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Antioxidative activity of chitosans with varying molecular weights ☆

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Abstract

Antioxidant activity of chitosans of different molecular weights (30, 90 and 120 kDa chitosan) in salmon (*Salmo salar*) was investigated. The progress of oxidation was monitored by employing the 2-thiobarbituric acid-reactive substances (TBARS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays. In general, all chitosans exhibited antioxidative activities in salmon. The addition of chitosans to salmon reduced lipid oxidation for seven days of storage. The TBARS values of salmon containing chitosan were significantly lower than those of the control ($p < 0.01$). At 0.2% (w/v) and 0.5% (w/v) concentrations, the TBARS with chitosan addition was decreased by 75% and 45%, respectively, over 15 days. At 1% concentration, the TBARS value with native chitosan addition was decreased by 32% after 15 days of storage. 90 kDa chitosan showed an increased DPPH free radical-scavenging activity with increasing concentration in the range of 0.2–1% (w/v). The free radical-scavenging activity of the 0.2 mM DPPH solution was saturated by 30 kDa chitosan at a concentration of $\geqslant 0.7\%$ (w/v), resulting in a strong antioxidant activity of approximately 85%. This was comparable to the DPPH free radical-scavenging activity of BHT.

Keywords: Chitosan; Molecular weight; Lipase; Antioxidative activity

1. Introduction

Chitosan (poly-β-1, 4-linked glucosamine) is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin. It has attracted attention as a biomedical material, owing to its unique biological activities which include antitumor, immuno-enhancing effects and antibacterial activity in combination with its low toxicity (Kim, Thomas, Lee, & Park, 2003; Suzuki et al., 1986; Suzuki, Tokoro, Okawa, Suzuki, & Suzuki, 1986). It can be used for the chelation of transition metals (Muzzarelli, Muzzarelli, & Terbojerich, 1997), edible coatings for fruit and vegetables (Castellanos-Perez, Maldanado-Vega, Fernandez-Villagomez, & Cafferal-Mendez, 1988; El-Ghaouth, Arul, Ponnampalam, & Castaigne, 1992; Park, 1999) packaging films (Butler, Vergano, Testin, Bunn, & Wiles, 1996; Caner, Vergano, & Wiles, 1998; Park et al., 1999; Rhim, Weller, & Ham, 1998) and waste water purification (Knorr, 1991). The application of this polysaccharide in the food industry and medicine is, however, limited because of its high molecular weight (MW) resulting in its low solubility in aqueous media (Ilyina, Tikhonov, Albulov, & Varlamov, 2000). It is important to improve the water solubility of chitosan so that it can be incorporated into different films which will expand its usefulness in the food industry.

Chitosan hydrolysate can be prepared by enzyme hydrolysis (Amano & Ito, 1978; Hirano & Nagano, 1989; Sashiwa, Uraki, Saimoto, Shigemasa, & Tokura, 1989) or acid hydrolysis (Horowitz, Roseman, & Blumenthal, 1957; Hwang et al., 2002). The enzymatic method has the advantage over chemical reactions due to the fact that it takes place under mild conditions and does not create environmental problems (Mekkriengkrai, Chirachanchai, & Pichyangkura, 2001). It also has the minimum effect on the chemical nature of the reaction product. There are several

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reports of different enzymes such as lipase, lysozyme, chitinase and chitosanase being utilized in this procedure (Aiba, 1994; Ilyina et al., 2000; Shin, Lee, & Lee, 2001; Stoyachenko, Varlamov, & Davankov, 1994).

Recently, the antioxidant activity of chitosan and its derivatives has attracted attention. Xie, Xu, and Liu (2001) studied the antioxidant activities of water-soluble chitosan derivatives which were considered to be hydroxyl radical scavengers. Antioxidant activities of different MW chitosans in salmon may be attributed to their metal-bonding capacities. Several sources of protein-bound iron exist in fish tissue, e.g., myoglobin, hemoglobin, ferritin and transferrin. The iron bound to these proteins may be released during storage thus activating oxygen and initiating lipid oxidation (St. Angelo, 1996). Xue, Yu, Hirata, Terao, and Lin (1998) reported that water-soluble chitosans may chelate metals or combine with lipids resulting in a significant antioxidative effect. Peng, Wang, and Tang (1998) and Winterowd and Sandford (1995) showed that chitosans retard lipid oxidation by chelating ferrous ions present in the system, thus eliminating their prooxidant activity or their conversion to ferric ion. Furthermore, amino groups in chitosans may participate in the chelation of metal ions. Kamil, Jeon, and Shahidi (2002) demonstrated that among the different viscosity chitosans, 14 cp chitosan was more effective than the higher viscosity chitosans in preventing lipid oxidation in the herring flesh model system. Darmadji and Izumimoto (1994) observed the effectiveness of chitosan treatment on the inhibition of lipid oxidation of beef. The addition of 1% chitosan resulted in a 70% reduction of thiobarbituric acid-reactive substances (TBARS) values in meat after 3 days of storage at 4 °C.

The highly unsaturated fatty acids commonly found in seafoods are particularly sensitive to oxidative change during storage (Hsieh & Kinsella, 1989a; Shahidi, 1997). It has been proposed that lipid oxidation in fish may be initiated and promoted by a number of mechanisms including autoxidation, photosensitized oxidation, lipoxygenase, peroxidase, and microsomal enzymes (Hsieh & Kinsella, 1989).

The heavy metal–polymer complexes are considered to form as a result of dative bonding with chitosan (Kamil et al., 2002). This involves the donation of nonbonding pairs of electrons from the nitrogen, and/or the oxygen of the hydroxyl groups, to a heavy metal ion (Tual, Espuche, Escoubes, & Domard, 2000).

Synthetic antioxidants and chelating agents can be added to food products to prevent lipid oxidation (Kamil et al., 2002). However, the growing consumer demand for food devoid of synthetic antioxidants has focused research on the development of new natural preservatives (Matsugo et al., 1998). Several sources of natural antioxidants are known (Shahidi, 1997), and some of them, such as those of rosemary and sage, are currently used in a variety of food products. Fundamental studies on chitosan as a natural antioxidative agent in fish have not been conducted (Kamil et al., 2002). The objective of this study was to examine the effect of chitosans of different molecular weights as antioxidative agents in salmon based on the measurement of 2-thiobarbituric acid-reactive substances (TBARS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity.

2. Materials and methods

2.1. Materials

All chemicals used in this study were obtained from commercial sources and were of the highest purity available. 2-Thiobarbituric acid (TBA) was purchased from Sigma Chemical Co. (St. Louis, MO). Native chitosan (MW 120 kDa, 84.71% deacetylation) and 40 cp (MW 80 kDa, 80.77% deacetylation) chitosan were purchased from Kimitsu Co. (Tokyo, Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical was obtained from Sigma–Aldrich Co. (St. Louis, MO).

2.2. Preparation of degraded chitosan

Degraded chitosan was prepared enzymatically from native chitosan using lipase from *Rhizopus japonicus* as described by Shin et al. (2001). Five fractions were obtained with MW varying from 30–50 kDa.

2.3. Determination of MWs of chitosan

High-performance size-exclusion chromatography together with Multiangle laser light scattering-refractive index system (MALLS-RI) was used to determine the MW of degraded chitosan, O-CM-chitosan from native chitosan and O-CM-chitosan from enzymatically degraded chitosan. The system consisted of a pump (P2000, Spectra System, San Jose, CA), an injection valve (model 7021, Rheodyne, Cotati, CA), a guard column (TSK PWH, Tosoh Corp., Tokyo, Japan), and SEC column (TSK Gel 3000PW, 7.8 × 600 mm, Tosoh Corp., Tokyo, Japan), Multiangle laser light-scattering (LS; Dawn DSP-F, Wyatt Technology, Santa Barbara, CA, USA), and refractive index (RI; Shodex SE71, Tokyo, Japan) detectors. Columns were maintained at 25 °C, and the detector was maintained at 35 °C. The mobile phase (water with 0.02% NaN_3) was filtered first through 0.2-μm, and then through 0.1-μm cellulose acetate filters. Flow rate of the mobile phase was 0.5 mL/min, and the sample injection volume was 500 μL. Output voltages of RI and LS at the 18 angles were collected and used to calculate MW using Astra 4.50 software (Kim et al., 2000).

2.4. Salmon sample preparation

Fresh salmon was obtained from a local supermarket and was ground using a blender. Ten grams of ground sample were placed in a vial and refrigerated at 4 °C for 15 days.

2.5. 2-Thiobarbituric acid test

The TBA test was conducted over a 15 day period on refrigerated samples using the method of Tarladgis, Watts, and Younathan (1960). Malondialdehyde (MDA) and other aldehydes, formed during lipid oxidation in the salmon were measured, and reported as TBARS values in units of MDA equivalent/kg salmon sample. The amount of the pink colored TBA complex was measured with a spectrophotometer at 538 nm. The maximum absorbance used in this study was by a modified version of methods of Tarladgis et al. (1960). Triplicate samples were analyzed.

2.6. DPPH radical assay

The free radical scavenging effect of chitosan was estimated according to the method of Blois (1958) with some modification. The chitosan sample (1 mL) was added into the 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma–Aldrich Co., St. Louis, MO; 1 mL). The mixture was shaken and incubated for 30 min at room temperature. The absorbance was then measured at 517 nm using a spectrophotometer (model UV-1601PC, Shimadzu Co., Tokyo, Japan). The DPPH radical-scavenging capacity was estimated based on the difference in absorbance with or without samples and expressed as a percentage of DPPH scavenging at 4 °C. Butylated hydroxytoluene (BHT) was used to compare the DPPH free radical-scavenging activity of chitosan (Fig. 5).

2.7. Statistical analysis

Analysis of variance was run using SAS software (Statistical Analysis Systems Institute, Cary, NC) and least significant difference (LSD) comparisons at $p < 0.01$ were made to determine the significant differences ($\alpha = 0.05$ level).

3. Results and discussion

3.1. IR spectra analyses

Infrared spectroscopy has been used to determine the structure of chitin and chitosan (Kurita, 1986). Fig. 1 shows the FTIR spectra of degraded chitosan and native chitosan. The spectral patterns of degraded and native chitosan used in this study were similar to that of chitosan reported by Lim, Khor, and Koo (1998). The main characteristic peaks of chitosan were at 3455 (O–H stretch), 2867 (C–H stretch), 1598 (N–H bend), 1154 (bridge O stretch), and 1094 cm^{-1} (C–O stretch). In the spectrum of degraded chitosan, the peaks between 1610 and 1410 cm^{-1} were from amine residues. The degree of deacetylation was calculated (Muzzarelli et al., 1997) to be 84.71% for native chitosan, 74.02% for degraded chitosan and 80.77% for 40 cp chitosan (Muzzarelli et al., 1997).

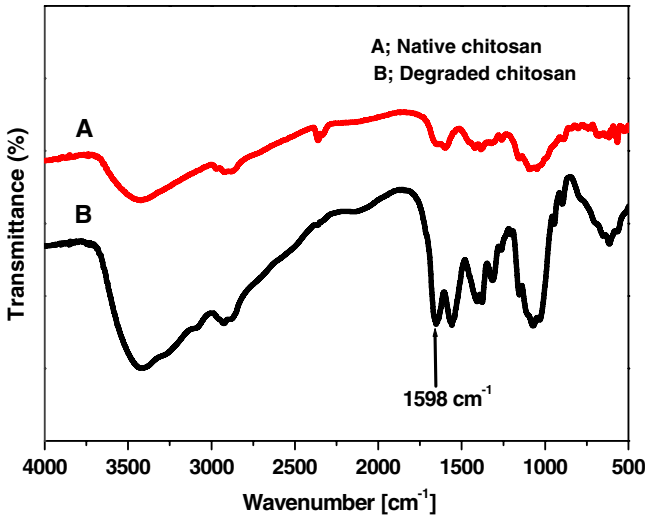

Fig. 1. IR spectra of native chitosan and degraded chitosan.

3.2. Values of TBA-reactive substances

The TBA test has been used widely to estimate the extent of lipid oxidation (Shahidi, 1994). Matsugo et al. (1998) reported that three different water-soluble chitosan derivatives inhibited TBARS formation in *t*-butylhydroperoxide and benzoyl peroxide induced lipid peroxidations. The TBARS values of salmon in control and salmon with chitosan addition from 0.2% (w/v) to 1% (w/v) were determined and compared. As seen in Figs. 2–4, the control containing no chitosan gave increasingly higher TBARS values over the 15 days of storage. In contrast the samples containing chitosans showed lower TBARS values over the same storage period except for 0.2% addition of 90 kDa chitosan at day 7 (Fig. 2), 0.5% addition of 120 kDa

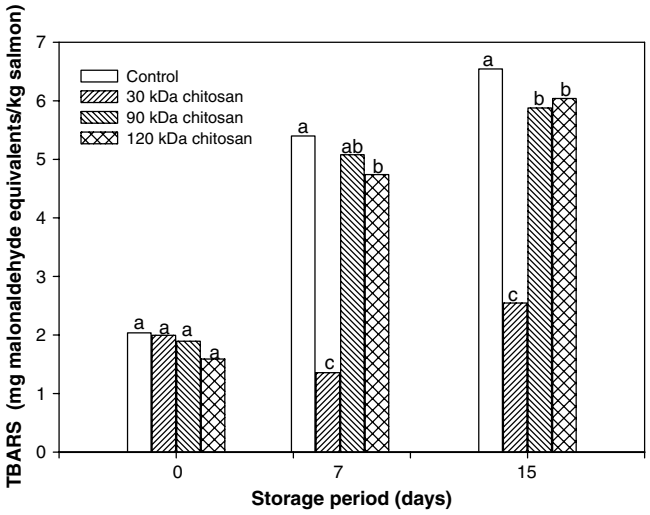

Fig. 2. Values of thiobarbituric acid-reactive substances (TBARS) of salmon at 7 and 15 days of storage at 4 °C with 0.2% chitosan addition. Each value is expressed as standard error of the mean of triplicate determinations. Bars with different letters are significantly different ($p < 0.01$).

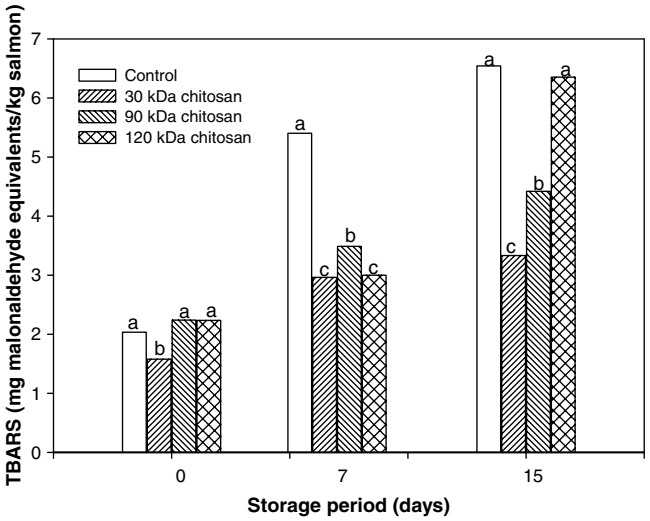

Fig. 3. Values of thiobarbituric acid-reactive substances (TBARS) of salmon at 7 and 15 days of storage at 4 °C with 0.5% chitosan addition. Each value is expressed as standard error of the mean of triplicate determinations. Bars with different letters are significantly different ($p < 0.01$).

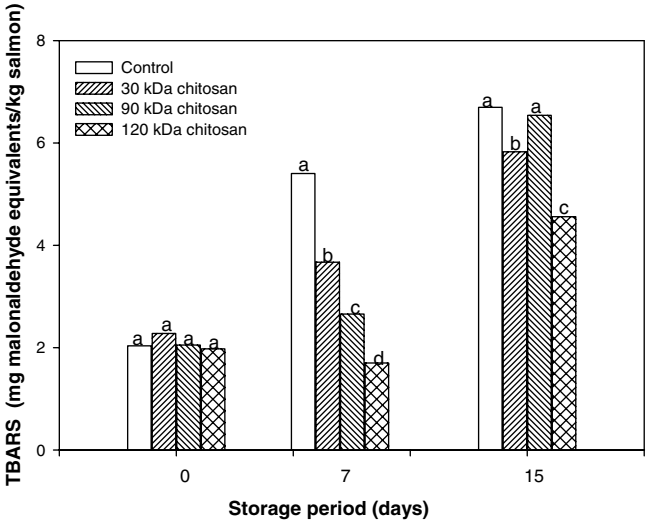

Fig. 4. Values of thiobarbituric acid-reactive substances (TBARS) of salmon at 7 and 15 days of storage at 4 °C with 1% chitosan addition. Each value is expressed as standard error of the mean of triplicate determinations. Bars with different letters are significantly different ($p < 0.01$).

chitosan at day 15 (Fig. 3) and 1% addition of 90 kDa chitosan at day 15 (Fig. 4). This suggests that the oxidation of salmon may be inhibited to some extent by the presence of chitosan.

The rate of lipid oxidation in salmon was reduced by addition of three chitosans of different MWs, as indicated by changes in the TBARS value of salmon during storage at 4 °C. However, the effect of the 30 kDa chitosan at 0.2% concentration on the TBARS value of salmon was significant ($p < 0.01$) showing a 75% reduction of lipid oxidation after 7 days of storage. The decrease in the TBARS value depended on the MW and concentration of chitosan. The chitosan with lowest MW (30 kDa) showed the most antioxidative activity at low concentrations. It was most effective at both 0.2% (w/v) and 0.5% (w/v) concentrations in reducing oxidation at 15 days (Figs. 2 and 3). At 0.2%, 0.5% and 1% (w/v) concentrations the TBARS value of 30 kDa chitosan decreased by 75%, 45% and 32% (w/v), respectively, at 7 days, and by 61%, 49% and 13%, respectively, at 15 days (Figs. 2–4). In contrast the TBARS value of 90 kDa chitosan decreased by 6%, 35% and 50% at 0.2%, 0.5% and 1% concentrations, respectively, at 7 days of storage, and by 10%, 32% and 2% at 0.2%, 0.5% and 1% concentrations, respectively, after 15 days of storage (Figs. 2–4). Similarly the TBARS value of 120 kDa chitosan decreased by 12%, 44% and 69% at 0.2%, 0.5% and 1% concentrations, respectively, after 7 days of storage, and by 7%, 3% and 32% at 0.2%, 0.5% and 1% concentrations, respectively, after 15 days of storage (Figs. 2–4). At 1% (w/v) concentration the 120 kDa chitosan was the most effective in reducing lipid oxidation (Fig. 4) while the 30 kDa chitosan, although not as effective at 1% concentration, did have some influence on reducing lipid oxidation.

The varying antioxidant effect of chitosans of different MW in salmon may be attributed to the MW differences which determine the chelation of metal ions (Kamil et al., 2002). The cationic amino groups of chitosans impart intramolecular electrostatic repulsive forces, which increase the hydrodynamic volume by extended chain conformation in their charged state (Kamil et al., 2002). This may be responsible for less chelation by high MW chitosans (Kamil et al., 2002). It seemed that high MW chitosan causes a higher repulsive force of NH_3^+ in the chitosan molecule resulting in reducing the antioxidative activity of chitosan. However, in this study, it would appear that high concentration of higher MW chitosans is better able to chelate the metal ions or may bind to the lipid and reduce its potential for lipid oxidation.

Darmadji and Izumimoto (1994) reported that addition of chitosan at 0.2%, 0.5% and 1% resulted in a decrease of 10%, 25%, and 40%, respectively, in the TBARS values of meat at first, but after 3 days of storage at 4 °C, 1% chitosan caused 70% reduction of TBARS value. St. Angelo and Vercellotti (1989) also reported that *N*-carboxymethychitosan was effective in controlling warmed-over flavor with 93% inhibition of TBARS, and 99% reduction in the hexanal observed in ground beef.

3.3. DPPH radical assay

The 30 kDa chitosan scavenged from 40% to 100% of DPPH radical while 90 kDa chitosan and 120 kDa chitosan showed less effect with a scavenging activity ranging from 9% to 37% (Fig. 5). The 90 kDa chitosan showed an increased DPPH free radical-scavenging activity with increasing concentration in the range of 0.2–1% (w/v) (Fig. 5). Furthermore, the free radical-scavenging activity of the 0.2 mM DPPH solution was saturated by 30 kDa chitosan at a concentration of $\geqslant$0.7% (w/v), resulting in

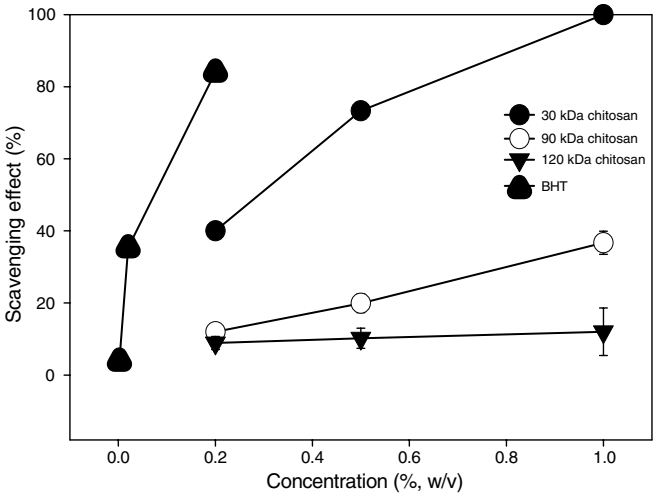

Fig. 5. DPPH-radical-scavenging activity of BHT and different MW chitosans at varying concentrations.

a strong antioxidant activity of approximately 85%. This was comparable to the DPPH free radical-scavenging activity of BHT (Fig. 5).

It is generally considered that the inhibition of lipid peroxidation by an antioxidant can be explained by various mechanisms. One is the free radical-scavenging activity. Park, Je, and Kim (2004) suggested that chitosan may eliminate various free radicals by the action of nitrogen on the C-2 position of the chitosan. Xie et al. (2001) reported that the scavenging mechanism of chitosan is related to the fact that the free radicals can react with the hydrogen ion from the ammonium ions (NH_3^+) to form a stable molecule. The NH_3^+ has been formed by the amine group absorbing a hydrogen ion from the solution.

Youn, Kim, and Ahn (2001) showed that antioxidative effects of chitosan were increased with larger MW and higher concentration as indicated by the results of DPPH radical assay. The present study revealed that the scavenging activities also depended on the MWs of chitosans. However, in this case the 30 kDa chitosan showed the highest scavenging activity compared to 90 and 120 kDa chitosan. The scavenging activities of chitosans increased with increasing concentration from 0.2% to 1% (w/v) except for the native chitosan which showed no significant difference with concentration ($p < 0.01$). The increase in concentration of 30 kDa chitosan resulted in the increase of total amine groups responsible for scavenging more radicals. The results indicate that the radical-scavenging activity of 120 kDa chitosan was not affected by different concentrations. The 120 kDa chitosan would have lower mobility than the lower MW chitosans which would increase the possibility of inter- and intramolecular bonding among the high MW chtiosan molecules. Therefore, the chance of exposure of their amine groups might be restricted which would account for less radical-scavenging activity. These results support the TBA results of salmon with 120 kDa chitosan addition at 0.2% or 0.5% (w/v) concentration found in this study. In the case of the addition of 1% (w/v) chitosan, the highest DPPH radical-scavenging activity was shown by addition of 30 kDa chitosan, while 90 kDa chitosan and 120 kDa chitosan showed lower DPPH radical-scavenging activities.

While the radical-scavenging activity of low MW chitosans appear to play a role in their antioxidant activities, this is not the case for the high MW chitosan. In the latter case, its antioxidant activity, as indicated by the result from the TBA assay, must be explained by other mechanism such as metal chelation or lipid binding. High MW chitosan may also have a synergistic effect in the presence of other natural materials with antioxidative activity in food systems.

4. Conclusions

Incorporation of 0.2%, 0.5% and 1% chitosan with various molecular weights into salmon resulted in reduced lipid oxidation. The 30 kDa chitosan which has high water solubility used in this study may be considered as a potential natural antioxidant for stabilizing lipid containing foods to prolong shelf life as well as being an excellent antimicrobial agent. The present study should provide a possible application of chitosan as a food additive to high lipid food systems.

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