Increased Hydrophobicity of Carboxymethyl Starch Film by Conjugation with Zein

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A carboxymethyl starch (CMS) film was prepared by a process in which gelatinized CMS was dried, and subsequently treated with water-soluble carbodiimide in the presence of zein in 70% ethanol or 70% acetone to form acid-amide cross-linkages in order to increase the hydrophobicity of the surface of the film. A small amount of zein protein was found to be present on the surface of the zein-CMS conjugate (Zein-CMS) film, resulting in its insolubility in hot water, low water vapor permeability, and resistance to digestion with $\alpha$-amylase and $\beta$-amylase. Digestion of the Zein-CMS film with protease rendered the film readily water-soluble, suggesting conjugation with zein as an effective means of increasing the hydrophobicity of biodegradable starch-based articles.

Key words: biodegradable material; starch; zein; conjugation

There is great potential demand for biodegradable materials, and starch is regarded as an attractive starting material for their production because it is a natural polymer which can be mass-produced from sustainable agricultural products such as corn, potato, cassava, and sago palm, and because it is relatively cheap with little fluctuation in its price. However, since starch can easily swell and dissolve in water, treatment of the starch to make it water-proof is required for use in food-packaging applications such as bottles, packages, and films. Suitable modification of starch itself may be an effective method for improving such properties. Since the modification of starch to obtain an insoluble derivative would result in its resistance to enzyme digestion, this is not an advantageous approach to produce biodegradable material. Another approach is to treat the surface of starch-based articles with a biodegradable substance which would serve to inhibit the swelling and dissolution of starch arising from the surface. Among such surface treatments, conjugation of a hydrophobic substance with the starch molecules on the surface of a starch-based article is expected to be more effective to obtain a chemically and physically stable water-proof material than just coating the surface with it. As suitable substances to be conjugated with starch, natural products are considered to be superior to synthetic products from the viewpoint of their biodegradability.

Zein is a family of alcohol-soluble storage proteins of maize which is rich in glutamine, proline, leucine, alanine, and phenylalanine residues. These hydrophobic proteins are categorized into four classes. $\alpha$-Zein is the major protein class (75–85% of the total zein fraction) consisting of two groups of proteins with molecular weights of about 23,000–24,000 and 26,000–27,000. These proteins appear as highly extended rigid asymmetric particles with a 50% $\alpha$-helix structure in a 70% methanol solution, and associate with each other to form aggregates, presumably through hydrogen-bonding via polar amino acid residues in the $\alpha$-helix.1,2) The size of the aggregates reaches 5–10 nm and 280–430 nm (a two-size population) in a 70% ethanol solution, and 640–1,000 nm in a 70% acetone solution.3) These structural features are indicative of the peculiar hydrophobicity of the surface of zein molecules and the aggregates. Biodegradable water-resistant films have been prepared from zein in an aqueous acetone solution by drying, and zein has been applied as a coating to improve the poor water resistance of an article such as a bowl-shaped wafer.4) This suggests that a stable water-proof surface could be obtained on starch-based articles through conjugation with zein, as compared with coating it, because zein chemically conjugated with starch on the surface would not be soluble in the medium containing an organic solvent such as an alcoholic drink. Lim and Jane5) have reported that water-resistant, biodegradable plastics could be prepared from mixtures of starch, zein, and a cross-linking agent such as formaldehyde or glutaraldehyde. However, since a residual amount of the aldehydes used as cross-linking agents may remain in the arti...
icles, their application for the production of food packaging materials would not be generally acceptable.

We have previously established a method for conjugating proteins and amino acids with starch by using water-soluble carbodiimide, which led to a marked decrease in the solubility, swelling power, degree of retrogradation, and digestibility with α- or β-amylase and an increase in the thermal stability of starch.6–7 In these studies, carboxymethyl starch (CMS) produced with a slight degree of modification was successfully conjugated through acid-amide linkages with proteins or amino acids. Notably, no residual carbodiimide, the cross-linking agent, remains in the reaction products, because carbodiimide reacts with the carboxyl groups of CMS and breaks down in the successive reaction involving the formation of acid-amide linkages.8

In the present study, a CMS film prepared from corn starch was conjugated with zein in a 70% ethanol or 70% acetone solution in order to increase the hydrophobicity of the surface of the CMS film, and the changes in its solubility, water vapor permeability, and digestibility with enzymes were investigated.

Materials and Methods

Materials. Corn starch (Nihon Shokuhin Kako Co., Tokyo, Japan) was used after being repeatedly washed with distilled water at 4°C and air-dried (13% moisture). CMS (the degree of modification was 60 carboxymethyl residues/1000 glucose residues) was prepared as previously described (Hattori et al., 1995; Yang et al., 1995). Zein was supplied by Showa Sangyo Co. (Tokyo, Japan), and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) was purchased from Dojindo (Kumamoto, Japan). All other reagents were commercially available reagent-grade products.

Preparation of the CMS film. CMS (2 g) was dispersed in 200 ml of distilled water and then gelatinized at 85°C for 30 min. After cooling to room temperature, the CMS solution was degassed for 30 min and poured into an acrylic resin tray (200 × 200 × 100 mm³). After drying at 40°C for 48 h, a CMS film was obtained.

Preparation of the zein-conjugated CMS film. The CMS film (2 g) was immersed in a bath of 200 ml of 70% ethanol or 70% acetone containing 4 g of EDC. A zein solution (4 g/200 ml of 70% ethanol or 70% acetone) was dropped into the bath, and the mixture was incubated at room temperature for 5 h. After thoroughly washing with the same solvent and air-drying, a zein-conjugated CMS (Zein-CMS) film was obtained.

Microscopic observation. The Zein-CMS film (5 mg) was immersed in 1 ml of distilled water and stained with 1 ml of a Coomassie brilliant blue solution (CBB, 10 mg/ml) for 30 min. After washing with distilled water by centrifuging five times at 3,000 rpm for 5 min, the cross-section of the film was observed under an optical microscope (Olympus, Tokyo, Japan). The surface and cross-section of the Zein-CMS film were examined using a JEOL JSM-6000F scanning electron microscope (SEM; Tokyo, Japan) at an accelerating voltage of 3.3 kV after coating with Pt by the ion-sputtering method.

Solubility measurement. The CMS, control and Zein-CMS films (10 × 10 mm²) were each weighed, suspended in 5 ml of distilled water, and heated at 50°C, 70°C, or 90°C for 15 min with stirring at 500 rpm. The solubility was evaluated on the basis of the saccharide concentration in the supernatant obtained by centrifugation (18,000 rpm at 20°C), as determined by the phenol-sulfuric acid method.9

Digestion with enzymes. The digestibility of the Zein-CMS film with α-amylase (EC 3.2.1.1, Sigma, St. Louis, MO, U.S.A.), β-amylase (EC 3.2.1.2, Sigma), or actinase (Kaken Pharmacy Co., Tokyo, Japan) was measured as described previously.5,7,10

Digestion with α-amylase: A sample (2 mg) was dispersed in 1.8 ml of a 0.02 M sodium citrate buffer (pH 6.5) containing 0.1 M sodium chloride. α-Amylase (0.2 units) in 0.2 ml of the buffer was then added, and the reaction mixture was incubated at 30°C for 60 min. The digestibility was evaluated on the basis of the saccharide concentration in the filtrate obtained by passing the sample through a 0.45 μm pore-size membrane filter; saccharides were determined by the phenol-sulfuric acid method.9

Digestion with β-amylase: A sample (2 mg) was dispersed in a mixture of 1.9 ml of distilled water and 0.1 ml of a 1 M sodium acetate buffer (pH 6.0), β-Amylase (10 units) in 0.1 ml of the buffer was then added, and the reaction mixture was incubated at 30°C for 60 min. The digestibility was evaluated as just described.

Digestion with actinase: A sample (100 mg) was dispersed in 0.1 M Tris- HCl buffer (pH 7.8) containing 5 mM calcium chloride. Actinase (5 mg) were then added, and the reaction mixture was incubated at 30°C for 24 h. The solubilized material was evaluated as already described.

Measurement of the water vapor permeability. The water vapor permeability of the Zein-CMS film was measured by using a 70 μl-silver capsule containing 50 μl of distilled water, this being covered with the film and sealed with an adhesive. The sealed capsule was incubated at 50°C for the indicated time (0–120 min) and weighed. The water vapor permeability
Fig. 1. Scanning Electron Micrographs of the Zein-CMS Film.

The Zein-CMS film, a zein-conjugated carboxymethyl corn starch film, was prepared by using 70% ethanol as the solvent. A, surface; B, cross-section.

Fig. 2. Optical Micrographs of the Cross-section of the CMS, Control, and Zein-CMS Films Stained with CBB.

CMS film: prepared from carboxymethyl corn starch; control film: prepared without conjugation by using 70% ethanol as the solvent; Zein-CMS film: a zein-conjugated CMS film prepared by using 70% ethanol. The arrow heads show the surface of the film. Direct magnification × 400.

Table 1. Protein Content of the Starch-Based Films

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<tr>
<th>Film</th>
<th>Protein content (%)</th>
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<tr>
<td>CMS(^a)</td>
<td>0.13</td>
</tr>
<tr>
<td>Control(^b)</td>
<td>0.18</td>
</tr>
<tr>
<td>Zein-CMS(E)(^c)</td>
<td>0.55</td>
</tr>
<tr>
<td>Zein-CMS(A)(^d)</td>
<td>0.71</td>
</tr>
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</table>

\(^a\) Prepared from carboxymethyl starch.
\(^b\) Prepared without conjugation by using 70% ethanol as the solvent.
\(^c\) Prepared by using 70% ethanol as the solvent.
\(^d\) Prepared by using 70% acetone as the solvent.

was evaluated on the basis of the decrease in weight.

**Analytical methods.** The total saccharide concentration was measured by the phenol-sulfuric acid method\(^9\) using glucose as the standard. The protein content was measured by the rapid micromethod for the determination of nitrogen,\(^11\) with 6.25 being used as the conversion factor for nitrogen to protein.

**Results and Discussion**

**Features of the zein-conjugated CMS film**

The CMS film was prepared from the CMS solution by drying it in a warm oven. The zein-conjugated CMS (Zein-CMS) film was prepared by acid-amide cross-linking between the carboxyl groups of the CMS film and the amino groups of zein with EDC in 70% ethanol or 70% acetone, and with subsequent air-drying. A control film was also prepared by treating it with zein dissolved in 70% ethanol in the absence of EDC. Every film containing the Zein-CMS film was colorless and transparent. The thickness of the films were 28–41 μm, the Zein-CMS films being slightly thicker than the CMS film, probably due to some shrinkage as a result of drying twice during their preparation. SEM images of the Zein-CMS films showed a smooth surface and a dense internal structure with few cavities (Fig. 1). In order to examine the degree of conjugation of zein and its location, cross-sections of the control film and the Zein-CMS film stained with CBB were observed under an optical microscope (Fig. 2). The CMS film and the control film showed no CBB-staining, whereas the surface of the Zein-CMS film was clearly stained with CBB. This observation indicates that there were zein molecules attached to the surface of the CMS film, presumably through a acid-amide linkage.

The protein content of the films is summarized in Table 1. The CMS film contained a small amount of protein derived from the original corn starch. Since the protein content of the control film was slightly greater than that of the CMS film, a small amount of adsorbed zein seems to have remained on the film after washing it well. The protein content of the Zein-CMS films was in the range of about 0.6–0.7%. The Zein-CMS film prepared by using acetone as the reaction solvent had a somewhat greater protein content than that prepared by using ethanol, probably due to the larger size of the aggregates in acetone than those in ethanol.\(^3\) The amount of conjugated zein was estimated to be only about 0.4–0.6%, this being less than expected.
Reduced solubility

The solubility of the films was examined by incubating each film in water at 50–90°C for 15 min. During the incubation period, the CMS film and the control film became extremely swollen within a short time, whereas the Zein-CMS film swelled to a very limited extent (data not shown). The solubility of the CMS film was about 25% at 50°C, indicating that the film was not water-proof, and it increased with increasing temperature (Fig. 3). The control film had somewhat lower solubility than the CMS film due to a small amount (0.05%) of residual adsorbed zein, but its solubility also increased to about 20% at 90°C. On the other hand, the Zein-CMS film was insoluble in water over the temperature range tested, indicating that it was practically stable in hot water. The limited swelling and insolubility of the Zein-CMS film were attributed to the small amount of hydrophobic protein on the surface of the hydrophilic CMS film that was conjugated through an acid-amide linkage.

Permeation of water vapor

The permeation of water vapor through the Zein-CMS film was measured to evaluate the increased hydrophobicity of the CMS film after its conjugation with the hydrophobic zein molecules and aggregates. As a control, a silver capsule containing distilled water was sealed with polyvinylidene chloride and heated at 50°C. The capsule showed no change in weight over a 120 min period (Fig. 4), indicating complete sealing of the capsule. It is thus considered that the permeation of water vapor through the CMS-based films could be evaluated in this manner. The amount of permeated water vapor through each film increased linearly with increasing incubation time. The CMS film showed the highest permeation (the initial permeation rate after 30 min, which was calculated as water vapor transfer rate, was about 0.057 g/sec·m²). Since the control film showed permeation similar to that of the CMS film, it is considered that the zein remaining on the CMS film did not influence the permeation. On the other hand, the CMS-Zein films, particularly that prepared with 70% ethanol as the solvent, showed remarkably low permeation (the initial permeation rate after 30 min was about 0.041 g/sec·m²) as compared with the CMS film. The aggregates of zein formed in 70% acetone are more bulky than those formed in 70% ethanol³. The film prepared with ethanol may have had smaller interstitial pores than the film prepared with acetone, and this might explain why the water vapor permeation for the film prepared with ethanol is lower than that of the film prepared with acetone. In any case, it was evident that the hydrophobic zein molecules conjugated with the starch molecules on the surface of the CMS film markedly reduced the permeation of water vapor through the film, confirming that the method employed would be effective for increasing the hydrophobicity of the CMS film, even though the amount of conjugated zein was relatively small (0.4–0.6%). Furthermore, an additional conjugating treatment of the film with zein may result in a further decrease in the water vapor permeation.

Digestibility with enzymes

The digestibility of the CMS film, control film, and Zein-CMS film with α-amylase, β-amylase, and α-amylase was examined. The CMS film and the control film were both susceptible to digestion with α-amylase and β-amylase (about 60–90% digestibility) (Fig. 5). As compared with those films, the Zein-CMS film showed extremely low digestibility by both
Fig. 5. Digestibility of the CMS, Control, and Zein-CMS Films by $\alpha$-Amylase (⅓) and $\beta$-Amylase (■).

CMS film: prepared from carboxymethyl corn starch; control film: prepared without conjugation using 70% ethanol as the solvent; Zein-CMS(E) film: a zein-conjugated CMS film prepared by using 70% ethanol; Zein-CMS(A) film: a zein-conjugated CMS film prepared by using 70% acetone. Digestibility is expressed as the percentage of digested saccharides.

Fig. 6. Change in Solubility of the CMS, Control, and Zein-CMS Films upon Digestion with Actinase.

■ before actinase-digestion; □ after actinase-digestion.

CMS film: prepared from carboxymethyl corn starch; control film: prepared without conjugation using 70% ethanol as the solvent; Zein-CMS(E) film: a zein-conjugated CMS film prepared by using 70% ethanol; Zein-CMS(A) film: a zein-conjugated CMS film prepared by using 70% acetone. Solubility is expressed as the percentage of dissolved saccharides.

Amylases. The conjugated protein moiety on the surface of the film is thought to have been responsible for the indigestibility of the Zein-CMS film by inhibiting the activity of the amylases. To eliminate the protein moiety from the surface, the Zein-CMS film was digested with actinase, an acidic protease, at 30°C for 24 h. The actinase-digested Zein-CMS film was found to be readily soluble in water (Fig. 6), whereas the solubility of the other films was not affected by actinase digestion. It is thus considered that the Zein-CMS film is a biodegradable material, and that zein-conjugation could be applied to achieve an increase in the hydrophobicity of such starch-based articles as compost bags, wrapping films, and molded articles. These findings demonstrate the feasibility of applying zein-conjugation as a useful method for preparing biodegradable starch-based articles.

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References