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Emulsifying properties and bactericidal action of chitosan–lysozyme conjugates

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Abstract

The solubility of chitosan was greatly improved by conjugation with lysozyme through Maillard-type protein–polysaccharide reaction. The emulsifying properties, especially emulsion stability, were improved by lysozyme–chitosan conjugation. The improvement in emulsifying properties was better in the conjugates of high molecular weight-type chitosan (HMC) than low molecular-type chitosan (LMC). The lysozyme–HMC conjugate has greatly improved emulsion stability at acidic pH. The conjugates have greatly enhanced bactericidal action against *Escherichia coil* K-12, a typical Gram-negative bacterium. Although the bactericidal activity of LMC was much lower than that of HMC, it was improved by conjugation with lysozyme. Thus, chitosan–lysozyme conjugate can be used as a new functional ingredient having excellent emulsifying properties and bactericidal action.

Keywords: Chitosan; Lysozyme; Conjugates; Functional properties; Bactericidal activity

1. Introduction

Protein–polysaccharide conjugates have been proposed to be used as a new functional biopolymer having excellent emulsifying properties and heat stability (Kato, Murata, & Kobayashi, 1988; Kato, Sato, & Kobayashi, 1989; Kato, Sasaki, Furuta, & Kobayashi, 1990; Nakamura, Kato, & Kobayashi, 1990, 1991, 1992). Ovalbumin–dextran conjugates prepared by covalent binding of the ε-amino groups in the protein to the reducing-end carbonyl group in the polysaccharide through controlled Maillard reaction revealed excellent emulsifying activity and emulsion stability even at higher salt concentration and in acidic pH condition when compared with commercial emulsifiers (Kato et al., 1990). Lysozyme–galactomannan conjugate also showed excellent emulsifying properties and enhanced bactericidal effect to Gram-negative bacteria (Nakamura et al., 1992).

Chitosan, a polyaminosaccharide, is a partially deacetylated polymer of N-acetyl glucosamine and is usually prepared from chitin (2 acetamido-2-deoxy beta-1, 4-D-glucan) which has been found in a wide range of natural sources such as crustaceans, fungi, and insects (Shepherd, Reader, & Falshaw, 1997). Chitosan is a natural hydrophilic biopolymer, non-toxic, and biodegradable (Kas, 1997).

The antimicrobial activity of chitosan is well observed in a wide variety of microorganisms including fungi, algae and some bacteria. However, the antimicrobial action is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g. plain or derivative), degree of chitosan polymerization, host natural nutrient constituency, substrate chemical and/or nutrient composition, and environmental conditions such as substrate water activity and/or moisture (Cuero, 1999). The effect of bacterial growth phase, reaction temperature, pH value, and salts on the inhibitory activity of shrimp chitosan (98% deacetylated) against *Escherichia coil* were investigated (Tsai & Su, 1999). Decrease in the degree of polymerization of chitosan reduced the number of inhibited microorganisms, indicating that the

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functional groups for the growth inhibition are the cationic amino groups of chitosan (Young & Kauss, 1983). Lysozyme, on the other hand, attacks only specific positions of glycosidic bonds between the N-acetylhexosamines of the peptidoglycan layer in bacterial cell walls. The walls of Gram-positive bacteria are generally quite sensitive to attack by lysozyme. However, Gram-negative bacteria are less susceptible to such attack, because the outer membrane excludes lysozyme and prevents its access to the site of action on the peptidoglycan in cell walls (Nakamura et al., 1991).

The improvement of chitosan properties such as solubility and antimicrobial activity should be investigated for industrial application. Therefore, the conjugation of lysozyme with chitosan through Maillard reaction was attempted in this study. The emulsifying properties and antimicrobial activity of the conjugates were determined.

2. Materials and methods

2.1. Materials

MacConkey medium was obtained from Nissui Seiyaku Co., Japan. *E. coil* K-12 was from the Institute for Fermentation, Osaka, Japan. Water soluble low-molecular weight chitosan (LMC, MW 3–30 kDa) was purchased from Wako Co., Japan. High-molecular weight chitosan (HMC, MW 400 kDa, the degree of substitution 0.95) was provided by Professor Daizo Koga, Department of Biological Chemistry, Yamaguchi University. Lysozyme was purified from fresh egg white by recrytallizing at pH 9.5 in the presence of 5% NaCl. HMC was solubilized in 0.1 M acetate buffer (pH 4), centrifuged and then dialyzed against distilled water at 4 °C for 24 h. A galactomannan preparation (average MW of 15 kDa) was obtained by dialyzing a mannase hydrolysate of guar gum from Taiyo Chemical Co. (Tokyo, Japan). Unless otherwise stated, all reagents used in this study were reagent grade.

2.2. Lysozyme–polysaccharide conjugation

The lysozyme–polysaccharide powder mixtures were prepared by saturating the partner polysaccharide to keep the mixing molar ratio of protein to polysaccharide 1:4 for HMC, LMC and galactomannan, respectively. The mixtures were dissolved in water at 10% (w/v) and freeze-dried. Powdered lysozyme–polysaccharide mixtures were dry-heated at 60 °C under 79% relative humidity in a desiccator containing saturated KBr solution in the bottom for a given time (Nakamura et al., 1992). The lysozyme–polysaccharide conjugates were separated from unbound protein and polysaccharide by cation exchange chromatography on a column of CM-Toyopearl (Shu, Sahara, Nakamura, & Kato, 1996).

2.3. SDS-polyacrylamide gel electrophoresis

SDS-potyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (1970) with a 15% acrylamide separating get and a 3% acrylamide stacking get containing 0.1% SDS. Samples (15 μl, 0.2%) were prepared in a Tris-glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was performed at a current of 10 mA for 5 h in Tris-glycine electrophoretic buffer containing 0.1% SDS. After electrophoresis, the gels were stained for proteins with 0.2% Coomassie brilliant blue-R250. Protein stain was destained with 10% acetic acid containing 20% methanol. Densitometric measurement of the lysozyme and lysozyme–chitosan conjugate band was performed using a Model GS-700 Imaging Densitometer (Bio Rad, California).

2.4. Measurement of solubility

Sample solutions (0.1%) at various pH values (pH 4 & 5, 0.1 M acetate buffer; pH 6 & 7, 0.1 M phosphate buffer) were prepared. Solubility was estimated by measuring the turbidity of the solutions at 500 nm (Babiker, Fujisawa, Matsudomi, & Kato, 1996).

2.5. Measurement of emulsifying properties

The emulsifying properties of the samples were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 ml of corn oil and 3.0 ml of protein and protein–polysaccharide conjugate solution (0.1%) in 0.1 M acetate (pH 4.0) or phosphate buffer (pH 7.0) were shaken together and homogenized in an Ultra Turrax instrument (Hansen & Co., West Germany) at 12 000 rpm for 1 min at 20 °C. A 50 μl aliqot of the emulsion was taken from the bottom of the container at different time intervals and diluted with *5* ml of a 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after emulsion formation (0 min). The emulsion stability was estimated by measuring the half-time of the initial turbidity of the emulsion.

2.6. Measurement of antibacterial activity to E. coil

E. coil K-12 was cultured for 10 h (mid-exponential-phase) in Luria-Bertani medium (LB), and diluted 10^3 times with 20 mM phosphate buffer (pH 7.0), and 4.5 ml of the cell suspension was mixed with 0.5 ml of lysozyme, lysozyme–polysaccharides mixtures, or conjugates (50 μg/ml) and incubated at different temperatures (4, 20, 37, or 50 °C) for 30 min. To investigate the antimicrobial

effect of the protein and its conjugates at various concentration, the cell suspension was mixed with lysozyme, lysozyme–polysaccharide mixtures, or conjugates at various concentrations (2.5, 12.5, 25, or 50 μg/ml) and incubated at 37 °C for 30 min. Then a 100 μl portion of each treatment was surface-plated onto agar plate. Colonies were counted after incubation at 37 °C overnight (Nakamura et al., 1992). Sample-free solution was used as a control.

3. Results and discussion

3.1. Formation of lysozyme–polysaccharide conjugates

The formation of covalently bound lysozyme–chitosan conjugates through the Maillard reaction was confirmed by SDS-PAGE. As shown in Fig. 1, LMC–lysozyme conjugates were gradually formed during dry-heating at 60 °C and 79% relative humidity for 5, 10 and 15 days. The LMC–lysozyme conjugates showed broad high molecular weight bands during dry-heating (Fig. 1A), since LMC has a wide range of molecular weight distribution (3–30 kDa). Densitometric measurement indicated that unbound lysozyme gradually decreased during dry-heating, while the broad high molecular weight band increased, and after one week new higher molecular weight bands emerged in the boundary between staking and separating gels, and at the top of staking (Fig. 1B). On the other hand, in HMC–lysozyme conjugates, the intensity of the band at the top of the stacking gel was increased progressively with increase in dry-heating time, indicating that HMC–lysozyme conjugate can not enter the gel due to its high molecular (Fig. 2A). To evaluate changes in band intensity of lysozyme and HMC–lysozyme conjugate, densitometric measurement of the SDS-PAGE patterns was carried out. The unbound lysozyme decreased and the HMC–lysozyme conjugate increased during dry-heating (Fig. 2B). The results demonstrate that lysozyme was covalently attached to chitosans through the Maillard reaction between the ε-amino groups in the protein and the reducing-end carbonyl groups in polysaccharides during dry heating at 60 °C and 79% relative humidity. Lysozyme–galactomannan conjugate was used as a control, and the SDS-PAGE pattern of the lysozyme–LMC conjugate was almost identical to that of the lysozyme–galactomannan conjugate (Nakamura, Kobayashi, & Kato, 1994).

3.2. Solubility of lysozyme–polysaccharide conjugates

The effect of pH on the solubility of lysozyme–polysaccharides conjugates is shown in Fig. 3. The solubility was represented as an index of turbidity at 500 nm (Babiker et al., 1996). HMC was found to be highly

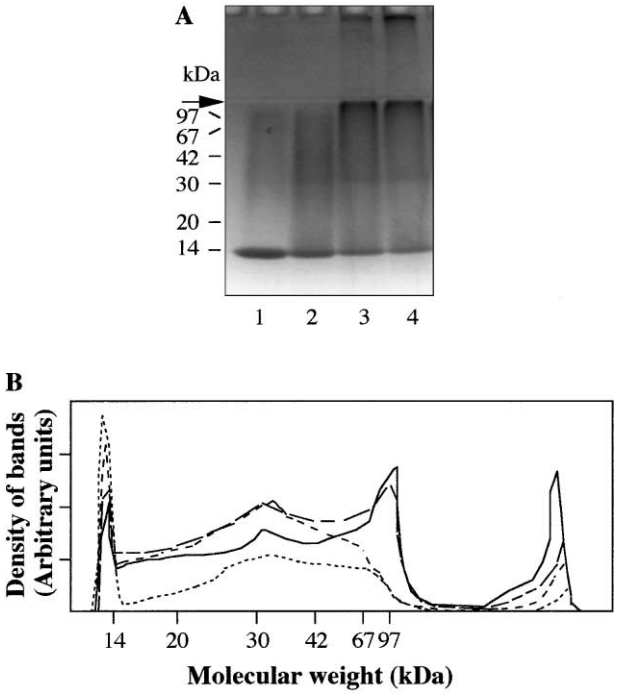

Fig. 1. SDS-PAGE pattern (A) and densitometric profile (B) of LMC-lysozyme conjugates. Lane 1–4, low molecular weight-type chitosan (LMC)–lysozyme conjugate dry-heated at 60 °C and 79% relative humidity for 1, 3, 7 and 14 days, respectively. Arrow in (A) indicates the boundary between the stacking and separating gels. Lines in (B): - - - - - - -, 1 day; -.-.-, 3 days; - - - -, 7 days; —, 14 days.

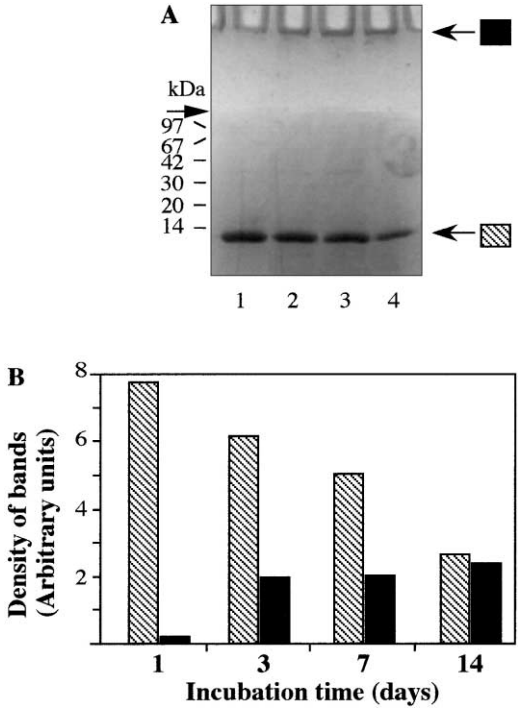

Fig. 2. SDS-PAGE pattern (A) and densitometric profile (B) of high molecular weight-type chitosan (HMC)–lysozyme conjugates. Lane 1–4, HMC-lysozyme conjugate dry-heated at 60 °C and 79% relative humidity for 1, 3, 7 and 14 days, respectively. Shadow bar indicates the lysozyme band and solid bar indicates the band of HMC–lysozyme conjugate at the top of stacking gel.

soluble at acidic pH, and less soluble and slightly viscous at neutral pH. However, when lysozyme was mixed with HMC, the mixture exhibited greatly increased solubility at neutral pH. This suggests noncovalent binding between chitosan and lysozyme, resulting in the solubilization of HMC. After conjugation with lysozyme, the solubitity further increased (Fig. 3). The lysozyme–HMC conjugate seems to form amphiphilic structure to suppress the aggregation of HMC molecules at neutral pH. In contrast, LMC has excellent solubility over a wide range of pH. The solubility of the lysozyme–LMC and lysozyme–galactomannan conjugates was as good as LMC and galactomannan.

3.3. Functional properties of lysozyme–polysaccharide conjugates

The effect of polysaccaharide conjugation on the emulsifying activity of lysozyme at pH 4.0 and 7.0 is shown in Fig. 4A and B, respectively. It is well known that most commercial emulsifiers show poor emulsifying properties at acidic pH. Therefore, the emulsifying properties of the conjugates were measured at both acidic and neutral pH. Although the emulsifying activity of lysozyme was greatly improved by mixing with HMC at pH 4.0 (Fig. 4A), further improvement was observed when the mixture was dry-heated at 60 °C and 79% relative humidity for 5, 10, and 15 days. LMC and galactomannan conjugates also exhibited improved emulsifying activity. The remarkable improvement in emulsifying activity in the lysozyme–HMC mixture may be due to noncovalent interactions. On the other hand, improvement in emulsifying activity in lysozyme–LMC mixture was not as marked, but pronounced in the lysozyme–LMC conjugate formed by dry-heating for 5 days. The emulsifying activity of lysozyme at pH 7.0 was also improved after conjugation with HMC, LMC, or galactomannan (Fig. 4B). The improvement of emulsifying activity in the lysozyme–chitosan conjugate at pH 4.0 and 7.0 was comparable to that of the lysozyme–galactomannan conjugate.

The effect of polysaccaharide conjugation on the emulsion stability of lysozyme at pH 4.0 and 7.0 are shown in Fig. 5A and B, respectively. The emulsion stability of lysozyme at pH 4.0 was greatly improved after conjugation with HMC when compared with LMC and galactomannan (Fig. 5A). The emulsion stability of chitosan–lysozyme conjugate dry-heated for 15 days was found to be 30, 20, and 10 min for HMC, LMC and galactomannan conjugates, respectively (Fig. 5A). On the other hand, the emulsion stability of lysozyme at pH 7.0 was greatly enhanced in the lysozyme–galactomannan conjugate (Fig. 5B). The emulsion stability of the HMC, LMC and galactomannan conjugates dry heated for 15 days were 5.0, 2.8, and 15 min, respectively.

The results indicate that the emulsion stability of lysozyme at pH 4.0 was greatly improved by chitosan conjugation and only slightly improved at pH 7.0 when compared with galactomannan conjugate. The differences in the emulsifying properties among polysaccharide conjugates may be attributed to differences in chemical structure, the positively charged state, the degree of polymerization and the solubility of the protein and/or polysaccharide moiety (Shu et al., 1996). It has been reported that the hydrophobic residues of protein denatured at the oil–water interface may be anchored to the surface of oil droplets in an emulsion and the hydrophilic parts of polysaccharide may be oriented to the water phase, thereby inhibiting the coalescence of the oil droplets (Kato, Minaki, & Kobayashi, 1993). Chitosan is a polycationic polymer soluble at acidic pH. Therefore, a stable emulsion may be formed in the presence of protein–chitosan conjugate due to suppression of coalescence of oil droplets by the increased cationic repulsive force. Chitosan–lysozyme

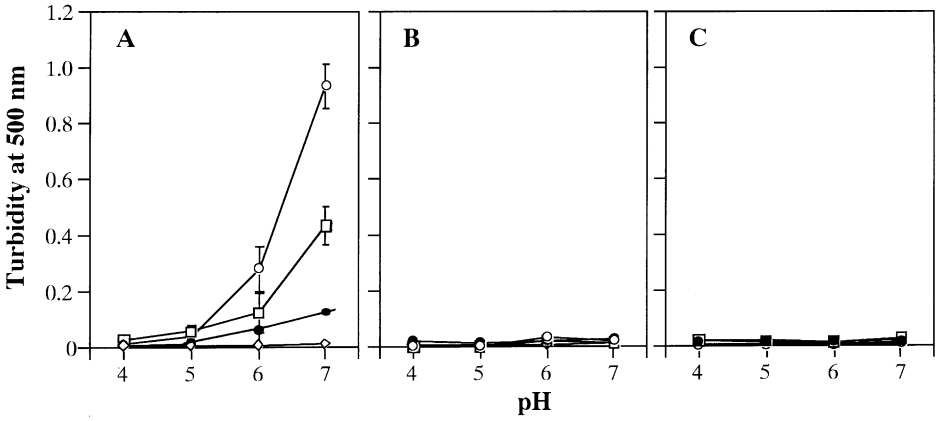

Fig. 3. Solubility of (A) high molecular weight-type chitosan (HMC), (B) low molecular weight-type chitosan (LMC) and (C) galactomannan conjugated with lysozyme. (◇) lysozyme, (○) HMC, LMC or galactomannan, (□) lysozyme–polysaccharide mixtures, (●) lysozyme-polysaccharide conjugates dry-heated for 14 days. Values are means of triplicates ± S.D.

conjugate may be the most promising compound as an emulsifier at acidic pH.

3.4. Bactericidal activity of lysozyme–polysaccharide conjugates

Bactericidal action against *E. coil* K-12 at pH 7.0 was measured at different temperatures (4, 20, 37, and 50 °C) for lysozyme, lysozyme–polysaccharide mixtures and conjugates at a final concentration of 50 μg/ml (Fig. 6). HMC-lysozyme conjugates (Fig. 6A) were found to be very effective in inhibiting the growth of the bacterium cells at all temperatures, except at 4 °C where the conjugates were observed to destroy about 80% of the bacterial cells. HMC–lysozyme conjugates revealed similar bactericidal action regardless of dry-heating time. Increases in the temperature enhanced the antibacterial activity of HMC conjugates. LMC conjugates were especially effective in reducing the number of survival cells when the temperature was increased (Fig. 6B). As shown in Fig. 5B, LMC–lysozyme conjugate caused about 80% reduction in the number of survival cells at 37 °C, and led to a slight decline in the numbers of *E. coli* cells at lower temperature (4 °C). Galactomannan conjugates (Fig. 6C) were found to have less effect on the bactericidal activity of lysozyme compared to HMC and LMC conjugates.

It has been reported (Tsai & Su, 1999) that *E. coil* cells in the mid-exponential phase at pH 7.0 are less susceptible to chitosan at lower temperature because chitosan is less soluble at pH 7.0 and lower temperature may cause changes in the numbers of available binding sites at the cell surface, thereby reducing the interaction between chitosan and the cells. However, our results

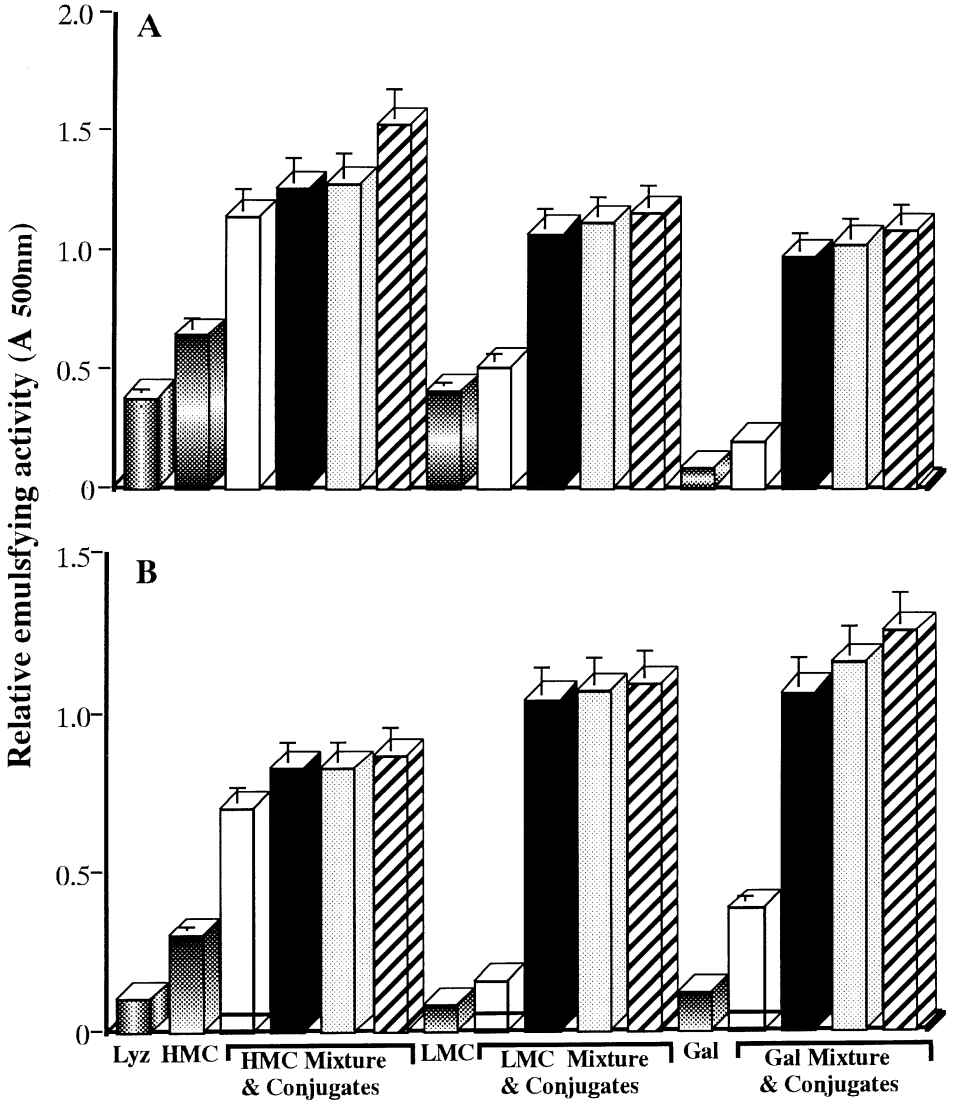

Fig. 4. Emulsifying activity of lysozyme–polysaccharide conjugates at pH 4.0 (A) and pH 7.0 (B). Lyz, lysozyme, HMC, high molecular weight-type chitosan, LMC, low molecular weight-type chitosan, Gal, galactomannan; *open bars:* lysozyme–polysaccharide mixture; *solid bars:* lysozyme–polysaccharide conjugates dry-heated for 5 days; *dotted bars:* 10 days; *dashed bars:* 15 days. Values are means of triplicates ± S.D.

demonstrate that low temperature stress had little influence on the bactericidal activity of HMC conjugated with lysozyme. This may be due to increases in the solubility of chitosan after conjugation with lysozyme. Our results confirmed that the number of cationic amino groups or the degree of polymerization greatly influenced the bactericidal activity of chitosan. As the number of cationic amino groups of HMC increased, the number of inhibited cells increased; and when the amino groups decreased in LMC, the number of inhibited cells decreased. The data indicate that the functional groups for growth inhibition are the cationic amino groups of chitosan (Young & Kauss, 1983).

The effect of the concentration of protein–polysaccaride conjugates on the growth of the bacterial cells is shown in Fig. 7. Increases in the concentration of HMC, HMC–lysozyme conjugates or HMC–lysozyme mixture (Fig. 7A) resulted in rapid increases in the rate of inhibition of the bacterial growth. It was observed that 50 μg/ml of HMC–lysozyme conjugates completely inhibited the growth of the bacterium. When compared to LMC (Fig. 7B), HMC conjugation was very effective even at lower concentration (2.5 μg/ml). Galactomannan conjugation (Fig. 7C) was found to have little effect even at higher concentration (50 μg/ml). These results indicate that lysozyme–HMC conjugates are very effective in inhibiting bacterial growth and are promising for industrial applications HMC since the conjugates also had improved emulsifying properties and solubility, especially at neutral pH.

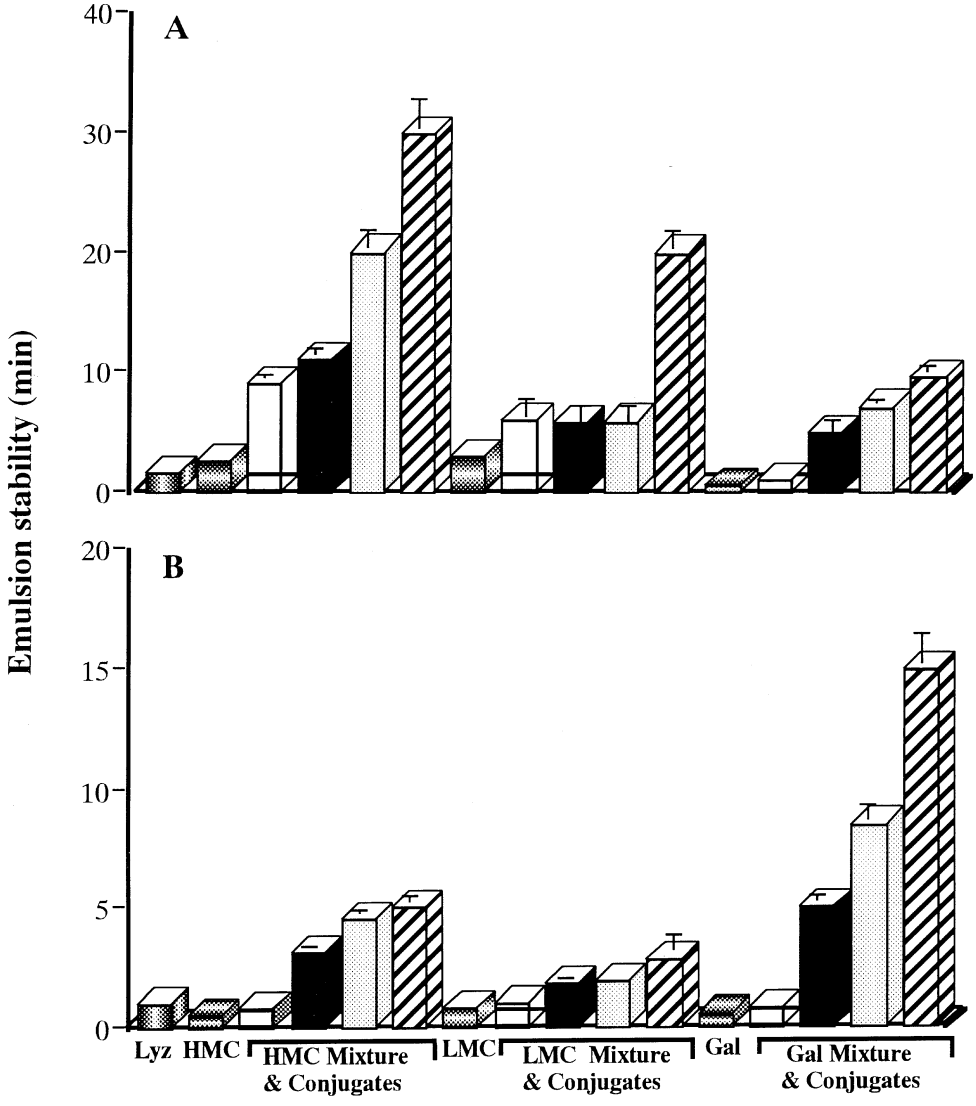

Fig. 5. Emulsion stability of lysozyme–polysaccharide conjugates at (A) pH 4.0 and (B) pH 7.0. Lyz, lysozyme; HMC, high molecular weight-type chitosan; LMC, low molecular weight-type chitosan; Gal, galactomannan; *open bars* : lysozyme–polysaccharide mixtures; *solid bars:* lysozyme-polysaccharide conjugates dry-heated for 5 days; *dotted bars:* 10 days; *dashed bars:* 15 days. Values are means of triplicates ± S.D.

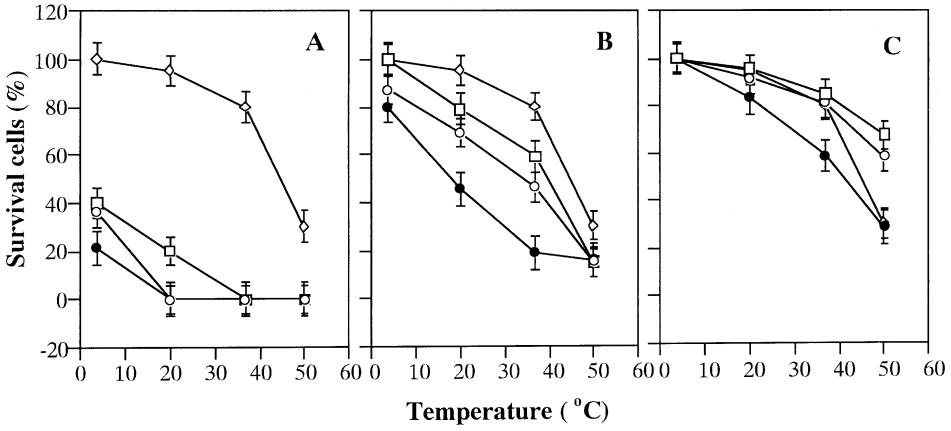

Fig. 6. Effect of polysaccharide conjugation on the bactericidal activity of lysozyme against *Escherichia coil* K-12 at different temperatures and at pH 7.0. (A) High molecular weight-type chitosan (HMC) conjugates, (B) low molecular weight-type chitosan (LMC) conjugates and (C) galactomannan conjugates. ◇, lysozyme; ○, HMC, LMC or galactomannan; □, lysozyme–polysaccharide mixtures; ●, lysozyme–polysaccharide conjugates dry-heated for 10 days. Values are means of triplicates ± S.D.

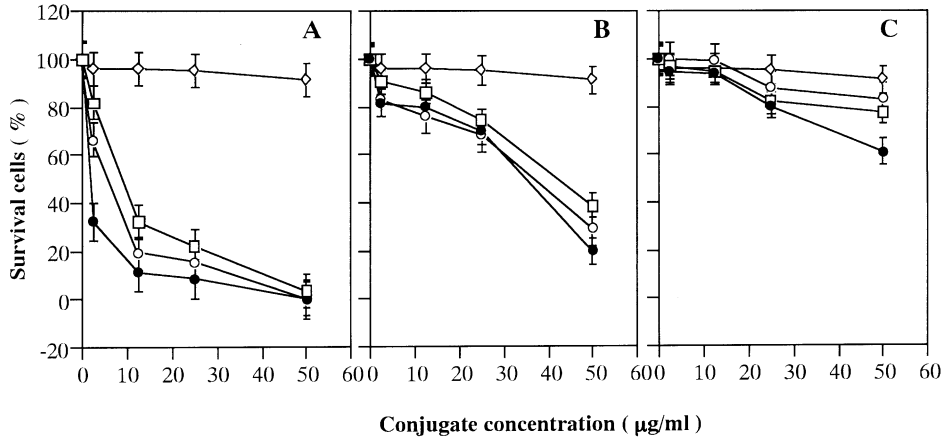

Fig. 7. Relationship between bactericidal activity and lysozyme–polysaccharides conjugate concentration at 37 °C. (A) high molecular weight-type chitosan (HMC) conjugates, (B) low molecular weight-type chitosan (LMC) conjugates and (C) galactomannan conjugates: ◇, lysozyme; ○, HMC, LMC or galactomannan; □, lysozyme–polysaccharide mixtures; ●, lysozyme–polysaccharide conjugates dry-heated for 10 days. Values are means of triplicates ± S.D.

4. Conclusion

Although lysozyme exerts antimicrobial effects only on Gram-positive bacteria, HMC conjugation extended the antimicrobial activity to Gram-negative bacteria and greatly improved its emulsifying properties. No cell toxicity has been reported in egg white–polysaccharide conjugate when tested with mammalian cells, and the protein structure in the conjugate was kept in the native form (Nakamura et al., 1992). Therefore, HMC–lysozyme conjugate can be potentially used in formulated food or drug systems since it possess novel bifunctional properties even under acidic conditions.

References

Cuero, R. G. (1999). Antimicrobial action of exogenous chitosan. *EXS*, *87*, 315–333.

Babiker, Elfadil E., Fujisawa, N., Matsudomi, N., & Kato, A. (1996). *Journal of Agricultural and Food Chemistry*, *44*, 3746–3750.

Kas, H. S. (1997). Chitosan, preparation and application to microparticulate systems. *Journal Microencapsulation*, *14*, 689–711.

Kato, A., Minaki, K., & Kobayashi, K. (1993). Improvement of emulsifying properties of egg white proteins by the attachment of polysaccharide through maillard reaction in a dry state. *Journal of Agricultural and Food Chemistry*, *41*, 540–543.

Kato, A., Murata, K., & Kobayashi, K. (1988). Preparation and characterization of ovalbumin–dextran conjugate having excellent emulsifying properties. *Journal of Agricultural and Food Chemistry*, *36*, 421–425.

Kato, A., Sasaki, Y., Furuta, R., & Kobayashi, K. (1990). Functional proteinpolysaccharide conjugate prepared by controlled dry-heating of ovalbumin–dextran mixture. *Agricultural Biological Chemistry*, *54*, 107–112.

Kato, A., Sato, T., & Kobayashi, K. (1989). Emulsifying properties of protein–polysaccharide complexes and hybrids. *Agricultural Biological Chemistry*, *53*, 2147–2152.

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

Nakamura, S., Kato, A., & Kobayashi, K. (1990). Novel bifunctional lysozyme–dextran conjugate that acts on both Gram-negative and Gram-positive bacteria. *Agricultural Biological Chemistry*, *54*, 3057–3059.

Nakamura, S., Kato, A., & Kobayashi, K. (1991). New antimicrobial characteristics of lysozyme–dextran conjugate. *Journal of Agricultural and Food Chemistry*, *39*, 647–650.

Nakamura, S., Kato, A., & Kobayashi, K. (1992). Bifunctional lysozymegalactomannan conjugate having excellent emulsifying properties and bactericidal effect. *Journal of Agricultural and Food Chemistry*, *40*, 735–739.

Nakamura, S., Kobayashi, K., & Kato, A. (1994). Role of positive charge of lysozyme in the excellent emulsifying properties of Maillard-type lysozyme–polysaccharide conjugate. *Journal of Agricultural and Food Chemistry*, *42*, 2688–2691.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, *26*, 716–723.

Shepherd, R., Reader, S., & Falshaw, A. (1997). Chitosan functional properties. *Glycoconjugate Journal*, *14*, 535–542.

Shu, Yu-Wei., Sahara, S., Nakamura, S., & Kato, A. (1996). Effects of the length of polysaccharide chains on the functional properties of the Maillard-type lysozymepolysaccharide conjugate. *Journal of Agricultural and Food Chemistry*, *44*, 2544–2548.

Tsai, G. J., & Su, W. H. (1999). Antibacterial activity of shrimp chitosan against *Escherichia coil*. *Journal of Food Protection*, *62*, 239–243.

Young, D. H., & Kauss, H. (1983). Release of calcium from suspension-cultured *Glycine* max cells by chitosan, other polycation, and polyamines in relation to effects on membrane permeability. *Plant Physiology*, *73*, 698–702.