Inhibition of Adhesion of Several Bacteria onto Microtiter Plate by Selected Food Additives

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(Received: November 25, 2010)
(Accepted: May 24, 2011)

Adhesion inhibitory effects of food additives, such as Polylysine (PL) and Whey protein (WP), as well as Sucrose fatty acid ester (SFE) with fatty acid of C8 to C18, Monoglycerin fatty acid ester (MFE) with fatty acid of C8 to C18, Gardenia yellow pigment (GY), Monascus pigment (MP), and Protamine (PT) that had been shown to inhibit adhesion of Salmonella Enteritidis onto microtiter plate, were determined on several bacteria. Among SFE tested, adhesion of S. Typhimurium onto microtiter plate was decreased to less than 50% of the control by SFE with fatty acid of C10, C12, C14, and C16 at 0.05% and that of C18 at 0.01%. MFE with fatty acid of C8, C10, C12, C16, C18 also inhibited the adhesion to less than 50% of the control. The adhesion of S. Typhimurium was also almost completely inhibited by PT and PL at 0.01%, 0.1% MP, 0.025% WP, and 1% GY. Adhesion of Pseudomonas aeruginosa was decreased to less than 50% of the control by SFE with C8 and C16 fatty acid at 0.05%, C12 and C14 at 0.01%, 0.1% MP, 0.01% PL, and 0.025% WP, but not by 0.05% MFE tested. 1% GY, and 1% MP. Adhesion of P. fluorescens was almost completely inhibited by SFE with fatty acid of C14, C16, and C18 at 0.05, 0.005, and 0.005%, respectively, and 1% GY, 1% MP, 0.1% PT, and 1% PL, but not by MFE and WP even at 0.05 and 0.25%, respectively. Adhesion of Listeria monocytogenes was decreased to less than 50% of the control by SFE with fatty acid of C10, C12, C16, and C18 at 0.05% and by MFE with fatty acid of C10, C14, C16, and C18 at 0.05, 0.05, 0.005, and 0.005%, respectively. The adhesion decreased to less than 50% of the control by 0.1% MP, 1% PT, and 1% PL, but not by 1% GY and 0.25% WP. Adhesion of Staphylococcus aureus decreased to less than 50% of the control by SFE with fatty acid of C10, C12, C14, C16, and C18 at 0.05, 0.01, 0.005, 0.005, and 0.005%, respectively. The adhesion was inhibited by more than 50% by 0.05% MFE with C8 fatty acid and by 0.005% MFE with C10 to C18 fatty acid. GY, MP, PT, PL, and WP decreased the adhesion by more than 50% at 0.1, 0.01, 0.01, 0.01, and 0.025%, respectively. It seems to be important to select suitable substances for inhibition of adhesion of each of the bacterial species.

Key words: adhesion inhibition, pathogen, fatty acid esters, protamine, polylysine, whey protein

Introduction

Food-borne illness might occur with some foods naturally contaminated with pathogen, but the others with secondary contaminated with pathogen exist in the food producing and processing environments. Prevention of secondary contamination of food-borne pathogen at the time of processing and the cooking is more important. Moreover, non-heated foods such as raw ready-to-eat vegetables and fruits carry high infection risk by pathogens com-
pared with the conventional heat-processed foods. Therefore, the development of effective non-thermal decontamination methods and the secondary adhesion prevention technology of food-borne pathogen are necessary.

Many of bacteria form biofilm in which bacteria stick to the solid surface rather than a planktonic state. Because it is difficult to remove the attached bacteria and biofilm, and it becomes more difficult to completely decontaminate with a sterilizer, inhibition of the secondary adhesion of food-borne pathogens on food processing environment and raw ready-to-eat foods have become more important1, 2, 16, 18).

In our previous study, adhesion of Salmonella Enteritidis IFO 3313 was effectively inhibited in the presence of Gardenia yellow, Protamine, Monascus pigment, Sucrose fatty acid ester and Monoglycerin fatty acid ester even at low concentration, in an adhesion test using crystal violet staining and microtiter plate11). Therefore, in this study, we have examined adhesion inhibition and antibacterial activities of these additives and Polylsine and Whey protein for adhesion of Listeria monocytogenes, Pseudomonas aeruginosa, P. fluorescens, S. Typhimurium, and Staphylococcus aureus and the interaction of additives with the surfaces of bacterial cell and a microtiter plate well is discussed.

Materials and Methods

1. Bacterial strain and culture condition

Salmonella Typhimurium IFO12529 and Pseudomonas aeruginosa IFO13275 were purchased from the Institute for Fermentation, Osaka, Japan. Listeria monocytogenes No. 185 was kindly provided from Public health center, Saku, Nagano, Japan. Pseudomonas fluorescens was isolated from lettuce and identified with RiboPrinter system (Dupont, USA) in our laboratory. Staphylococcus aureus NBRC13276 was obtained from NITE Biological Research Center (NBRC), Kazusakamata, Kisarazu-shi, Chiba, Japan. Luria Broth (LB, Becton, Dickison and Company) was used to culture S. Typhimurium. Tryptic Soy Broth (TSB, Becton, Dickison and Company) was used to culture L. monocytogenes, P. aeruginosa, and P. fluorescens. These bacteria were cultured overnight at 30°C with shaking at 130 rpm to obtain cells in stationary phase of growth. S. aureus was cultured overnight in TSB supplemented with 2% NaCl at 37°C with shaking at 130 rpm to obtain cells in stationary phase of growth.

For adhesion or adhesion inhibition tests, 0.1% Bacto-Soytont (Difco Lacoratories) was used for S. Typhimurium, Brain Heart Infusion Broth (BHI, OXOID) for L. monocytogenes, 1/5 BHI Broth for P. aeruginosa and P. fluorescens, and 1/5 TSB supplemented with 2% NaCl for S. aureus.

2. Adhesion inhibition test

Adhesion inhibition test was done according to the method of Miyamoto et al.11) Each of the bacterial cultures was adjusted to OD$_{600}$ = 0.7 with a suitable medium mentioned above. After 150 µl of the bacterial suspension were inoculated into a well of 96-well microtiter plate (made of polystyrene, SANPLATEC Co., Ltd., Osaka, Japan), 50 µl of adhesion inhibitor was added to the well. The mixture was incubated at 30°C for 24 hr for S. Typhimurium and L. monocytogenes, at 30°C for 48 hr for P. aeruginosa and P. fluorescens, 37°C for 24 hr for S. aureus. Protamine (PT) and Whey protein ASAMA (WP) were provided by Asama Chemical Co., Ltd., Tokyo, Japan, Polylsine (PL) from Chisso Corporation, Tokyo, Japan, Sucrose fatty acid esters (SFE) from Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan. Monascus pigment (MP) and Gardenia yellow (GY) were obtained from Wako Pure Chemicals Co., Ltd., Tokyo, Japan. Monoglycerin fatty acid esters (MFE) were the products of Taiyo Kagaku Co., Ltd., Tokyo, Japan.

The fatty acid esters were dissolved in water and sterilized in an autoclave at 121°C for 20 min. The other additives were dissolved in water and filter-sterilized with EB-DISK 25 (pore size 0.2 µm, Kanto Chemical Co., Ltd., Tokyo, Japan). The concentrations used in this study (0.005%, 0.01%, and 0.05% for fatty acid esters; 0.01, 0.1, and 1.0% for GY, MP, PT, and PL) were based on the effective concentrations previously reported for S. Enteritidis11) and for WP (0.0025, 0.025, and 0.25%) were based on the preliminary experiment (data not shown).
3. Measurement of quantity of adhesion bacteria by the crystal violet method

Method of Mireles et al.\(^9\) was used to measure adhesion of bacteria. After the incubation, supernatant in each of the wells was removed by suction with a pipette. Wells retain the bacterial cells were washed twice with sterile ultrapure water. After the well was dried for 15 min at room temp., 150 \(\mu\)l of 1% crystal violet solution were added to each well and reacted at room temperature for 20 min. After the supernatant was removed by suction with a pipette, each well was washed with sterile water 4 times and dried for 15 min at room temp. To each well, 200 \(\mu\)l of 99% ethanol were added to extract crystal violet retained in the well. To measure absorbance of the ethanol extracts, 100 \(\mu\)l of the solution was withdrawn and transferred to a new well of 96-well microtiter plate and measured absorbance at 595 nm with Microplate Reader Model 450 (Bio-Rad Laboratories Japan, Tokyo, Japan). Relative adhesion (%) in the presence of additive was expressed in percent of the control value. Values were the mean±SD for 5 separate experiments.

4. Measurement of viable counts

Viable counts of bacteria were measured after incubation with additives by conventional plating method as described previously\(^11\). After incubation with additives, the bacterial cells in a well were collected by pipetting. The cell suspension was diluted with phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM Na\(_2\)HPO\(_4\), 2.68 mM KCl, 1.47 mM KH\(_2\)PO\(_4\)) and 100 \(\mu\)l of the diluted solution was streaked onto Tryptic Soy Agar (TSA, Becton, Dickinson and Company) plate. After incubation at 30°C for 48 h, colonies were counted. To measure viable counts of \textit{S. aureus}, TSA supplemented with 2% NaCl was used. The results were shown as an average of two separate experiments.

Results

1. Effects on \textit{S. Typhimurium}

Figure 1 shows the effects of additives on adhesion and viability of \textit{S. Typhimurium}. The adhesion inhibition effect of SFE was the strongest in C18 fatty acid ester; it almost completely inhibited the adhesion at 0.01%. In contrast, the C8 ester did not inhibit even at 0.05%.

SFE with C12 and C16 fatty acids showed almost 100% adhesion inhibition at 0.05%, and C14 and C10 around 90 and 75%, respectively, at the same concentration. SFE with C12 fatty acid seems a little more effective than C14, but C14 also showed effectiveness in reducing the
adhesion to around 50% at 0.01%. It seems that there is a relationship between the increase in length of the fatty acid and inhibition effect. In general, the effect of MFE was weaker than SFE. But in contrast with SFE, MFE with C8 fatty acid ester showed the highest adhesion inhibition effect among MFE tested, though it did not completely inhibit adhesion of S. Typhimurium even at 0.05%. The adhesion of Typhimurium was almost completely inhibited by PT and PL at 0.01%, and by MP, WP, and GY at 0.1%, 0.025%, and 1%, respectively. In most of the additives, viable bacterial counts decreased with increase of the concentration of the additives, but, except for PL with which at 0.01% the viable bacterial count reached the detection limit, the extent was one or two log decrease.

2. Effects on P. aeruginosa

Figure 2 shows the effects of additives on adhesion and viability of P. aeruginosa. All the SFE inhibited the adhesion of the test strain to some extent. SFE with fatty acids of C10 and C16, at 0.05%, decreased the adhesion of the bacterium to less than 50% of the control, and C12 and C14 at 0.01%. Ratio of adhesion increased to up to around two and half times of the control at 0.005% in all the SFE tested except that with C12 fatty acid. For SFE with C14 fatty acid, although adhesion decreased to less than 50% of the control at 0.05%, a slight increase in adhesion was observed when it is compared to 0.01%. In the case of SFE with C12 fatty acid, adhesion of the bacterium decreased with increase of the concentration of SFE. Moreover, any of the SFE did not inhibit the adhesion completely even at 0.05%. In contrast to S. Typhimurium, the length of the fatty acid did not affect on the adhesion inhibition activity of SFE. PT inhibited the adhesion of P. aeruginosa in a dose-dependent manner. PL and WP inhibited adhesion, but the increase of efficiency of adhesion inhibition was not completely correlated to the concentration. A slight inhibition was observed with MFE of C10 fatty acid, but any other of MFE, GY and MP did not affect adhesion of P. aeruginosa even at the highest concentration tested. Viable bacterial counts largely decreased in the presence of PT and PL and reached to the limit of detection at the concentration above 0.1%, but for the other additives, variation were around one or two log.

3. Effects on P. fluorescens

Figure 3 shows the effects of additives on adhesion and viability of P. fluorescens. The adhesion inhibition effect of SFE was the
strongest in C16 and C18 fatty acid esters; they almost completely inhibited the adhesion at 0.005%. SFE with C14 fatty acid also almost completely inhibited the adhesion at 0.05%. However, C10 ester reduced the adhesion only to around 50% at 0.05% and the C8 and C12 esters did not inhibited adhesion even at 0.05%.

In contrast to SFE, MFE with fatty acid of C12 to C18 did not show any adhesion-inhibition effect even at 0.05%. The C10 ester showed adhesion inhibition effect similar to that of SFE of C10 ester. The C8 ester showed a weak
adhesion inhibition, such as only around 20% at 0.05%. Adhesion of *P. fluorescens* was inhibited by more than 80% with GY at 1.0%, and almost completely by PT at the same concentration. PT also showed strong antibacterial activity at 0.1%, and viable bacterial count reached the detection limit. The variations of viable bacterial count by the other additives were around one or two log. MP and PL inhibited adhesion by around 45%, and WP by only around 20% even at the highest concentration tested.

4. **Effects on *L. monocytogenes***

Figure 4 shows the effects of additives on adhesion and viability of *L. monocytogenes*. Among SFE tested, the adhesion inhibition effect was the strongest in C18 fatty acid ester; it almost completely inhibited the adhesion at 0.05%. The C10, C12 and C16 esters inhibited the adhesion by around 60% at 0.05%. MFE with C10, C16 and C18 fatty acid esters inhibited the adhesion by more than 80% at 0.05%. The C12 ester also inhibited adhesion by around 60% at 0.01%, but less than 40% at 0.05%. Adhesion of *L. monocytogenes* was also largely inhibited with MP, PT and PL at 0.1%, 1%, and 1%, respectively, and slightly with WP at 0.25%. The SFE of C10 ester decreased the viable counts of *L. monocytogenes* by 4 log, but for all other additives the variation were around one or two log.

5. **Effects on *S. aureus***

Figure 5 shows the effects of additives on adhesion and viability of *S. aureus*. The adhesion inhibition effect of SFE was the strongest in C16 and C18 fatty acid esters; they completely inhibited the adhesion at 0.005%. The C14 ester completely inhibited the adhesion at 0.01% and C12 and C10 at 0.05%. Adhesion inhibition effects increased with increase of the fatty acid chain length and concentration of fatty acid esters tested and only a slight decrease in viable counts was found. MFE of C8 ester showed adhesion inhibition at 0.05%, reducing the adhesion by around 70%. But, adhesion inhibition of MFE was more effective in the fatty acid ester with fatty acid chain length more than 10. They inhibited the adhesion by around 80%, or even more, at 0.005%. MFE of C10, C12, and C14 fatty acids showed a noticeable antibacterial activity, reducing the variable bacterial count by around 4 log, but the C16 and C18 esters showed no antibacterial activity in even at the highest concentration tested. GY and MP almost completely inhibited the adhe-
sion at 0.1% and 0.01%, respectively. PT and PL inhibited adhesion by more than 60% at 0.01%, and, at 1.0%, PT almost completely inhibited the adhesion and PL more than 80%. WP also inhibited adhesion by more than 80% at 0.025% and 0.25%.

6. Comparison of adhesion inhibitory effects of selected food additives

Table 1 shows the concentration of additives that inhibited the adhesion of each bacterium more than 50% of that of the control at least at one of the concentrations tested. Among the 6 SFE tested, five, C10 to C18, showed adhesion inhibition of more than 50% against S. Typhimurium and S. aureus; four, C10, C12, C16 and C18, against L. monocytogenes; and, C10 to C16, against P. aeruginosa; and three, C14 to C18, against P. fluorescens. Among the 6 MFE tested, all showed effectiveness of more than 50% inhibition against S. aureus; five, C8, C10, C12, C16 and C18, against S. Typhimurium, and C10 to C18, against L. monocytogenes. But, any of the MFE did not show inhibition activity more than 50% against the two Pseudomonas species strains tested. Of the other additives tested, all showed inhibition activity more than 50% against S. Typhimurium and S. aureus; GY, MP, PT and PL against P. fluorescens; PT, PL and WP against P. aeruginosa; and MP, PT and PL against L. monocytogenes.

Discussion

It is known that the fatty acid ester has antibacterial activity[13]. In this experiment, SFE did not show strong antibacterial activity against bacteria tested, and with the exception of C10 fatty acid ester against L. monocytogenes, which induced decrease around 4 log, only up to 2 log decrease was found at the highest concentration tested. In the case of MFE that showed adhesion inhibition, noticeable decrease on the bacterial counts was observed only with C10, C12 and C14 esters against S. aureus at 0.01% and above. However, these esters showed adhesion inhibition around 80%, or more, at 0.005% without decrease of the bacterial counts, and, the decrease was around 4 log even at the highest concentration tested (Fig. 5). Thus, it seems that adhesion inhibition effect of SFE, with exception of C10 against L. monocytogenes, which the relationship between the antimicrobial activity and adhesion inhibition is not clear, and of MFE did not depend on the antibacterial activity of the substance.

The adhesion inhibition effects increased with increase in the length of fatty acid chain in SFE against S. Typhimurium, P. fluorescens and S. aureus, and in MFE against L. monocytogenes and S. aureus (Figs. 1–5). The adhesion inhibition activity of SFE and MFE seems to be attributed to the increase in the hydrophobicity of the esters[6]. The HLB (Hydrophile–Lipophile Balance), which expresses hydrophobicity and hydrophilicity balance, is about 19 for SFE and 4–7 for MFE[6]. This high HLB might be one of the reasons why the adhesion inhibition effect of SFE was stronger than that of MFE. Both SFE and MFE showed strong adhesion inhibition effects on S. aureus (Fig. 5), but weakly on L. monocytogenes (Fig. 4) in Gram-positive bacteria tested. Further study is required to reveal the reason of this difference of adhesion inhibition in Gram-positive bacteria.

MFE, GY, and MP showed no inhibitory effects on the adhesion of P. aeruginosa. In P. aeruginosa, it has been shown that extracellular EPS matrix and glucan of the cell envelope inhibit the invasion of chemicals from the surrounding environment[4, 8, 22], and low metabolic activity of the stationary phase cell contributes to the resistance to chemicals and adhesion inhibitor[7, 17, 23]. This seems one of the reasons that adhesion of P. aeruginosa was not effectively inhibited by the additives tested in this study (Fig. 2). The reason why the adhesion of P. aeruginosa was promoted by SFE at 0.005% and MFE is not known. The elucidation of mechanism for the adhesion promotion by these substances seems also important to develop effective method to inhibit adhesion of P. aeruginosa.

PT is a basic protein including 21 residues of arginine among 31 constitution amino acid residues[12]. PL is also the basic protein[14]. Therefore, they seem to bind strongly to the negatively charged bacterial cell surface with electrostatic interaction and the adhesion of the cell to the microtiter plate has been inhibited[8, 15]. It is known that the antibacterial activity of PL increase with increase in the number of lysine residue[15]. Although both proteins are basic
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Concentration caused more than 50% adhesion inhibition (%)</th>
<th>Gardenia yellow</th>
<th>Monascus pigment</th>
<th>Protamine</th>
<th>Polylysine</th>
<th>Whey protein</th>
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<tr>
<td></td>
<td>Sucrose fatty acid ester</td>
<td>Monoglycerin fatty acid ester</td>
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<td></td>
<td>C8</td>
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<td>C18</td>
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<tr>
<td>S. Typhimurium</td>
<td>&gt;0.05*</td>
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<td>P. aeruginosa</td>
<td>&gt;0.05*</td>
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<td>0.01</td>
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<td>0.05</td>
<td>&gt;0.05*</td>
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<tr>
<td>P. fluorescens</td>
<td>&gt;0.05*</td>
<td>0.05</td>
<td>0.05</td>
<td>&gt;0.05*</td>
<td>0.05</td>
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<td>L. monocytogenes</td>
<td>&gt;0.05*</td>
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<tr>
<td>S. aureus</td>
<td>&gt;0.05*</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
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*: Adhesion was not inhibited by the additive more than 50% at the highest concentration tested.
proteins, the mechanisms for adhesion inhibition and for interaction with surface of bacteria and microtiter plate well seem to be slightly different each other. It is interesting that adhesion inhibition activity and antibacterial activity were different in PT and PL against different bacterial species among Gram-negative bacteria. It seems necessary to examine the difference of electric charge and hydrophobicity between these bacterial cell surfaces to elucidate the species specificity in the adhesion inhibition.

GY has polyene structure specific to carotenoid. A polyene compound has been reported to inhibit binding of S. Enteritidis cell to a microtiter plate and collagen. In addition, GY also has disaccharide, gentiobiose that glucose was β-1,6-linked, and is hydrophilic. According to the structure, it is presumed that GY has high HLB value, and seems to bind to the surfaces of both bacterial cell and microtiter plate well, resulting in the inhibition of electrostatic and hydrophobic interactions between bacterial cells and a microtiter plate well in S. Typhimurium, P. fluorescens, and S. aureus. GY did not inhibit adhesion of P. aeruginosa (Fig. 2) and L. monocytogenes (Fig. 4) to microtiter plate. To clarify the reason for the no effects of GY on adhesion in P. aeruginosa and L. monocytogenes, further experiments showing the amount of binding of these substances onto both of the surface of these bacteria and microtiter plate well are required.

MP has both hydrophilic ketone group and hydrophobic fatty acid chain with C5 or C7. It has been shown that it strongly inhibited adhesion of S. Enteritidis to microtiter plate in previous study using various natural pigments and food additives. In this study, it inhibited the adhesion of S. Typhimurium, L. monocytogenes, and S. aureus to microtiter plate (Fig. 1, 4, and 5) to less than 50% of the control at 0.01, 0.1, and 0.01%, respectively. Amphiphilic property of MP seems important for adhesion inhibition. However, MP did not inhibit the adhesion of P. aeruginosa even at 0.25%. Further detailed studies are required to elucidate the reason for the no effects of MP on P. aeruginosa.

WP did not decrease viable counts of all the bacteria tested at the highest concentration, 0.25%, but it strongly inhibited adhesion of S. Typhimurium, P. aeruginosa, and S. aureus at 0.025% (Figs. 1, 2, and 5). It has been reported that the WP used in this experiment includes a natural immunity antibody abundantly. Sun et al. have reported that an antibody for AAP, which is protein related to adhesion of Staphylococcus epidermidis, inhibited adhesion of S. epidermidis. Therefore, it seems that the antibodies in the whey protein bound to adhesion factors including flagella and pili to inhibit their function resulting in adhesion inhibition of bacterial cells. However, because these antibodies were included in only a very small amount, the other ingredients of whey protein, such as β-lactoglobulin and α-lactalbumin also seem to be involved in an adhesion inhibition. These antibodies and other factors in WP seem to have no effects on adhesion of P. fluorescens and L. monocytogenes. WP inhibited the adhesion of P. aeruginosa by 75% at 0.025 but not at 0.25%. To reveal the reason for the decrease of adhesion inhibition activity of WP at 0.25%, further investigation using detailed range of concentration is necessary.

The results obtained in this study suggest that the treatment of the surface of the food processing environments, addition of these adhesion inhibitors to rinse water of food and feed-water in hydroponic culture, and spray of the inhibitors onto fresh produce would effectively decrease the secondary bacterial contamination or increase the efficacy of subsequent decontamination treatment of the secondary contaminated bacteria by both rinsing with water and treatment with sterilizers. Detailed mechanism for adhesion inhibition by each additive is under investigation and will be reported in the near future.

Acknowledgments

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Research project for ensuring food safety from farm to table DI-7201). The authors are grateful to Mr. Takashi Kaji, Mitsubishi Chemical Corp. Foods Co., Ltd., and Dr. Toyoki Sato, Asama Chemical Co., Ltd. for their kind supply of sucrose fatty acid esters, and prolamine and whey protein, respectively.
References


