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Citric acid pretreatment for suppressing adhesion of major egg allergens to a stainless steel surface

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ABSTRACT

Adhesion of proteins to solid surfaces can cause a number of problems in food manufacturing. In particular, a tiny amount of adherent allergenic proteins may pose a cross-contamination risk. To explore a strategy for suppressing adhesion of major egg allergens to stainless steel surfaces, the effect of citric acid pretreatment of stainless steel surfaces on the adhesion of ovalbumin and ovomucoid was studied under various conditions. Stainless steel powder was subjected to adhesion experiments after treatment with 50 mM citric acid solution, rinsing with water, and drying. Results from ovalbumin adhesion at 30 °C and pH 7.4 demonstrate that citric acid was more effective against ovalbumin adhesion than any univalent or divalent organic acid tested. This supports the idea that the suppression of adhesion is ascribed to the acid anions attaching to the stainless steel surface and yielding an effective negative surface charge. Citric acid pretreatment also suppressed the adhesion of ovalbumin at higher pH of 9.0 and temperature of 80 °C. The adhesion of ovomucoid was also suppressed at both 30 and 80 °C by citric acid pretreatment. These results suggest that the citric acid pretreatment is effective against the adhesion of major egg allergens to stainless steel surfaces.

1. Introduction

Adhesion of proteins to solid surfaces can occur under various conditions and cause a number of problems in food processing and manufacturing. For example, adherent proteins can form a fouling layer on the surface of food processing equipment and interfere with heat transfer (de Jong, 1997; Wallhäußer, Hussein, & Becker, 2012). Proteins may provide a nutritional environment, and insufficient cleaning may result in bacterial growth on the equipment surface (Dat, Hamanaka, Tanaka, & Uchino, 2010). These bacteria can contaminate subsequent product manufacture. Moreover, if the adherent proteins are allergenic, they may pose a cross-contamination risk. Even a very small amount of cross-contamination may cause serious problems in respect of undeclared allergens. Food manufacturers have to minimize cross-contamination risk by sufficient cleaning. Allergen removal through cleaning has been recognized as one of the critical points for effective allergen control (Jackson et al., 2008). To lower the cross-contamination risk further, methods to suppress the adhesion of allergenic proteins to food contact surfaces are required.

Hen egg is one of the most frequently implicated causes of rapid allergic responses to food in children (Bush & Hefle, 1996). The estimated frequency of food allergy to egg in North America is 1.5% for infants and children, second after that to milk (Sicherer & Sampson, 2010). Egg has been reported as an undeclared allergen found frequently in processed foods. A survey of food recall actions because of the presence of undeclared allergens reported to the US Food and Drug Administration showed that egg accounted for the greatest number of food recalls among common food allergens (Vierk, Falci, Wolyniak, & Klontz, 2002). Although inadequate ingredient statements including omissions and errors were the most common cases, cross-contamination from manufacturing equipment accounted for 40% of all recalled products. A recent study on the pre-packaged food products available commercially in Thailand also showed that 17 out of 129 products contained egg as an undeclared allergen at levels greater than 10 ppm (Surojanametakul et al., 2012). Thus, information on the adhesion behavior of egg proteins to processing equipment surfaces is essential to the control of undeclared egg allergens in processed foods. However, the literature information on their adhesion is rather limited.

In our previous study (Sugiyama, Hagiwara, Watanabe, & Sakiyama, 2012), the adhesion behavior of three egg white

Abbrevations: ANOVA, analysis of valiance; CA, citric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OVA, ovalbumin; OVM, ovomucoid; SS, stainless steel; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.

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proteins, ovalbumin (OVA), ovomucoid (OVM), and lysozyme, to a stainless steel (SS) surface was studied at 30 °C and pH 7.4 with consideration of the effect of coexisting ionic species. OVA (45 kDa, pI 4.5) and OVM (28 kDa, pI 4.1) are major allergens and constitute 54 and 11% of total egg white protein, respectively (Mine & Yang, 2008). SS is one of the most common materials used in food processing equipment. Our results demonstrated that the presence of such multivalent anions as phosphate and citrate reduced the adhesion of OVA and OVM to the SS surface. A low level adhesion of OVA was also observed by pretreating the SS surface with a phosphate buffer. This suggests that the low level adhesion resulted from phosphate ions attaching to the SS surface even after water rinsing. Based on these results, we hypothesize that treating a SS surface with an appropriate anionic substance can reduce the adhesion of major egg allergens.

This study is focused on the effect of pretreatment of a SS surface with citric acid (CA) on the adhesion of OVA and OVM. The adhesion of OVA is studied more extensively because most undeclared egg-positive products are found to contain OVA (Surojanametakul et al., 2012). The adhesion to CA-treated surfaces is compared to untreated surfaces at different temperature and pH values. The effect of pretreatment conditions is also studied.

2. Materials and methods

2.1. Stainless steel powder

Fine 316L SS powder (PF-5F; specific surface area: 0.57 m^2/g) obtained from Epson Atmix Co. (Hachinohe, Aomori, Japan) was used as the substrate surface for the adsorption experiments. Its large specific surface area is favorable for the precision required in adsorption measurements. As in our previous work (Thammathongchat, Hagiwara, & Sakiyama, 2010), the powder was washed successively with 0.1 M NaOH, distilled water, and ethanol, before being dried at 50 °C and stored at room temperature until needed.

2.2. Egg allergens

OVA (Sigma A5503) purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) was used without further purification. OVM was purified from hen egg white as described in our previous work (Sugiyama et al., 2012), through precipitation by the addition of trichloroacetic acid/acetone and gel filtration chromatography on Sephadex G-100 (fractionation range for globular proteins: 4–150 kDa). The solution of purified OVM was dialyzed against distilled water, freeze-dried, and stored at −20 °C until needed.

2.3. Pretreatment of stainless steel powder

SS powder (10 g) was mixed with 20 ml of 50 mM CA solution in a glass vial. After being tightly sealed with an aluminum cap, the glass vial was incubated at 30 °C for 120 min with vigorous shaking. The SS powder was collected by filtration on a hydrophilic polytetrafluoroethylene membrane (Millipore, Billerica, MA, USA). The powder on the membrane filter was rinsed repeatedly with distilled water. After being dried at 50 °C, the powder was stored at room temperature until needed for the adsorption experiments.

For comparison of the pretreatment conditions, a univalent or divalent acid was used instead of CA (trivalent acid) without changing any other conditions. The acids used for the pretreatment are listed in Table 1 with their pKa values (Lide, 2009). In other experiments, CA concentration and pretreatment time were changed as described in later text.

Table 1
Organic acids tested for the pretreatment of stainless steel surface.

Name	Structure	pKa
Acetic acid	CH_3–COOH	4.76
Succinic acid	HOOC–CH_2–CH_2–COOH	4.21, 5.64
L-Malic acid	HOOC–CH_2–CHOH–COOH	3.40, 5.11
L-Tartaric acid	HOOC–CHOH–CHOH–COOH	2.98, 4.34
Citric acid	HOOC–CH_2–C(OH)(COOH)–CH_2–COOH	3.13, 4.76, 6.40

2.4. Adsorption experiments

OVA or OVM was dissolved at 2 mg/ml in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4). One milliliter of the protein solution was added to a glass vial containing 2 g of the SS powder with or without pretreatment. After being sealed tightly with an aluminum cap, the glass vial was incubated at 30 °C for 120 min with vigorous shaking. The supernatant was withdrawn to measure the protein concentration by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The amount of adherent protein was calculated from the difference between the protein concentrations before and after incubation. In some adhesion experiments, pH or temperature was altered to check the effect of adhesion conditions. For the adhesion experiments at pH 8.5 and 9.0, 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer was used instead of HEPES buffer. The adhesion experiment was repeated at least three times.

2.5. Statistical analysis

The significance in difference between the data sets was tested, if necessary, using Student's *t*-test or a one-way analysis of variance (ANOVA) in GraphPad Prism 5.04 (GraphPad Software, CA, USA). The statistical significance threshold was set to $p \leq 0.05$.

3. Results and discussion

3.1. Effect of organic acid pretreatment on OVA adhesion

Fig. 1 compares the effect of various organic acids used in the pretreatment of the SS surface on OVA adhesion at 30 °C in 50 mM HEPES buffer (pH 7.4). The mean value and standard deviation of the experimental data are shown for each organic acid. The

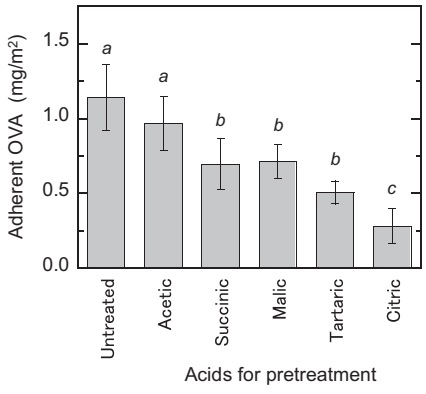

Fig. 1. Effect of pretreatment of SS surface with different types of organic acid on OVA adhesion. Adhesion experiments were performed in 50 mM HEPES buffer (pH 7.4) at 30 °C. Error bars represent standard deviations. Different italic letters indicate significant differences in adherent amount of OVA ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test).

statistical significance of differences among the results was tested by a one-way ANOVA and subsequent Tukey's multiple comparison test. Pretreatment with acetic acid resulted in no significant change in OVA adhesion ($p > 0.05$), although the mean value of the acetic data was numerically lower than that of the no acid control. Pretreatment with other organic acids suppressed OVA adhesion significantly. A statistical comparison showed that the suppressive effect of the three types of divalent acid was not significantly different ($p > 0.05$) but was less than that of CA ($p < 0.05$). Thus the suppressive effect on OVA adhesion depended on the acid valency used in pretreatment. According to the pKa values listed in Table 1, each acid is considered as almost fully dissociated at the adhesion pH of 7.4. Therefore the more negative charge the acid anion carried at the adhesion pH, the higher the suppressive effect.

A passive film consisting mainly of oxides and hydroxides of chromium and iron exists on the SS surface (Asami & Hashimoto, 1979; Elsener & Rossi, 1995). Upon contact with an aqueous solution, the hydroxyl groups (M−OH) can be positively charged by protonation ($M-OH_2^+$) under low pH conditions and negatively charged by deprotonation ($M-O^-$) under high pH conditions (Parks, 1965). The apparent zero charge of SS type 316L is reported to occur at pH 8.5 (Takahashi & Fukuzaki, 2008). Therefore, a bare SS type 316L surface is positively charged at pH 7.4. The positively charged surface attracts anions. If anions, especially multivalent ones, attach to the surface, the effective surface charge would decrease and become negative. This leads to a repulsion against negatively charged proteins such as OVA. This is a probable explanation for the suppression of OVA adhesion by acid pretreatment. It seems reasonable that the adhesion suppression depended on the organic acid valency because anionic species with more negative charge would cause a larger decrease in effective surface charge.

Based on the results in Fig. 1, we selected CA as the best pretreatment agent. Fig. 2 shows the effect of concentration and time for CA pretreatment on the adhesion of OVA at 30 °C. In a 50 mM CA solution, the change in pretreatment time from 120 to 30 min did not affect OVA adhesion significantly ($p > 0.05$, one-way ANOVA), although the mean adherent amount numerically decreased with pretreatment time. In a 100 mM CA solution, a comparable suppressive effect was observed after 10 min of pretreatment. These results indicate that the pretreatment time can be shortened to obtain a similar suppression effect on OVA adhesion. However, we continued using the pretreatment conditions of 50 mM CA for 120 min to ensure sufficient attachment of citrate anions to the SS surface.

3.2. Suppression of OVA adhesion at different pH and temperature

We conducted most adhesion experiments at pH 7.4, which is the typical pH of liquid whole egg. Liquid egg white normally has a higher pH value. Our measurement of egg white pH yielded a value of 8.9, which is similar to a reported one (Nemeth et al., 2011). Therefore, the adhesion of OVA to the CA-treated SS surface was also studied at higher pH values of 8.5 and 9.0. The concentration of OVA adherent to the CA-treated surface (Fig. 3) was lower than that of the untreated surface at every pH tested ($p < 0.05$, Student's t-test). These results suggest that CA pretreatment would be effective for suppressing OVA adhesion not only for liquid whole egg but also for liquid egg white.

Egg products should be subjected to a microbicidal treatment to ensure the products are safe and suitable to eat (Codex Alimentarius Commission, 2007). Where heat treatment is used for this purpose, a combination of temperature and treatment time should be considered. According to Japanese regulations, for example, the temperature and time combination for liquid whole egg should not be less effective than that achieved at 64 °C for 2.5 min. Through heat treatment, the equipment surfaces frequently suffer from severe adhesion of egg proteins because of thermal aggregation. Thus we studied the effect of CA pretreatment on OVA adhesion at higher temperatures. OVA adhesion to the untreated and CA-treated surfaces increased with temperature with a lower adherence to the latter (Fig. 4). Thus CA pretreatment was effective in suppressing OVA adhesion even under hot conditions.

Elevation of the adhesion temperature resulted in increased OVA adhesion even for the CA-treated surface. This is probably because of the promotion of the thermal aggregation of OVA. As shown from the results of the adhesion experiments at 30 °C, citrate anions attached to the surface cannot prevent the adhesion of OVA completely. Even though the number of OVA molecules adhering directly to the surface is small, these molecules can form a base to link a number of OVA molecules aggregated through disulfide linkages that are known to form also in cooked egg (Fig. 5). Another possible reason is the detachment of citrate anions from the SS surface at high temperature. To examine the latter possibility, CA-treated SS particles were heated at 80 °C in 50 mM HEPES buffer (pH 7.4) for 120 min before being subjected to the adhesion experiment at 30 °C. The results (Fig. 6) showed that the preliminary heating of the CA-treated surface yielded no significant increase in OVA adhesion at 30 °C ($p > 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). Thus a significant

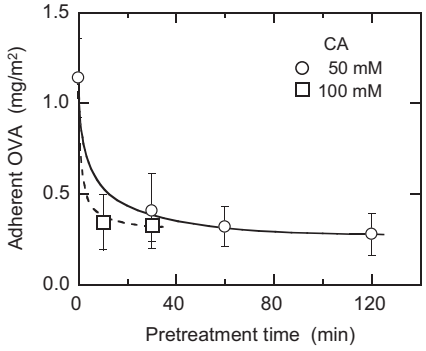

Fig. 2. Effect of CA concentration and time for SS surface pretreatment on OVA adhesion. Adhesion experiments were performed in 50 mM HEPES buffer (pH 7.4) at 30 °C. Error bars represent standard deviations. Curves are presented to guide the eye.

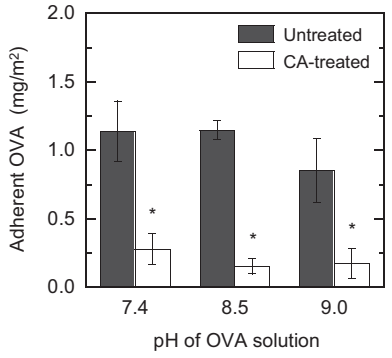

Fig. 3. Efficacy of CA pretreatment against OVA adhesion at different pH values. Adhesion experiments were performed in 50 mM HEPES buffer (pH 7.4) or 50 mM Tris buffer (pH 8.5 and 9.0) at 30 °C. Error bars represent standard deviations. An asterisk indicates that the value is significantly different from that for the untreated surface ($p < 0.05$, Student's t-test).

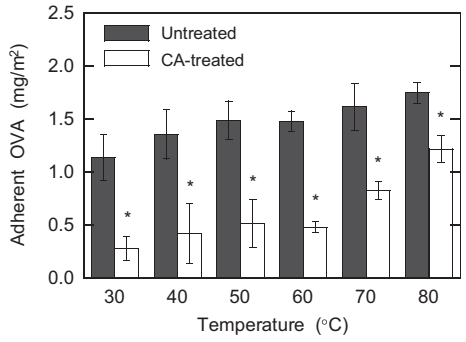

Fig. 4. Efficacy of CA pretreatment against OVA adhesion at different temperatures. Adhesion experiments were performed in 50 mM HEPES buffer (pH 7.4). Error bars represent standard deviations. An asterisk indicates that the value is significantly different from that for the untreated surface ($p < 0.05$, Student's *t*-test).

suppression of OVA adhesion was observed still after heating compared with the case without CA treatment. Therefore, citrate anions cannot be considered to detach from the SS surface even at high temperature.

3.3. Suppression of OVM adhesion

The effect of CA pretreatment on OVM adhesion, another major allergen of hen egg, was also studied at 30 and 80 °C. As shown in Fig. 7, the concentration of OVM adherent to the CA-treated surface was negligible at 30 °C. The elevation of adhesion temperature from 30 to 80 °C increased OVM adhesion to both surfaces considerably. However, the concentration of OVM adherent to the CA-treated surface at 80 °C was still significantly lower than that to the untreated surface. Thus, CA pretreatment of the SS surface was also effective against OVM adhesion.

Based on the idea that CA pretreatment makes the effective surface charge negative, the adhesion of other proteins with net negative charges would also be suppressed. Egg white contains lysozyme (14.3 kDa, pI 11.0) as an allergen (Mine & Yang, 2008). A lysozyme molecule has a net positive charge in the pH range of liquid egg products. In our previous work (Sugiyama et al., 2012), the presence of multivalent anions increased the adhesion of lysozyme to SS surfaces. This suggests that CA pretreatment promotes the adhesion of lysozyme or other proteins with a net positive charge if any and could be a drawback. However, the content of positively charged proteins is normally low in food materials. Lysozyme constitutes only 3.4% of total egg white protein. Thus the increased adhesion of positively charged proteins may not so much influence the total amount of adherent proteins, although confirmation of this assumption is necessary.

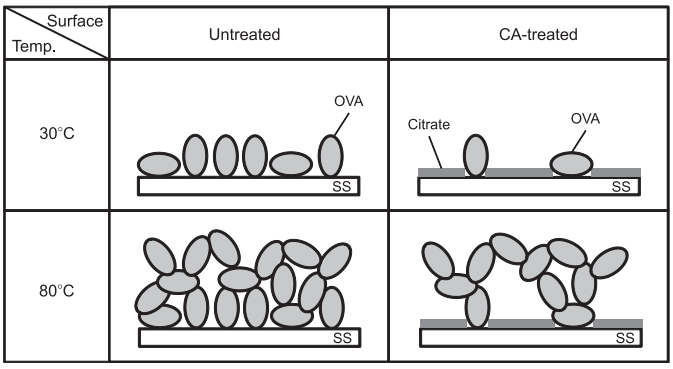

Fig. 5. Schematic illustration of possible modes of protein adhesion to SS surfaces with and without CA pretreatment at low and high temperatures.

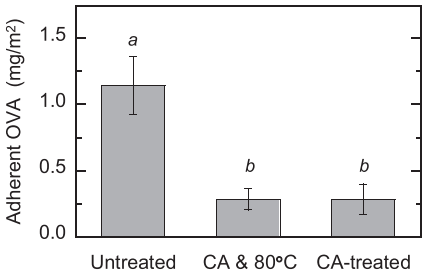

Fig. 6. Effect of preheating of CA-treated SS surface on OVA adhesion. Adhesion experiments were performed in 50 mM HEPES buffer (pH 7.4) at 30 °C. Error bars represent standard deviations. Different italic letters indicate significant differences in adherent amount of OVA ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test).

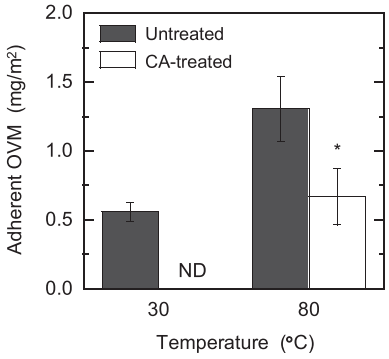

Fig. 7. Efficacy of CA pretreatment against OVM adhesion at 30 and 80 °C. Adhesion experiments performed in 50 mM HEPES buffer (pH 7.4). ND: not detected. Error bars represent standard deviations. An asterisk indicates that the value is significantly different from that for the untreated surface ($p < 0.05$, Student's *t*-test).

4. Conclusions

The CA pretreatment proposed in this study reduced the adhesion of OVA and OVM to a SS surface from 30 to 80 °C. The suppressive effect probably results from citrate anions attaching to the SS surface and influencing the effective surface charge. Although various other factors affecting adhesion are to be studied further, CA pretreatment may be an excellent choice for reducing total egg allergens, or total proteins, adherent to SS surfaces.

Acknowledgments

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