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Effects of Ionic Substances on the Adsorption of Egg White Proteins to a Stainless Steel Surface

Hiroki SUGIYAMA, Tomoaki HAGIWARA, Hisahiko WATANABE, and Takaharu SAKIYAMA†

Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

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The surface fouling of food processing equipment by proteins was studied by investigating the adsorption of egg white proteins to the surface of stainless steel (SS) at pH 7.4 and 30 °C, and particularly the effects of different types of ionic substances. Ovalbumin and ovomucoid, acidic egg white proteins, were less adsorbed in the presence of phosphate (P_i), a multivalent anion, than in the presence of HEPES, an amphoteric ion. On the other hand, lysozyme, a basic egg white protein, was more adsorbed in the presence of P_i than in the presence of HEPES. Citrate as another multivalent anion and taurine as another amphoteric ion affected the respective adsorption of those egg white proteins similarly to P_i and HEPES. The adsorption of an egg white protein to an SS surface therefore depended on the combination of the type of protein and the effective charge of the coexisting ionic substance. This behaviour can be well explained by assuming that a small ionic substance precedes a protein in attaching to an SS surface, resulting in an alteration to the effective surface charge. Pretreating SS with a P_i buffer lowered the amount of ovalbumin adsorbed with the HEPES buffer, demonstrating that P_i can attach to and remain on the SS surface to affect the subsequent protein adsorption.

Key words: egg white protein; stainless steel surface; adsorption; phosphate; citrate

Foods and/or their components inevitably adhere to the surfaces of processing equipment in food manufacturing processes; for example, heat exchangers used for sterilizing milk usually suffer from the deposition of milk components, mainly proteins and minerals.[1,2] Proteins are known to adhere or be adsorbed to such solid surfaces as stainless steel (SS), even at room temperature.[3] The proteins attached to equipment surfaces may contaminate the product to be subsequently manufactured; if the contaminant is allergenic, a tiny degree of contamination might cause a serious problem in respect of undeclared allergenic components. Although food manufacturers have to resolve such contamination by sufficient cleaning, the means for effectively reducing allergens adhering to food contact surfaces would be helpful. Information on the adsorption and desorption characteristics of allergenic proteins is therefore intrinsically important.

Hen egg is one of the most frequently implicated causes of immediate food-allergic reactions in children.[4] A food allergy monitoring investigation[5] conducted in Japan from 2000 to 2001, collecting 3882 cases of doctor-diagnosed immediate-type food allergic response, showed the hen egg to be the most common causative food for all ages, and to maintain the top ranking from age 0 up to age 6. It is also known that egg white is more allergenic than egg yolk, the allergens in egg white including ovomucoid (OVM), ovalbumin (OVA), ovotransferrin, and lysozyme (LYZ).[4]

Literature information is relatively limited about the adsorption behavior of egg white proteins to solid surfaces. The adsorption isotherms for OVA and LYZ to a titanium surface have been reported at various pH values.[6] The adsorption of LYZ has also been studied to surfaces of silica (SiO_2),[7–10] polystyrene,[10] acrylic acid/2-hydroxyethyl methacrylate co-polymer gels (contact lenses),[11] hematite (α-Fe_2O_3),[7] and titania.[12] The adsorption of OVA has been studied to the surfaces of silica,[13] hydroxyapatite/chitosan composite,[14] titanium,[15] alumina,[15] and activated carbon.[15] Although an attempt to measure the adhesive force between an SS surface and OVA deposited by heating has been reported,[16] no literature could be found on the adsorption of OVM to a solid surface. Moreover, information on the adsorption of egg white proteins to an SS surface is scarce, although SS is one of the most common materials used for food processing equipment.

This study is focused on the adsorption behavior of OVA, OVM, and LYZ to an SS surface at a normal temperature. OVA (45 kDa, pI 4.5) and OVM (28 kDa, pI 4.1), respectively constituting 54% and 11% of total egg white protein, are major allergens, while LYZ (14.3 kDa, pI 11.0), constituting only 3.4% of total egg white protein, is a minor allergen.[17] LYZ is however characterized by its basic nature. Previous studies[18–20] have demonstrated the important role of acidic amino acid residues in the adsorption of proteins to an SS surface, indicating the large contribution of electrostatic interaction between a protein and SS surface to the adsorption. LYZ would thus stand in contrast to OVA and OVM due to the large difference in pI. Although the ionic substances contained in a protein solution may possibly influence the adsorption behavior of protein, the influence of those ionic substances on protein adsorption has hardly been addressed in the literature.

† To whom correspondence should be addressed. Tel: +81-3-5463-0619; Fax: +81-3-5463-0699; E-mail: sakiyama@kaiyodai.ac.jp
Abbreviations: ANOVA, analysis of variance; Cit, citrate; LYZ, lysozyme; OVM, ovomucoid; OVA, ovalbumin; SS, stainless steel; Tau, taurine

We compare in this study the adsorption behavior of OVA, OVM, and LYZ in the presence of different types of ionic substance. The adsorption experiments were performed at 30 °C and at pH 7.4, the typical pH value for whole egg.

Materials and Methods

SS particles. Fine SS particles of type 316L obtained from Yasui Kikai Co. (Osaka, Japan) were used as the substrate surface for the adsorption experiments. The large specific surface area of such fine particles is favorable to the precision of adsorption measurements. The particles were successively washed with 0.1 N NaOH, distilled water, and ethanol, before being dried at 50 °C and stored at room temperature until needed for the adsorption experiments. The specific surface area of the particles was estimated to be 0.58 m^2/g based on the results of a nitrogen adsorption experiment.

Egg white proteins. OVA (Sigma A5503) and LYZ (Sigma L6876) purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) were used without further purification. OVM was purified from hen egg white according to the method reported by Lineweaver and Murray.[21] Briefly, OVM was precipitated by lowering the pH value to 3.5 with the gradual addition of a mixture of 0.5 M trichloroacetic acid/acetone (1:2 v/v), and further purified by gel filtration chromatography (Sephadex G-100, 26 mmϕ × 700 mm), using a 0.15 M NaCl-0.1 M phosphate buffer at pH 7.4 as the eluent. The solution of purified OVM thus obtained was dialyzed against distilled water, freeze-dried, and stored at −20 °C until needed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the OVM preparation showed a broad band at around 30 kDa, this being in good agreement with the result reported by Juià *et al.*[22]

Protein solutions. To study the adsorption isotherms of the proteins, each protein was dissolved at various concentrations in a 50 mM phosphate (P_i) buffer (pH 7.4), or in a 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4). An aqueous solution of 50 mM citrate (Cit) or 50 mM taurine (Tau) was also employed in some experiments to dissolve the protein samples, adjusting the pH value to 7.4 with NaOH. Figure 1 shows the chemical structure and pK_a value of each substance contained in the protein solutions. P_i and Cit exist mainly as multivalent anions at pH 7.4, whereas HEPES and Tau exist mainly as amphoteric ions.

Adsorption experiments. The protein solutions prepared as just presented were subjected to adsorption experiments similarly to those described by Ito *et al.*[23] One milliliter of a protein solution was added to a glass vial containing 2 g of SS particles. After being tightly sealed with an aluminum cap, the glass vial was incubated at 30 °C for 2 h while vigorously shaking. The supernatant was taken out for measuring the protein concentration by a BCA protein assay (Pierce, Rockford, IL, USA). The adsorbed amount was calculated from the difference between the protein concentrations before and after incubation.

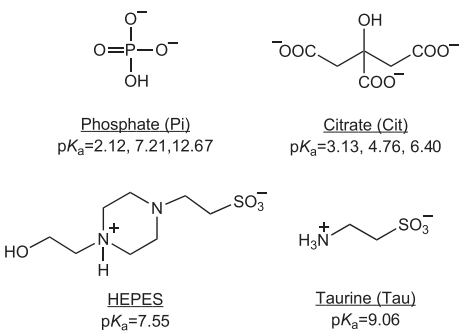

Fig. 1. Chemical Structures and pK_a Values for the Ionic Substances Used in This Study.

Desorption experiments. To examine the removability of adsorbed OVA, desorption experiments were conducted by a method similar to that employed by Imamura *et al.*[19] The adsorption experiment was first conducted with 2 mg/mL of an OVA solution in a 50 mM HEPES buffer at pH 7.4. Half (0.5 mL) of the supernatant was withdrawn for measuring its OVA concentration by the BCA assay to determine the adsorbed amount. To the rest of the mixture, 0.5 mL of a 50 mM HEPES buffer (pH 7.4) or 50 mM P_i buffer (pH 7.4) was added. Since the addition of a fresh buffer solution lowered the OVA concentration in the supernatant, reversibly adsorbed protein was expected to become detached from the surface of the SS particles. After the mixture had been incubated at 30 °C while vigorously shaking for 2 h, the supernatant was again withdrawn to measure the protein concentration by the BCA assay. The amount of protein remaining on the stainless steel surface was calculated from the values for protein concentration according to the mass balance.

Pre-treatment of the SS particles with a buffer solution. SS particles were used in some adsorption experiments after being pre-treated with the 50 mM P_i buffer (pH 7.4) or 50 mM HEPES buffer (pH 7.4). Fifteen grams of the SS particles were mixed with 15 mL of one of the buffers in a glass vial. After being tightly sealed with an aluminum cap, the glass vial was incubated at 30 °C for 2 h while vigorously shaking. The SS particles were then collected by filtration on a hydrophilic PTFE membrane (Millipore, Billerica, MA, USA). The particles on the membrane filter were repeatedly rinsed with distilled water until the effluent showed negligible absorbance at 200 nm. After being dried at 50 °C, the particles were stored at room temperature until being used for the adsorption experiments.

Results and Discussion

Adsorption behavior in different types of buffer solution

The relationship between the adsorbed amount and the final concentration of the adsorbate at a constant temperature, namely the adsorption isotherm, provides the basic data to investigate the adsorption characteristics. Adsorption isotherms of OVA to the SS surface at 30 °C in the 50 mM HEPES buffer (pH 7.4) and in the 50 mM P_i buffer (pH 7.4) are shown in Fig. 2. The amount of OVA adsorbed was affected by both its concentration in the supernatant and the type of buffer used. In the HEPES buffer, OVA was almost completely adsorbed to the SS surface at very low OVA concentrations, as illustrated by the steep increase in the adsorbed amount along the vertical axis. The adsorbed amount then increased more moderately with increasing OVA concentration. Adsorption isotherms with a steep

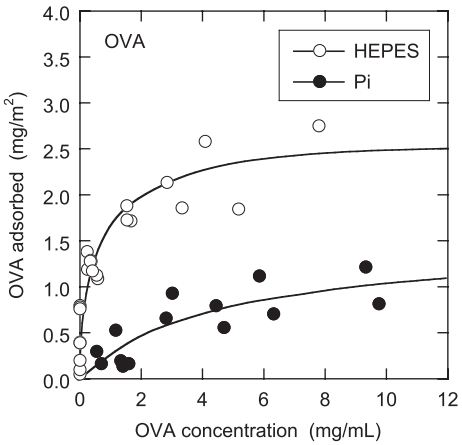

Fig. 2. Adsorption Isotherms of OVA in the 50 mM P_i Buffer (pH 7.4) and 50 mM HEPES Buffer (pH 7.4) to the Surface of SS Particles at 30 °C.

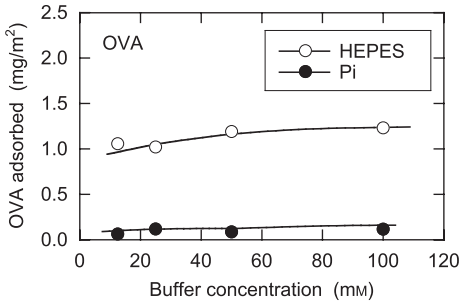

Fig. 3. Effect of Buffer Concentration on the Adsorption of OVA in the P_i Buffer (pH 7.4) and HEPES Buffer (pH 7.4) to the Surface of SS Particles at 30 °C.
The initial OVA concentration was 2 mg/mL.

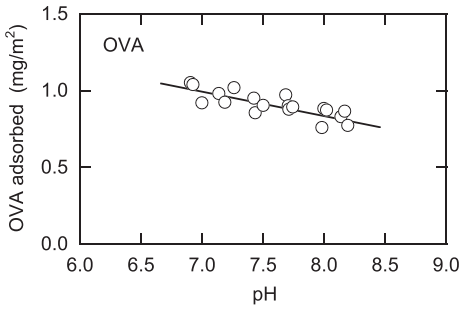

Fig. 4. Effect of pH Value on the Adsorption of OVA in the 50 mM HEPES Buffer to the Surface of SS Particles at 30 °C.
The initial OVA concentration was 2 mg/mL.

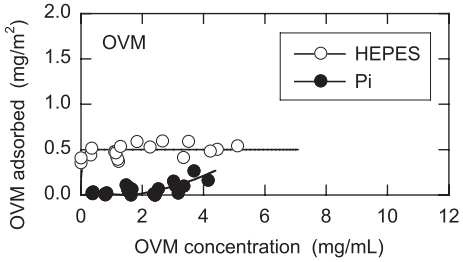

Fig. 5. Adsorption Isotherms of OVM in the 50 mM P_i Buffer (pH 7.4) and 50 mM HEPES Buffer (pH 7.4) to the Surface of SS Particles at 30 °C.

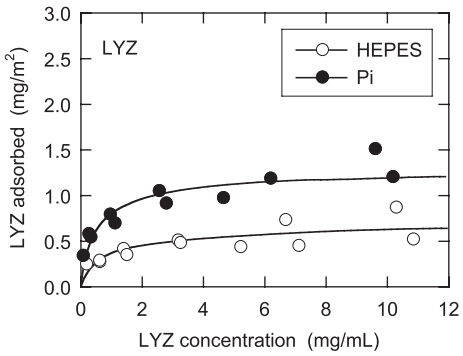

Fig. 6. Adsorption Isotherms of LYS in the 50 mM P_i Buffer (pH 7.4) and 50 mM HEPES Buffer (pH 7.4) to the Surface of SS Particles at 30 °C.

increase along the vertical axis have also been reported for the adsorption of OVA to a titanium surface at pH 3.0–6.0 (100 mM KNO_3) and at pH 8.0 (a 5 mM sodium borate buffer).[6] The adsorbed amount of OVA in the P_i buffer was less than that in the HEPES buffer at all OVA concentrations, and the adsorbed amount did not increase steeply along the vertical axis. This indicates that the affinity of OVA in the P_i buffer was much lower than that in the HEPES buffer.

The effect of buffer concentration on the adsorption of OVA was studied to confirm the difference in the adsorbed amount caused by the two types of buffer. Figure 3 shows the results of adsorption experiments when using 2 mg/mL of OVA dissolved in buffers of different concentrations. The adsorbed amount of OVA in the HEPES buffer was much greater than that in the P_i buffer irrespective of the buffer concentration, although the adsorbed amount slightly decreased with decreasing concentration of both buffers. The buffer concentration therefore had nothing to do with the large difference between the adsorbed amounts in the two types of buffer. The use of a buffer solution of low concentration led to a slight increase in pH during the adsorption experiment, this increase in pH being larger in the P_i buffer than in the HEPES buffer if their concentrations were the same. For example, the final pH value was 7.52 in the 12.5 mM HEPES buffer, whereas it was 7.90 in the 12.5 mM P_i buffer. The amount of OVA adsorbed in the 50 mM HEPES buffer at various pH values was measured and is compared in Fig. 4 to evaluate the effect of pH variation on the adsorption. The adsorbed amount decreased steadily but slightly with increasing value of pH, the increase in pH from 6.9 to 8.2 resulting in no more than a 20% decrease in the adsorbed amount. The variation of pH during the adsorption experiments thus did not seem to be responsible for the large difference between adsorbed amounts in the two types of buffer.

Figure 5 shows the adsorption isotherms of OVM in the two types of buffer. Although the adsorbed amount of OVM was less than that of OVA in each buffer solution, the effect of buffer type on the adsorption was qualitatively similar to the case of OVA adsorption, the adsorbed amount being greater in the HEPES buffer than in the P_i buffer at any OVM concentration. The adsorption isotherm for OVM in the HEPES buffer was nearly rectangular, indicating the strong affinity of OVM to the SS surface. However, OVM was hardly adsorbed to the SS surface in the P_i buffer, particularly at low concentrations, suggesting relatively low affinity to the SS surface in the presence of P_i. Figure 6 shows the adsorption isotherms for LYZ. The effects of the two types of buffer on the adsorption of LYZ were the reverse of the cases of OVA and OVM, the adsorbed amount of LYZ being less in the HEPES buffer than in the P_i buffer at any LYZ concentration. The effect of the type of buffer on the adsorption behavior thus depended on the type of protein. Taking the adsorbed amounts in the HEPES buffer as control data, the presence of P_i decreased the adsorbed amount of each acidic protein (OVA and OVM) and increased that of the basic protein (LYZ).

To discuss the difference between the amounts adsorbed in the presence of HEPES and P_i, we have to start with the characteristics of the SS surface. The SS surface has a passive film consisting mainly of oxides and hydroxides of chromium and iron.[24,25] Upon contact

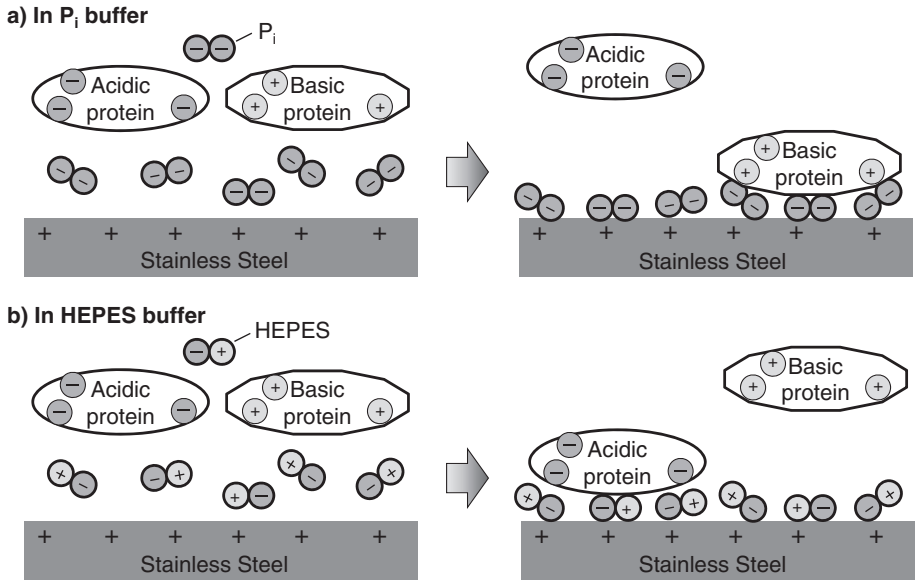

Fig. 7. Schematic Illustration of the Adsorption Mode for Proteins in Different Types of Buffer Solution.

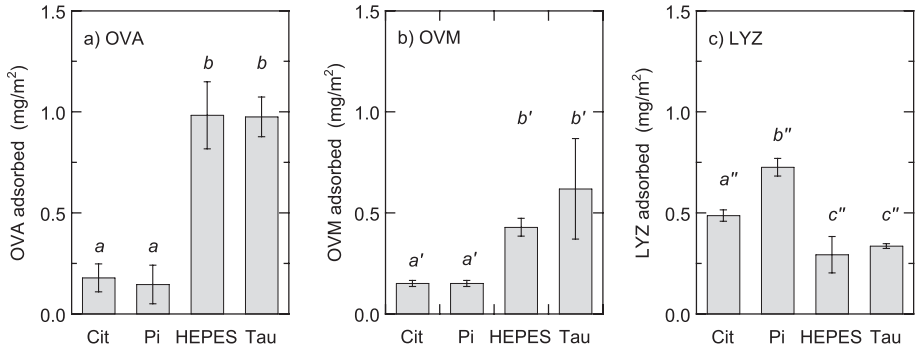

Fig. 8. Amounts of (a) OVA, (b) OVM, and (c) LYZ Adsorbed in the Presence of Cit, P_i, HEPES, and Tau at 50 mM.
The initial protein concentration was 2 mg/mL and the initial pH value was adjusted to 7.4 in all the experiments. Bars show standard deviation (OVA and OVM, $n = 4$ for Cit and Tau, $n = 6$ for P_i and HEPES; LYZ, $n = 3$ for Cit and Tau, $n = 5$ for P_i and HEPES). Different italic letters indicate the significant difference in the adsorbed amount of each protein ($p < 0.05$, one-way ANOVA and Tukey-Kramer test).

with an aqueous solution, the hydroxyl groups (M-OH) can be positively charged by protonation ($M\text{-}OH_2^+$) at low pH values and negatively charged by deprotonation ($M\text{-}O^-$) at high pH values.[26] The apparent point of zero charge has been reported to be at pH 8.5 for SS of type 316L.[27] A bare surface of type 316L SS is therefore positively charged at pH 7.4. This positively charged surface attracts acidic protein molecules and repels basic ones. Similarly, small anions in an aqueous environment can be attracted by the positively charged surface. They may precede proteins in attaching to the stainless steel surface, because they have a higher molar concentration and a larger diffusion coefficient than proteins. Attached multivalent anions (P_i) can make the effective surface charge negative, leading to repulsion toward acidic protein molecules and attraction toward basic ones (Fig. 7a). In contrast, if amphoteric ions (HEPES) attach to the surface, there is no alteration to the effective surface charge, the positively charged surface attracting acidic protein molecules and repelling basic ones (Fig. 7b). The results of the adsorption experiments described here can be well explained by assuming that coexisting ionic substances become attached to the SS surface.

Adsorption behavior in the presence of Cit and Tau

To further investigate the effects of ionic substances on the protein adsorption behavior, other ionic substances, Cit and Tau, were employed for the adsorption experiments. Cit exists mainly as a multivalent anion at pH 7.4, whereas Tau exists mainly as an amphoteric ion. Figure 8a compares the amounts of OVA adsorbed in the presence of different types of ionic substance at 50 mM. The initial OVA concentration was 2 mg/mL. As already shown, the amount of OVA adsorbed in the presence of P_i was lower than that in the presence of HEPES. The amounts of OVA adsorbed in the presence of Cit and P_i were not significantly different from each other, although being significantly lower than in the presence of Tau and HEPES. The statistical significance of the differences ($p < 0.05$) was confirmed by a one-way analysis of variance (ANOVA) and subsequent Tukey-Kramer multiple comparison test conducted with GraphPad Prism 5.04 (GraphPad Software, CA, USA). Similar results were obtained for the adsorbed amount of OVM in the presence of different types of ionic substance (Fig. 8b). Cit gave a significantly greater adsorbed amount of LYZ than in the presence of HEPES or Tau (Fig. 8c). These results suggest that not

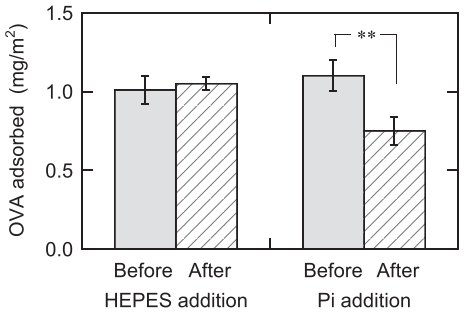

Fig. 9. Comparison between the Amounts of OVA on the SS Surface Before and After Partially Replacing the Supernatant with a Fresh Buffer Solution.

Preliminary adsorption was conducted with 2 mg/mL of OVA dissolved in a 50 mM HEPES buffer at pH 7.4. Half of the supernatant was then replaced with a fresh buffer of 50 mM HEPES (pH 7.4) or 50 mM P_i (pH 7.4). Bars show the standard deviation ($n = 4$). $^{**}p < 0.01$ (*t*-test).

only P_i, but also other multivalent anions could reduce the adsorption of OVA and OVM and enhance the adsorption of LYZ.

Desorption by adding a fresh buffer solution

After the OVA adsorption experiment in the presence of 50 mM HEPES at pH 7.4, half of the supernatant was replaced with a fresh buffer solution of 50 mM HEPES (pH 7.4) or 50 mM P_i (pH 7.4) to check the removability of OVA adsorbed. The results are shown in Fig. 9. No significant decrease was apparent in the adsorbed amount of OVA when half of the supernatant was replaced with the HEPES buffer. Reducing the OVA concentration in the supernatant without changing other environmental conditions therefore resulted in no detectable detachment of OVA from the SS surface, indicating that the adsorption of OVA was irreversible in the HEPES buffer. This is consistent with its strong affinity to the SS surface shown by the adsorption isotherm in the HEPES buffer (Fig. 2). In contrast, the amount of adsorbed OVA was decreased significantly ($p < 0.01$, *t*-test) when half of the supernatant was replaced with the P_i buffer. The presence of P_i thus caused detachment of OVA from the SS surface. This partial removal of OVA with P_i can be explained by assuming that a change occurred in the ionic substances attached to the SS surface. HEPES molecules attached to the SS surface during the adsorption experiment could be partially replaced by P_i when its concentration in the supernatant was increased. The replacement by P_i made the local effective surface charge negative and induced the desorption of neighboring OVA molecules through electrostatic repulsion.

Effect of pretreating the SS particles with buffers

The ability of the buffer components to attach to the SS surface was demonstrated by treating the SS particles with a buffer solution before the adsorption experiment. The SS particles were first soaked in a 50 mM P_i buffer (pH 7.4) and then washed with distilled water. After being dried, the particles were subjected to the adsorption experiment in a 50 mM HEPES buffer (pH 7.4). As shown in Table 1, the pretreatment with P_i reduced the adsorbed amount of OVA by about 30% when compared to the case without any pretreatment. A similar pretreatment with the 50 mM HEPES buffer (pH 7.4) increased the amount of OVA adsorbed with the 50 mM P_i buffer (pH 7.4). These results indicate that each of the buffer components was adsorbed to the surface of the SS particles during the pretreatment and remained on the surface after rinsing with water to influence the adsorption of OVA in the other buffer solution. The results also suggest that pretreating with an appropriate ionic substance may act to reduce OVA adsorption. Further study is required on the effects of pretreating with various multivalent anions and on the stability of the remaining anions when in contact with various types of aqueous solution.

Table 1. Effect of Pretreating with P_i and HEPES on the Amount of OVA Adsorbed to the SS Surface

Pretreatment	Adsorption buffer	OVA adsorbed (mg/m²)*
No	HEPES	0.97 ± 0.21
P_i	HEPES	0.61 ± 0.03
No	P_i	0.13 ± 0.08
HEPES	P_i	0.56 ± 0.01

*Mean ± standard deviation.

Conclusions

The results of this study have revealed that the presence of ionic substances affected the adsorption behavior of egg white proteins. For example, the multivalent anions, P_i and Cit, suppressed the adsorption of the major egg white allergens, OVA and OVM. This behaviour was well explained by assuming that small ionic substances would precede proteins in becoming attached to the SS surface to influence the effective charge of the SS surface. The ability of ionic substances to attach to and remain on the SS surface was demonstrated by the pretreatment experiments with P_i and HEPES.

Acknowledgment

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