against *E. coli* K12. N, LPa_1, LPa_2, and LPa_4 are the same as in Figure 3. Mix2 and Mix5 indicate mixtures of lysozyme and perillaldehyde at mole ratios of 2 and 5, respectively.

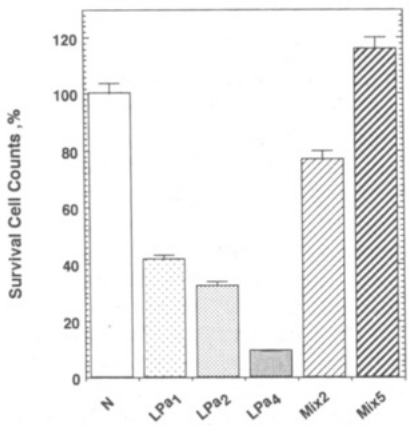

Figure 6. Antimicrobial activities of lysozyme derivatives against *S. aureus*. The symbols are the same as in Figure 5.

of lysozyme used in this study (data not shown). The results suggest that the attachment of the naturally occurring aromatic aldehyde, perillaldehyde, to the catalytic function of lysozyme has rendered the protein active in killing Gram-negative bacteria (*E. coli*) which are normally resistant to the bacteriolytic action of lysozyme. Furthermore, these data imply a direct involvement of both compounds of the conjugate in the killing action, but they could not operate synergistically when they were mixed without being conjugated. In fact, we cannot exclude the possibility that the enhanced antimicrobial activity of the modified lysozyme may be partially attributed to the conformational changes of lysozyme molecule, as revealed by fluorescence spectra (Figure 3).

Since both lysozyme and perillaldehyde have antimicrobial activity against Gram-positive bacteria, we have examined the antimicrobial effects of their conjugates on *S. aureus,* and the data are shown in Figure 6. Similar results were obtained with *S. aureus*, but the antimicrobial activities of the modified lysozymes against *S. aureus* were markedly stronger than their effects against *E. coli*. Indeed, these results are very intriguing, since the LPa_4 lysozyme derivative possesses less enzymatic activity than the unmodified lysozyme (Figure 4). Thus, one point that emerges from these results is that lysozyme and perillaldehyde act differently, because together (in the conjugate) they are able to exert antimicrobial effects that neither is able to achieve alone or in free mixture. It would seem, therefore, that their mode of action as a conjugate differs operationally and that each impinges on a distinct cellular target when they are coupled together. Thus, it can be concluded that perillaldehyde, as a hydrophobic compound, might be excluded or entrapped by the outer membrane but that this exclusion effect is circumvented when its hydrophilicity is enhanced by lysozyme. Our results do not exclude the possibility that the exposure of such a hydrophobic compound to the surface of lysozyme may have facilitated the interaction of lysozyme with the bacterial membrane through hydrophobic and electrostatic interactions between perillaldehyde with the hydrophobic core and the positively charged lysozyme molecule with the negatively charged phosphate groups of the lipid bilayer or membrane-bound proteins that are necessary for cellular metabolism or cell growth, thus altering biosynthetic reactions that normally occur at this intracellular site of the bacteria.

DISCUSSION

The major problem in developing effective antibiotics is the limited antimicrobial action to certain strains. This phenomenon is prominent among polysaccharides degrading enzymes, such as hen egg white lysozyme (Funatsu and Tsuru, 1977) and *N*-acetylmuramidase of microbial origin (Hayashi et al., 1989), which have been widely used as antimicrobial or anti-inflammatory agents in formulated food or drug systems. However, the antimicrobial activities of these enzymes are limited to certain Gram-positive bacteria, and they do not act on Gram-negative ones. The data of the present study clearly indicate that lysozyme incorporating two or more residues of a weak bactericidal phenolic aldehyde, perillaldehyde, was converted into a potent bactericidal agent against both Gram-negative and Gram-positive bacteria. Many of the medical properties of the green leaves as well as seeds of *P. frutescens* have been reported (Duke 1988; Terao et al., 1991). It has been reported that perillaldehyde exhibits antimicrobial activity against a broad spectrum of microorganisms. Its minimal inhibitory concentrations were found to be 1000 and 500 μg/mL against *S. aureus* and *E. coli*, respectively (Kang et al., 1992). Accordingly, perillaldehyde appears to be relatively more effective against Gram-negative bacteria than against Gram-positive ones. On the contrary, lysozyme is well-known to be a potent bactericidal protein against Gram-positive bacteria, but its effect on Gram-negative strains is limited. In light of these facts, we have postulated that lysozyme can act synergistically with perillaldehyde against either type of bacteria if they are coupled together. However, the dual antimicrobial action of lysozyme and perillaldehyde was remarkable when they were attached to each other but was undetectable with their free mixture even at a higher mole ratio than in the conjugate. An explanation could be the water insolubility of perillaldehyde, which became readily soluble when bound to the soluble protein. It is well-known that Gram-negative bacteria are highly resistant to many hydrophobic antibiotics, which are thought to be excluded by the intact membrane. Therefore, conjugation of such a hydrophobic compound to the protoplasting function of lysozyme was sufficient to cause the observed effect on the viability of *E. coli*, probably because it may facilitate the internalization and concentration of the phenolic groups of the perillaldehyde (a lethal structural motif to the membrane function; Nicholls, 1982) in the vicinity of the cytoplasmic membrane of the bacteria. These fascinating results are promising in terms of plausible

involvement of such conjugates in food and drug applications. The merit of this finding would come from the fact that perillaldehyde alone is an unfavorable antibiotic either in food or in drug manufacturing because of its weak antimicrobial activity and its strong flavor, which may not be acceptable by worldwide consumers. Moreover, perillaldehyde is a water-insoluble compound, a property that would limit its involvement in practical applications. Thus, the lysozyme–perillaldehyde conjugate elaborated in the present study, having reasonable water solvation and enzymatic activity, heralds an opportunity to introduce an alternative antimicrobial agent to develop better and safer foods.

The fact that modification of lysozyme with a hydrophobic bactericidal compound can effectively inhibit the growth of *E. coli* would suggest that such an approach might merit additional study by employing lysozyme to deliver various potent hydrophobic bactericidal compounds, such as cinnamic aldehyde, or many other known phenolic compounds, to the killing site of Gram-negative bacteria. In addition, the present study introduces a new conceptual utilization of lysozyme not only as an antimicrobial agent, to extend its narrow antibacterial spectrum, but also as a carrier protein for the efficient delivery of different substances to Gram-negative bacteria.

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Received for review November 15, 1993. Revised manuscript received April 26, 1994. Accepted May 18, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1994.