ELSEVIER

Food Chemistry 79 (2002) 367–372

Food Chemistry

www.elsevier.com/locate/foodchem

Effect of chitosan conjugation on the functional properties and bactericidal activity of gluten peptides

Elfadil E. Babiker

Department of Food Science & Technology, Faculty of Agriculture, University of Khartoum, Shambat, Sudan

Received 5 February 2002; accepted 5 March 2002

Abstract

Gluten was solubilized by chymotrypsin and the soluble peptides were purified and conjugated with chitosans of different degrees of polymerization through the Maillard reaction. High molecular weight-type chitosan (HMC) was found to be soluble at acidic pH and least soluble at neutral pH. However, after conjugation with gluten peptides, its solubility considerably improved at neutral pH while low molecular weight-type chitosan (LMC) conjugates were found to be soluble at both pHs. The emulsifying activity and emulsion stability of HMC conjugate were greatly improved at acidic pH compared to that of LMC. On the other hand, LMC conjugation slightly improved the emulsifying activity of gluten peptides at neutral pH compared to that of HMC. Despite its great efficiency in inhibiting the growth of *Escherichia coli* cells at high temperature, HMC conjugated with gluten peptides inhibited the growth of the bacterium cells, even at low temperature, significantly better than LMC conjugate.

Keywords: Chitosan; Gluten peptides; Functional properties; Bactericidal activity

1. Introduction

Being a non-toxic, biocompatible and biodegradable polymer, chitosan has been widely used for pharmaceutical and medical applications. Several biomedical applications for chitosan have been reported over last two decades due to its wound healing, antimicrobial and hemostatic properties (Kas, 1997). Chitosan, a polyaminosaccharide, is a partially deacetylated polymer of N-acetyl glucosamine and is usually prepared from chitin (2 acetamido-2-deoxy β-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 mixture of water-insoluble β,1–4 glucosaminopolymer and a soluble derivative prepared by nitrous acid modification (Hadwiger & Beckman, 1980). The antimicrobial activity of chitosan is well observed on 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 effects of cell age, reaction temperature, pH value, and salts on the inhibitory activity of shrimp chitosan (98% deacetylated) against *Escherichia coli* (CCRC 10674) were investigated (Tsai & Su, 1999). It was observed that the age of a bacterial culture affected its susceptibility to chitosan, with cells in the late exponential phase being most sensitive to chitosan. Higher temperatures (25 and 37 °C) and acidic pH increased the bactericidal effects of chitosan, while sodium ions might complex with chitosan and accordingly reduce chitosan activity against *E. coli* (Tsai & Su, 1999). Chitosan was found to cause leakage of glucose and lactate dehydrogenase from *E. coli* cells, the mechanism involving a cross-linking between the polycations of chitosan and the anions on the bacterial surface that changes the membrane permeability (Tsai & Su, 1999). Accordingly, decreases in the degree of polymerization of chitosan resulted in decreases in the number of inhibited microorganisms, which indicated that the functional groups for the growth inhibition are the cationized amino

E-mail address: elfadilbabiker@hotmail.com

0308-8146/02/$ - see front matter
PII: S0308-8146(02)00188-7

groups of chitosan (Young & Kauss, 1983). Protein–polysaccharide conjugates have been proposed to be useful as new functional biopolymers having excellent emulsifying properties and antimicrobial effects (Kato, Murata, & Kobayashi, 1988; Kato, Sato, & Kobayashi, 1989; Kato, Sasaki, Furuta, & Kobayashi, 1990; Nakamura, Kato, & Kobayashi, 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 high salt concentration and acidic pH, comparable to commercial emulsifiers (Kato et al., 1990). Lysozyme–galactomannan conjugates showed excellent emulsifying properties and significant bactericidal effects on Gram-negative bacteria (Nakamura et al., 1992). In this study, and in order to develop a bifunctional biopolymer as an excellent emulsifier and food preservative with broad antimicrobial effects without any food toxicity, chitosan of different degrees of polymerization was mixed with gluten peptides and conjugated through controlled Maillard reaction at 60 °C and 79% relative humidity for different time intervals (5, 10, and 15 days).

2. Materials and methods

2.1. Materials

MacConkey medium was obtained from Nissui Seiyaku Co., Japan. *E. coli* K-12 was from the Institute for Fermentation, Osaka, Japan. Water-soluble low molecular weight chitosan (MW 3–30 kDa) and high molecular weight chitosan (Crab shell, MW 400 kDa, 98.0% D.A.) were purchased from Wako Co., Japan. Chymotrypsin (52 units/mg) was purchased from the Sigma Chemical Co. (St. Louis, MO). Unless otherwise stated, all reagents used in this study were of reagent grade. High-molecular weight chitosan (HMC) was solubilized in 0.1 M acetate buffer (pH 4.0), centrifuged and then dialyzed against distilled water at 4 °C for 24 h.

2.2. Methods

2.2.1. Wheat gluten preparation

Gluten was prepared by washing flour dough with water until the washings were free of soluble proteins. The gluten ball was dialyzed against distilled water and then freeze-dried in a freeze-dryer (VD-80, Taitec Co., Japan).

2.2.2. Preparation of chymotrypsin-digested gluten

A freeze-dried sample (4 g) of gluten was suspended in 400 ml of 0.05 M Tris–HCl (pH 8.0) containing 0.05% sodium azide, and then 40 mg of chymotrypsin were added. The mixture was incubated at 37 °C for 24 h. After incubation, chymotrypsin was inactivated by heating at 100 °C for 3 min. The obtained chymotrypsin-treated gluten was centrifuged (8000 rpm for 10 min) to remove a small amount of undigested protein, and then the supernatant was dialyzed (3000–4000, molecular weight cut-off) against distilled water at 4 °C for 24 h and then freeze-dried.

2.2.3. Gluten peptides–chitosan conjugation

Chitosan–gluten peptide powder mixtures in the weight ratios of 1:4 and 1:2 for HMC and LMC, respectively, were used. The mixture was dissolved in water at 10% (w/v) and freeze-dried. Powdered chitosan–gluten peptide 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 (5, 10, and 15 days).

2.2.4. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the method of Laemmli (1970) with a 15% acrylamide separating gel and a 3% acrylamide staking gel 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 done at a current of 10 mA for 5 h in electrophoretic Tris–glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins and carbohydrate with 0.2% Coomassie brilliant blue-R250 and 0.5% periodate-funchsin solution (Zacharius, Zell, Morrison, & Woodlock, 1969), respectively. Protein stain was destained with 10% acetic acid containing 20% methanol.

2.2.5. Measurement of solubility

Sample solutions (0.1%) at various pH values (pH 4 and 5, 0.1 M acetate buffer; 6 and 7, 0.1 M phosphate buffer) were mixed thoroughly. Solubility was indicated by measuring the turbidity of the solutions at 500 nm.

2.2.6. 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 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 $12000\times\boldsymbol{g}$ for 1 min at 20 °C. A 50-μl sample of the emulsion was taken from the bottom of the container at different times and diluted with 5 ml of a 0.1% sodium dodecylsulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after the emulsion formation (0 min). The emulsion stability was

estimated by measuring the half-time of the initial turbidity of the emulsion.

2.2.7. Measurement of bactericidal activity against E. coli

A modification of the microassay of Nonnke and Smith (1984) was used to test antibacterial activity. *E. coli,* K-12, cultured for 10 h (mid-exponential-phase) in Luria-Bertani (LB) medium, was diluted 10^3 times with LB medium or 20 mM phosphate buffer (pH 7.0) and used for inoculation of the assay. Assays were carried out in sterile test tubes. To each test tube, 4.5 ml of the LB diluted medium was mixed with 0.5 ml of gluten peptides, gluten peptide–chitosan mixtures or conjugates at different concentrations (2.5, 12.5, 25 or 50μg/ml). Test tubes were covered with sterile lids and incubated at 37 °C for 3.0 h. The absorbance of the assay mixture at 620 nm was determined. To determine the effect of temperature, 4.5 ml of the phosphate buffer-diluted medium was mixed with 0.5 ml of gluten peptides, gluten peptide–chitosan mixtures or conjugates, with a final concentration of 50 μg/ml. The mixture was incubated at different temperatures (4, 20, 37, or 50 °C) for 30 min. Then, a 100-μl portion of each treatment was surface-plated onto a MacConkey agar plate. Colonies were counted after incubation at 37 °C overnight. Control assays contained all components except gluten peptide–chitosan, gluten peptide–chitosan mixture or conjugates.

3. Results and discussion

3.1. Molecular weight of gluten peptides–chitosan conjugates

Regardless of any heating time (5, 10 or 15 days), gluten peptides conjugated with HMC and LMC showed higher molecular weight bands in the top of the separating and stacking gel after protein staining (lanes 3–8, Fig. 1). However, the conjugates were slightly cross-reacted with the carbohydrate stain (data not shown) and showed very faint bands. This may be due to the presence of the amino groups in chitosan, a polyaminosaccharide. Densitometer measurement of the SDS–PAGE bands showed that about 60% of gluten peptides were attached to LMC. On the other hand, a small amount of gluten peptides were attached to HMC. This difference is likely due to the fact that the molar ratios of the available binding sites are much larger in LMC than HMC. Since the molar ratios of LMC to HMC is 1:26, only 2–3% gluten peptides can be attached to HMC. Nevertheless, the results of SDS–PAGE demonstrate that gluten peptides were covalently attached to chitosans through the Maillard reaction between the ϵ-amino groups in protein and the reducing-end carbonyl groups in polysaccharides during dry heating at 60 °C and 79% relative humidity. Similar results were reported for soy protein–galactomannan conjugates (Babiker et al., 1998), and a lysozyme-dextran conjugate (Nakamura, Kato, & Kobayashi, 1990).

3.2. Solubility of gluten peptides–chitosan conjugates

The effect of pH on the solubility of gluten peptides, before and after conjugation with chitosan, is shown in Fig. 2. Gluten peptides were found to be soluble at acidic and neutral pH. HMC was found to be highly soluble at acidic pH and least soluble and slightly viscous at neutral pH. However, after conjugated with gluten peptides, its solubility considerably improved at neutral pH (Fig. 2A). LMC was found to be soluble

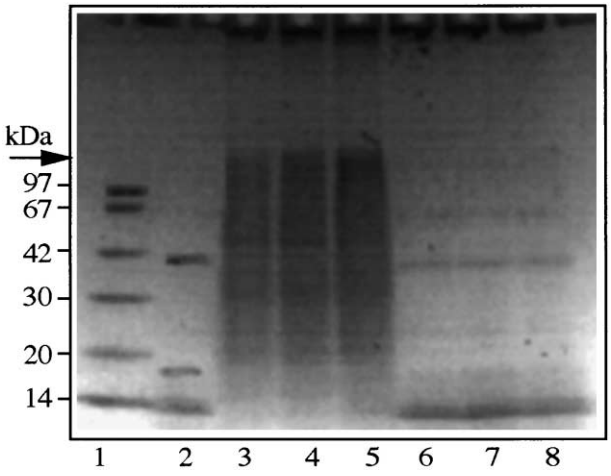

Fig. 1. SDS–PAGE pattern of gluten peptides and gluten peptide–chitosan conjugates: lane 1, molecular marker; lane 2, gluten peptides; lanes 3–5, low molecular weight-type chitosan (LMC) conjugates; lanes 6–8, high molecular weight-type chitosan (HMC) conjugates. All conjugates were dry-heated for 5, 10, or 15 days. Arrow indicates the boundary between the stacking (upper) and separating (lower) gels.

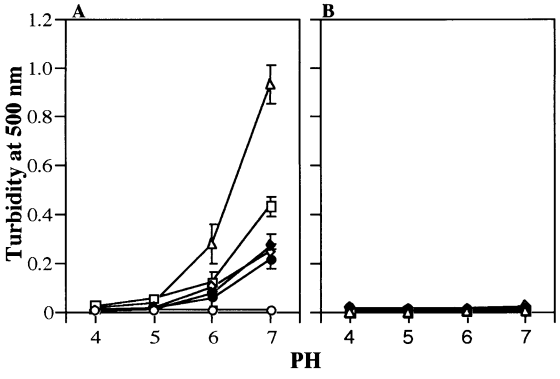

Fig. 2. Solubility of (**A**) HMC and (**B**) LMC conjugated with gluten peptides at different pH values: (○) gluten peptides, (△) HMC or LMC, (□) gluten peptide–chitosan mixtures, gluten peptide–chitosan conjugates dry-heated for (◇) 5, (●) 10, or (◆) 15 days. Values are means of triplicates (±S.D.).

before and after conjugation with gluten peptides (Fig. 2B). The improvement in the solubility of HMC at neutral pH is apparently due to the attachment of gluten peptides through the Maillard reaction.

3.3. Functional properties of gluten peptide–chitosan conjugates

The effect of chitosan conjugation on the emulsifying activity of gluten peptides at pH 4.0 and 7.0 is shown in Fig. 3. Although the emulsifying activity of gluten peptides at pH 4.0 was slightly improved by mixing the peptides with HMC (Fig. 3A), further improvement was observed when the conjugates were dry-heated for 10 and 15 days at 60 °C and 79% relative humidity, compared to LMC conjugates. The rate of improvement in the emulsifying activity at pH 4.0 was found to be slightly affected by the dry-heating time during the conjugation process (Fig. 3A). The emulsifying activity of gluten peptides (0.815), which is estimated by the turbidity of the emulsion measured immediately after emulsion formation, was highly increased when HMC conjugate was dry-heated for 15 days, while LMC conjugate, even after being heated for 15 days, slightly reduced the emulsifying activity of gluten peptides. The emulsifying activities of the conjugate were found to be 1.523 and 0.769 for HMC and LMC conjugates dry-heated for 15 days, respectively (Fig. 3A). The effect of chitosan conjugation on the emulsifying activity of gluten peptides at pH 7.0 (0.755) was slightly improved after conjugation with LMC compared to HMC conjugates (Fig. 3B). The rate of improvement in the emulsifying activity at pH 7.0 was found to be slightly affected by the dry-heating time during the conjugation process. When gluten peptide–chitosan conjugates were dry-heated for 15 days, the emulsifying activity was increased to 1.029 for LMC conjugate. However, the emulsifying activity of gluten peptides at pH 7.0 was slightly reduced even after being heated for 15 days (0.717) when HMC was conjugated with gluten peptides (Fig. 3B). The emulsion stability (the half time of the initial turbidity) of gluten peptides at pH 4.0 (1.0 min) was greatly improved after conjugation with HMC compared to LMC conjugation (Fig. 4A). The emulsion stabilities of chitosan–gluten peptides, dry-heated for 15 days, were found to be 15.6 and 3.5 min for HMC and LMC conjugates, respectively. The emulsion stabilities of gluten peptides at pH 7.0 (1.0 min) were slightly increased when HMC and LMC conjugates were dry-heated for 15 days and found to be 2.0 and 2.1 for the conjugates, respectively (Fig. 4B). The results revealed that, at pH 4.0, the emulsifying properties of gluten

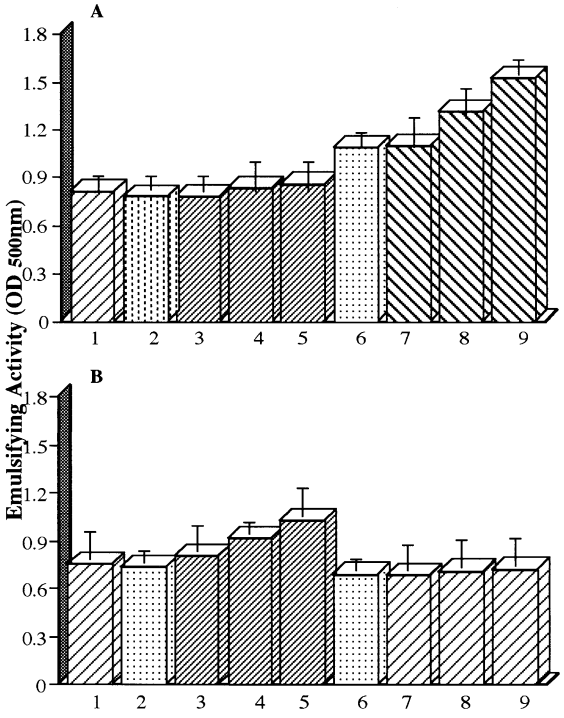

Fig. 3. Emulsifying activity at (**A**) pH 4.0 and (**B**) pH 7.0 of gluten peptides, HMC and LMC conjugates: bar 1, gluten peptide; bar 2, gluten peptide–LMC mixtures; bars 3–5, LMC conjugates; bar 6, gluten peptides–HMC mixtures; bars 7–9, HMC conjugates. All conjugates were respectively dry-heated for 5, 10, or 15 days. Values are means of triplicates (±S.D.).

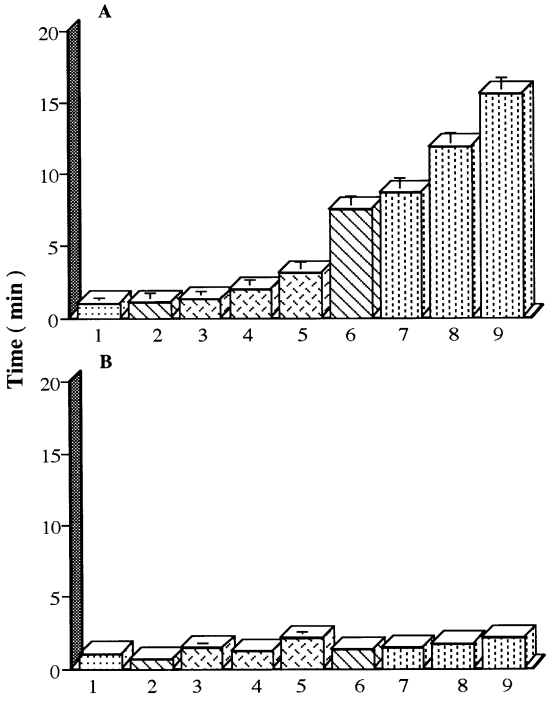

Fig. 4. Emulsion stability at (**A**) pH 4.0 and (**B**) pH 7.0 of gluten peptides, HMC and LMC conjugates: bar 1, gluten peptides; bar 2, gluten peptide–LMC mixtures; bars 3–5, LMC conjugates; bar 6, gluten peptide–HMC mixtures; bars 7–9, HMC conjugates. All conjugates were respectively dry-heated for 5, 10, or 15 days. Values are means of triplicates (±S.D.).

peptides were greatly improved by HMC conjugation while at pH 7.0 LMC conjugation slightly improved the emulsifying properties of the peptides. Despite both polysaccharides being covalently attached to gluten peptides through the Maillard reaction and both soluble at pH 4.0, the rate of improvement in the emulsifying properties was found to differ between the polysaccharides conjugates. This difference is basically attributed to the difference in the degree of polymerization, indicating that the number of cationized groups in chitosan plays a great role in inhibiting the coalescence of the oil droplets and a stable emulsion is then formed. The properties of protein–polysaccharide conjugate as an excellent emulsifier are attributed to the amphiphilic nature of the conjugated molecule. 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 the polysaccharide may be oriented to the water phase, thereby inhibiting the coalescence of the oil droplets (Babiker et al., 1998). Thus, a stable emulsion may be formed in the presence of protein–polysaccharide conjugate.

3.4. Bactericidal activity of gluten peptide–chitosan conjugates

Bactericidal activity of gluten peptides, HMC, LMC and the conjugates, at different concentrations toward *E. coli* K-12 was investigated (Fig. 5). Gluten peptides, HMC, LMC and the conjugates were tested in serial dilutions from 50 to 2.5 μg/ml. As shown in Fig. 5A, the activity of gluten peptides toward the bacterium cells at 37 °C was much weaker even at high concentration. However, after conjugation with HMC, it was observed that as the concentration increased, the absorbance at 620 nm rapidly decreased (Fig. 5A). It was found that about 50 μg/ml of the conjugate dry-heated for 15 days completely inhibited the growth of the bacterial cells ($OD_{620} = 0.0$). Compared to HMC and its conjugates, LMC and its conjugates gradually decreased the number of surviving cells, even at a concentration of 50 μg/ml (Fig. 5B). The results demonstrate that HMC conjugates were very effective in improving the bactericidal activity of gluten peptides compared to LMC conjugates. To further demonstrate the effect of temperature on bactericidal activity of gluten peptide–chitosan conjugates toward *E. coli* K-12, gluten peptides, chitosan–gluten peptides mixture or conjugates were separately incubated with the bacterium cells at different temperatures (4.20, 37, and 50 °C) for 30 min. As shown in Fig. 6, and regardless of any heating time (5, 10, and 15 days), HMC–gluten peptide conjugates (Fig. 6A) were found to be very effective in inhibiting the growth of the bacterial cells at all temperature, except at 4 °C at which about 80% of the bacterial cells were destroyed by the conjugates compared to HMC and gluten peptides. It was observed that, at 20 °C, HMC conjugates significantly inhibited the growth of the bacterial cells. Results demonstrate that the temperature had little influence on the bactericidal activity of HMC conjugates. On the other hand, LMC conjugates (Fig. 6B) were also found to be effective in reducing the number of surviving cells, especially when the temperature was elevated. As shown in Fig. 6B, LMC–gluten peptide conjugates caused about 84–86% reduction in the number of surviving cells at 50 °C. However, at lower temperature (4 °C), LMC conjugates were observed to decrease the numbers of *E. coli* cells very slightly. These results indicated that low temperature stress greatly

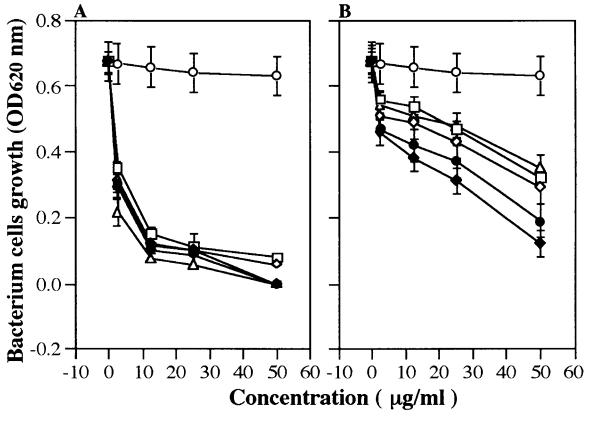

Fig. 5. Bactericidal activity at 37 °C of (**A**) HMC–(**B**) LMC–gluten peptide conjugates at different concentrations against *Escherichia coli* K-12: (○) gluten peptides, (△) HMC or LMC, (□) chitosan–gluten peptide mixtures, chitosan–gluten peptide conjugates dry-heated for (◇) 5, (●) 10, or (◆) 15 days. Values are means of triplicates (±S.D.).

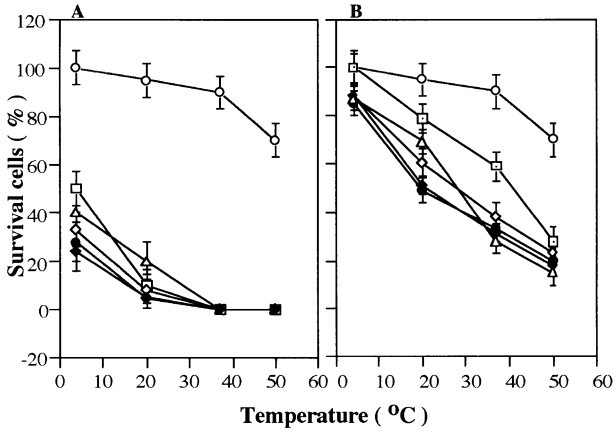

Fig. 6. Bactericidal activity at pH 7.0 of (**A**) HMC and (**B**) LMC conjugated with gluten peptides against *Escherichia coli* K-12 at different temperatures: (○) gluten peptides, (△) HMC or LMC, (□) gluten peptide–polysaccharide mixtures, gluten peptide–chitosan conjugates dry-heated for (◇) 5, (●) 10, or (◆) 15 days. Values are means of triplicates (±S.D.).

influenced the bactericidal activity of LMC and its conjugates. This may be due to the fact that, changes in the numbers of available binding sites at the cell surface might have occurred as a result of low temperature stress. It has been reported (Tsai and Su, 1999) that *E. coli* cells in the mid-exponential phase at pH 7.0 are less susceptible to HMC, especially at low temperature, because at pH 7.0 chitosan is least soluble and lower temperature may cause changes in the numbers of available binding sites at the cell surface; accordingly, the reaction rate between chitosan and the bacterial cells will be changed. However, our results demonstrate that low temperature stress had little influence on the bactericidal activity of HMC after conjugation with gluten peptides compared to LMC conjugates. Our results confirmed that the number of cationized amino groups, or the degree of polymerisation, greatly influenced the bactericidal activity of chitosan. It was found that, as the number of cationized amino groups of polysaccharide increases, the number of inhibited cells increases and when it decreases the number of inhibited cells decreases, indicating that the functional groups for the growth inhibition are the cationized amino groups of chitosan (Young & Kauss, 1983). It has been reported that chitosan causes leakage of glucose and lactate dehydrogenase from *E. coli* cells, the mechanism involving a cross-linkage between the polycation of chitosan and the anions on the bacterial surface that changes the membrane permeability (Tsai & Su, 1999). Conjugation of HMC with gluten peptides greatly improved the antimicrobial efficiency of the peptides to Gram-negative bacteria. Moreover, low temperature stress was found to have little effect on the bactericidal activity of the conjugates.

In conclusion, although gluten peptides have no antimicrobial effects against Gram negative bacteria, HMC conjugation greatly enhanced antimicrobial efficiency toward Gram-negative bacteria and greatly improved emulsifying properties, especially at acidic pH. In addition, this conjugation was achieved without the use of chemicals. Moreover, it has been reported that there is no cell toxicity in the conjugate, tested using mammalian cells, and that the protein structure in the conjugate is kept in the native from (Kato, Minaki, & Kobayashi, 1993). Therefore, it can be potentially used in formulated food or drug systems possessing novel bifunctional properties, emulsifier and/or antimicrobial reagent even under acidic conditions.

References

Babiker, E. E., Azakami, H., Matsudomi, N., Iwata, H., Ogawa, T., Bando, N., & Kato, A. (1998). Effect of polysaccharide conjugation or transglutaminase treatment on the allergenicity and functional properties of soy protein. *Journal of Agricultural and Food Chemistry*, *46*, 866–871.

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

Hadwiger, L. A., & Beckman, J. M. (1980). Chitosan as a component of pea–*Fusarium solani* interactions. *Plant Physiology*, *66*, 205–211.

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

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

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

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.

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-positive bacteria. *Agricultural and 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 lysozyme-galactomannan conjugate having excellent emulsifying properties and bactericidal effect. *Journal of Agricultural and Food Chemistry*, *40*, 735–739.

Nonnke, B. J., & Smith, K. L. (1984). Inhibition of mastitis bacteria by bovine milk apo-lactoferrin evaluated in an *in vitro* microassay of bacterial growth. *Journal of Dairy Science*, *67*, 606–611.

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. *Glycoconjuate Journal*, *14*, 535–542.

Tsai, G. J., & Su, W. H. (1999). Antibacterial activity of shrimp chitosan against *Escherichia coli*. *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.

Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Annals of Biochemistry*, *30*, 148–152.