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# Adhesion of *Listeria monocytogenes* to Silica Surfaces after Sequential and Competitive Adsorption of Bovine Serum Albumin and β-Lactoglobulin

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**Adsorbed bovine serum albumin was resistant to exchange with β-lactoglobulin, and when albumin was adsorbed from a mixture, its surface concentration increased with time. The passivating character of adsorbed albumin and its resistance to desorption were consistent with the level of *Listeria monocytogenes* adhesion evoked by albumin-containing protein films.**

Adsorption of soluble macromolecules such as proteins to solid surfaces may affect adhesion of microorganisms and other cells (15). Adsorbed proteins can inhibit or facilitate attachment of subsequently arriving microorganisms or be displaced by other surface-active species (17). *Listeria monocytogenes* is a pathogenic bacterium that can exist in a variety of food processing environments (3, 5) and is capable of adhering to food contact surfaces (6, 16). Problems for food processors arise because adhered microorganisms may exhibit an increased resistance to sanitizers and other antimicrobial agents (13, 14). Consequently, the microbial-macromolecular matrix (i.e., the biofilm) can serve as a continuing source of contamination in food processing systems. Recently, Al-Makhlafi et al. (1) reported the effect of four milk proteins (α-lactalbumin, β-lactoglobulin [β-Lg], β-casein, and bovine serum albumin [BSA]) on the adhesion of *L. monocytogenes* to silica surfaces exhibiting either high or low hydrophobicity. The protein-specific effects on adhesion were different, with β-Lg and BSA evoking the most dissimilar responses. The purpose of this study was to evaluate bacterial adhesion following adsorption of β-Lg and BSA in sequence and from mixtures to surfaces exhibiting high and low hydrophobicities. Such a study has practical relevance, as processes involving contact between surfaces and biological fluids can involve many different surface-active species.

**Protein adsorption and cell adhesion.** All materials used in the experiments were described previously, as were the procedures for silica surface modification, cell culture and adhesion, rinsing, image analysis, and statistical analysis (1). In the present experiments, however, surfaces were allowed to contact BSA and β-Lg either sequentially or simultaneously before cell culture contact. Individual proteins were always used at the molar equivalent of 1.000 mg/ml of β-Lg. In sequential adsorption, surfaces were allowed to contact the second protein for 8 h if the second contact had been preceded by an 8-h contact with the first protein and for 1 h if preceded by a 1-h contact. For the cell adhesion experiments, cultures of *L. monocytogenes* were diluted to a cell density of $10^9$ CFU/ml after reaching the stationary phase.

**Sequential and competitive adsorption with $^{14}$C-BSA.** In order to provide an independent, more direct indication of adsorption and exchange events undergone by BSA and β-Lg at the interface, adsorption experiments for which BSA was labeled by reductive methylation of lysine residues with [$^{14}$C]formaldehyde according to the methods of Jentoft and Dearborn (10) were conducted. These techniques have been used to study protein adsorption from mixed solutions as well as protein exchange reactions at solid-water and air-water interfaces (2, 7–9, 19). Alkylation of lysine residues in this way minimally affects their basicity (18) and does not alter protein hydrophobicity insofar as it relates to surface activity (7–9). In these tests, all surfaces were made hydrophobic by silanization with 0.100% dichlorodiethylsilane, as opposed to dichlorodimethylsilane (1), dissolved in xylene.

In a typical sequential adsorption experiment, 250 μl of $^{14}$C-BSA solution was placed in one well of a Nunclon multiwell dish (Nunc Inc., Naperville, Ill.). A silanized silica plate of known surface area was placed onto the surface of the solution. Adsorption was allowed to proceed for 1 or 4 h, and the surface was rinsed by introducing 250 μl of buffer and then withdrawing 250 μl of the solution, with this sequence being repeated 10 times. After 30 min, 250 μl of β-Lg (1 mg/ml) was introduced and then 250 μl of the solution was withdrawn, and this sequence was repeated 10 times. Contact with β-Lg was maintained for the same period of time as that allowed for contact with $^{14}$C-BSA. The surface was then rinsed as before and left in buffer for an additional 30 min. The adsorbed protein layer was removed from the surface by immersion of the silica plate in 1 ml of 48% hydrofluoric acid for 5 min (19). To this was added 15 ml of Gold AB scintillation fluid (Packard Instrument Co.) and 1 ml of Triton X. The sample was well mixed and then analyzed within 1 day with a Beckman scintillation counter.

Competitive adsorption tests were run similarly, with adsorption from a solution of 250 μl of $^{14}$C-BSA combined with 250 μl of β-Lg solution occurring for 1 or 4 h. The surface was rinsed, and the adsorbed layer was removed and analyzed, as described above. The results of the experiments were compared with those of the controls, which proceeded exactly as described above, with the exception that the β-Lg solutions were replaced with equal volumes of protein-free phosphate buffer. Interpretation of adsorption kinetics for BSA and β-Lg with reference to first-order kinetic models shows data recorded for 4 and 8 h to be indistinguishable (11, 12).

**Adhesion following sequential protein adsorption.** The numbers of cells adhered to hydrophobic and hydrophilic silica surfaces following sequential contact with proteins for 8 h are

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TABLE 1. Population of *L. monocytogenes* adhered to each type of surface immediately following sequential contact with β-Lg and BSA for 1 and 8 h

| Surface | Mean number of adhered cells/cm$^2$ | | | |
|---|---|---|---|---|
| | β-Lg–BSA | | BSA–β-Lg | |
| | 1 h | 8 h | 1 h | 8 h |
| Hydrophobic[a] | $1.7 \times 10^6$ | $1.3 \times 10^7$ | $1.5 \times 10^6$ | $2.2 \times 10^6$ |
| Hydrophilic[b] | $1.4 \times 10^6$ | $1.4 \times 10^7$ | $1.3 \times 10^6$ | $9.0 \times 10^5$ |

[a] Mean number of adhered cells on protein-free surface = $1.6 \times 10^7$ cells per cm$^2$.
[b] Mean number of adhered cells on protein-free surface = $2.9 \times 10^6$ cells per cm$^2$.

shown in Table 1, along with those measured on protein-free surfaces. These data indicate that the film formed by adsorption of β-Lg followed by BSA (β-Lg–BSA) encouraged adhesion more than did the film formed by adsorption of BSA followed by β-Lg (BSA–β-Lg) ($P < 0.05$), although the extent of adhesion to β-Lg–BSA remained lower than that measured on bare hydrophobic surfaces. Moreover, BSA–β-Lg reduced the number of adhered cells to values well below that evoked by the bare hydrophilic surfaces, with BSA–β-Lg inhibition of cell adhesion on hydrophilic surfaces being more extensive than that observed on hydrophobic surfaces ($P < 0.05$).

The mechanisms by which these two proteins may affect adhesion to both hydrophobic and hydrophilic silica surfaces when each adsorbs from a single-component solution for 1 and 8 h were discussed previously (1). The surfaces treated with β-Lg–BSA evoked an adhesion response similar to that measured following β-Lg adsorption alone, while those treated with BSA–β-Lg evoked a response closer to that measured following adsorption of BSA alone, although the extents of cell adhesion are higher in the present case. Al-Makhlafi et al. (1) measured about $4 \times 10^5$ cells per cm$^2$ following adsorption of BSA for 8 h to each type of surface. If only on the basis of the relative rates at which each of these proteins, once adsorbed, achieves a nonexchangeable (or more tightly bound) state, we would expect β-Lg to experience more resistance in exchanging with adsorbed BSA than BSA would experience in exchanging with adsorbed β-Lg (1, 12). Table 1 suggests that 8 h is sufficient for rendering the first protein effectively nonremovable, i.e., the kinetics associated with the adoption of a more tightly bound form are not relevant after 8 h of protein-surface contact.

Results of sequential adsorption tests performed with hydrophobic silica and $^{14}$C-BSA are shown in Table 2, which shows that about 72% of adsorbed $^{14}$C-BSA resisted exchange with β-Lg when protein contact was allowed for 4 h.

The fact that greater inhibition of *Listeria* adhesion is seen with hydrophilic surfaces in the BSA–β-Lg tests is consistent with the facts that (i) less adhesion would be expected on exposed hydrophilic silica than on exposed hydrophobic silica and (ii) while BSA shows roughly equal affinities for the two surfaces, β-Lg shows greater affinity for hydrophobic surfaces and may exchange more readily with BSA adsorbed to hydrophobic surfaces than to hydrophilic surfaces (12). These two points are also consistent with the increase in adhesion measured on both surfaces following BSA–β-Lg contact relative to that measured following adsorption of BSA alone (1).

Table 1 also shows results from the 1-h sequential adsorption tests. For both surfaces, cell adhesion to β-Lg–BSA as well as to BSA–β-Lg was lower than that observed with bare hydrophilic surfaces. These data would suggest that adsorbed

TABLE 2. Average radioactivity detected in the protein layers removed from the silica surfaces at the end of each experiment

| Experiment | Average radioactivity (dpm/cm$^2$)[a] |
|---|---|
| Control, 1-h sequential adsorption[b] | 2,695 (21) |
| 1-h sequential adsorption | 1,935 (7) |
| Control, 4-h sequential adsorption | 2,217 (11) |
| 4-h sequential adsorption | 1,599 (4) |
| Control, 1-h competitive adsorption[c] | 5,861 (14) |
| 1-h competitive adsorption | 1,480 (1) |
| Control, 4-h competitive adsorption | 5,717 (14) |
| 4-h competitive adsorption | 2,295 (27) |

[a] Values shown are the means of duplicate experiments; the percent deviation of each replicate from the mean is in parentheses.
[b] Adsorption of $^{14}$C-BSA, followed by adsorption of β-Lg.
[c] Adsorption of a mixture of $^{14}$C-BSA and β-Lg.

β-Lg was readily exchanged with BSA while adsorbed BSA more effectively resisted exchange with β-Lg, at least to the extent that the surfaces evoked an adhesion response similar to that seen for the 8-h tests. Table 2 shows that about 72% of adsorbed $^{14}$C-BSA resisted exchange with β-Lg when protein contact was allowed for 1 h. The similarity of the numbers of adhered cells with hydrophobic silica following the 8-h and 1-h BSA–β-Lg adsorptions is consistent with the similar BSA contents of these layers.

Earlier, we reported adhesion following a 1-h contact with BSA to be greater than that measured in the present tests (1). It was argued that because of its low initial binding constant relative to that of β-Lg (1, 12), the population of BSA molecules tightly adsorbed in the passivating orientation would be relatively low. Apparently, the presence of β-Lg in this case facilitated BSA adsorption in the most passivating orientation. In the cases in which BSA was adsorbed first, the results of Table 1 would suggest that after 2 h of contact, the BSA film is more similar to one formed during 8 h of contact than to one formed during 1 h of contact for films formed from a single-component solution on either surface (1).

**Adhesion following competitive adsorption.** Following adsorption from a mixture of β-Lg and BSA for 8 h, adhesion to protein-coated hydrophobic silica was greater than that observed on protein-coated hydrophilic silica ($1.0 \times 10^6$ versus $3.4 \times 10^5$ cells per cm$^2$; $P < 0.05$), but it remained below that recorded on bare hydrophilic silica. The adhesion behavior measured on the hydrophilic surface is quantitatively similar to that observed in the case of BSA adsorbed alone on each surface (1).

The reduced number of cells in the case of the hydrophilic surfaces could be related to the faster conformational rearrangement undergone by BSA at that surface relative to that of β-Lg (4, 12) as well as to the expectation of less adhesion on regions of exposed hydrophilic surfaces compared with that on hydrophobic surfaces. The concentrations of these two proteins in solution are equal, and these concentrations are greater than those corresponding to diffusion-limited adsorption. It is expected that β-Lg would initially bind to the surface faster than BSA, but it might readily exchange with BSA as its rate of conversion to a nonexchangeable form is relatively low (1, 12). Adsorbed BSA, on the other hand, may be much more difficult to displace.

In competitive adsorption experiments with $^{14}$C-BSA, the amount of $^{14}$C-BSA present on the surface after 4 h was about 40% of what it would have been in the absence of β-Lg (Table 2). This value is lower than expected, since the extents of

adhesion recorded following adsorption from a mixture of β-Lg and BSA for 8 h are not only similar to those recorded following BSA–β-Lg sequential contact (Table 1) but also are close to that measured following 8 h of contact with BSA alone. This may be due in part to the concentration of $^{14}$C-BSA being of a molarity about 17% lower than that of β-Lg (which was used at 1 mg/ml), while the concentrations in the cell adhesion experiments were equimolar. While the fact of the lower concentration would have had little effect on adsorption from single-component solutions, it may have compromised $^{14}$C-BSA's ability to compete with β-Lg for surface sites when they were mixed, especially considering that the diffusion coefficient of BSA is nearly half that estimated for β-Lg (11).

Following 1 h of protein contact with either surface, the mean numbers of adhered cells recorded for hydrophobic and hydrophilic silica were $1.6 \times 10^6$ and $7.3 \times 10^5$ cells per cm$^2$, respectively. These levels are greater than those recorded for each surface after contact with the protein mixture for 8 h but are lower than that recorded on the bare hydrophilic surface. The amount of $^{14}$C-BSA present on the surface after 1 h was about 25% of what it would have been in the absence of β-Lg, according to Table 2. Again, this is lower than expected. But both proteins show high affinities for hydrophobic silica. Resolving the adsorption of each protein into two steps (arrival in an exchangeable form followed by conversion to a nonexchangeable form), Krisdhasima et al. (12) observed that among the four milk proteins tested, β-Lg was fastest in completing the first step and slowest in completing the second while the opposite was true for BSA. Thus, domination of the interface by β-Lg at a short contact time with the presence of BSA increasing with time should be expected, and that was observed. The higher number of cells adhered after 1 h as opposed to 8 h of contact with these protein solutions could also be related to both the lower surface coverage and the higher rinsability of the protein following a 1-h contact, which would leave bare hydrophobic and hydrophilic surface regions.

The adhesion response following 1 h of contact with the protein mixture was lower than that recorded following BSA contact from a single-component solution for 1 h (1). The reason for this is not obvious, but the finding is analogous to that associated with the β-Lg–BSA tests of Table 1. These results suggest that adsorption of BSA with concomitant exchange of adsorbed β-Lg may facilitate BSA adsorption in a favorable, passivating orientation.

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