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Improving emulsifying activity of ε-polylysine by conjugation with dextran through the Maillard reaction

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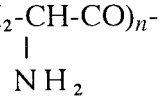
Abstract

ε-Polylysine (PL) was conjugated with dextran through the Maillard reaction to improve its emulsifying activity. The covalent attachment of dextran to PL was confirmed by Sephadex G-150 gel filtration chromatography and SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The resulting PL–dextran conjugate possessed an excellent emulsifying activity as compared with commercial emulsifiers. The emulsifying activity of conjugate was not affected even in the presence of 1.0 M NaCl and above pH 7. In addition, the PL–dextran conjugate retained most of the original antimicrobial activities of PL. The PL–dextran conjugate thus prepared could be used for the formulation of processed foods as a bifunctional food additive, emulsifier and antibacterial reagent.

1. Introduction

There is a growing awareness that the continued widespread use of chemical preservatives in industrial and consumer products may pose a serious health problem. Therefore, it is relevant to consider natural alternatives for preserving foods. The use of ε-polylysine (PL) as a natural food preservative has been increasing in Japan, because it has an excellent antimicrobial activity and heat stability (Hiraki, 1995; Shima, Matsuoka, Iwamoto & Sakai, 1984). A homopolymer of L-lysine, ε-poly-L-lysine with the structure described below, was discovered and isolated from the culture medium of *Streptomyces albulus* by Shima and Sakai (1977). The degree of polymerization (*n*) is



25–30. PL behaves like a cationic surface active agent, because the amino groups of PL have positive charges in water. Therefore, PL can be used as a natural antimicrobial agent (Delihas, Riley, Berkowitz & Polttoratskaia, 1995; Shima et al., 1984).

The practical problem when applying PL in processed foods is that PL tends to interact with proteins and acidic polysaccharides, leading to the possible loss of antimicrobial activity (Hiraki, 1995). Moreover, PL has poor emulsifying properties. Therefore, the usage of PL has been restricted mostly to starch-based foods (Hiraki, 1995; Otsuka, Kuwahara & Manabe, 1992). On the other hand, many investigations have been carried out to improve emulsifying properties of food proteins by conjugating with polysaccharides (Dickinson & Galazka, 1991; Fujiwara, Oosawa & Saeki, 1998; Kato, Minaki & Kobiyashi, 1993; Matsudomi, Inoue, Nakashima, Kato & Kobayashi, 1995; Matsudomi, Tsujimoto, Kato & Kobayashi, 1994; Nakamura, Kato & Kobayashi, 1992). In these studies, polysaccharides are covalently attached to proteins through the Maillard reation between ε-amino groups of food proteins and the reducing-end carbonyl groups of polysaccharides. The resulting protein–polysaccharide conjugates confer excellent emulsifying properties, superior to those of commercial emulsifiers (Kato et al., 1993).

In the present study, the improvement of emulsifying activity of PL by conjugating with dextran was attempted in order to prepare commendable emulsifiers having excellent antibacterial activity.

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2. Materials and methods

2.1. Materials

PL (average molecular weight: 4700) used in this study was obtained from Chisso Co. (Yakohama, Japan). Dextran (molecular weight: 60,000–90,000) was purchased from Wako Pure Chemicals Ind. (Tokyo, Japan). Commercial emulsifiers, Sunsoft SE-11 (sucrose–fatty acid ester) and Sunsoft Q-18S (deca-glyceryl monoesterate) were supplied from Taiyo Chemicals Co. (Tokyo, Japan). Corn oil was purchased from Hayashi Chem. Co. (Tokyo, Japan).

2.2. Methods

2.2.1. Preparation of PL–dextran conjugate

After a mixture of PL and dextran (1:9, w/w) was dissolved in distilled water, it was frozen rapidly with the aid of liquid nitrogen and freeze-dried. The freeze-dried sample powders were kept in desiccators over saturated $Mg(NO_3)_2$ for 3 days to equilibrate the water activity at 0.65, then they were placed in glass vials (1.0 cm id×4.2 cm) and sealed. Heating treatment to enhance the formation of PL–dextran conjugate through the Maillard reaction was conducted between 50 and 100°C for up to 4 h. After cooling in ice water bath, heated samples were dissolved in distilled water (0.1%, w/v as PL) and the absorbance at 420 nm was determined as an index of browning.

2.2.2. Gel filtration chromatography

The PL–dextran conjugate was separated from the unreacted PL by gel filtration on a Sephadex G-150 column (2.8 × 65 cm). Elution was carried out with 0.066 M carbonate buffer (pH 10.0) containing 0.2 M NaCl and 0.02% (w/v) sodium azide as eluent (Matsudomi et al., 1995) with a flow rate of 0.4 ml min^{-1} and 5 ml of fractions were collected automatically. PL was detected by the ninhydrin reaction, while dextran was detected by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The standard proteins used for calibration were human β-globulin (160 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), cytochrome C (12.4 kDa). The void volume was determined with Blue Dextran 2000.

2.2.3. Gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) using 10% acrylamide separating gel and 4.5% acrylamide stacking gel containing 0.1% SDS. After electrophoresis, gel sheets were stained with Coomassie brilliant blue R-250 for PL and with periodic acid-Schiff (PAS)-silver for dextran (Gradilone, Arranz & Cabada, 1998).

2.2.4. Emulsifying activity

The emulsifying activity of PL–dextran conjugate was determined by the spectroturbidimetric method of Pearce and Kinsella (1978). For the emulsion preparation, 0.5 g of corn oil and 1.5 ml of conjugate solution (containing 0.1% PL) were homogenized by a Hitachi homogenizer HG30 at 12,000 rpm for 1 min. A 50 μl aliquot of sample emulsion was taken from the bottom of the container after different intervals and diluted with 5.0 ml of 0.1% SDS solution. Absorbance of the diluted emulsion was determined at 500 nm. Absorbance at 0 min was expressed as the emulsifying activity of conjugate. Effect of pH on the emulsifying activity was determined by using 0.2 M acetic acid buffer (pH 3), 0.2 M maleic acid–Tris–NaOH buffer (pH 5–8) and 0.1 M boric acid–NaOH buffer (pH 9–10). Effect of NaCl concentration on the emulsifying activity was measured in the range up to 1.0 M at pH 7.4 (0.2 M maleic acid–Tris–NaOH buffer).

2.2.5. Antimicrobial activity

The minimum inhibitory concentrations (MIC, μg ml^{-1}) of PL and its conjugate with dextran were determined by the broth dilution method as follows; bacteria (Table 1) were inoculated into nutrient broth (Difco Lab.) with 1×10^6 cells ml^{-1} and aerobically incubated. Growth was recorded turbidimetrically after incubation at 30°C for 20 h. MIC were recorded as the lowest concentration which completely inhibited the growth of bacteria. Each test was conducted in triplicate.

3. Results and discussion

3.1. Conjugation of PL with dextran

Effect of heating temperature on the development of the Maillard reaction between PL and dextran (1:9, w/w) was first investigated (Fig. 1). It is apparent that the Maillard reaction proceeded rapidly above 80°C, but very slowly below 60°C.

The elution profiles of the PL–dextran conjugate separated by Sephadex G-150 gel chromatography were compared (Fig. 2). When freeze-dried PL–dextran mixture was heated at 80°C for 5 or 90 min, the ninhydrin-positive peak shifted towards the higher molecular weight fraction and overlapped with the dextran peak, suggesting that PL was convalently bound to dextran to form the PL–dextran conjugate. It is noteworthy that the PL–dextran conjugate was formed even during the period of adjusting water activity (Fig. 2), suggesting that the reaction between PL and dextran takes place even at room temperature, but it requires the presence of a certain amount of water.

Covalent bonding of dextran to PL was also confirmed by SDS–PAGE. Fig. 3 presents SDS–PAGE

Table 1
MIC of PL and PL–dextran conjugate

Bacteria species		Minimum inhibitory concentration (μg ml^{-1})						
		PL	PL–dextran conjugates[a]					
			0 min	5 min	10 min	30 min	60 min	90 min
Bacillus cereus	IAM 12605	25	35	40	40	40	40	40
Bacillus subtilis	IAM 1026	20	30	30	30	30	30	30
Listeria monocytogenes	Serotype 1/2a	30	50	50	50	50	50	50
Listeria monocytogenes	Serotype 4b	10	10	10	10	10	10	10
Staphylococcus aureus	IAM 1011	10	10	10	10	10	10	10
Staphylococcus aureus	IAM 12544	15	20	20	20	20	20	20
Escherichia coli	JCM 1649	20	20	45	45	45	45	45
Morganella morganii	Kouno strain	25	35	35	35	40	40	40
Pseudomanas aeruginosa	IAM 1514	10	25	50	50	50	50	50
Pseudomanas fluorescens	IAM 12022	50	60	60	60	60	60	60
Pseudomanas putida	IAM 1236	25	35	35	35	35	35	35
Salmonella typhimurium	Saheki strain	25	35	35	35	40	40	40

[a] PL–dextran conjugates were prepared by heating the mixture of ε-polylysine and dextran (1:9, w/w) at 80°C.

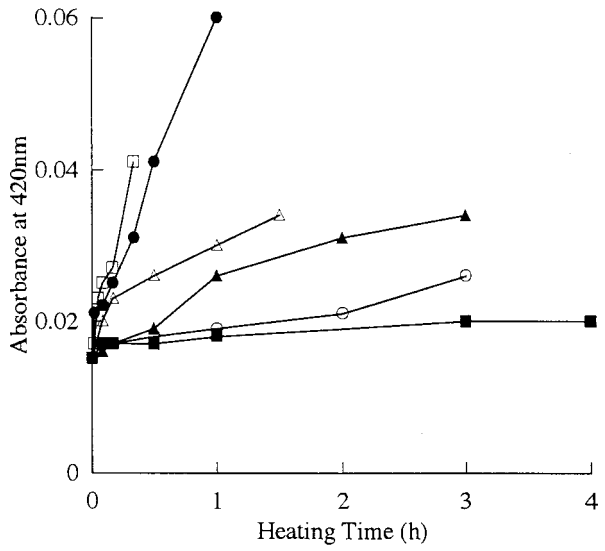

Fig. 1. Development of the Maillard reaction between ε-polylysine and dextran (1:9, w/w). ■: 50°C; ○: 60°C; ▲: 70°C; △ 80°C; ●: 90°C; □: 100°C.

patterns of PL incubated with dextran at 80°C for up to 90 min. Heating of PL–dextran mixture (1:9, w/w) at 80°C increased the PL band remaining on the top of the stacking gel and a dispersed PL band near the top of the separating gel, suggesting a wide distribution of polymerized PL. The PAS-silver staining also showed similar patterns. These results suggest that the PL molecule was covalently attached to the dextran molecule through a Maillard reaction between the amino groups of PL and the reducing-end carbonyl group of dextran, which is only one active functional group per molecule. The number of dextran molecules bound to one molecule of PL was not determined in this study, but it might be limited because of steric hindrance caused by attached dextran molecules. These findings are in support of those reported by Matsudomi et al. (1994, 1995), who modified protamine and plasma protein through the Maillard reaction.

3.2. Emulsifying activity

Fig. 4 presents the development of the emulsifying activity of the PL–dextran conjugate prepared by heating between 50 and 100°C. It is obvious from Fig. 4 that both PL and dextran had very poor emulsifying activities (abs. about 0.2), whereas the heat treatment above 50°C markedly improved the emulsifying activity of PL, especially at the early period of heating. The improved emulsifying activity could be due to the formation of PL–dextran conjugate as shown in Figs. 2 and 3. The macromolecule branched structure of dextran in the PL–dextran conjugate may efficiently cover the oil droplets to result in the superb emulsifying activity.

It is interesting to note that the emulsifying activity of PL increased about 5-fold (abs. about 1.0) during the adjustment of water activity, confirming the formation of PL–dextran conjugate as shown by the Sephadex G-150 gel filtration chromatography (Fig. 2). Furthermore, the emulsifying activity of PL–dextran conjugate, right after the water activity adjustment, was already superior to those of the commercial emulsifiers (Sunsoft SE-11 and Q-18S), a sucrose–fatty acid ester and a polyglycerine ester.

Effect of pH on the emulsifying activity of PL–dextran conjugate was compared with those of commercial emulsifiers, SE-11 and Q-18S (Fig. 5). The PL–dextran conjugate used in this experiment was prepared by heating the freeze-dried mixture of PL and dextran at

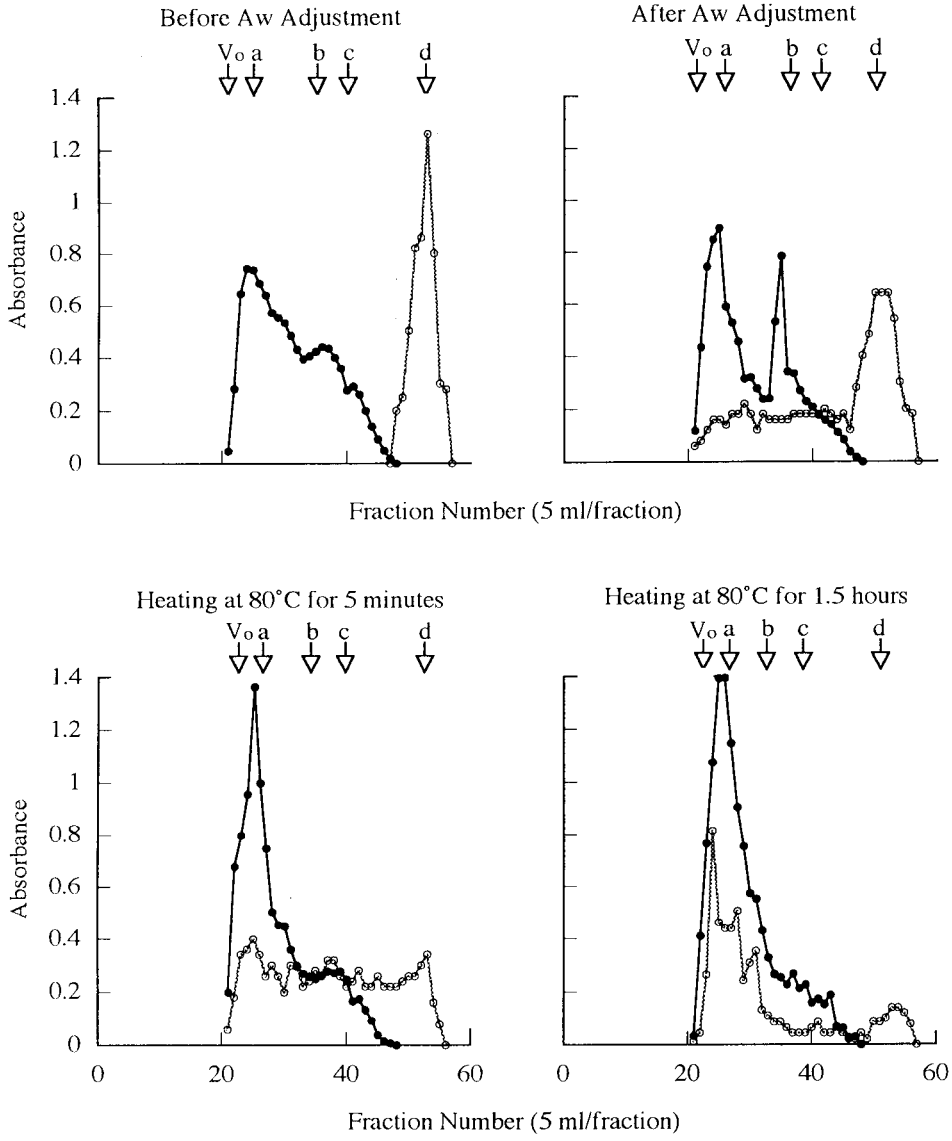

Fig. 2. Sephadex G-150 gel filtration chromatography profiles of ε-polylysine–dextran mixtures before and after the Maillard reaction. ●: phenol–sulphuric acid reaction (abs. at 490 nm); ○: ninhydrin reaction (abs. at 570 nm). Void volume (Vo) and elution times of markers (a: 160 kDa; b: 67 kDa; c: 45 kDa; d: 12.4 kDa) are indicated by arrows.

80°C for 1.5 h. The concentrations of conjugate and commercial emulsifiers were fixed at 0.1%. The emulsifying activity of conjugate was higher than those of commercial emulsifiers at the neutral and alkaline pH range (pH 7–10), but it was lowered with decreasing pH. SE-11 showed a similar tendency to the conjugate with respect to pH, while the emulsifying activity of Q-18S was not much affected by pH between 3 and 10. Those findings are similar to the report by Matsudomi et al. (1995) who compared the emulsifying stability of plasma protein–galactomannan conjugate with the commercial emulsifiers.

Fig. 6 shows the effect of NaCl on the emulsifying activities of PL–dextran conjugate and commercial emulsifiers. The emulsifying activity was determined by homogenizing 0.5 g of corn oil and 1.5 ml of 0.1% conjugates or commercial emulsifiers in the presence of NaCl up to 1.0 M at pH 7.4. The PL–dextran conjugate retained excellent emulsifying activity even in the presence of 1.0 M NaCl. The emulsifying activity of Q-18S was almost constant irrespective of NaCl concentration, but its activity was far lower than that of the conjugates. On the other hand, the emulsifying activity of SE-11 decreased gradually with increasing NaCl level. These findings are in agreement with the results of Kato, Sasaki, Furuta and Kobayashi (1990). Such outstanding emulsifying activity of the PL–dextran conjugate at neutral pH range and in high NaCl condition is advantageous and beneficial for the formulation of various processed foods.

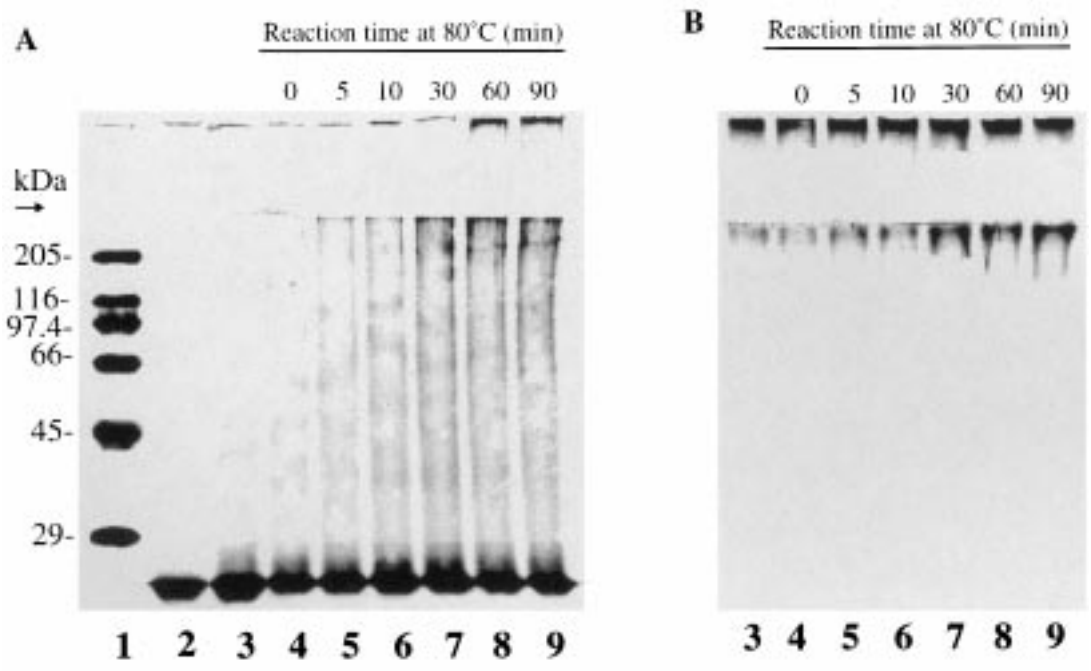

Fig. 3. SDS–polyacrylamide gel electrophoretic patterns of ε-polylysine–dextran mixture (1:9, w/w). (A) protein stain with Coomassie brilliant blue; (B) carbohydrate stain with periodic acid-Schiff (PAS)-silver; arrow indicates the boundary between stacking and separating gels. (Lane 1) molecular weight markers; (lane 2) ε-polylysine; (lane 3) ε-polylysine–dextran mixture; (lanes 4–9) ε-polylysine–dextran conjugate prepared by heating at 80°C for up to 90 min.

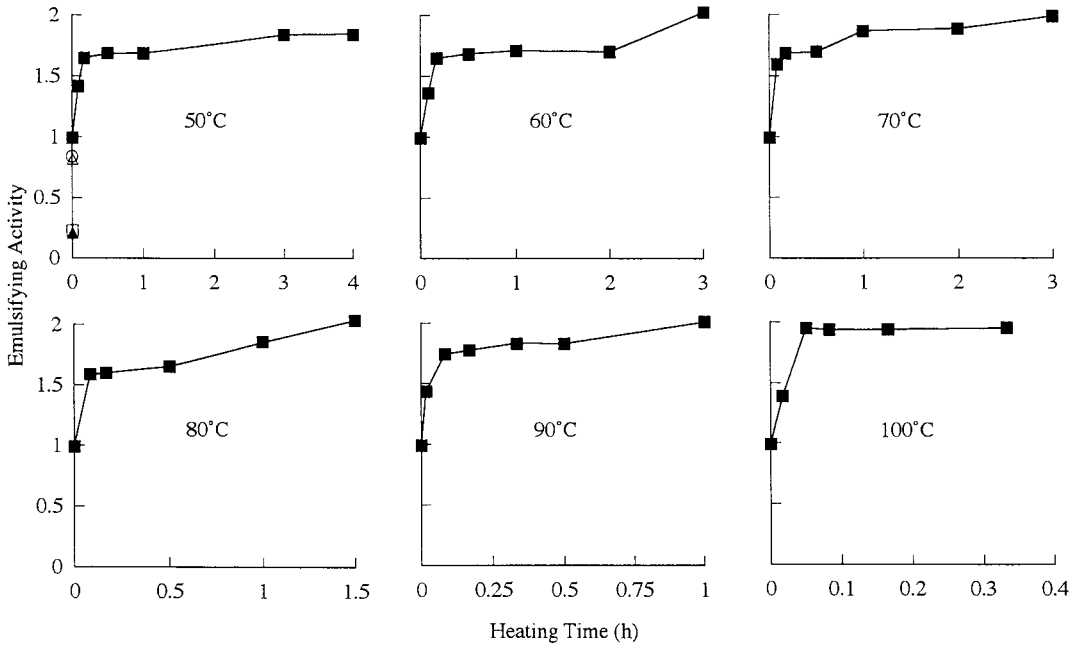

Fig. 4. Changes in emulsifying activities (pH 7.4) of ε-polylysine–dextran mixture (1:9, w/w) during heating at 50–100°C. ■: ε-polylysine–dextran conjugate; ▲: 0.1% ε-polylysine; □: 0.9% dextran; ○: Sunsoft SE-11; △: Sunsoft Q-18S.

3.3. Antimicrobial activity

Antimicrobial activities of PL on different bacterial species have been reported by Shima et al. (1984), Delihas et al. (1995) and Ting, Ishizaki and Tanaka (1997). According to the study of Vaara (1992), polycationic compounds disrupt the outer membrane of Gram-negative bacteria and PL disrupts the outer membrane by binding to it and discharging a large quantity of lipopolysaccharides. Since PL binds to the membrane

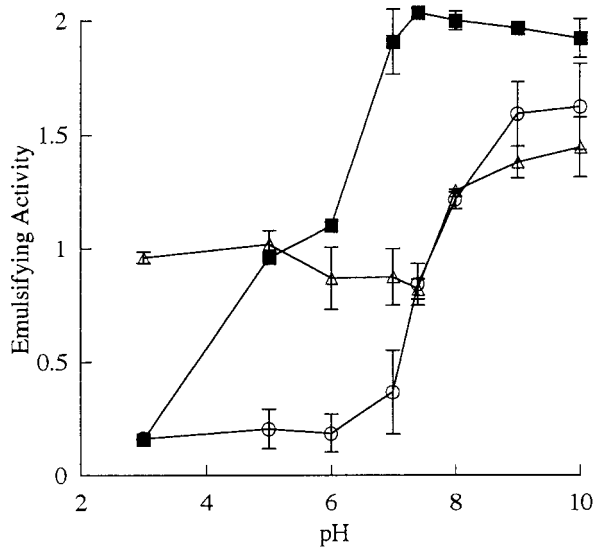

Fig. 5. Effect of pH on the emulsifying activities. ε-Polylysine–dextran conjugate was prepared by heating at 80°C for 90 min. ■: ε-polylysine–dextran conjugate; ○: Sunsoft SE-11; △: Sunsoft Q-18S. Vertical lines represent standard deviations of the mean.

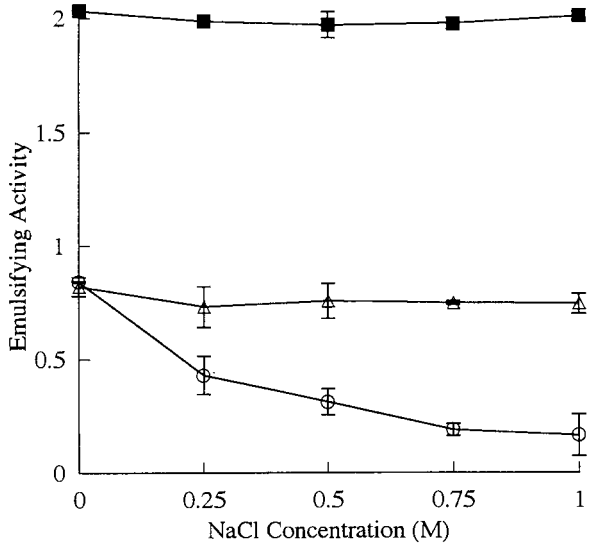

Fig. 6. Effect of NaCl concentration on the emulsifying activities (pH 7.4). ε-Polylysine–dextran conjugate was prepared by heating at 80°C for 90 min. ■: ε-polylysine–dextran conjugate; ○: Sunsoft SE- 11; △: Sunsoft Q-18S. Vertical lines represent standard deviations of the mean.

through free amino groups of the molecule, a reduction or loss of the antimicrobial activities of PL might be taking place as the result of the formation of conjugate through the Maillard reaction with dextran.

Table 1 summarizes MIC of PL and PL–dextran conjugate formed by heating the mixture of PL and dextran (1:9, w/w) at 80°C for up to 90 min on six Gram-positive bacteria and six Gram-negative bacteria. As shown in Table 1, PL had a wide antibacterial spectrum, inhibiting both Gram-positive and Gram-negative bacterial growth at concentrations of 10–30 μg ml^{-1} except *Pseudomonas fluorescens* IAM 12022. It is interesting to note that the MIC value varied with different strains of the same bacterium (*Listeria monocytogenes* serotype 1/2a and serotype 4b; *Staphylococcus aureus* IAM 1011 and IAM 12544). It can be summarized from these results that PL can kill a wide variety of bacterial species irrespective of the type of cell envelope structure and the sensitivity towards PL is quite different between bacterial species. These results are comparable to the findings of Delihas et al. (1995).

On the other hand, the antimicrobial activities of PL were only slightly lost against most of the bacteria used in this study after the conjugation with dextran at 80°C by the Maillard reaction. Prolonged heating did not cause the decrease of MIC. According to the studies reported previously (Nakamura & Mizushima, 1975; Schnaitman, 1971; Sekizawa & Fukui, 1973), amphiphilic media such as Triton X-100 and SDS can be used as an antimicrobial agent, because they dissolve the bacterial outer membrane. Therefore, the antimicrobial activities of commercial emulsifiers, SE-11 and Q-18S, were also determined. However, their MIC were found out to be larger than 1000 μg ml^{-1}, suggesting that antibactericidal activity of those emulsifiers cannot be expected.

4. Conclusion

The results of this study showed that the PL–dextran conjugate was successfully prepared by heating the freeze-dried mixture of PL and dextran through the Maillard reaction. The PL–dextran conjugate had more excellent emulsifying activity than commercial emulsifers. The emulsifying activity of conjugate was especially superb in the neutral pH range and was not influenced by a high NaCl concentration (1.0 M). Furthermore, the antimicrobial activity of PL was almost retained in the conjugate with dextran. From these findings, the possible development of a new emulsifying agent with an excellent antimicrobial activity from PL and dextran through the Maillard reaction is proposed.

Acknowledgements

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