Enhancing Effect of $\beta$-Lactoglobulin on the Antioxidative Activity of $\alpha$-Tocopherol in an Emulsion of Linoleic Acid

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The antioxidative activity of $\alpha$-tocopherol and Trolox, a water-soluble carboxylic acid derivative of $\alpha$-tocopherol, in an emulsion stabilized with the hydrophilic emulsifier, polyoxyethylene (20) sorbitan monolaurate, was evaluated by measuring the changes in linoleic acid content, peroxide value, and thiobarbituric acid value. The activity of $\alpha$-tocopherol and Trolox to depress the antioxidation of linoleic acid was stronger when they were added in the aqueous phase than when added in the oil phase, and Trolox in the aqueous phase was the most effective. The results of interfacial-tension measurements support the idea that the affinity of phenolic antioxidants to the surface layer of oil droplets might be related to their antioxidative activity. The antioxidative activity of $\alpha$-tocopherol and Trolox added in the oil phase was markedly enhanced by using $\beta$-lactoglobulin together with Tween 20 to stabilize a linoleic acid emulsion. The results of a linoleic acid determination with cold and radioactive linoleic acid indicate that linoleic acid in a complex with the $\beta$-lactoglobulin molecule would be effectively protected by the phenolic antioxidants, $\alpha$-tocopherol and Trolox, against antioxidation.

Key words: $\alpha$-tocopherol; Trolox; linoleic acid emulsion; antioxidative activity; $\beta$-lactoglobulin

Food proteins and amino acids are known to be potent inhibitors of the antioxidation of lipids in different systems like a powder model,17-20 oil-in-water emulsion7-9 and solution (dispersion).12,13 Among the food proteins, milk casein,12,13 soy protein,10 bovine serum albumin,14 oil seed proteins,15 wheat gliadin,16 and maize zein40 have been shown to be effective antioxidants. The experimental results of the increased antioxidative activity of ovalbumin by heat treatment27 or by covalently binding with polysaccharides3 suggest that the surface properties of a protein are important for it to exhibit antioxidative activity in an emulsion. Whey proteins are of substantial and growing importance to the food industry. Among them, $\beta$-lactoglobulin is a major protein and is well known to have excellent surface and emulsifying properties.27-30 However, we have not found any results showing the effective antioxidative activity of this protein.

The activity of tocopherol phenolic antioxidants has been widely studied in different systems including bulk oil, emulsion, and micellar solution.27-32 Frankel et al. have recently suggested the importance of the interfacial properties of a phenolic antioxidant for its activity by showing that the activity of lipophilic $\alpha$-tocopherol or hydrophilic Trolox depended on the affinity of each toward bulk oil and oil droplets in an emulsion.30,32

Tocopherols are known to decrease free radicals like peroxyl and alkoxyl radicals by donating phenolic hydrogen.33-35 Hopia et al. have recently shown that Trolox also acts as a hydrogen donor to alkoxyl radicals and inhibits the accumulation of the secondary cleavage product, hexanal.30 The polar lipid radicals are easy to diffuse away from center of an oil phase into the more polar surface region of oil droplets in an oil-in-water emulsion. $\alpha$-Tocopherol and Trolox can be expected to be surface active from their molecular structure, and then to react with polar lipid radicals at the oil-water interface. The affinity of these phenolic antioxidants to the surface of oil droplets thus seems to be important for their activity in an emulsion.

In this present experiment, we studied the antioxidative activity of $\alpha$-tocopherol and Trolox added to the oil or water phase of a linoleic acid emulsion made from Tween 20, and the effects of whey protein $\beta$-lactoglobulin, which is known as a highly hydrophobic protein, on the activity of these phenolic antioxidants in a linoleic acid emulsion.

Materials and Methods

Materials. $\alpha$-Tocopherol and linoleic acid were purchased from Wako Pure Chemical Industries (Japan), having a purity of more than 98% and 99%, respectively. The purity of linoleic acid was confirmed by thin-layer chromatography. Trolox was purchased from Aldrich Chemical Co., and polyoxyethylene (20) sorbitan monolaurate (Tween 20), $\beta$-lactoglobulin and n-tetradecane were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [1-14C]Linoleic acid was purchased from American Radiolabeled Chemicals, and Soluene-350 tissue solubilizer and the scintillation cocktail were obtained from Packard Instrument Co.

Preparation of the linoleic acid emulsions. An oil-in-water emulsion stabilized with Tween 20 was prepared with 25 mg of linoleic acid and 10 ml of a 0.1 M sodium phosphate buffer (pH 7.0) containing 100 mg of Tween 20 by sonicating in ice-cold water for 5 min with a soni-
cator (Nissei US-50). To make an emulsion stabilized with \( \beta \)-lactoglobulin or a combination of \( \beta \)-lactoglobulin and Tween 20, \( \beta \)-lactoglobulin was added to the sodium phosphate buffer to make a final concentration of 0.2, 0.5 or 1.0 wt\%. \( \alpha \)-Tocopherol and Trolox (10\(^{-2}\) wt\%) were then mixed with linoleic acid before emulsifying, when the antioxidative effects were to be examined in the oil phase, or were added to the prepared emulsion when to be examined in the aqueous phase. These emulsions were degassed in ice-cold water for 15 min to remove any entrapped air bubbles. The emulsion stability was assessed by visually monitoring the distinct oil layers at the top of the stored emulsion samples.

**PV and TBA values.** An aliquot (4.0 ml) of the linoleic acid emulsion was transferred into a 15-mm (i.d.) test tube, loosely capped and incubated at 40\(^\circ\)C while continuously shaking. The extent of oxidation is expressed as the amount of hydroperoxide and thiobarbituric acid-reactive substances (TBARS). Accumulated hydroperoxide was measured by the ferric thiocyanate method,\(^{20}\) and the results are expressed as the peroxide value (PV) in mg of hydroperoxide per kg of oil. TBARS was measured spectrophotometrically\(^{28}\) and the results are expressed as the TBA value in mg of malondialdehyde (MDA) per kg of oil.

**Linoleic acid determination.** The emulsions were freeze-dried after adding 0.02 ml of 0.1% butyylated hydroxytoluene, and submitted to extraction with chloroform/methanol (2:1, v/v; C/M mixture). Linoleic acid was then determined by a gas-liquid chromatography (Hitachi G3000 instrument) after methyl-esterification with BF\(_3\) and methanol. A calibration curve was made with standard methyl linoleate.

**Determination of radioactive linoleic acid.** [1\(^{14}\)C] Linoleic acid diluted to a specific activity of 2.5 KBq/mmol was emulsified with Tween 20, \( \beta \)-lactoglobulin, or a combination of them as already described. After standing for 30 min in ice-cold water, the solvent extract at the bottom, cream in the middle, and aqueous extract of the top were separated by centrifuging at 16,000 \( \times \) g for 20 min after being mixed with the C/M mixture. The cream and aqueous extract were dissolved in Soluene-350, and the radioactivity was measured with a scintillation counter (Beckman LS 6500).

**Interface-tension measurement.** Interfacial tension at the oil-water interface was measured with a Wilhelmy plate surface tensiometer (Kyowa Interface Science Co., CBVP-A3) for 24 hr with a cell maintained at 25\(^\circ\)C. n-Tetradeacne and a bis-tris buffer (0.002 M, pH 7.0) were used for the oil and aqueous phases, respectively. \( \alpha \)-Tocopherol and Trolox were respectively dissolved in the oil and aqueous phases at a concentration of 10\(^{-2}\) wt\%.

**Results and Discussion**

**Oxidative stability of linoleic acid emulsified with Tween 20**

PV and the TBA value of linoleic acid in an emulsion at day 0 were both about 0. In an emulsion with Tween 20 hydrophilic emulsifier, the accumulation of hydroperoxide was weakly depressed by the addition of \( \alpha \)-tocopherol in the oil or aqueous phase (Table 1). Trolox was more effective than \( \alpha \)-tocopherol in depressing PV. The effects of \( \alpha \)-tocopherol and Trolox were stronger when they were added in the aqueous phase than in the oil phase. While the effects of these antioxidants on the TBA value showed a similar trend to that of PV, the measured values were much lower than those of PV.

The changes in linoleic acid content are shown in Table 2. More linoleic acid was detected in the emulsion with added \( \alpha \)-tocopherol or Trolox than in the emulsion without any antioxidant throughout the experimental 5 days. The effects of these antioxidants were stronger when they were added to the aqueous phase than to the oil phase, and the addition of Trolox to the aqueous phase was the most effective. Both the detected linoleic acid and the amount of hydroperoxide calculated from PV suggest that most of the decreased linoleic acid was recovered as hydroperoxide.

The foregoing results show that, in a Tween 20-stabilized emulsion, the activity of \( \alpha \)-tocopherol and Trolox to depress the autooxidation of linoleic acid was stronger when they were added to the aqueous phase than when added to the oil phase, and that adding Trolox to the aqueous phase was the most effective.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>PV</th>
<th>TBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>2 day</td>
</tr>
<tr>
<td>Not added</td>
<td>144</td>
<td>1199</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol (in oil)</td>
<td>89.0</td>
<td>1026</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol (in water)</td>
<td>102</td>
<td>700</td>
</tr>
<tr>
<td>Trolox (in oil)</td>
<td>7.1</td>
<td>116</td>
</tr>
<tr>
<td>Trolox (in water)</td>
<td>4.6</td>
<td>108</td>
</tr>
</tbody>
</table>

A linoleic acid emulsion made with Tween 20 (0.25 wt% of oil, 1.0 wt% of emulsifier) was prepared with 10\(^{-2}\) wt% of \( \alpha \)-tocopherol or Trolox dissolved in the oil phase before emulsification or in the water phase after emulsification.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Content of Emulsion Made with Tween 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 day</td>
</tr>
<tr>
<td>Not added</td>
<td>65.1</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol (in oil)</td>
<td>82.2</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol (in water)</td>
<td>94.7</td>
</tr>
<tr>
<td>Trolox (in oil)</td>
<td>104</td>
</tr>
<tr>
<td>Trolox (in water)</td>
<td>102</td>
</tr>
</tbody>
</table>

Emulsions were prepared with the same composition as those in Table 1. The changes in linoleic acid content are expressed as a percentage of the initial content before incubation.
Interfacial tension of α-tocopherol and Trolox

α-Tocopherol and Trolox can be expected to be surface active because they have both hydrophilic and hydrophobic groups in their molecules. By measuring the time-dependent change in interfacial tension, we could show that both α-tocopherol and Trolox in the oil or water phase had surface activity (Figure). The activity was higher when they were dissolved in the water phase than in the oil phase, and the activity of Trolox in the water phase was the highest.

These results suggest that α-tocopherol and Trolox oriented more effectively from the water phase to the oil-water interface than from the oil phase, the most effective case being Trolox in the water phase. The tendency of these antioxidants to diffuse from the water phase into the oil-water interface might be related to their antioxidative activity when added to the water phase. At the oil-water interface, α-tocopherol and Trolox would react with polar lipid radicals which have diffused from the center of oil droplets in the emulsion.

Oxidative stability of linoleic acid emulsified with β-lactoglobulin or by a combination of β-lactoglobulin and Tween 20

Next, the antioxidative activity of α-tocopherol in an emulsion made with hydrophobic protein β-lactoglobulin was studied (Table 3). A stable emulsion could not be made with 0.2% β-lactoglobulin, and a distinct oil layer separated within 1 day. With 0.5% or 1.0% β-lactoglobulin, and with a combination of 0.2% β-lactoglobulin and 1.0% Tween 20, a stable emulsion was formed, and no phase separation was detected for 3 days.

Table 3 shows that, in the emulsions stabilized with β-lactoglobulin or with a combination of β-lactoglobulin and Tween 20, α-tocopherol added to the oil phase depressed PV and the TBA value effectively for 3 days. Trolox was much more effective than α-tocopherol in the emulsion made with a combination of β-lactoglobulin and Tween 20. These results show that the antioxidative activity of α-tocopherol and Trolox was markedly increased when oil droplets were covered with a sufficient amount of β-lactoglobulin or with a mixture of β-lactoglobulin and Tween 20.

Determination of linoleic acid content in an emulsion stabilized with β-lactoglobulin or with a combination of β-lactoglobulin and Tween 20

The anionic surfactant, sodium dodecyl sulfate (SDS), is well known to form a complex with globular proteins and is useful to solubilize membrane proteins. SDS was thus used for the determination of linoleic acid content in the emulsion made with a combination of Tween 20 and β-lactoglobulin. Linoleic acid extracted with the C/M mixture before and after heating with SDS is called the C/M extract and SDS extract, respectively. Table 4 shows that almost all the linoleic acid was in the C/M extract of the emulsion made with Tween 20 or β-lactoglobulin, while only 40% of the linoleic acid was in the C/M extract of the emulsion made with a combination of Tween 20 and β-lactoglobulin.

Table 4 also shows changes in the linoleic acid content by incubating for 3 days. Total linoleic acid was decreased to about 50% in the emulsion without any antioxidant, and 62% was recovered in the Tween 20-stabilized emulsion with α-Tocopherol. In contrast, more than 90% of linoleic acid was detected by the addition of Trolox or α-tocopherol to the emulsion made with a combination of Tween 20 and β-lactoglobulin. In particular, linoleic acid extracted with SDS was effectively maintained, suggesting that these antioxidants would be effective for protecting β-lactoglobulin in a complex with linoleic acid against autoxidation.
Table 4. Effects of α-Tocopherol and Trolox on the Change in Linoleic Acid Content of an Emulsion Stabilized with Tween 20 and/or β-Lactoglobulin

<table>
<thead>
<tr>
<th>Emulsifier</th>
<th>Antioxidant</th>
<th>C/M extract (%)</th>
<th>SDS extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>Tween 20</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>β-lactoglobulin</td>
<td>—</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td>Tween 20+β-lactoglobulin</td>
<td>—</td>
<td>40.5</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>38.6</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>Tween 20+β-lactoglobulin</td>
<td>Trolox</td>
<td>38.9</td>
</tr>
<tr>
<td>After 3 days</td>
<td>Tween 20</td>
<td>—</td>
<td>54.3</td>
</tr>
<tr>
<td></td>
<td>Tween 20+β-lactoglobulin</td>
<td>—</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>62.4</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>Tween 20+β-lactoglobulin</td>
<td>α-Tocopherol</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>65.4</td>
<td>54.3</td>
</tr>
</tbody>
</table>

Emulsions were prepared with linoleic acid (0.25 wt%) and Tween 20 (1.0 wt%) and/or β-lactoglobulin (0.2 wt%). α-Tocopherol and Trolox were dissolved in linoleic acid at a concentration of 10−4 M. The linoleic acid content in each emulsion was measured by extracting with a C/M mixture with or without SDS treatment as described in the text, and is expressed as a percentage of the initial contents before incubation.

Table 5. Distribution of [1-14C]Linoleic Acid Extracted from an Emulsion Made with Tween 20 and/or β-Lactoglobulin

<table>
<thead>
<tr>
<th>Emulsifier</th>
<th>Distribution of [1-14C]linoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/M extract (%)</td>
</tr>
<tr>
<td>Tween 20</td>
<td>93.5</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>94.1</td>
</tr>
<tr>
<td>Tween 20+β-lactoglobulin</td>
<td>43.6</td>
</tr>
</tbody>
</table>

Linoleic acid containing [1-14C]linoleic acid in an emulsion (0.25 wt% oil) made with Tween 20 (1.0 wt%) and/or β-lactoglobulin (0.2 wt%) was extracted with a C/M mixture and is expressed as a percentage of the initial content.

TWEEN 20 or both of them. Although most of the linoleic acid in the emulsion made with Tween 20 or β-lactoglobulin was in the C/M extract, 54% of linoleic acid was found in the cream layer of the emulsion made with both Tween 20 and β-lactoglobulin. These results show that most of the linoleic acid other than that in the C/M extract was in the cream as a complex with β-lactoglobulin.

These results enable us to conclude that linoleic acid in a complex with the β-lactoglobulin molecule at the oil-water interface was effectively protected by α-tocopherol or Trolox against autoxidation.

References


