Adsorption of β-Lactoglobulin onto the Surface of Stainless Steel Particles

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The adsorption of β-lactoglobulin (β-Lg), one of the main constituents of fouling deposits in milk processing, onto the surface of stainless steel particles was studied under various conditions. The adsorption isotherm of β-Lg at 25°C was of the Langmuir type, and the plateau suggested that the surface was covered by a monolayer of β-Lg. The amount of β-Lg adsorbed steeply increased above 65°C. At 75°C, it increased almost linearly with the protein concentration in the bulk solution. Heating and chemical modification of the SH-group caused a much smaller amount of β-Lg to be adsorbed at 75°C. These findings indicate that the thermal aggregation of denatured β-Lg at the surface is important in the adsorption. More β-Lg was adsorbed at pH 4 than at pH 6.85. This suggests that the electrostatic interaction between β-Lg and the surface contributes to the adsorption behavior.

Thermal processing of milk causes the formation of fouling deposits on stainless steel surfaces of heat exchangers. This deposit formation is a serious problem because it reduces the efficiency and performance of heat exchangers and provides a place for possible microbial growth. Although a number of studies have been concerned with the fouling deposits, the mechanism of deposit formation is not yet understood.

β-Lactoglobulin (β-Lg), a whey protein, is one of the main constituents of milk deposits on heat exchangers. Although many other substances are involved in milk deposit formation, knowledge of the adsorption behavior of β-Lg onto solid surfaces would contribute to the understanding of the deposit formation mechanism. The adsorption behavior of β-Lg has been studied for several kinds of solid surfaces: chrom[ium,† stainless steel, polystyrene, polyethylene, and silicon. However, most of these studies were concerned with the adsorption at room temperature. Information on the adsorption behavior of β-Lg at higher temperatures is lacking.

Here, the adsorption of β-Lg onto the surface of stainless steel was studied under various conditions, especially at a higher temperature. To measure the amount of β-Lg adsorbed with accuracy, a large surface area of stainless steel should be used in the adsorption experiments. Therefore, fine particles of stainless steel were used in this study. Chemically modified β-Lg was used in addition to native β-Lg to identify the factors controlling the adsorption behavior.

Materials and Methods

Stainless steel particles. Fine particles of SUS316 stainless steel (<100 mesh) were obtained from Yasui Kikai Co., Ltd. (Osaka, Japan). They were thoroughly washed with 0.1 M NaOH, deionized water, and then methanol, then dried at 50°C. Based on the result of the nitorgen adsorption experiment, the specific surface area of the particles was 0.170 m²/g.

Reagents. β-Lg from bovine milk (3 times crystallized) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Ethyl-3-(3-dimethylamino)propyl]carbodiimide hydrochloride (EDC) and glycineamide hydrochloride were products of Dojin Laboratories (Kumamoto, Japan) and Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.), respectively. Iodoacetic acid, guanidine hydrochloride, and acetic anhydride were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Chemical modification of sulphydryl groups of β-Lg. A monomer of β-Lg contains five cystein residues; one is free, and the others are involved in two intramolecular disulfide bridges. In this study, the free sulphydryl group of the β-Lg molecule was carboxymethylated (—SH—SCH₂COOH) by a modification of the method used by Yamada et al. β-Lg (0.45 g) was dissolved in 16 ml of 0.575 M Tris–HCl buffer (pH 8.6) containing 8 mM urea and 5.25 mM EDTA. After the solution was degassed, 0.6 g of iodoacetic acid dissolved in 3 ml of 1 M NaOH was added to the solution. The mixture was left in the dark at room temperature for 1 h. The solution of carboxymethylated β-Lg thus obtained was dialyzed at 4°C against 35 mM phosphate buffer (pH 6.85) containing 0.1 M NaCl.

Chemical modification of amino and carboxyl groups of β-Lg. Carboxyl groups or amino groups of β-Lg molecules were chemically modified to alter the net charge of the protein. Glycineamide was chemically linked to carboxyl groups of β-Lg molecules (—COOH—CONH₂CH₂CONH₂) by a carbodiimide reagent. β-Lg and glycineamide hydrochloride were dissolved in 6 M guanidine hydrochloride. The concentrations of β-Lg and glycineamide were 10 mg/ml and 1 M, respectively. After the solution pH was adjusted to 4.75 with 1 N HCl, EDC was added to the solution. The final concentration of EDC was 0.1 M. The solution pH was again adjusted to 4.75 with 1 N HCl. After 1 h, 1 M sodium acetate was added to the solution to remove unreacted EDC. Finally, the reaction mixture containing COOH-modified β-Lg was dialyzed at 4°C against McIlvaine buffer at a specified pH, which was prepared by mixture of 0.1 M citric acid and 0.2 M Na₃HPO₄ at an appropriate ratio.

To acetyl the amino groups in the β-Lg molecules (—NH₂—NHCOCH₃), β-Lg was dissolved at the concentration of 4 mg/ml in a half saturated sodium acetate solution. Into this solution, acetic anhydride was added dropwise until the solution pH ceased to change. The solution of NH₂-modified β-Lg thus obtained was then dialyzed against McIlvaine buffer of a specified pH at 4°C.

Adsorption experiment. Native β-Lg was dissolved in 35 mM phosphate (K₂HPO₄/NaH₂PO₄) buffer (pH 6.85) containing 0.1 M NaCl. The concentration of β-Lg was varied from 0.4 to 10 mg/ml. In some experiments, McIlvaine buffer (pH 4.0 or 6.85) was used instead of the

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Abbreviations: β-Lg, β-lactoglobulin; EDC, 1-ethyl-3-(3-dimethylaminopropyl]carbodiimide hydrochloride.
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phosphate buffer. Two milliliters of this β-Lg solution was poured into a 20-ml glass bottle, and then 2 g of the stainless steel particles was suspended. The glass bottle was tightly sealed with a gum stopper and an aluminum cap, and then laid down horizontally on the bottom of a cage which was vigorously shaken in a constant temperature bath. After the incubation, the concentration of β-Lg in the supernatant solution was measured by the method of Lowry et al. The amount of β-Lg adsorbed was calculated from the difference in β-Lg concentration before and after the incubation.

In some experiments, the solution of native β-Lg was heated at 75°C for 60 min before the adsorption experiment. The solution of chemically modified β-Lg prepared as mentioned above was also used for the adsorption experiments after appropriate dilution.

ζ-Potential of stainless steel particles. To study the surface charge of stainless steel particles in the buffer solutions, the ζ-potential of the particles was measured with a streaming potential analyzer (Shimadzu, ZP-10B).

Results and Discussion

Figure 1 shows the courses of adsorption of β-Lg onto the stainless steel particles. The amount of β-Lg adsorbed increased at first, and reached a constant value within 15 min at 25°C, and within 30 min at 75°C. Therefore, the incubation time was fixed at 60 min in the following adsorption experiments.

Figure 2 shows the effects of temperature on the adsorption of β-Lg at pH 6.85. In each experiment, the initial concentration of β-Lg was 10 mg/ml. At a temperature below 60°C, the amount of β-Lg adsorbed was small and approximately constant. A steep increase in the adsorbed amount was observed above 65°C. Arnebrant et al. also reported a similar temperature dependence of the amount of β-Lg adsorbed onto a hydrophilic chromium surface using an ellipsometric measurement. The increase in amount adsorbed at an elevated temperature is probably related to the thermal denaturation and aggregation of β-Lg. From the reported DSC data, thermal denaturation of β-Lg begins at 60–70°C.

The contribution of the denaturation and aggregation of β-Lg to its adsorption was also indicated by the difference in the adsorption isotherms at different temperatures. Figure 3 shows the adsorption isotherms at 25°C and at 75°C. The adsorption isotherm of β-Lg at 25°C was of the Langmuir type, which is in agreement with the results in several published reports. According to the estimation by Wahlgen and Arnebrant, a close-packed layer of β-Lg corresponds to 2.7 mg/m² if a hexagonally packed spherical monomer is assumed. The plateau value obtained in Fig. 3(a) is comparable with this estimated value. Thus, the plateau in the adsorption isotherm at 25°C suggests that the surface of the stainless steel particles was almost completely covered by β-Lg. On the other hand, the amount of β-Lg adsorbed at 75°C increased almost linearly with its concentration up to 10 mg/ml. At concentrations above 10 mg/ml, large precipitates were formed in the bulk solution and interfered with the measurement. The amount of β-Lg adsorbed at 75°C exceeded the plateau value observed at 25°C, indicating multilayer adsorption of β-Lg at 75°C.

As shown in Fig. 1, the amount of β-Lg adsorbed became constant after 30 min of incubation even at 75°C. This indicates that the stainless steel particles did not adsorb β-Lg any more even though a considerable amount of β-Lg remained in the bulk solution. However, when the bulk solution was replaced by a fresh solution and incubated at 75°C with the same stainless steel particles, the adsorption of β-Lg was again observed. Figure 4 shows the results obtained from the incubations repeated 5 times. The total amount of β-Lg adsorbed increased nearly constantly with the repetition of incubation. Similar experiments were done using preheated β-Lg instead of native β-Lg. The results

![Fig. 1. Courses of the Adsorption of β-Lg onto Stainless Steel Particles.](image1)

![Fig. 2. Effects of Temperature on the Adsorption of β-Lg at pH 6.85.](image2)

![Fig. 3. Adsorption Isotherms of β-Lg at pH 6.85.](image3)
are compared with those for native \( \beta \)-Lg in Fig. 4. In the first 2 incubations, less of the preheated \( \beta \)-Lg was adsorbed onto the stainless steel particles than the native \( \beta \)-Lg. In the following 3 incubations, little increase in the adsorbed amount was observed for the preheated \( \beta \)-Lg. These findings indicate that \( \beta \)-Lg once denatured and aggregated in the bulk solution is also adsorbed onto the surface to some extent, but does not make an appreciable contribution to the increase in the adsorbed amount. Therefore, the adsorption of \( \beta \)-Lg at an elevated temperature is considered to be a process in which aggregation of denatured \( \beta \)-Lg occurs at the surface. Since the formation of intermolecular disulfide linkages may be involved in this aggregation process, the effect of chemical modification of free sulfhydryl groups on the adsorption of \( \beta \)-Lg was examined. As Fig. 4 shows, the amount of SH-modified \( \beta \)-Lg adsorbed during the 5 repeated incubations was quite small, and comparable with that of the preheated \( \beta \)-Lg. This indicates the important role of free sulfhydryl groups in the adsorption of \( \beta \)-Lg. The increase in the amount of \( \beta \)-Lg adsorbed during the repeated incubations was thus ascribed to the presence of free sulfhydryl groups, which could form intermolecular disulfide linkages.

The adsorption of \( \beta \)-Lg was affected also by the pH of the solution. Figure 5 shows the amount of \( \beta \)-Lg adsorbed when the stainless steel particles were repeatedly incubated 5 times with a \( \beta \)-Lg solution at pH 4.0 or pH 6.85 at 75°C.

In each incubation, the initial concentration of \( \beta \)-Lg was lowered to 0.5 mg/ml to avoid the precipitation of \( \beta \)-Lg at the lower pH. Although the dimer–monomer transition of \( \beta \)-Lg occurs according to pH change, \( \beta \)-Lg is known to associate to form dimers in the range of pH 3.5 to 7 at ordinary temperatures. Thus, in each incubation, the initial associated state of \( \beta \)-Lg was considered to be the same at both pHs. As shown in Fig. 5, the total amount of \( \beta \)-Lg adsorbed was much greater at pH 4.0 than at pH 6.85. However, as the incubation was repeated at pH 4.0, the increase in the amount adsorbed in each incubation became smaller. Especially in the fifth incubation, almost the same amount of \( \beta \)-Lg was adsorbed at both pHs. Thus, the effect of pH on the adsorbed amount became smaller as the surface became covered by \( \beta \)-Lg, suggesting that the effect of pH was mainly ascribed to the interaction between \( \beta \)-Lg and the stainless steel surface.

Because the isoelectric point of \( \beta \)-Lg is 5.1, the net charge of \( \beta \)-Lg is negative at pH 6.85 and positive at pH 4.0. On the other hand, the \( \zeta \)-potential measurements for the stainless steel particles in the buffer solutions showed that the surface of the stainless steel particles was negatively charged at both pHs. The increase in the amount adsorbed at pH 4.0 suggests electrostatic interaction between \( \beta \)-Lg and the stainless steel surface. To confirm this, adsorption experiments were done with two kinds of chemically modified \( \beta \)-Lg at pH 6.85 and at 75°C. Figure 6 shows the results in comparison with those obtained for the native \( \beta \)-Lg. The initial concentration of each protein was 1.0 or 0.5 mg/ml. When the positive charge of \( \beta \)-Lg was diminished by acetylation of amino groups, no adsorption was observed. When the negative charge of \( \beta \)-Lg was diminished by the modification of carboxyl groups, the adsorbed amount increased markedly. These findings can be explained by the electrostatic interaction between \( \beta \)-Lg and the negatively charged surface. The NH\(_2\)-modified \( \beta \)-Lg, having a negative charge, is hardly adsorbed onto the stainless steel surface because of the electrostatic repulsion between them, but COOH-modified \( \beta \)-Lg, with a positive charge, is adsorbed in large amounts because of the electrostatic attraction.

Figure 7 shows the mode of adsorption of \( \beta \)-Lg onto the stainless steel surface. At a low temperature (25°C), \( \beta \)-Lg molecules were adsorbed onto the stainless steel surface until a monolayer was formed. At a high temperature (75°C), native or denatured protein was first adsorbed onto

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**Fig. 4.** Comparison between the Adsorbed Amounts of Native, Preheated, and SH-modified \( \beta \)-Lg in Repeated Batch Incubations at pH 6.85, 75°C. After each incubation, the bulk solution was replaced by a fresh solution containing 10 mg/ml native, preheated, or SH-modified \( \beta \)-Lg.

**Fig. 5.** Comparison between the Adsorbed Amounts of \( \beta \)-Lg in Repeated Batch Incubations at Different pHs. The incubation temperature was 75°C. After each incubation, the bulk solution was replaced by a fresh solution containing 0.5 mg/ml \( \beta \)-Lg.

**Fig. 6.** Comparison between the Amounts of Native and Chemically Modified \( \beta \)-Lg Adsorbed at pH 6.85, 75°C. Initial concentration of \( \beta \)-Lg: (a) 0.5 mg/ml; (b) 1 mg/ml.
the bare surface of the stainless steel particles. Subsequently, aggregation of denatured β-Lg molecules occurred at the surface, resulting in a much higher amount of adsorption. In this aggregation process, free sulfhydryl groups of β-Lg molecules are important. Although the aggregation of β-Lg molecules also occurs in the bulk solution, those aggregated molecules are hardly adsorbed on the surface. In addition, electrostatic interaction between β-Lg and the surface is suggested to be one of the factors affecting the adsorption behavior. However, the participation of some other interactions cannot be excluded, because β-Lg was adsorbed even at pH 6.85, where the electrostatic interaction results in repulsion between β-Lg and the stainless steel surface.

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