Functional Casein–Polysaccharide Conjugates Prepared by Controlled Dry Heating

Akio Kato, Ryusuke Mifuru, Naotoshi Matsudomi, and Kunihiko Kobayashi

Department of Biochemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan
Received August 19, 1991

Casein was conjugated with dextran and galactomannan in a controlled dry state at a relative humidity of 79% and at 60°C for 24 hr. The covalent attachment of polysaccharides to casein was confirmed by SDS-PAGE and HPLC. The emulsifying activity of the casein–dextran and casein–galactomannan conjugates was 1.5 times higher than that of casein. The emulsion stability of the casein–dextran and casein–galactomannan conjugates was 10 times higher than that of casein. The improvement in these emulsifying properties reached a steady state when the conjugation of casein with polysaccharide was done for 24 hr in a controlled dry state, suggesting the rapid formation of conjugates through a Maillard reaction in the case of casein. Compared to commercial emulsifiers, the casein–polysaccharide conjugates showed better emulsifying properties in acidic and high-salt concentration systems.

Protein–polysaccharide conjugates with outstanding emulsifying properties have been developed by attaching dextran or galactomannan to tightly folded proteins through a naturally occurring Maillard reaction. Although such proteins have favorable surface activity due to their amphiphilicity, they are not suitable as an emulsifier in their native form. This is because the native form is unstable to heating for the pasteurization and homogenization necessary to prepare an emulsion and, as a result, the proteins coagulate during the emulsifying process. These problems could be overcome by conjugating proteins with polysaccharides. Thus, ovalbumin and lysozyme with poor emulsifying properties in their native forms have already been converted to highly emulsifying proteins by conjugating with dextran or galactomannan.

Casein is one of the most surface-active proteins due to its amphiphilic properties and unfolded conformation. However, the emulsifying properties of casein are not good enough for commercial use, because casein may easily coalesce with oil droplets due to its capacity to form a micelle structure or coagulate. Therefore, it is desirable for industrial application to enhance the emulsifying properties of casein by conjugating with polysaccharides. In addition, it is interesting to study the conjugation of unfolded protein with polysaccharides under controlled dry heating. This paper describes the effects of conjugating with polysaccharides on the functional properties of casein.

Materials and Methods

Materials. Dextran (average molecular weight of 60,000–90,000) was obtained from Wako Pure Chemicals. A galactomannan preparation was obtained by dialyzing a mannose hydrolysate of guar gum (average molecular weight of 15,000–25,000) supplied by Taiyo Chemicals Co. α-casein was prepared by the method of Zittle and Custer, and the commercial emulsifiers, Sunsoft SE-11 and Q-188, were supplied by Taiyo Chemicals Co.

Preparation of casein–polysaccharide conjugates. α-casein and dextran or galactomannan were mixed in water in a weight ratio of 1:3 and then lyophilized. Each mixture was incubated at 60°C in a relative humidity of 78.9% in a desiccator containing a saturated KBr solution for 24 hr.

Emulsifying properties. The emulsifying properties were determined by the method of Pearce and Kinsella. To form an emulsion, 1.0 ml of corn oil and 3.0 ml of a sample solution in 100 mM sodium phosphate buffer at pH 7.4 were shaken together and then homogenized in a Polytron (Kinematica, Switzerland) at 12,000 rpm for 1 min at 20°C. One hundred microliters of the emulsion was taken from the bottom of a test tube at different times and diluted with 5 ml of a 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm, the emulsifying activity being determined from the absorbance measured immediately after the emulsion had formed. The emulsion stability was estimated by measuring the half-time of the turbidity measured immediately after the emulsion had formed.

Results

The browning color development of casein–polysaccharide mixtures during dry heating at 60°C and at 78.9% relative humidity was followed to deduce the optimum storage time, as shown in Fig. 1. The browning color of the casein–glucose mixture increased rapidly after 12 hr of incubation, while that of the casein–polysaccharide mixtures increased only slightly even after 24 hr of incubation. The casein–glucose mixture became insoluble after 12 hr of incubation, while there were no detectable changes in solubility of the casein–polysaccharides. From the rapid browning reaction of the casein–glucose mixture, an adequate Maillard reaction seems to have occurred in casein–
Fig. 1. Browning Color Development of Casein–Carbohydrate Mixtures during Dry Heating at 60°C and 78.9% Relative Humidity.
- ●, casein-glucose mixture in a weight ratio of 10:1; ▲, casein-dextran mixture in a weight ratio of 1:3; ■, casein-galactomannan mixture in a weight ratio of 1:3.

Fig. 3. Emulsifying Properties of Casein–Dextran Conjugates Obtained by Dry Heating at 60°C for a Given Time (hr).
- ○, casein-glucose conjugate (24 hr) in a weight ratio of 10:1; ●, ▲, ■, casein-dextran conjugates heated for 0, 6, 12, and 24 hr, respectively, in a weight ratio of 1:3.

Table 1. Free Amino Groups in Casein–Polysaccharide Conjugates

<table>
<thead>
<tr>
<th>% modification of free amino groups</th>
<th>Number of free amino groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated casein</td>
<td>0</td>
</tr>
<tr>
<td>Casein–dextran conjugate</td>
<td>25.6</td>
</tr>
<tr>
<td>Casein–galactomannan conjugate</td>
<td>31.6</td>
</tr>
</tbody>
</table>

Fig. 2. SDS Polyacrylamide Gel Electrophoresis of Casein–Polysaccharide Conjugates.
- A, protein stain of casein–dextran conjugates; B, carbohydrate stain of casein–dextran conjugates; C, protein stain of casein–galactomannan conjugates; D, carbohydrate stain of casein–galactomannan conjugates. Lane 1, untreated casein; Lanes 2, 3, 4, 5, and 6, casein–polysaccharide conjugates dry-heated at 60°C for 0, 6, 12, 18, and 24 hr, respectively. The arrow indicates the boundary between the stacking and separating gels. The casein–polysaccharide conjugates were obtained by dry heating at 60°C and 78.9% relative humidity for a given time.

Gel electrophoresis.

Figure 2 shows the SDS–polyacrylamide gel electrophoretic patterns of the casein–polysaccharide conjugates. The appearance of polyanions at the top of the separating gel that was observed in the conjugates suggests the formation of higher molecular weight materials. These polyanions indicate consistent with carbohydrate bands, indicating the covalent linkage between protein and the polysaccharides.

Table 1 shows the number of free amino groups in the casein–polysaccharide conjugates obtained after 24 hr of incubation in a dry state at 79% relative humidity and 60°C. The rate of decrease of free amino groups in the conjugate with galactomannan is larger than that in the conjugate with dextran. From the rate of decrease in free amino groups, three to four lysine residues seem to have bound covalently with the polysaccharides. This covalent linkage has already been confirmed by the electrophoretic patterns (Fig. 2).

Figure 3 shows the emulsifying properties of casein–dextran conjugates dry-heated at 60°C for a given time (hr) in 78.9% relative humidity. The turbidity of the emulsion is plotted as the ordinate, and the standing time after emulsion formation as the abscissa. The optimum mixing ratio for the casein–polysaccharide conjugates was
Fig. 4. Emulsifying Properties of Casein–Galactomannan Conjugates Obtained by Dry Heating at 60°C for a Given Time (hr).
- △, ▲, ■, casein–galactomannan conjugates heated for 0, 6, 12, and 24 hr, respectively, in a weight ratio of 1:3.

Fig. 5. Comparison between the Emulsifying Properties of Casein–Polysaccharide Conjugates Obtained by Dry Heating at 60°C for 24 hr and Those of Commercial Emulsifiers in a Water System.
• casein–dextran conjugate; ■, casein–galactomannan conjugate; △, Sunsoft SE-11; ▲, Sunsoft Q-185.

Fig. 6. Comparison between the Emulsifying Properties of Casein–Polysaccharide Conjugates Obtained by Dry Heating at 60°C for 24 hr and Those of Commercial Emulsifiers in a High-salt Solution System.
• casein–dextran conjugate; ■, casein–galactomannan conjugate; △, Sunsoft SE-11; ▲, Sunsoft Q-185.

Fig. 7. Comparison between the Emulsifying Properties of Casein–Polysaccharide Conjugates Obtained by Dry Heating at 60°C for 24 hr and Those of Commercial Emulsifiers.
• casein–dextran conjugate; ■, casein–galactomannan conjugate; △, Sunsoft SE-11; ▲, Sunsoft Q-185.

dextran by dry heating for 24 hr. On the other hand, the emulsifying properties of the casein–glucose conjugate were much less due to the loss of solubility.

The emulsifying properties of the casein–galactomannan conjugate were similarly investigated (Fig. 4). The emulsifying activity was 1.5 times better and the emulsion stability more than ten times better than that of the control casein–dextran mixture (without incubation, 0 hr) by conjugating casein with...
conjugates were compared with those of the commercial emulsifiers, Sunsoft SE-11 and Q-18. The former is a sucrose-fatty acid ester and the latter is a polyglycerin ester. As shown in Fig. 5, the emulsifying properties of the casein–polysaccharide conjugates were comparable to those of the commercial emulsifiers in a water emulsion system. On the other hand, the casein–polysaccharide conjugates had better emulsifying properties than the commercial emulsifiers in a salt solution (0.1 M phosphate buffer, pH 7.4), as shown in Fig. 6. In addition, the emulsifying properties of the casein–polysaccharide conjugates were not reduced as much as those of Sunsoft SE-11 in an acidic emulsion (0.1 M acetate buffer, pH 3), and retained the same high values as those of Sunsoft Q-18$ (Fig. 7).

The effects of casein–polysaccharide conjugates on protease treatment are shown in Fig. 8. The HPLC patterns reveal that the casein–polysaccharide conjugates were completely digested by trypsin within 5 min as well as the unmodified casein. The heat stability of the casein–polysaccharide conjugates was also examined, as shown in Fig. 9. Although the casein–polysaccharide conjugates were heated at 100°C for 5 min, there were no effects on the emulsifying properties, suggesting the possibility for heat pasteurization in industrial applications.

**Discussion**

Improvement to the emulsifying properties of proteins has been extensively studied by many researchers to utilize proteins as an emulsifier. Since proteins are usually amphiphilic, the emulsifying properties are particularly good. However, their instability to the heating required for pasteurization and homogenizing during emulsion formation restricts the use of proteins as industrial emulsifiers. As described here, casein–polysaccharide conjugates were stable to heat treatment and revealed emulsifying properties that were superior to those of commercial emulsifiers.

Compared to ovalbumin- or lysozyme–polysaccharide conjugates, casein–polysaccharide conjugates were more rapidly formed through a Maillard reaction.$^{1,2}$ It took only 24 hr to get functional casein–polysaccharide conjugates, while it took two weeks for ovalbumin- or lysozyme–polysaccharide conjugates to produce comparable emulsifying properties to those of commercial emulsifiers. This may be due to the unfolding structure in which amino groups easily react with the reducing-end carbonyl groups in polysaccharides. This rapid formation of casein–polysaccharide conjugates is a favorable feature for industrial applications. Another advantage with protein–polysaccharide conjugates is their emulsion stability in acidic and high-salt concentration systems. Casein–polysaccharide conjugates also revealed this characteristic as shown in this paper.

We confirmed that a protein–polysaccharide conjugate was non-toxic by an oral administration test on rats, and negative by an Ames test and Rec assay.$^{3-5}$ As shown in Fig. 8, the peptide portions of casein–polysaccharide conjugates were digested by intestinal protease, and the undigested peptide–polysaccharide may be excreted, because galactomannan is an undigestible dietary fiber that decreases the lipid content in rat liver.$^{8}$ In addition to the good emulsifying properties, the beneficial effect of dietary fiber can also be expected.

Thus, casein–polysaccharide conjugates are one of the most promising approaches for developing new protein emulsifiers.

**References**

40. in press.