Iron-Binding Property and Antioxidative Activity of Xanthan on the Autoxidation of Soybean Oil in Emulsion

Kazuko Shimada,*,† Hiromi Muta,† Yukiko Nakamura,† Hiromi Okada,† Kayoko Matsuo,† Sawako Yoshioka,† Taeko Matsudaira,‡ and Takashi Nakamura‡

Department of Food and Life Science, Yamaguchi Women's University, 3-2-1 Sakurabatake, Yamaguchi 753, Japan, and Honen Corporation, 1-2-3 Ohtemachi, Chiyoda-ku, Tokyo 100, Japan

The Fe^{2+}-binding property and its binding site on xanthan were investigated in connection with the synergistic antioxidation to soybean oil in the presence of tocopherols. Xanthan significantly inhibited Fe^{2+}-induced oxidation of soybean oil in emulsion at pH 7.0 or 4.0. The binding parameters (K_d, n) between xanthan and Fe^{2+} ion were obtained at pH values of 4.0, 5.0, 6.0, and 6.5 by using the Scatchard equation. The maximum number (about 0.6 mol/kg of xanthan) of high-affinity binding sites agreed with the pyruvate residue content in xanthan. ^{1}H-NMR spectra indicated that xanthan bound Fe^{2+} through a pyruvate residue. These results suggest that xanthan chelates Fe^{2+} ions and consequently suppresses oil peroxidation synergistically in the presence of tocopherols by inactivation of the Fe^{2+} ion.

Keywords: *Xanthan; natural antioxidant; chelation; pyruvate*

INTRODUCTION

Oil-in-water (O/W) emulsions such as mayonnaise, salad dressings, and coffee creamers are widely used in food products. Various polysaccharide stabilizers are added to food emulsions to enhance the emulsion stability by increasing the viscosity of the aqueous phase. The polysaccharide xanthan contributes to O/W emulsion stability and is now used to stabilize most salad dressings as a natural food additive (Hennock *et al.*, 1984; Coia and Stauffer, 1987; Yilmazer and Kokini, 1991). Xanthan is an anionic polysaccharide produced by the microorganism *Xanthomonas campestris*, whose primary structure consists of a pentasaccharide repeating unit with a (1→4)-β-D-glucopyranan backbone with *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyluronic acid−(1→2)-6-*O*-acetyl-α-D-mannopyranosyl side chains 3-linked to alternate glucose residues (Jansson *et al.*, 1975). The terminal mannose of the side chain is substituted with a pyruvate ketal, but the degree of substitution is not stoichiometric. About half or one-third of the side chains possess pyruvate substituents, depending on the culture conditions (Cadmus *et al.*, 1978).

The autoxidation of oils in food emulsions results in deterioration of flavor and taste. Several synthetic phenolic antioxidants have been used successfully to prevent lipid oxidation in food products. The most commonly used are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Consumers are concerned about the safety of synthetic food additives. The food industry needs effective "natural" antioxidants. This has led to renewed interest in natural products and increased research using natural antioxidants. The effects of mono- or disaccharides on lipid peroxidation in emulsions have been investigated extensively (Mabrouk and Dugan, 1961; Mabrouk, 1964; Sims *et al.*, 1979; Yamaguchi and Yamada, 1981; Yamauchi *et al.*, 1982, 1984), but little was known of the effects of polysaccharides used widely as emulsion stabilizers in food. Recently, Shimada *et al.* (1992) reported that xanthan strongly inhibited the autoxidation of soybean oil containing tocopherols in a cyclodextrin (CD) emulsion system. It was also elucidated that xanthan suppresses oil peroxidation by the inactivation of metal ion such as Fe^{2+} and may chelate a metal ion between two side chains with a pyruvate residue.

In this study, the Fe^{2+}-binding property and its binding site on xanthan were investigated in detail to confirm its synergistic antioxidation in the presence of tocopherols by the inactivation of Fe^{2+}.

MATERIALS AND METHODS

Materials. β-CD was provided by Nihon Shokuhin Kako Co. (Tokyo, Japan). Soybean oil was obtained from Honen Corp. (Tokyo, Japan). α-, β-, γ-, and δ-tocopherols in the soybean oil were present at 18.6 ± 0.4, 2.5 ± 1.2, 80.1 ± 0.3, and 17.6 ± 0.6 (mean ± SD, n = 3) mg/100 g of oil, respectively, as measured by HPLC (Shimada *et al.*, 1992). Xanthan (sodium salt) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Deuterium oxide (D_2O) was obtained from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Determination of the Pyruvate Group in Xanthan. Pyruvate group content in xanthan was measured using the 2,4-dinitrophenyl derivative (Duggan, 1969). Xanthan (0.2% w/v) in 2 N H_2SO_4 was hydrolyzed at 100 °C for 6 h. Two milliliters of the hydrolysate containing free pyruvate was mixed with 1 mL of 0.5% 2,4-dinitrophenylhydrazine (2,4-DNPH)/2 N HCl solution for the formation of its derivative. The derivative was extracted with 5 mL of ethyl acetate and then with 5 mL of 10% Na_2CO_3 three times. The total volume of the collected extract was adjusted to 100 mL with 10% Na_2CO_3. The absorbance of the extract was determined at 375 nm. Sodium pyruvate was used as a standard.

Measurement of Antioxidative Activity of Xanthan on Fe^{2+}-Induced Soybean Oil Peroxidation in Emulsion. β-CD was used as an emulsifier. β-CD is capable of producing a stable emulsion even under an acidic condition at pH 4.0. For the preparation of the Fe^{2+}/ascorbate system, a mixture of 0.8 mL of soybean oil and 3.2 mL of 50 mM Tris-HCl buffer (pH 7.0) or 50 mM sodium acetate buffer (pH 4.0) containing 1.5% β-CD, 20 μM $FeSO_4$, and 100 μM sodium ascorbate with or without 0.5% xanthan was homogenized at 22 000 rpm for 2 min at 20 °C with a laboratory disperser (Ystral-Mitamura

* Author to whom correspondence should be addressed.
† Yamaguchi Women's University.
‡ Honen Corp.

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Riken Kogyo, Germany). For the Fe^{2+}/fructose system, an emulsion was prepared as described above except for use of 10 mM fructose instead of 100 μM sodium ascorbate. Sodium ascorbate or fructose was added to the emulsion system to maintain iron in the Fe^{2+} state. The emulsions were incubated at 37 °C for 50 days. At intervals, the peroxide value (POV) was determined directly for each 100 μL of emulsion according to the iodometric method (Yamauchi *et al.*, 1984). POV determined according to the AOCS method (1964) for autoxidized soybean oil was used to prepare a standard curve. POV was expressed in milliequivalents per kilogram of oil. Thiobarbituric acid (TBA) value was measured according to the method of Asakawa *et al.* (1975) by adding 0.1 mL of 10 mM butylated hydroxytoluene to the reaction mixture. TBA value was calculated from the standard curve obtained using 1,1,3,3-tetraethoxypropane and expressed as millimoles of malondialdehyde per kilogram of oil.

Determination of Total Iron and Fe^{2+}. Iron analyses were done according to the *o*-phenanthroline method of Sandel (1959), with or without ascorbate, depending on whether data for total Fe or only Fe^{2+} were desired. Total Fe content was determined to verify that it agreed with Fe^{2+} content; that is, all iron was in the Fe^{2+} state.

Measurement of Fe^{2+} Binding to Xanthan under Different pH Conditions. Xanthan solutions (0.05% w/v) were incubated with variable amounts of $FeSO_4$ for 1 h at 37 °C unless otherwise indicated. The concentration of Fe^{2+} added was from 0.25 to 5.00 mM; the media in xanthan solution were 50 mM sodium acetate buffers (pH 4.0 and 5.0) and 50 mM Tris-HCl buffers (pH 6.0 and 6.5). The xanthan solution was centrifuged at 2500 rpm (1000*g*) for 10 min to remove xanthan using Amicon ultrafiltration cones (Amicon Corp., Lexington, MA) with a 50 000 molecular weight cutoff. The ultrafiltrate and the xanthan solution without ultrafiltration treatment were analyzed by the *o*-phenanthroline method for Fe content. Fe^{2+} bound to xanthan was calculated as the difference between the Fe^{2+} concentrations of the solutions before and after the ultrafiltration treatment.

The data obtained from these experiments were plotted according to the Scatchard (1949) method to examine the kinetic characteristics of binding.

Measurement of Apparent Viscosity. After 0.05% xanthan solutions (w/v) containing various concentrations of Fe^{2+} ion were incubated at 37 °C for 1 h, apparent viscosity was measured at a constant shear rate of 19.2 s^{-1}. The apparent viscosity was measured at 37 °C using a cone-and-plate type viscometer (Model DVM-E2, Tokimec Inc.).

Nuclear Magnetic Resonance (NMR). ^{1}H-NMR spectra were obtained at 90 °C using a JEOL JNM-GSX270L FT spectrometer at 500 MHz. Xanthan was lyophilized twice in D_2O, dissolved in the same solvent, and placed in 5-mm tubes at a concentration of about 0.5 mg/0.6 mL of D_2O. $FeSO_4$ (0.33 mM) was added immediately prior to analysis. Fe^{2+} added was about 0.4 mol/kg of xanthan. Reference was internal acetone (2.21 ppm). A few drops of acetone were added to each solution.

Each result reported in this study is from a single experiment.

RESULTS AND DISCUSSION

Effect of Xanthan on Fe^{2+}-Induced Oxidation of Soybean Oil in the Aqueous Emulsion System. Although the autoxidation of soybean oil in an emulsion was investigated in the addition of $FeSO_4$ alone, the rate of oxidation was not accelerated (data not shown here). The added Fe^{2+} ions may have been oxidized rapidly, so that resulting Fe^{3+} ions became insoluble and were unable to contribute to oil peroxidation. Therefore, to maintain iron in the Fe^{2+} state, $FeSO_4$ was added to the emulsion system in the presence of ascorbate or fructose. It is known that ascorbate is a strong reducing agent, which readily reduces Fe^{3+} to the Fe^{2+} state. Yamauchi *et al.* (1984) reported that reducing sugars

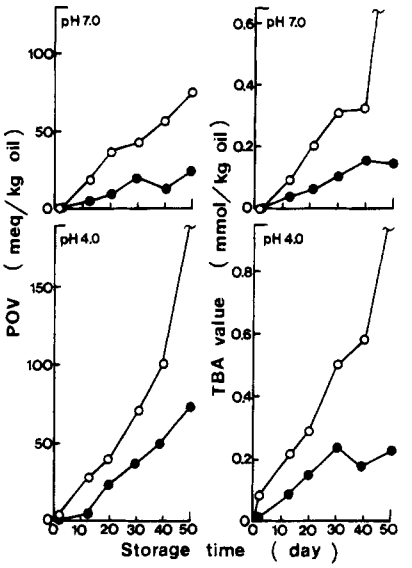

Figure 1. Effect of xanthan on the oxidation of soybean oil in an Fe^{2+}/ascorbate emulsion system. β-CD was used as an emulsifier. pH 7.0, 50 mM Tris-HCl buffer; pH 4.0, 50 mM sodium acetate buffer; (○) CD; (●) CD plus xanthan.

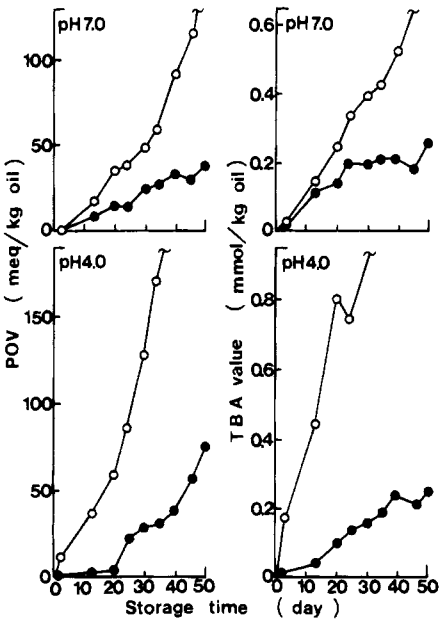

Figure 2. Effect of xanthan on the oxidation of soybean oil in an Fe^{2+}/fructose emulsion system. β-CD was used as an emulsifier. pH 7.0, 50 mM Tris-HCl buffer; pH 4.0, 50 mM sodium acetate buffer; (○) CD; (●) CD plus xanthan.

such as fructose reduced Fe^{3+} to Fe^{2+} and that the resulting Fe^{2+} ions accelerated oil peroxidation.

In an Fe^{2+}/ascorbate emulsion system, the effect of xanthan on the oxidation of soybean oil was investigated by measuring the POV and TBA value. The Fe^{2+}-induced oxidation of soybean oil proceeded rapidly in the absence of xanthan during storage but was retarded by the addition of xanthan at pH 7.0 or 4.0 (Figure 1). The rate of oil peroxidation in the emulsion at pH 4.0 was much faster than that at pH 7.0. Lipid peroxidation is accelerated at acidic pH by metal ions such as Fe^{2+} ion (O'Brien, 1969; Yamauchi *et al.*, 1988). In an Fe^{2+}/fructose system, the oxidation of soybean oil in emulsion was inhibited by the addition of xanthan at pH 7.0 or 4.0 compared with that in the absence of xanthan (Figure 2). These results indicate that xanthan contributes to the inactivation of Fe^{2+} ions by chelation and

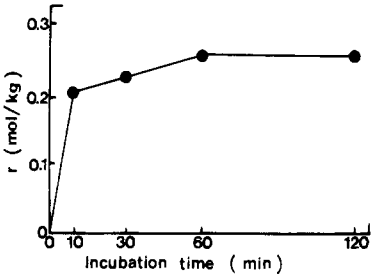

Figure 3. Time course of Fe^{2+}-binding to xanthan. Xanthan (0.05%) was incubated in Tris-HCl buffer (pH 6.0) containing 1 mM $FeSO_4$ at 37 °C.

inhibits the Fe^{2+}-induced oxidation of soybean oil in the emulsion. The rates of oil oxidation in the Fe^{2+}/fructose emulsion were faster than those in the Fe^{2+}/ascorbate emulsion except for that of the emulsion in the presence of xanthan at pH 4.0. This may indicate that ascorbate is unstable, especially at neutral pH, compared with fructose. In a previous paper, it was reported that xanthan inhibited the autoxidation of soybean oil caused by trace metal ions such as Fe^{2+} contaminating the emulsion and suppressed oil peroxidation synergistically in the presence of tocopherols (Shimada *et al.*, 1992). The inactivation of Fe^{2+} ions by xanthan was confirmed in this experiment.

In O/W emulsion, xanthan contributed to the increase of viscosity in the continuous water phase and the decrease of average droplet size (Hennock *et al.*, 1984). The investigation of the effects of the increased viscosity and the decreased droplet size by xanthan on oil oxidation in the emulsion is in progress in our laboratory. Although the POV and TBA values are determined for evaluating the rate of oil oxidation in this study, it is thought that the measurement of oil oxidation products, such as oil hydroperoxides, is required for the more detailed investigation of the antioxidative mechanism of xanthan.

Fe^{2+}-Binding Property of Xanthan at Different pH. The Scatchard equation (eq 1) has been used to describe the binding property of Fe^{2+} ion with xanthan. The equation is

$$r/[Fe^{2+}] = n/K_d - r/K_d \quad (1)$$

where r is the fraction of available xanthan binding sites occupied by Fe^{2+}, $[Fe^{2+}]$ is the concentration of free Fe^{2+} ion after binding equilibrium, K_d is the dissociation constant, and n is the number of binding sites per unit weight of xanthan. When $r/[Fe^{2+}]$ is plotted against r, extrapolation to intersection on the r-axis yields the value of n and extrapolation to the $r/[Fe^{2+}]$-axis yields n/K_d. Calculation of the slope gives the reciprocal of the dissociation constant (K_d) of Fe^{2+}–xanthan interaction. Little is known of the binding parameters (K_d, n) between xanthan and Fe^{2+}, although the kinetic characteristics of Ca^{2+} or Fe^{2+} binding to several polysaccharides (cellulose, locust bean gum, guar gum, gum karaya, gum ghatti, gum arabic, alginate) were studied (Fernandez and Phillips, 1982; Ha *et al.*, 1989).

Xanthan (0.05% w/v) was incubated in Tris-HCl buffer (pH 6.0) containing 1 mM $FeSO_4$ at 37 °C (Figure 3). The binding amount of Fe^{2+} (r, mol/kg of xanthan) increased with the incubation time and reached constant level after 1 h of incubation. Subsequent experiments were carried out using a 1-h incubation time. Fixed amounts of xanthan (0.05% w/v) were incubated with variable amounts of Fe^{2+} ion in different pH

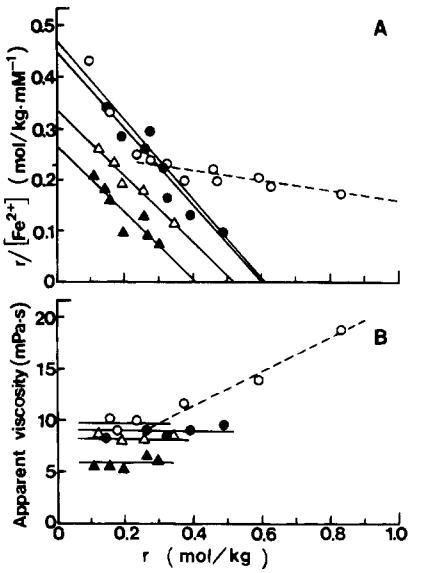

Figure 4. Scatchard plot of Fe^{2+} binding to xanthan (A) and relationship between apparent viscosity of the xanthan solution and r (amount of Fe^{2+} binding) (B) at pH 4.0, 5.0, 6.0, and 6.5. Xanthan concentration was 0.05%. Apparent viscosity was measured at 37 °C and a shear rate of 19.2 s^{-1}. (−▲−) pH 4.0; (−△−) pH 5.0; (−●−) pH 6.0; (−○−) pH 6.5; (- -○- -) pH 6.5.

Table 1. Kinetic Characteristics of Fe^{2+} Binding by Xanthan at pH 4.0, 5.0, 6.0, and 6.5

pH	K_{d1} (M)	n_1 (mol/kg)	K_{d2} (M)	n_2 (mol/kg)
4.0	1.56×10^{-3}	0.41		
5.0	1.56×10^{-3}	0.52		
6.0	1.35×10^{-3}	0.60		
6.5	1.31×10^{-3}	0.61	9.62×10^{-3}	2.48

solutions (pH 4.0, 5.0, 6.0, and 6.5), and $r/[Fe^{2+}]$ was plotted against r (Figure 4A). Scatchard plots gave straight lines at pH values of 4.0, 5.0, and 6.0, and xanthan had only one type of binding site (high Fe^{2+} affinity) in this pH range. On the other hand, the Scatchard plot was clearly biphasic at pH 6.5; two different types of binding sites (high and low Fe^{2+} affinities) were detected in xanthan. Because the oxidation of Fe^{2+} to Fe^{3+} was detected above pH 7.0 after 1 h of incubation at 37 °C, the experiment for the Fe^{2+}-binding property of xanthan was carried out below pH 6.5. The binding parameters (K_d and n) obtained from Scatchard plots are shown in Table 1. The dissociation constants (K_{d1}) for the high-affinity binding site at each pH were relatively constant in the range of 1.56×10^{-3} to 1.31×10^{-3} M. This indicated that the affinities of the binding sites were of the same magnitude even under the different pH conditions. On the other hand, the numbers (n_1) of high-affinity binding sites increased as the pH increased from acidic to neutral medium and reached a constant value of about 0.6 mol/kg of xanthan under the condition of nearly neutral pH (pH 6.0 and 6.5). This may have been due to the increased ionization of functional groups, which is dependent on pH. The ability of functional groups in xanthan to bind Fe^{2+} ions may be greatest in the neutral pH range due to the maximum ionization of these functional groups. Xanthan is an anionic polysaccharide containing negatively charged groups of glucuronic acid and pyruvate. The pyruvate content of the xanthan used was 0.61 mol/kg of xanthan, which was determined by using 2,4-DNPH. The pyruvate group content (0.61 mol/kg) agreed very closely with the maximum number (about 0.6 mol/kg)

of high-affinity binding sites obtained under the condition of nearly neutral pH. This suggests that the high-affinity binding site of xanthan consists of a structure containing a pyruvate group and the mole ratio of the maximally bound Fe^{2+} ion to pyruvate group was 1:1. Because limited binding of Fe^{2+} ion to xanthan removed [by the method of Holzwarth and Ogletree (1979)] about 79% of the pyruvate detected, a Scatchard plot of the depyruvate xanthan could not be obtained (data not shown here).

The second binding site for Fe^{2+} ion at pH 6.5 showed low affinity (K_{d2} = 9.62 × 10^{-3} M) (Table 1). The difference of xanthan–Fe^{2+} interactions at high- and low-affinity binding sites was investigated by measuring apparent viscosity at 37 °C and a shear rate of 19.2 s^{-1}. After the xanthan solutions (0.05% w/v) containing various concentrations of Fe^{2+} ion were incubated at 37 °C for 1 h under the same condition used for determining the binding parameters, the apparent viscosity and the binding amount of Fe^{2+} (r) were measured. The apparent viscosity was constant in the range of r measured at pH values of 4.0, 5.0, and 6.0, while the viscosity increased with increasing r at pH 6.5 when r was more than about 0.3 (Figure 4B). When the interaction between xanthan and Fe^{2+} ion occurred only on the high-affinity binding site, the apparent viscosity was constant. Under the condition of interaction between xanthan and Fe^{2+} ion on low-affinity binding site, the apparent viscosity increased with r. It is known that the viscosity of xanthan–Ca salt solution is higher than that of univalent cations such as K^+ and Na^+ ions below 40 °C, which suggests the formation of intermolecular Ca^{2+} bridges (Tako and Nakamura, 1987). The increase of viscosity may be attributed to the formation of intermolecular Fe^{2+} bridges on xanthan molecules, which may be formed between carboxyl groups of glucuronic acid residue(s) and/or pyruvate residue(s). Intermolecular binding by Fe^{2+} ion may show low Fe^{2+} affinity. On the other hand, the high-affinity binding site may contribute to intramolecular Fe^{2+}-binding and may be the structure containing the pyruvate residue.

Although buffers containing components that do not chelate metal strongly are used in this study, the binding property between xanthan and Fe^{2+} ion may be influenced to some extent by the components in buffers.

Fe^{2+}-Binding Site of Xanthan. The ^{1}H-NMR spectra of xanthan and xanthan plus Fe^{2+} are shown in Figure 5. Peaks at 1.45 and 2.15 ppm represent the methyl protons of pyruvate and *O*-acetate residues, respectively (Morris *et al.*, 1977). The peak observed at 2.21 ppm represents the proton of acetone used as the internal standard. The peak at 1.87 ppm was assigned to the methyl proton of sodium acetate, which was an impurity in the xanthan sample, on the basis of the ^{1}H-NMR spectrum of sodium acetate (Lopes *et al.*, 1992). The ^{1}H-NMR spectrum of xanthan plus Fe^{2+} was different from that of xanthan alone in that peaks at 1.45 and 1.87 ppm were absent with the appearance of new peaks at 2.08 and 2.35 ppm (Figure 5A,B). It is known that proton signals of solutions containing Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, or most of the lanthanide ions (class 1) are not broad compared to those of solutions containing other transition metal ions such as Cu^{2+} and Mn^{2+} (class 2) and are shifted by the paramagnetism of these ions (Iwaki *et al.*, 1973; Dwek, 1973). The paramagnetic shift is observed in the neighboring protons of atom coordinated to metal ion such as class 1 (McDonald and Phillips, 1963). The new peaks at 2.08 and 2.35 ppm might appear on the spectrum of xanthan plus Fe^{2+} as a result of a shift of the peaks at 1.45 and 1.87 ppm observed on the spectrum of xanthan (Figure 5B). The fact that the areas of shifted peaks are smaller than those of original peaks may result from the broadening of the line widths. ^{1}H-NMR spectra of xanthan and xanthan plus Fe^{2+} indicated that xanthan bound Fe^{2+} through a pyruvate residue.

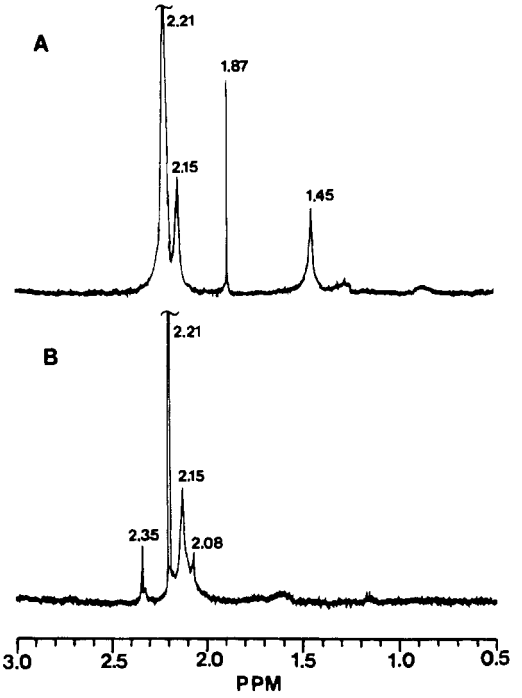

Figure 5. Partial ^{1}H-NMR spectra of xanthan (A) and xanthan plus Fe^{2+} (B) in D_2O. The peaks at 1.45 and 2.15 ppm represent the methyl protons of pyruvate and *O*-acetate residues, respectively. The peak at 1.87 ppm represents the methyl proton of sodium acetate contained as an impurity in the xanthan sample. The peak at 2.21 ppm represents the proton of acetone used as the internal standard.

The structure of the xanthan–Fe^{2+} complex assembled with a molecular structure model was proposed in a previous study (Shimada *et al.*, 1992). The proposed structure was the six-coordinate complex created when the two charged oxygen atoms (of the pyruvate residue and D-glucuronic acid in an adjacent side chain) chelated an Fe^{2+} ion in combination with a hemiacetal oxygen atom of the D-glucuronic acid residue, an oxygen atom of HO-3 of internal D-mannose residue, an acetal oxygen atom of the pyruvate residue, and an oxygen atom of a water molecule. This study showed that the interaction between Fe^{2+} ion and pyruvate residue was detected by ^{1}H-NMR analysis and xanthan bound an Fe^{2+} ion for each pyruvate residue. For these data, it is suggested that xanthan chelates Fe^{2+} ions by forming the proposed six-coordinate structure and consequently suppresses oil peroxidation synergistically in the presence of tocopherols by the inactivation of Fe^{2+} ions. However, other antioxidative mechanisms of xanthan may exist in the emulsion system in addition to the metal chelation. Further research is needed to determine in detail the antioxidative properties of xanthan.

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