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Inhibitory effects of peptide-bound polysaccharides on lipid oxidation in emulsions

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Abstract

The inhibitory effects of polysaccharides on lipid oxidation in emulsions were investigated. Methyl linoleate was emulsified by β-casein or surfactants to prepare emulsions, and the oxidation was induced by an azo-compound or $FeSO_4$ addition. The initial oxidation was followed by measuring the oxygen consumption in the emulsions for 30 min, and the extent of the lipid oxidation over a long time (~48 h) was evaluated by the determination of the unoxidized lipid by gas chromatography and the thiobarbituric acid test. Peptide-bound polysaccharides, such as gum arabic and soluble soybean polysaccharides (SSPS), showed an ability to inhibit lipid oxidation, whereas maltodextrin and pullulan exhibited no inhibitory effects. Especially, SSPS almost perfectly suppressed the lipid oxidation from the initial to the late stages, irrespective of the emulsifiers and oxidation-inducing reagents. The mechanism whereby the peptide-bound polysaccharides inhibited the lipid oxidation in the emulsions is considered in terms of the chemical composition and other properties of the polysaccharides.

Keywords: Peptide-bound polysaccharides; Gum arabic; Soluble soybean polysaccharide; Lipid oxidation in emulsions; Antioxidative activity

1. Introduction

Polysaccharides play important roles as thickening, stabilizing and gelling agents in many foods. Also, for emulsion systems, polysaccharides are very often used to improve the emulsion stability and textural properties (Dickinson, 1998; Robins, 1991). In addition to such ordinary applications relating to physical properties, a new aspect of polysaccharide role in emulsion systems, i.e. the inhibitory effects of polysaccharides on lipid oxidation, was pointed out by Shimada, Fujikawa, Yahara, and Nakamura (1992). The antioxidative activity of polysaccharides seems to be mainly due to the viscosity increase in the continuous phase of an emulsion, leading to a reduction in the oxygen diffusion rate and oil droplet collision probability (Shimada, Okada, Matsuo, & Yoshioka, 1996). It was also suggested that their chelating ability contributed to the antioxidative activity of polysaccharides (Shimada et al., 1992, 1996).

Normally, natural and synthesized antioxidants are added to food emulsions to protect lipids from oxidation. Most antioxidants are low-molecular weight compounds with a strong radical-scavenging activity (Halliwell, Murcia, Chirico, & Aruoma, 1995). The proper formulation or use of such antioxidants in foods is sometimes difficult because the antioxidants also act as prooxidants under some conditions (Porter, 1993). Furthermore, it is possible that an excess intake of oil-soluble antioxidants disorders physiological conditions due to the easy accumulation of such antioxidants in biomembranes (Hayashi, Morimoto, Miyata, & Sato, 1993). Therefore, the use of hydrocolloids, such as polysaccharides and proteins with mild antioxidative activity, is desirable to produce safe food products including lipids.

In a previous paper (Matsumura, Satake, Egami, & Mori, 2000), we found that gum arabic (GA) had an

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inhibitory effect on lipid oxidation in an emulsion induced by the addition of an azo-compound or soybean lipoxygenase, whereas pullulan (PL) and maltodextrin (MD) showed no such inhibitory effects. In this case, the degree of lipid oxidation was determined by measuring the oxygen consumption in the emulsion for a short period (approximately 20 min), i.e. the initial stage. It remains unclear whether the propagation process in lipid oxidation and the degradation of lipid peroxides in the late stage are affected by the presence of the polysaccharides or not. Therefore, in the present study, the effects of the polysaccharides on lipid oxidation were examined for longer periods (48 h), using gas chromatography and the thiobarbituric acid (TBA) method.

GA is an arabinogalactan-protein (Williams & Phillips, 2000), whereas PL and MD have no covalently attached peptide moieties. It is possible that the inhibitory effects of GA on lipid oxidation originate from its peptide moieties. Recently, a water-soluble polysaccharide was extracted from soybean and called soluble soybean polysaccharide (SSPS). SSPS has various functions, such as dispersion, stabilization, emusification, and adhesion (Maeda, 2000). SSPS includes peptides which are supposed to be covalently attached to the rhamnogalacturonan main back-bone. Since SSPS has similar characteristics to GA, such as the presence of covalently-bound peptides and good emulsifying activity, effects on lipid oxidation can also be expected. Therefore, we compared the antioxidative activity of SSPS with that of GA in the present study.

2. Materials and methods

2.1. Materials

β-Casein was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The sugar ester (S-1670, HLB = 16) was obtained from Mitsubishi Kagaku Co., Ltd. (Tokyo, Japan). PL (PI-20) was obtained from the Hayashibara Co., Ltd (Okayama, Japan). MD, with dextrose equivalent of 2–5, was purchased from Matsutani Chemical Industries (Osaka, Japan). GA (Vistop D-2041) was a kind gift by San-ei Chemical Industries (Osaka, Japan). SSPS (Soya Fibe SY-4024) was supplied by Fuji Oil Co., Ltd. (Osaka, Japan). Pectin from citrus was obtained from Nakarai Tesque Co., Ltd. (Kyoto, Japan). Methyl linoleate, methyl laurate, Tween 20 and the other chemicals of analytical grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Emulsion preparation

An oil-in-water emulsion was prepared from a 5 wt.% oil phase and 95 wt.% aqueous phase. The aqueous phase was β-casein solution (0.5 wt.% β-casein in 10 mM sodium phosphate buffer, pH 7.0) or surfactants solutions (2 wt.% sugar ester S-1670 or Tween 20 in 10 mM sodium phosphate buffer, pH 7.0). The oil phase was methyl linoleate for the oxygen electrode experiment and TBA test, whereas the mixture of methyl linoleate and methyl laurate (7:3) was used for the determination of the remaining methyl linoleate after oxidation by gas chromatography in which the methyl laurate was added as an internal standard. The oil and aqueous phases were mixed and homogenized for 3 min in a high-speed blender (Nichion Irikagakukiki Seisakusho, Tokyo, Japan) operated at 22,000 rpm. The average droplet diameter was further reduced using an ultrasonic homogenizer (Nihonseiki Kaisha, Tokyo, Japan) operated at maximum power for 2 min. A laser diffraction particle-size analyzer (Model LA-500, Horiba Saisakusho, Kyoto, Japan) was used to determine the droplet diameter distribution from which the specific surface area was derived.

2.3. Dilution of emulsions and initiation of lipid oxidation

The prepared oil-in-water emulsions described in the previous section were diluted by a phosphate buffer (10 mM sodium phosphate buffer, pH 7.0). For the oxygen electrode experiment, the final concentration of the oil phase was 1.0% (w/w). For the gas chromatography experiment and TBA test, the concentration of the oil phase in the diluted emulsion was 0.3% (w/w). When testing the inhibitory effects of the polysaccharides on lipid oxidation, the polysaccharides were solubilized in the phosphate buffer before dilution of the emulsions. The final concentration of the polysaccharide in the emulsion was 2.5 or 5%(w/w). The oxidation of methyl linoleate in the emulsion was initiated by adding 5 μM 2,2′-azobis (2-aminopropane)hydrochloride (AAPH) or 5 μM ferrous sulfate ($FeSO_4$) at 37 °C.

2.4. Oxygen electrode

The degree of lipid oxidation during the initial stage was monitored by measuring the oxygen consumption in the emulsion with a Clark-type oxygen electrode (Hansatech, D.W. Oxygen Electrode Unit, UK) at 37 °C. The lipid oxidation rate was calculated from the slope of the linear part of the reaction curve.

2.5. Gas chromatography

The emulsion was stored at 37 °C for 48 h after the initiation of lipid oxidation as described in Section 2.3. An aliquot of the emulsion (2 ml) was removed periodically, to which was added 6 ml of solvent (chloroform/methanol, 2:1). The mixture was shaken

vigorously and centrifuged for 10 min at 1.7×10^3 g. After removing the top layer, 3 ml of solvent (methanol/water, 1:1) was added to the bottom layer. The mixture was shaken vigorously and re-centrifuged for 10 min. The bottom layer was recovered and evaporated to dryness under a stream of nitrogen gas, followed by being dissolved in 1ml hexane. Its aliquot (1 μl) was directly injected into the column inlet of the Shimadzu GC-14B apparatus (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a hydrogen flame ionization detector. The analytical conditions were as follows: a glass column (0.32×210 cm) packed with Chromosorb W (60–80 mesh); temperature programme, 60–240 °C at 4 °C/min; injection temperature, 260 °C; carrier gas (N_2) at a flow rate of 60 ml/min; N_2 pressure, 6 kg/cm^2; H_2 pressure, 0.6 kg/cm^2; air pressure, 0.5 kg/cm^2. The oxidation degree was calculated, based on the ratio of the amounts of methyl linoleate and methyl laurate (internal standard).

2.6. TBA test

The TBA value was measured by the method of Buege and Aust (1978). An aliquot (0.5 ml) of emulsion after oxidation was mixed with 2.0 ml of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.04% butylated hydroxytoluene in 0.25 N HCl) and 0.5 ml sodium phosphate buffer (10 mM, pH 7.0). The mixture was heated at 95 °C for 15 min. After cooling, the mixture was centrifuged for 1.7×10^3 g. The absorbance of the supernatant was determined at 535 nm, using a Shimadzu UN-2400PC recording spectrometer (Shimadzu Co., Ltd., Kyoto, Japan). The results were expressed as the amounts of malondialdehyde (MDA) which were quantitated using the molecular absorbance of MDA, that is, 1.56×10^5.

2.7. Radical-scavenging ability

The radical-sacavenging ability of the polysaccharides was tested as follows: the polysaccharide (50 mg) was dissolved in 2 ml sodium phosphate buffer (10 mM, pH 7.0). To the polysaccharide solution, 2 ml ethanol and 1 ml 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (0.5 mM DPPH in ethanol) were added. The absorbance of the DPPH was measured at 517 nm using a Shimadzu UN-2400PC recording spectrometer (Shimadzu Co., Ltd., Kyoto, Japan) 30 min after the addition of the DPPH solution.

2.8. Viscosity measurements of emulsions

The viscosities of the oil-in-water emulsions (0.3% methyl linoleate, w/w) were determined at 37 °C at various shear rates in the presence or absence of the polysaccharides (5%, w/w), using a controlled-strain rheometer (Rheosol G-300; UBM, Kyoto, Japan).

2.9. Protein determination of polysaccharides

The protein contents of the polysaccharides were determined according to the method of Lowry, Rowebrough, Farr, and Randall (1951).

2.10. Amino acid analysis

The powdered samples of GA and SSPS were hydrolyzed with 6 N HCl containing 0.1% phenol in a sealed and evacuated tube at 110 °C for 24 h for analyses of the amino acids, except for cysteine and tryptophan. For the detection of cysteine, the samples were treated with a performic acid solution (formic acid/hydrogen peroxide, 9:1) at 0 °C for 16 h prior to the hydrolysis by 6 N HCl. The hydrolyzates were analyzed using an amino acid analyzer (L-8500A, Hitachi). The wavelength for the detection was 440 nm (for proline and hydroxyproline) and 570 nm (for the other amino acids). For the analysis of tryptophan, the powdered sample was hydrolyzed in 4.2 N barium hydroxide including 3% thiodiethylene glycol in a sealed tube at 110 °C for 12 h. The hydrolyzate was neutralized and analyzed by HPLC (Shimadzu LC-10AS) on a Inertsil ODS column (GL Science). Elution was carried out by a solution of 10 mM perchloric acid and methanol (92:8), and the eluent was monitored by measuring the fluorescence (excitation at 285 nm, emission at 348 nm).

3. Results

3.1. Chemical composition of GA and SSPS

Although both GA and SSPS are polysaccharides, which have covalently attached peptides, previous reports indicate different chemical compositions between the two polysaccharides (Williams & Phillips, 2000; Maeda, 2000). The data for the sugar composition of GA and SSPS were supplied by San-ei Chemical Industries (Osaka, Japan) and Fuji Oil Co. Ltd. (Osaka, Japan), respectively. GA included galactose (44% of total sugars), arabinose (27%), rhamnose (13%) and glucuronic acid (16%), which were consistent with the previous data (Williams & Phillips, 2000). The major sugar components of SSPS were also galactose (46%), arabinose (23%) and rhamnose (5%), but galacturonic acid (18%) was included instead of glucuronic acid. Other minor components such as fucose (3%), xylose (4%) and glucose (1%) were also found in SSPS.

The protein contents of GA and SSPS were 1.7 and 4.9%, respectively. The amino acid compositions of the polypeptides are shown in Table 1. GA included large amounts of hydroxyproline (24.7 mol.%) and serine (14.6 mol.%), corresponding to the previous reports (Akiyama, Eda, & Kato, 1984). On the other hand, a

Table 1
Amino acid composition of gum arabic and soluble soybean polysaccharide

Amino acid[a]	GA	SSPS
Hyp[b]	24.7	0.6
Asp	5.5	12.3
Thr	7.6	6.3
Ser	14.6	6.4
Glu	3.5	17.3
Pro[b]	7.7	6.3
Gly	6.4	8.6
Ala	2.8	9.4
Cys[c]	0.9	n.d.
Val	3.7	5.3
Met	0.2	0.9
Ile	1.4	3.0
Leu	8.2	5.0
Tyr	0.9	1.5
Phe	3.1	2.2
Lys	2.6	7.3
His	5.1	2.9
Arg	0.9	4.4
Trp[d]	0.2	0.3

[a] Amounts of amino acids are expressed as mol.%. All the amino acids except hydroxyproline, proline and tryptophan were determined by measuring absorbance at 570 nm with an amino acid analyzer.

[b] Hydroxyproline and proline were determined by measuring the absorbance at 440 nm with the amino acid analyzer.

[c] Cysteine was converted to cysteic acid by the treatment with performic acid prior to the hydrolysis with 6N HCl.

[d] Tryptophan was analyzed by HPLC with a fluorescence detector after the alkaline hydrolysis.

trace amount of hydroxyproline and a lower amount of serine were detected in the peptide component of SSPS. The major amino acids of SSPS were glutamic acid (17.3 mol%), aspartic acid (12.3 mol.%) and lysine (7.3 mol.%), although the contents of these three amino acids were low in GA. Therefore, it is obvious that the type of peptide component of SSPS is quite different from that of GA. For GA, it is well known that the *O*-galactosylhydroxyproline linkage acts as an attachment site of the carbohydrate chain and peptide (Cynthia Fong & Lamport, 1990). The lack of hydroxyproline suggests a different glycopeptide linkage style in SSPS. Studies of the overall peptide-saccharide arrangement, including the linkage site of SSPS, are now ongoing.

3.2. Effects of polysaccharide addition on oxygen consumption in emulsions

Fig. 1 shows the time-dependent oxygen consumption in the emulsions. The lipid oxidation was initialized by the addition of AAPH. For the β-casein-stabilized emulsion, the oxygen consumption started without a lag (pattern A), which enabled us to calculate the rate of oxygen consumption as the slope of the linear part of the increasing curve. When the β-casein-stabilized emulsion included 5% GA (pattern A′), the oxygen consumption was inhibited. The calculated relative rate of oxygen consumption in the presence of 5% GA was approximately 40% of that in the absence of the polysaccharide (Fig. 2). The effect of GA was concentration-dependent, that is, 2.5% GA only decreased the oxygen consumption rate to 58% of the control value without polysaccharides. SSPS was more effective in decreasing the oxygen consumption rate, decreasing the values to 15% and 9% of the control by the addition of 2.5 and 5% SSPS, respectively. On the other hand, MD and PL showed slight inhibitory effects, even if the addition was increased to 5%. The reproducibility of results of Fig. 2 was good, and the standard deviation of the value was within 6 (%) at most.

Fig. 1 also shows the results of the emulsions stabilized by surfactants (Tween 20 and sugar ester). The specific surface area of the surfactant-stabilized emulsions was slightly greater than that of the β-casein-stabilized emulsions, indicating the production of finer emulsions (data not shown). In the absence of the polysaccharides (patterns B and C), after the initialization by the addition of AAPH, a slower induction of oxygen consumption was observed compared with the pattern of the β-casein-stabilized emulsion. However, the sudden increase in oxygen consumption started after 7–10 min. After the onset of such a sudden increase, the slopes of the consumption curves were similar to the initial slope of the curve for β-casein-stabilized emulsion. The addition of 5% GA to the surfactants-stabilized emulsions inhibited the onset of the second induction of oxygen consumption, but did not affect the initial rate (patterns B′ and C′). In the presence of 5% SSPS, no increase in the oxygen consumption was observed over 30 min after the initiation (data not

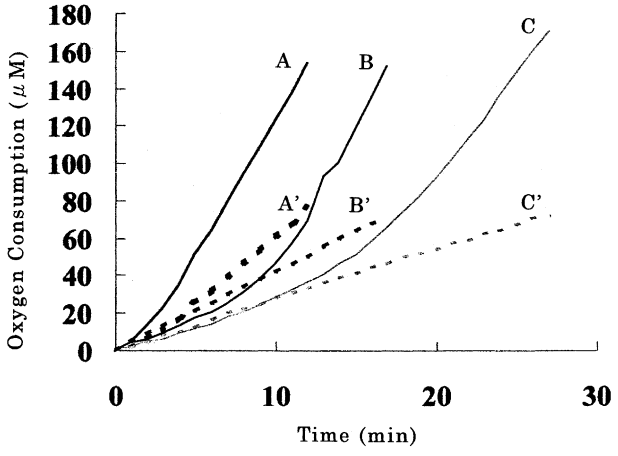

Fig. 1. Time-dependent oxygen consumption in emulsions in the presence or absence of GA. Methyl linoleate was emulsified by β-casein (A and A′), Tween 20 (B and B′) and sugar ester S-1670 (C and C′), respectively. Oxygen consumption was measured by oxygen electrode after the induction by AAPH at 0 min. Solid lines (A, B and C) and dotted lines (A′, B′ and C′) indicate the absence and presence of GA, respectively.

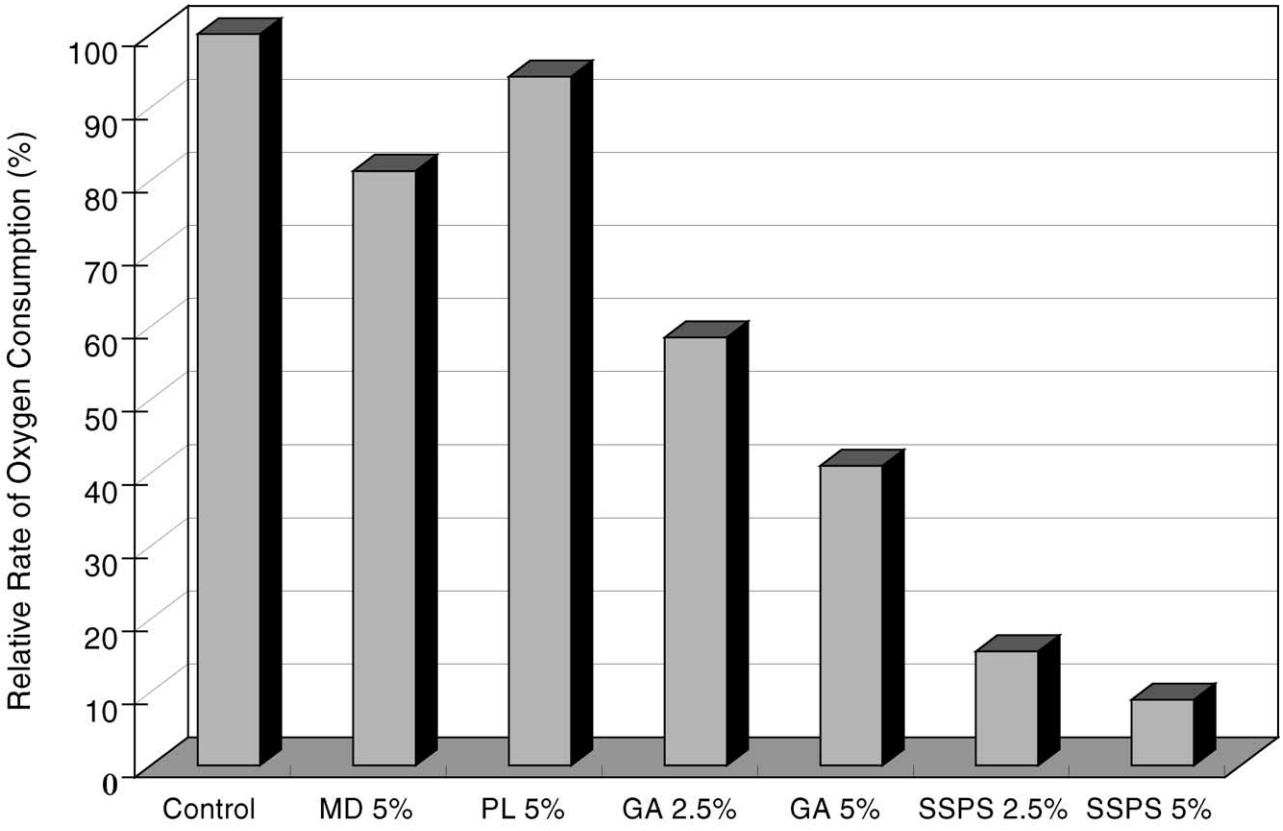

Fig. 2. Effects of polysaccharide addition on the relative rate of oxygen consumption in β-casein-stabilized emulsions. The rate of oxygen consumption in the β-casein-stabilized emulsion without polysaccharides (control) was calculated as the slope of the linear part of the increasing curve (A) in Fig. 1. The relative rates in the presence of polysaccharides are expressed as the percentage values of the control. The amounts of GA or SSPS addition were 2.5 and 5%, but that of MA or PL was only 5%. The numbers reported are the means of four measurements.

shown). From these results, the inhibitory effect of GA and SSPS, with respect to the lipid oxidation is clear, even in the emulsions stabilized by the surfactants. However, we gave up the idea that we could calculate the relative rate of oxygen consumption as in Fig. 2 for the surfactant-stabilized emulsions because of the ambiguity concerning the determination of the initial slope in the consumption curves.

In addition to AAPH, $FeSO_4$ was used to initiate the lipid oxidation. However, the increments of oxygen consumption were much slower than those induced by the initiation by AAPH (data not shown). As a result, for 30 min, it was difficult to find any significant difference in the rate with and without the polysaccharides.

3.3. Determination of unoxidized lipid in emulsions by gas chromatography

To evaluate the degree of lipid oxidation during longer periods, the amounts of unoxidized methyl linoleate were determined by gas chromatography. The results were expressed as the relative amounts of oxidized lipids, i.e. the percentage of oxidized lipid in the total lipid (Figs. 3–6). The reproducibility of the results of these figures was good, and the standard deviation of the value was within 8 (%) at most.

Fig. 3 shows the results of the oxidation induced by AAPH in β-casein-stabilized emulsions in the presence or absence of polysaccharides (5%). The amount of oxidized lipid steadily increased when no polysaccharides were included in the emulsion, finally reaching 72% after 48 h. MD and PL showed slight inhibitory effects on the oxidation (for instance, the result of MD for 12 h or that of PL for 48 h), but the oxidation level was still high. The addition of GA decreased the oxidation degree, and the final amount after 48 h (37%) was approximately half that in the absence of polysaccharides. SSPS suppressed the oxidation almost completely during storage.

Fig. 4 also shows the results of the oxidized lipid amounts in the β-casein-stabilized emulsions, but $FeSO_4$ was used to initiate the oxidation in this case. Comparing the results in the absence of polysaccharides of Fig. 4 with those of Fig. 3, the oxidation degrees were lower for 12 h and 24 h for the induction by $FeSO_4$. This may correspond to the slow oxidation induced by $FeSO_4$, as described in the previous section. However, there was no difference in the final value after 48 h between the

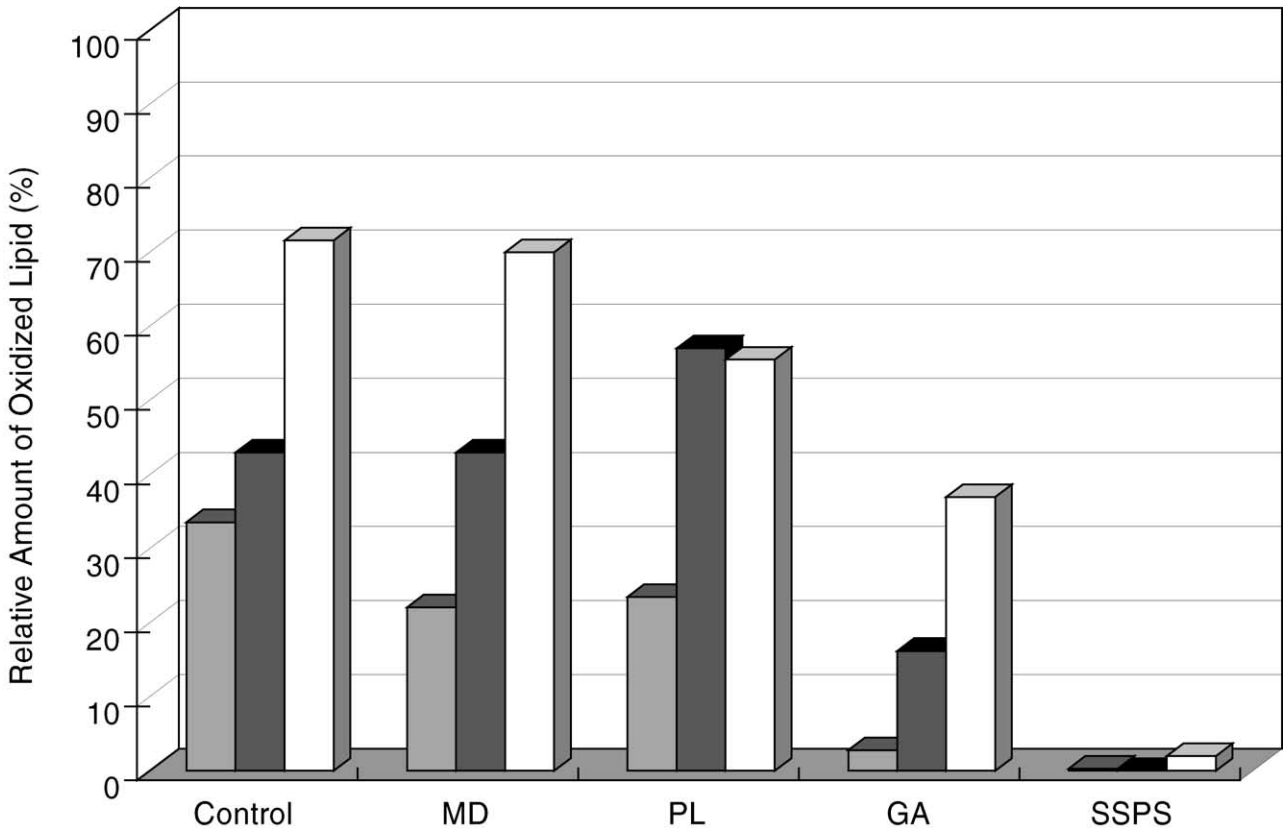

Fig. 3. Amounts of oxidized lipid by AAPH in β-casein-stabilized emulsions in the presence or absence of polysaccharides. AAPH was added to the emulsion to initiate the oxidation. Aliquots of the emulsion were removed after 12 h (▒), 24 h (■) and 48 h (□), and the amounts of unoxidized lipid were determined by using gas chromatography. The result is expressed as the relative amount of oxidized lipid, i.e., the percentage of oxidized lipid in the total lipid. Control means no addition of polysaccharides to the emulsion. MD, PL, GA and SSPS indicate the addition of these polysaccharides (5%) to the emulsion prior to the initiation of oxidation. The numbers reported are the means of three measurements.

AAPH and the $FeSO_4$ inductions. MD and PL seemed to enhance the oxidation for 12 h and 24 h in Fig. 4, but the oxidation level after 48 h was similar to that in the absence of polysaccharides. The inhibitory effects of GA on lipid oxidation were not observed during 24 h, but the oxidation level decreased to half that of the control after 48 h. SSPS showed the strongest inhibitory effects on lipid oxidation.

The results of Fig. 1 suggest a different mode in the lipid oxidation of β-casein-stabilized and surfactants-stabilized emulsions. Therefore, the degree of lipid oxidation was also determined for the surfactants-stabilized emulsions. The results for the oxidized lipid amounts in the sugar ester S-1670-stabilized emulsions are presented in Fig. 5. Similar results were also obtained for the Tween 20-stabilized emulsions (data not shown). Fig. 5A and B show the results of inductions by AAPH and $FeSO_4$, respectively. Comparing the results of Fig. 5A and B in the absence of polysaccharides, it is obvious that the oxidation degree was very low for the induction by $FeSO_4$.

For both the results in Fig. 5A and B, GA partially inhibited the lipid oxidation, whereas SSPS suppressed the oxidation almost completely as in the β casein-stabilized emulsions. However, MD and PL had no inhibitory effects on the lipid oxidation (data not shown).

Based on all the gas chromatography results, it was demonstrated that GA and SSPS had inhibitory effects on lipid oxidation in oil-in-water emulsions, while MD and PL had no or a very weak activity of inhibition. Especially, SSPS almost completely suppressed the lipid oxidation during storage for 48 h. The effects of SSPS were found to be dramatic in all the systems, irrespective of the emulsifiers (β-casein or the surfactants) and induction reagents (AAPH and $FeSO_4$).

3.4. Determination of TBA-reactive products

TBA tests were carried out to determine the breakdown of the lipid peroxidation in the emulsions. Figs. 6 and 7 show the results of the amounts of TBA-reactive products in the β-casein-stabilized emulsions in which the lipid oxidation was induced by AAPH and $FeSO_4$, respectively. The results are expressed as the amounts of MDA. The reproducibility of the results of TBA tests (Figs. 6–8) was good, and the standard deviation of the value was 5% at most.

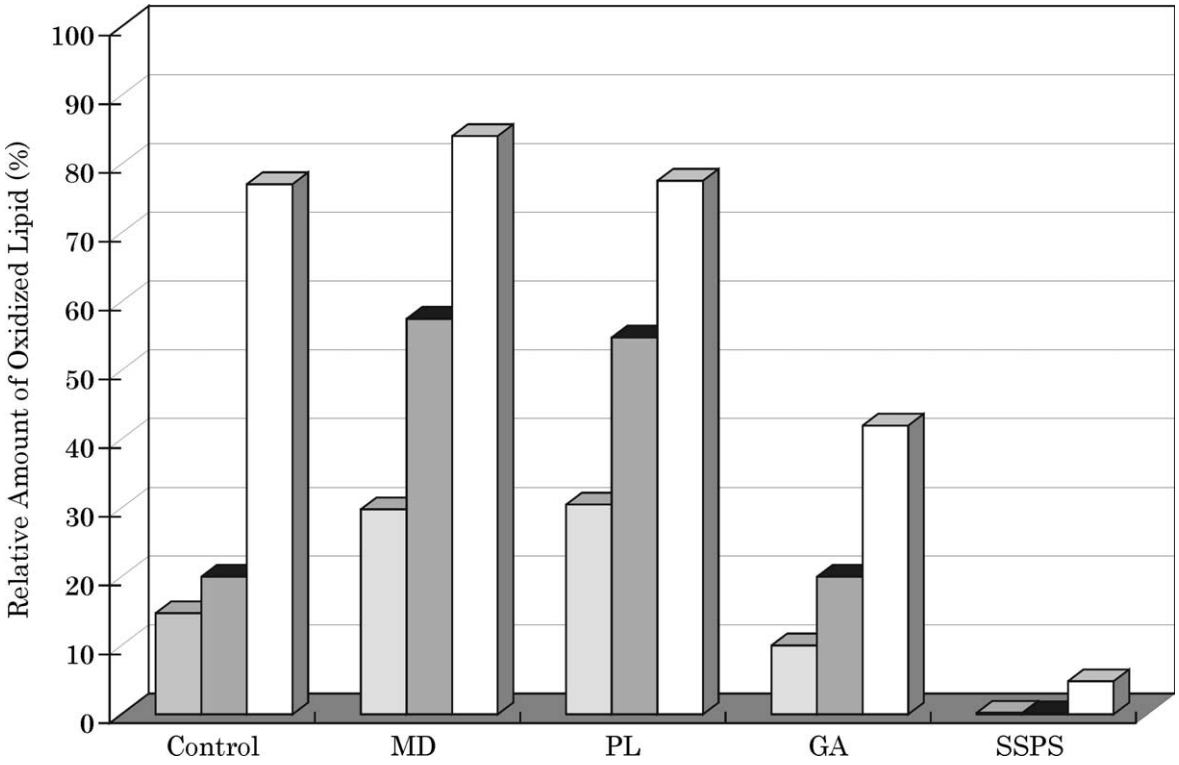

Fig. 4. Amounts of oxidized lipid by $FeSO_4$ in β-casein-stabilized emulsions in the presence or absence of polysaccharides. $FeSO_4$ was added to the emulsion to initiate the oxidation. Aliquots of the emulsion were removed after 12 (▒), 24 h (■) and 48 h (□), and the amounts of unoxidized lipid were determined using gas chromatography. The result is expressed as the relative amount of oxidized lipid, i.e., the percentage of oxidized lipid in the total lipid. Control means no addition of polysaccharides to the emulsion. MD, PL, GA and SSPS indicate the addition of these polysaccharides (5%) to the emulsion prior to the initiation of oxidation. The numbers reported are the means of three measurements.

When the oxidation was started by the addition of AAPH (Fig. 6), 21 μM and 37μM MDA were generated after 24 h and 48 h, respectively. MD and PL suppressed the generation of MDA; in particular a reduction of approx. 25% was detected for MD after 24h and 48h. Since no inhibition of lipid oxidation was observed by MD in the gas chromatography experiment (Fig. 3), it is possible that MD could inhibit the breakdown of the lipid peroxides. On the other hand, MD and PL enhanced the generation of MDA when the oxidation was induced by $FeSO_4$ (Fig. 7), corresponding to an enhanced lipid oxidation by MD and LA which was shown in a gas chromatography experiment (Fig. 4).

GA and SSPS demonstrated inhibitory effects with respect to the generation of TBA-reactive products in both the results of Fig. 6 and Fig. 7. However, the effects were more dramatic for the results of the AAPH induction case (Fig. 6); that is, GA reduced the production of MDA to approximately 10 μM after 48 h, and no TBA-reactive products were detected in the presence of SSPS. In contrast, 25 μM and 3 μM MDA were produced after 48 h, even in the presence of GA and SSPS, respectively, for the $FeSO_4$ induction (Fig. 7).

Fig. 8 shows the effects of GA and SSPS addition on the generation of TBA-reactive products in sugar ester S-1470-stabilized emulsions. Fig. 8A and B indicate the results of the AAPH and $FeSO_4$ induction systems, respectively. When the polysaccharides were not present in the emulsions, $FeSO_4$ caused the generation of more MDA (35 μM) after 48 h than AAPH (25 μM). GA reduced the amount of MDA in both systems, but the values in Fig. 8B (7 μM after 24 h and 23 μM after 48 h) were still higher than those of Fig. 8A (2 and 13 μM). These results are in contrast to those of Fig. 5A and B, in which AAPH induced the lipid oxidation to larger extents. The contradiction between the results of Fig. 5 and Fig. 8 suggests that the lipid peroxides in the sugar ester-stabilized emulsion were more unstable or breakable in the $FeSO_4$-catalyzed system. SSPS almost suppressed the generation of TBA-reactive products, irrespective of the induction by AAPH and $FeSO_4$. The production of the TBA-reactive products in the sugar ester-stabilized emulsions was not affected by the presence of MD and PL (data not shown).

All the results of the TBA tests are basically consistent with those of gas chromatography with respect to the inhibitory effects of the polysaccharides on the lipid oxidation process in the emulsions. Namely, MD and PL had no or a slight effect, while GA and SSPS suppressed the generation of breakdown products of the lipid peroxides. Confirming the results of gas chromatography, it was again demonstrated that SSPS was more effective than GA in suppressing the generation of

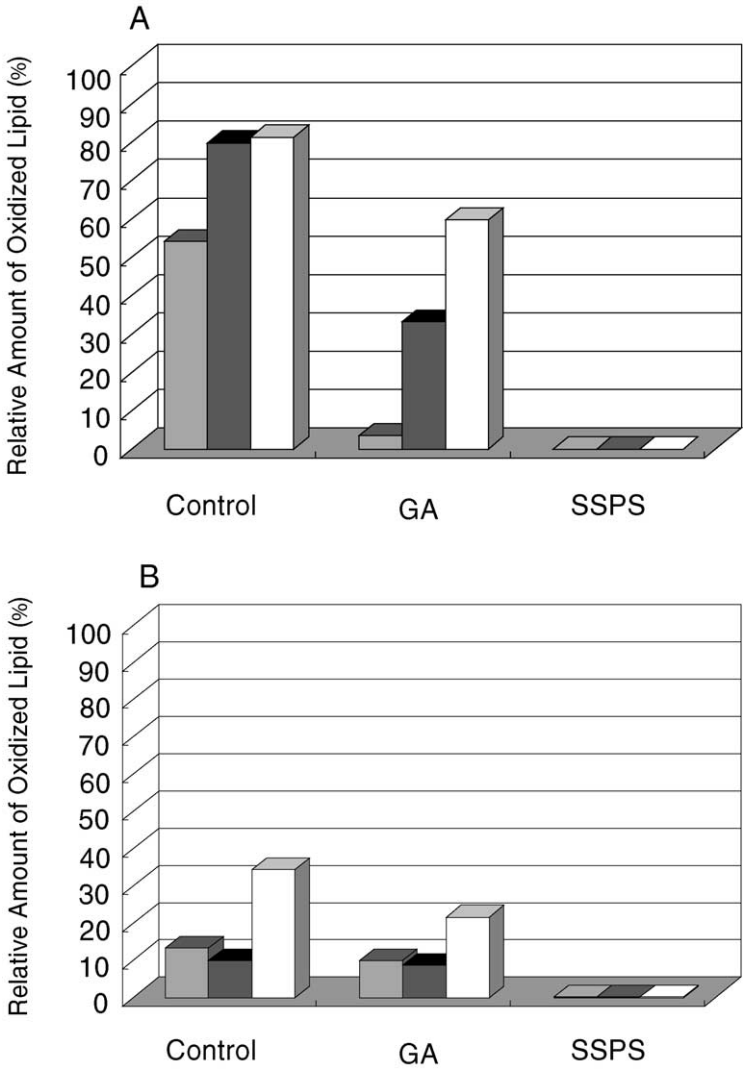

Fig. 5. Amounts of oxidized lipid in sugar ester (S-1670)-stabilized emulsions in the presence or absence of GA and SSPS. AAPH (A) and $FeSO_4$ (B) were added to the emulsion to initiate the oxidation. Aliquots of the emulsion were removed after 12 (▒), 24 h (■) and 48 h (□), and the amounts of unoxidized lipid were determined by gas chromatography. The result is expressed as the relative amount of oxidized lipid, i.e., the percentage of oxidized lipid in the total lipid. Control means no addition of polysaccharides to the emulsion. GA and SSPS indicate the addition of 5% of these polysaccharides prior to the initiation of oxidation. The numbers reported are the means of three measurements.

TBA-reactive products almost completely, despite the detection of small amounts of products in the β-casein-stabilized emulsions by adding $FeSO_4$ (Fig. 7).

3.5. Radical scavenging activity of polysaccharides

To test the activity of GA and SSPS as radical scavengers, the disappearance of the DPPH radical (stable and water-soluble radical) was followed for 30 min after the addition of these polysaccharides (Fig. 9). For comparison, the activities of MD, PL and pectin were also measured. It is known that pectin has the ability to react with free radicals (Gilbert, King, & Thomas, 1984; Uchida & Kawakishi, 1986). The reproducibility of the results was good, and the standard deviation of the value was 0.03 at most.

The absorbance values after 30 min were not affected by the MD and PL addition, indicating no radical scavenging ability of these polysaccharides. As compared to the control, a slight decrease in the absorbance was observed by the addition of GA. SSPS reduced the absorbance to half that in the absence of the polysaccharides, showing the ability to scavenge radicals. However, the scavenging activity of SSPS was lower than that of pectin, which caused a dramatic decrease (approximately 70%) in the absorbance of DPPH.

3.6. Viscosity of emulsions with polysaccharides

The effects of polysaccharide addition on the viscosity of the β-casein-stabilized emulsions were investigated. The measurements were done at various shear rates, and

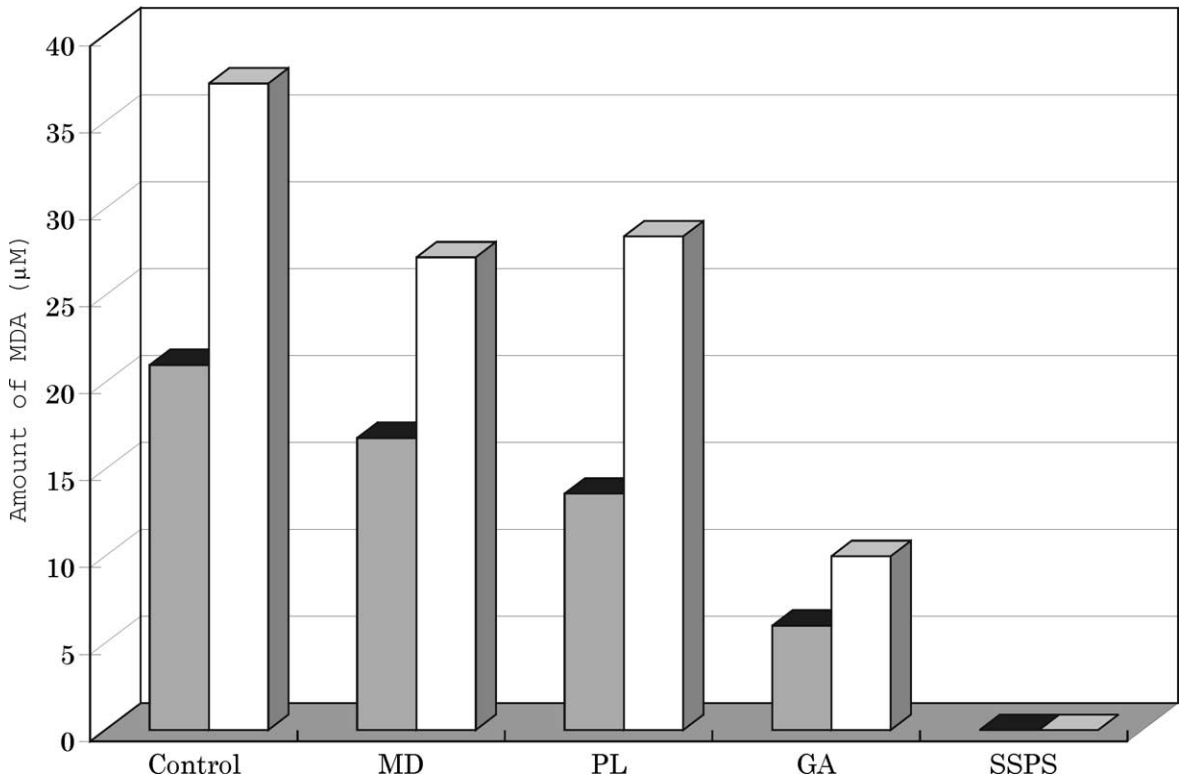

Fig. 6. Effects of polysaccharide addition on generation of TBA-reactive products induced by AAPH in β-casein-stabilized emulsions. AAPH was added to the emulsion to initiate the oxidation. Aliquots of the emulsion were removed after 24 h (■) and 48 h (□), and TBA-reactive products were determined. The result is expressed as the amount of MDA. Control means no addition of polysaccharides to the emulsion. MD, PL, GA and SSPS indicate the addition of these polysaccharides (5%) to the emulsion prior to the initiation of oxidation. The numbers reported are the means of three measurements.

the emulsions in the presence of the polysaccharides showed shear-thinning behaviour. Since it is thought that the experiments on lipid oxidation in the present study were carried out under medium shear rates, we compared the viscosity at 5 s^{-1}. The viscosity at this shear rate for the emulsion without the polysaccharide was 0.05 Pa·s. The addition of 5% PL increased the viscosity to 0.6 Pa·s, but no or only a slight increase in the viscosity was caused by the addition of the other polysaccharides, i.e. MD (0.06 Pa·s), GA (0.08 Pa·s), and SSPS (0.05 Pa·s). At low shear rates, the emulsions in the presence of the polysaccharides exhibited an increased viscosity, but a viscosity greater than 5 Pa·s was only attained by the PL addition. These results indicate that PL only functions as a thickening agent among the polysaccharides.

4. Discussion

In the present study, the inhibitory effects of four polysaccharides, i.e. MD, PL, GA and SSPS, on the lipid oxidation in emulsions were investigated. The polysaccharides are categorized into two groups according to the inclusion of proteinaceous components. GA and SSPS, which have covalently-attached peptide moieties, exhibited the ability to inhibit the oxidation of methyl linoleate in emulsions. Especially, SSPS suppressed the lipid oxidation almost completely from the initial stage (oxygen consumption) to the late stage (gas chromatographic and TBA experiments). The inhibition by SSPS was effective for the oxidation of the eicosapentaenoic acid ester as well as mehyl linoleate (unpublished data). However, MD and PL, polysaccharides without peptide moieties, showed little or no inhibition of lipid oxidation, although some reduction was observed for the generation of TBA-reactive product (Fig. 6).

The important question is whether the strong ability of SSPS to inhibit lipid oxidation is attributable to the SSPS molecule itself or to its contaminants. Since SSPS is extracted by hot water from ‘okara’, which is the residue after oil and protein extraction from soybean, it is possible that SSPS contains low molecular weight antioxidants, such as polyphenols (Furuta, Takahashi, Tobe, Kiwata, & Maeda, 1998). Therefore, extensive dialysis or washing by ethyl acetate was attempted to remove such antioxidative contaminants. However, these treatments could not alter the ability of SSPS to inhibit the lipid oxidation at all (data not shown). Therefore, at present, it seems that SSPS itself possesses the ability to inhibit the lipid oxidation in emulsions,

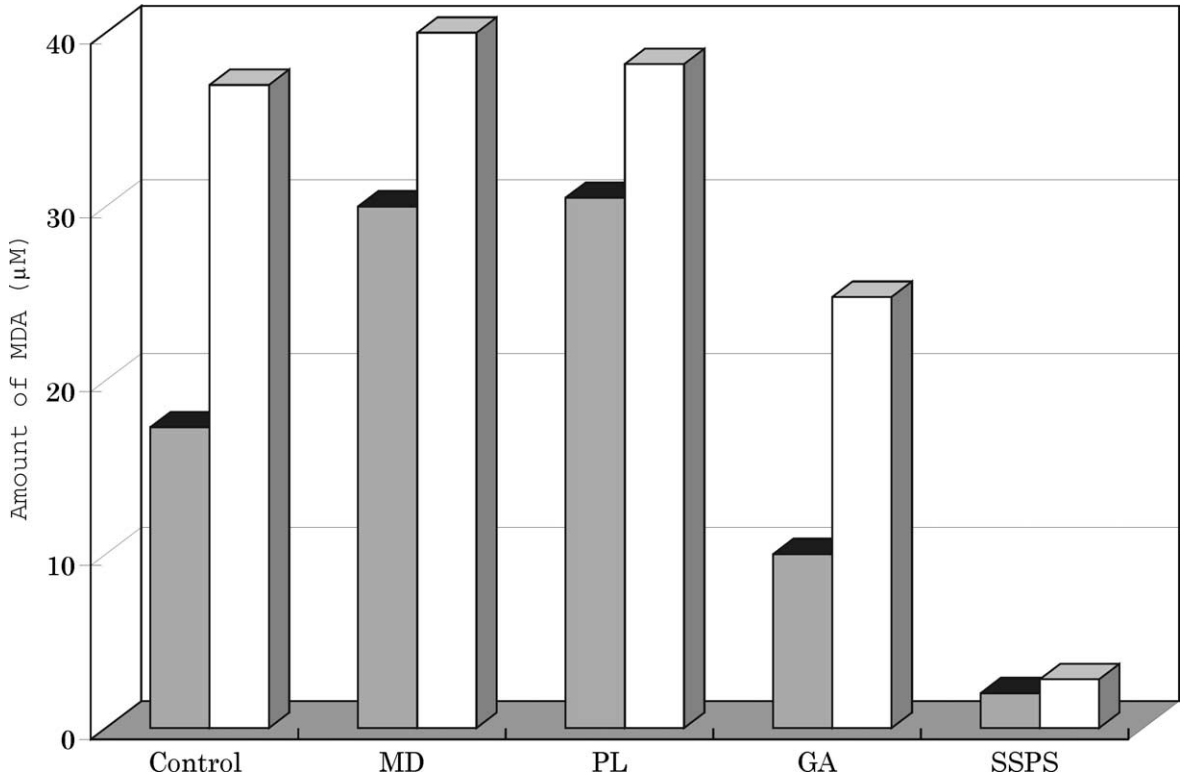

Fig. 7. Effects of polysaccharide addition on generation of TBA-reactive products induced by $FeSO_4$ in β-casein-stabilized emulsions. $FeSO_4$ was added to the emulsion to initiate the oxidation. Aliquots of the emulsion were removed after 24 h (■) and 48 h (□), and TBA-reactive products were determined. The result is expressed as the amount of MDA. Control means no addition of polysaccharides to the emulsion. MD, PL, GA and SSPS indicate the addition of these polysaccharides (5%) to the emulsion prior to the initiation of oxidation. The numbers reported are the means of three measurements.

although further analyses are needed on the potential antioxidants in SSPS.

Several experiments were done to understand the mechanism of the antioxidative activity of GA and SSPS. It has been suggested that polysaccharides reduce the oxidation rate by increasing the viscosity of the continuous phase in the emulsions (Shimada et al., 1996). The effects of the polysaccharide addition on the viscosity of the emulsions were also examined in the present study, in order to test the contribution of the viscosity increase to the inhibition of the lipid oxidation. The results showed that PL only could elevate the viscosity of the emulsion, whereas no dramatic increase in the viscosity was detected by the addition of the other polysaccharides, including GA and SSPS. Therefore, the ability of GA and SSPS, with respect to the inhibition of lipid oxidation in emulsions, cannot be attributed to the viscosity increases by these polysaccharides. GA and SSPS also did not exhibit a strong metal-chelating ability, which significantly decreases the possibility of metal-catalyzed lipid oxidation (data not shown).

Some macromolecules have the ability to scavenge or trap radicals, thereby inhibiting the lipid oxidation, although low molecular weight compounds are normally more effective for reaction with radicals. It was shown that SSPS had a significant radical scavenging activity, while no or little activity was detected for MD, PL and GA (Fig. 9). Pectin exhibited an excellent ability to scavenge the DPPH radical, being consistent with the previous results (Gilbert et al., 1984; Uchida & Kawakishi, 1986). The constituent sugars are common to SSPS and pectin, i.e. galacturonic acid, galactose, rhamnose, xylose, and arabinose, although the content of galacturonic acid in pectin is much higher (65%) than that of SSPS (18%) (May, 2000). It is postulated that the degradation of pectin by radicals occurs via breakage of the glucoside linkage among the galacturonic acids, in other words, radicals can be efficiently trapped by the galacturonic acids (Gilbert et al., 1984). This may be the reason for the high radical scavenging ability of pectin. Therefore, the radical scavenging ability of SSPS is also probably attributed to the presence of the galacturonic acids.

Although it is certain that the radical-scavenging activity contributes to the inhibitory effects of SSPS on lipid oxidation, the other mechanism may also occur. The first reason for this speculation is that pectin exhibited only a slight inhibitory effect on lipid oxidation in the same emulsion system (data not shown). Since the ability of pectin to scavenge radicals is higher than SSPS, as shown in Fig. 9, more or (at least) similar activity, with respect to the inhibition of the lipid oxi-

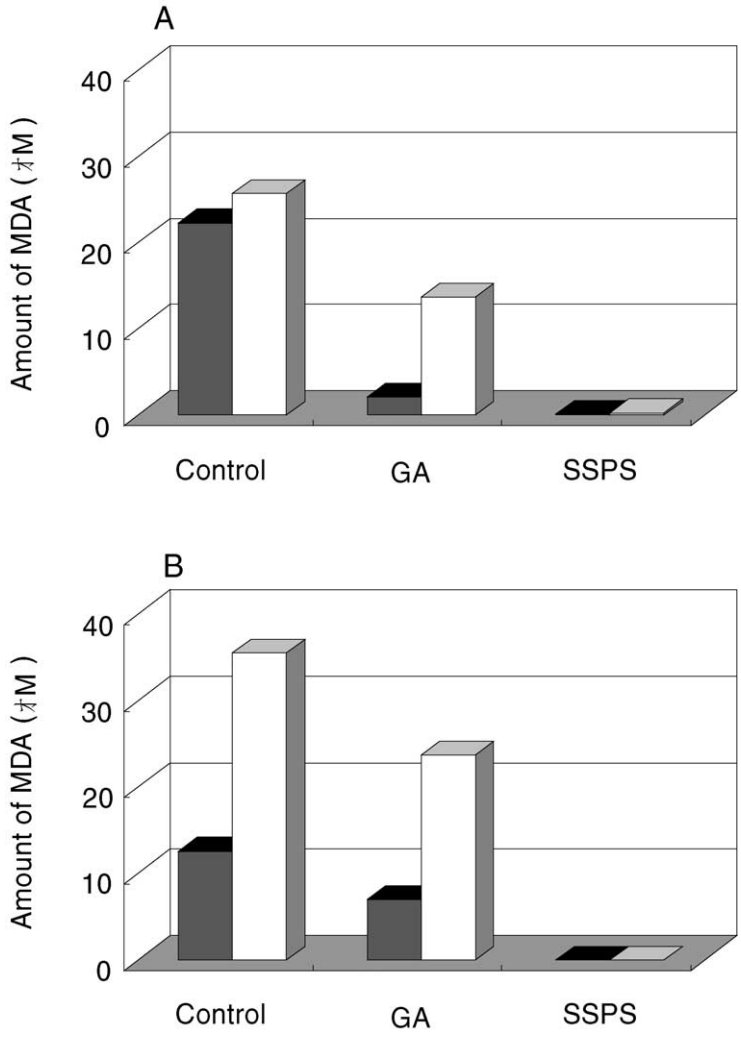

Fig. 8. Effects of GA and SSPS addition on generation of TBA-reactive products in sugar ester (S1670)-stabilized emulsions. AAPH (A) and $FeSO_4$ (B) were added to the emulsion to initiate the oxidation. Aliquots of the emulsion were taken out after 24 h (■) and 48 h (□), and TBA-reactive products were determined. The result is expressed as the amount of MDA. Control means no addition of polysaccharides to the emulsion. GA and SSPS indicate the addition of these polysaccharides (5%) to the emulsion prior to the initiation of oxidation. The numbers reported are the means of three measurements.

dation, can be expected for pectin if the radical-scavenging activity is the only crucial factor for the antioxidative activity of polysaccharides. The second reason is that GA, without a strong radical scavenging ability, induced reduction of the lipid oxidation by approximately 50%, as shown in the results from the oxygen consumption and gas chromatography experiments.

Considering the characteristics of the chemical compositions of GA and SSPS, it is reasonable to speculate that protein moieties make some contributions to their antioxidative activity. GA has a weak surface activity, but SSPS is more surface-active than GA. In particular, the reduction in the interfacial tension at the oil–water interface by SSPS was five-fold greater than that by GA when 0.01% solutions of both polysaccharides were used for interfacial tension experiments (unpublished data). Such surface activity is attributable to protein moieties, which adsorb and anchor GA (Randall, Phillips, & Williams, 1988) and SSPS (Maeda, 2000) molecules to the oil droplet surface. Since the emulsions were prepared from the oil and β-casein or the surfactant solutions before the addition of the polysaccharides in the present study, it is natural to think that GA and SSPS cannot easily cover the oil droplet surfaces. However, because of the excess amount of the polysaccharides (5%) as compared to those of oil (0.3%) and emulsifiers (β-casein, 0.03%; the surfactants, 0.12%) in our experimental conditions, GA and SSPS may access the parts of the oil droplet surface accumulation layer, which is not very thick with emulsifiers. Occasional displacements between the polysaccharides and the emulsifiers are also possible. As a result, it is likely that lipid molecules within the oil droplets can be protected from radical attack by GA and SSPS which

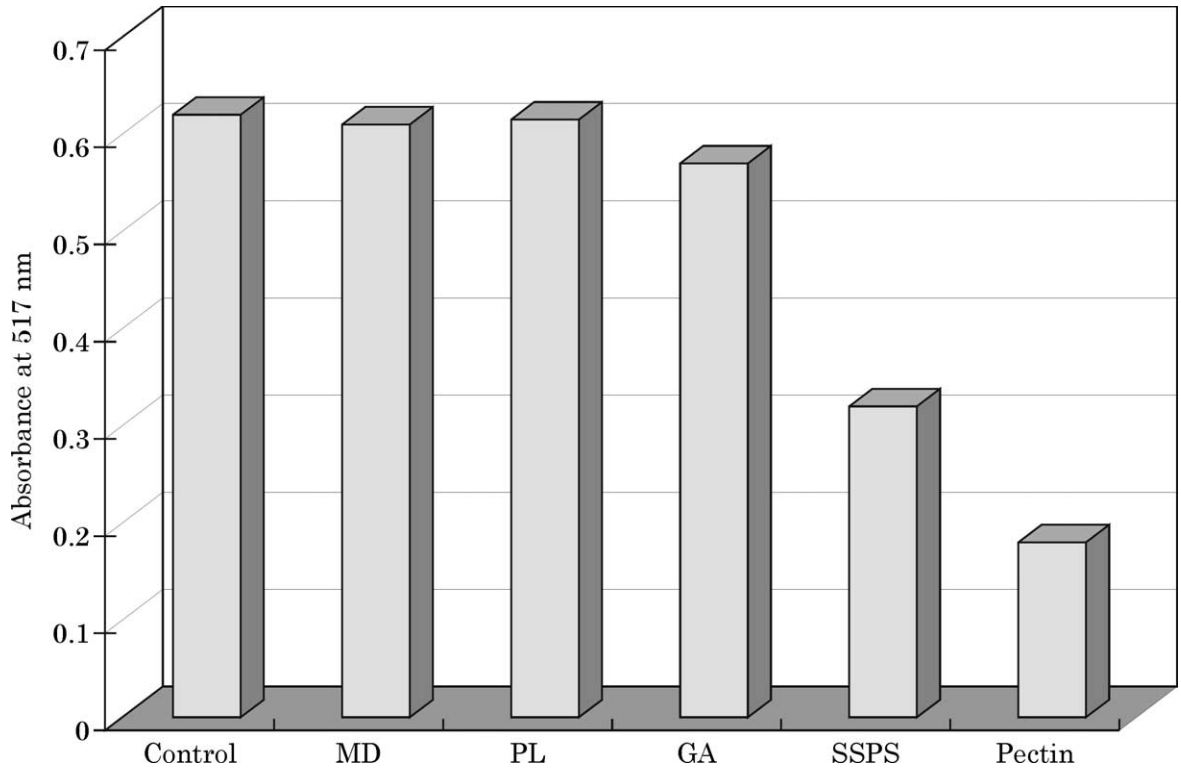

Fig. 9. Radical-scavenging activity of the polysaccharides. MD, PL, GA, SSPS and pectin were mixed with DPPH, and the absorbance at 517 nm was measured after 30 min. Control means the absorbance of DPPH in the absence of the polysaccharides. The numbers reported are the means of four measurements.

are in contact with the oil droplet surface. This protection may be mainly based on the physical mechanism, whereby the extending carbohydrate blocks, from the oil droplet surface into the aqueous phase, suffer radical attack. For SSPS, however, the chemical mechanism, that is, the radical scavenging by the reactive sugar component (galacturonic acid), should also function. The high activity of SSPS concerning the inhibitory effects on lipid oxidation may be due to such localization of the reactive sugar components on the droplet surfaces. On the other hand, perhaps, pectin without anchoring peptides could not efficiently trap free radicals near the oil droplet surface, thereby showing little antioxidative activity.

It remains unclear whether the peptide moieties of SSPS and GA also have radical- scavenging ability, thereby suppressing lipid oxidation in emulsions. Our preliminary experiments suggest that SSPS is not a naturally occurring material, but is formed by the manufacturing process, i.e. extraction from ''okara'' by hot water under weak acidic conditions. Despite the separation of the oil and protein fractions prior to the extraction of SSPS from okara, it is suggested that a small amount of soy isolate protein remains and is covalently attached to the carbohydrate chains via acid catalysis during the extraction process (unpublished data). The amino acid composition of SSPS in Table 1 is similar to that of soy proteins reported in the literature (Utsumi, Matsumura, & Mori, 1997), supporting this idea. There have been several reports about the antioxidative activity of soy protein or its hydrolyzate in emulsions (Chen, Muramoto, & Yamauchi, 1995; Hirose & Miyashita, 1999). Based on these findings, it is very likely that the peptide moieties of SSPS themselves have some ability to inhibit the lipid oxidation. On the other hand, the radical-scavenging activity of the peptide moieties of GA cannot be discussed at present because of the lack of data. To test the contribution of the peptide moieties of GA and SSPS, studies of the change in the antioxidative activity, by the protease treatments of the polysaccharides, are now ongoing.

As a conclusion, the peptide-bound polysaccharides, GA and SSPS, were found to have the ability to inhibit the lipid oxidation induced by AAPH and $FeSO_4$ in emulsions. Particularly, the activity of SSPS is excellent, almost perfectly suppressing the oxidation from the initial stage to the late stage. However, the lipid oxidation and its inhibition in emulsions are influenced by various conditions such as the combination of polysaccharides and emulsifiers (for instance, the difference in the results of Figs. 1–3), concentration and structure or composition of the lipids, the generation of free radicals, and the presence of metal ion. Further studies in model systems, which are more related to real emulsion foods, are needed to prove the actual antioxidative activity of SSPS and GA. Like GA, SSPS has potential

for use as an emulsifier, stabilizer or encapsulant for volatile flavours (Maeda, 2000). Because of the various functions, plus the new possible role as an antioxidant, SSPS should be used for the design of new, safe and good-quality emulsion products.

Acknowledgements

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