Influence of Succinylation on the Properties of Cast Films from Red Bean Protein Isolate at Various Plasticizer Levels

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ABSTRACT: The effects of succinylation at three anhydride levels (0.2, 0.4, and 0.6 g g^{-1}) on the properties of cast films from red bean protein isolate (RPI) at three glycerol levels of 0.2, 0.4, and 0.6 g g^{-1} were investigated. The tested properties included tensile strength (TS) and elongation at break (EB), surface hydrophobicity, moisture content (MC), total soluble matter (TSM), water vapor transmission rate, and permeability (WVTR and WVP), permeability coefficient of oil (PO). The results showed that the succinylation greatly improved the mechanical properties (especially the EB), but decreased the surface hydrophobicity of cast films. The MC, TSM, WVTR, WVP, and PO were considerably increased by the succinylation. The size exclusion chromatography analysis indicated that the succinylation resulted in protein aggregation or association, and transformation of insoluble precipitates (initially present) to soluble protein components. The dependence of the influence of succinylation upon some selected properties on the plasticizer level suggests interactions between introduced anionic succinic moieties and the hydroxyl groups of the plasticizer. These results suggest that the succinylation treatment could be applied to modify the mechanical properties of legume proteins, especially in the case that requires excellent flexibility of cast films. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 1934–1941, 2011

Key words: cast protein film; red bean protein isolate; legume; succinylation; plasticizer level

INTRODUCTION

There is an increasing interest in developing environment-friendly and even edible materials on natural biopolymers (e.g., protein and polysaccharide), in an attempt to substitute petroleum-based plastics or films. A number of food proteins, derived from both animal and plant origins, have been applied as material matrices for preparing biodegradable and even edible films, including casein, milk whey proteins, gelatin, corn zein, wheat gluten, soy protein, peanut protein, legume protein, hemp protein, and others.[1–11] In general, protein-based films exhibited good barrier properties against oxygen and organic vapors, especially at low relative humidity (RH) conditions, but poor water vapor barriers. The poor vapor barrier property is due to inherent hydrophilic nature of proteins. Additionally, the mechanical properties of these films are relatively weak relative to those synthetic biopolymers. These poor properties of protein films greatly limit their applications in food packaging.

A number of modifications using physical, chemical, and enzymatic treatments have been applied to improve the mechanical and barrier properties of protein films. Of all these treatments, heat curing and crosslinking treatments are the two most widely investigated techniques.[12–15] The mechanical strength and barrier properties of protein films can be to a variable extent improved by these two types of treatments. However, in many cases, the flexibility or elongation properties of the films on the contrary decreased. The decreases in elongation of the films would be unfavorable for the processing and application of the films, since the films with decreased elongation will be brittle in nature. To date, all these studies of property modification of the films are carried out during the film-forming process, which seems to be not easily controlled. For example, the addition of acetic and succinic anhydrides to film-forming solutions increased the water solubility, but did not affect other properties of soy protein films, whereas the addition of formaldehyde resulted in considerable increases in mechanical strength and concurrent decreases in water solubility and water vapor permeability.[16] Another strategy is to modify

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the conformational flexibility of the proteins before film casting, in an attempt to improve the properties of final cast films. Surprisingly, there are few available literatures addressing this issue.

On the other hand, the influence of acylation and succinylation in particular on the physicochemical and conformational properties of many food proteins has been widely investigated.[17–20] It was shown that the acylation treatment results in considerable changes in physicochemical and conformational properties, due to the substitution of cationic amino groups with anionic succinic moieties or neutral acetyl moieties. Some of the changes, e.g., increases in net negative charge and protein solubility at around pH of film-forming solutions would be favorable for the film-forming properties of the proteins.

In our previous work, we investigated the properties of three selected protein isolates from *Phaseolus* legumes to form films, and compared these properties with those of soy protein isolate (SPI) films, and found that the mechanical properties of these protein isolate films are much poorer than those of SPI films, but the hydrophobic properties are similar.[11] A heating curing treatment remarkably improved the tensile strength (TS) of these protein isolate films, with the extent much higher than that of SPI films, and concomitantly, the surface hydrophobicity considerably decreased. In this case, the elongation at break (EB) was almost unaffected by the heating. This seems to be the first report addressing the cast films from legume storage proteins. With enhanced interest in the utilization of these legume proteins in the food industry, it would be interesting to further develop edible films based on these legume proteins.

The objective of the present work was undertaken to investigate the influence of succinylation treatment at various anhydride-to-protein ratios on the properties of cast films from red bean protein isolate (RPI). The RPI was chosen, due to the consideration that the mechanical properties of the corresponding films were poorest among the three protein isolates from kidney, mung, and red beans.[11] The tested film properties included TS and EB, surface hydrophobicity, moisture content, and total soluble matter, as well as water vapor barrier properties. The size exclusion chromatography was applied to indicate the extent of succcinylation-induced protein aggregation in the cast films.

EXPERIMENTAL

Preparation of red bean protein isolate (RPI)

Red bean (*Phaseolus angularis*), cultivated in Shandong province of China were purchased in a supermarket in Guangzhou of China. The RPI were prepared from the defatted flour, according to the same process as described by our previous paper.[21] Briefly, the defatted flour was dispersed with 10-fold distilled water. The dispersion was then adjusted to about pH 8.0 with 2 mol/L NaOH, and magnetic-stirred at room temperature for more than 2 h, and then centrifuged at 9000*g* for 30 min to obtain the protein supernatant. The supernatant was further adjusted to pH 4.6 using 2 mol/L HCl, and placed at 4°C for 2 h, and then centrifuged at the same condition. The obtained precipitate was washed with precooled water for several times, and redispersed in distilled water, and adjusted to neutral pH. Finally, the protein dispersion was dialyzed three times at 4°C against desalted water (1 : 100, v/v, three times), and then lyophilized to yield the protein isolate (RPI). The protein content for the protein isolate was about 92.5% (dry basis), as determined by micro-Kjeldahl method, using a nitrogen conversion factor of 6.25.

Preparation of succinylated RPI products and determination of *N*-succinylation

Succinylated RPI products were prepared according to the method described by El-Adawy.[22] RPI (4%, w/v) was dispersed in distilled water, with 1 h of magnetic stirring. The pH of the dispersions was adjusted to about 8.0 with 2*M* NaOH. The succinylation was accomplished by gradual addition of small amounts of solid succinate anhydride up to the required levels (0.2, 0.4, and 0.6 g/g of protein) to rapidly stirred protein dispersions. The pH was maintained between 7.5 and 8.5 by the addition of 2*M* NaOH, during the reaction. After the pH was balanced at around 8.0, the dispersions were kept for 2 h to make the reaction go to completion. Then, the pH of the dispersions was adjusted to 7.0 using 2*M* HCl to stop the reaction. Last, the dispersions were dialyzed against deionized water for 48 h at 4°C to remove the impurities, and freeze-dried to produce succinylated RPI samples.

The extent of succinylation was determined using the method with 2, 4, 6-trinitrophene sulfonic acid (TNBS; purchased from Sigma Co., St. Louis, MO).[22] In brief, aliquots (1 mL) of protein dispersions (0.1%, w/v) in 50 m*M* NaCl solution (pH 9.0) containing 0.29% (w/v) sodium dodecyl sulfate (SDS), were mixed with 1 mL of 50 m*M* Na_2HPO_4 and 1 mL of 0.1% (w/v) TNBS solution. The resultant mixtures were incubated in a 60°C water bath for 2 h in the dark, and then cooled to room temperature. One milliliter of 10% SDS solution and 0.5 mL of 1*M* HCl solution were added to the individual mixtures. The absorbance of the final mixtures was read at 335 nm in a spectrophotometer against a reagent blank without protein. The absorbance of the control protein was defined as 100% free amino groups, and the degree of succinylation for various succinylated RPI

samples was calculated by percent decrease in absorbance relative to that of the control.

Film preparation

The film-forming solutions were prepared by dispersing individual protein products (5%, w/w) and 0.2–0.6 g of glycerol per gram of protein in distilled water. The dispersions were magnetically stirred for 30 min at room temperature. The pH of the dispersions was adjusted to 9.0 (±0.1) with 1*M* NaOH. The resultant film-forming solutions were incubated at 90°C for 30 min in a shaking water bath, and then centrifuged at low speed (100*g*, 10 min) to remove the bubbles in the solutions (after cooling to room temperature). Last, the film-forming solutions were cast into rimmed, leveled glass plates coated with polyethylene films (Clorox Cbina, Guangzhou, China). The film thickness was controlled by casting the same volume of the solutions on each plate (18 × 20 cm). The castings were air-dried at room conditions [25 ± 1°C, 50 ± 5% relative humidity (RH)] for 36 h, and then the films were peeled off the plates and various specimens for physical properties testing cut. Specimens of 2.5 × 10 cm rectangular strips were cut for tensile testing. All the films were stored in a desiccator containing magnesium nitrate saturated solution [$Mg(NO_3)_2.6H_2O$] with a RH of 50% for at least 2 days, before the measurements of individual properties.

Film thickness determination

Film thickness was measured with a hand-held micrometer to the nearest 0.001 mm. Five measurements were taken on each tensile testing specimen along the length of the strip with the mean value used in tensile strength calculations.

Tensile strength (TS) and elongation at break (EB)

TS and EB of the films were measured using a TA-XT2i texture analyzer (Stable Micro Systems, London, UK). Initial gap separation and cross-head speed were set at 50 mm and 1 mm/s, respectively. TS was calculated by dividing the maximum load at break by initial specimen cross-sectional area.[23] EB was calculated by dividing by the extension at break of the specimen by the initial gauge length of the specimen (50 mm) and multiplying by 100.[23] Each data was the mean and standard deviation of at least six determinations.

Surface hydrophobicity

The surface hydrophobicity of the films was estimated by the sessile drop method, based on optical contact angle method. Contact angle measurements were carried out with an OCA 20 AMP (Dataphiscis Instruments GmbH, Germany). A droplet of deionized water (4 μL) was deposited on the film surface with a precision syringe. The drop image was recorded by a video camera, and the profile of droplet was numerically solved and fitted to laplace-young equation. Ten parallel measurements were performed for each film. The surface in contact with LDPE support during drying will be referred as the "bottom side" in this study and the other side in contact with the air during drying will be referred as "top side." At least 10 parallel measurements were performed for each side each film.

High-performance size exclusion chromatography (HPSEC)

HPSEC experiments were performed using a TSK G4000SW column (TosoH Biosep, Montgomeryville, PA), connected to a waters high-performance liquid chromatography (HPLC) equipped with a Waters 2487 HPLC pump and a waters 1525 UV-visible detector operating at 280 nm. The column was equilibrated and eluted with 50 m*M* phosphate buffer (pH 7.2) containing 50 m*M* NaCl at an elution rate of 0.8 mL/min at room temperature. The protein samples for HPSEC analysis were prepared by solving dry untreated and succinylated RPI cast films (after storage for 3 weeks under P_2O_5) into the same buffer. The protein concentration of all the samples was adjusted to about 10.0 mg/mL, and the amount of injected samples was 20 μL.

Moisture content (MC) and total soluble matter (TSM)

The MC was determined from preweighed film samples (±0.0001 g) dried in an air-circulating oven at 105°C for 24 h, as the percentage of initial film weight lost during drying and reported on wet basis.

The TSM was measured by immersing three broken specimens (obtained from MC measurements) in 30 m*M* of distilled water (25°C) for 24 h, with occasional gentle stirring. The insoluble dry matter was measured by removing the film pieces from the beakers, gently rinsing them with distilled water, and then drying them in an air-circulating oven (at 105°C for 24 h). The weight of soluble dry matter was calculated by subtracting the weight of insoluble dry matter from the initial weight of dry matter.

Water vapor transmission rate (WVTR), water vapor permeability (WVP)

The WVTR and WVP of films were measured using the ASTM method.[24] Circular plastic cups with

diameter of 3 cm and depth of 5 cm were used. Three grams of $CaCl_2$ were placed in each cup, and the cups were covered with circular films with diameter of 7 cm. Sealed cups were preweighed with their contents and placed in a desiccator kept at 25°C. One liter of pure water was placed in the bottom for providing 100% RH at 25°C. Then, the cups were weighed every 12 h for a week. The WVTR and WVP of films were measured from the weight gain of the cups. The WVTR (g h^{-1} m^{-2}) and WVP (g mm m^{-2} h^{-1} kPa^{-1}) was calculated as eqs. (1) and (2):

$$\mathrm{WVTR} = W/t \times S \tag{1}$$

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \cdot L}{\Delta P} \tag{2}$$

where W is the increased water weight (g), t is the time (h), S is the film area covered in the mouth of cups (m^2), L is the mean film thickness (mm); $\triangle p$ was the partial water vapor pressure difference (kPa) across the two sides of the film specimen (the vapor pressure of pure water at 25°C = 3.1671 kPa).

Lipid barrier property

The permeability coefficient of oil (PO) was used as the indication for lipid barrier property of films. Tubules with 5 mL salad oil were sealed with circular film specimen with diameter of 4 cm and upside down on filter papers, and then stored in a chamber at 40% RH and 25°C with filter papers together. The weight of filter papers was recorded every day for a week. The PO value was calculated from Eq. (3):

$$\mathrm{PO} = \frac{\Delta W \times FT}{ST} \tag{3}$$

where $\triangle W$ is the weight variance with time (g); FT is the mean film thickness (mm); S is the film area covered in the mouth of tubules (mm^2); T is the time (days). Slopes of the steady state (linear) portion of weight gain versus time curves were used to estimate $\frac{\Delta W}{T}$.

Statistical analysis

An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) or Tamhane's with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Characterization of succinylated RPI products

The protein content of RPI applied in the present study was about 92% (dry basis). Treatment of this RPI with 0.2, 0.4, and 0.6 g g^{-1} protein of succinic anhydrides resulted in 74.5, 87.3, and 91.8% succinylation of the available amino groups, especially the ε-amino groups of lysine residues (data not shown). The extent of chemical modification for RPI succinylated at anhydride level of 0.4 g g^{-1} protein was slightly but insignificantly lower than that at anhydride level of 0.6 g g^{-1} protein, indicating that the anhydride level of 0.4 g g^{-1} protein might be high enough to ensure most of available amino groups to be succinylated.

The extent of chemical modification for succinylated RPI at anhydride level of 0.2 g g^{-1} protein was much less than that for kidney protein isolate (KPI) at the same anhydride level,[20] but higher than that for soy protein[17] and canola 12S globulin.[18] The differences are associated with the differences in extent of availability of the amino groups between various proteins.

Mechanical properties (TS and EB)

Table I shows the TS and EB of cast films from untreated and succinylated RPI samples at three glycerol levels (0.2, 0.4, and 0.6 g/g of protein), respectively. As expected, the TS of tested films from a given protein sample progressively decreased with increasing the applied glycerol level, while the EB on the contrary increased. This is in agreement with the general viewpoint that lower TS and higher EB are observed for protein films at higher plasticizer level. Plasticizers usually increase film flexibility due to their ability to reduce internal hydrogen bonding between polymer chains while increasing spacing.[25] However, the extent of decrease or increase varied with the type of applied RPI sample and the level of applied plasticizer. For example, the TS of unheated RPI films progressively and significantly ($P < 0.05$) decreased with the glycerol level increasing from 0.2 to 0.6 g g^{-1}, whereas in succinylated RPI cases, the TS was significantly decreased when the glycerol level was significantly increased from 0.2 to 0.4 g g^{-1}, but there was no significant decrease in TS upon further increasing the glycerol level to 0.6 g g^{-1} (Table I). A prominent increase in EB upon increasing the glycerol was also observed for untreated RPI film, while in the succinylated RPI films, there were no significant differences among various glycerol levels. The results indicated that the succinylation treatment remarkably changed the dependence behaviors of the mechanical properties of RPI films upon the plasticizer level.

At any test glycerol level, the TS of RPI films was significantly ($P < 0.05$) increased by the succinylation only at anhydride levels higher than 0.2 g g^{-1}, as compared with that of control. However, the extent of improvement was relatively limited (about

TABLE I
The Mechanical (TS and EB) and Surface Hydrophobic Properties (Contact Angle) of Cast Films from Untreated and Succinylated RPI Products

Items		Glycerol level (g g^{-1})	Untreated RPI films (control)	Succinylated RPI films (anhydride level, g g^{-1}) 0.2	0.4	0.6
TS (MPa)		0.2	$1.63 \pm 0.15^{b,e}$	$1.63 \pm 0.12^{b,e}$	$1.82 \pm 0.10^{a,e}$	$1.82 \pm 0.09^{a,e}$
		0.4	$1.22 \pm 0.08^{b,f}$	$1.17 \pm 0.07^{b,f}$	$1.48 \pm 0.11^{a,f}$	$1.45 \pm 0.11^{a,f}$
		0.6	$1.01 \pm 0.10^{b,g}$	$0.98 \pm 0.11^{b,f}$	$1.33 \pm 0.14^{a,f}$	$1.37 \pm 0.12^{a,f}$
EB (%)		0.2	$58.2 \pm 8.2^{c,g}$	$199.6 \pm 21.1^{a,e}$	$172.1 \pm 25.6^{a,e}$	$132.1 \pm 18.6^{b,e}$
		0.4	$75.4 \pm 7.5^{c,f}$	$217.4 \pm 14.3^{a,e}$	$201.4 \pm 17.2^{a,e}$	$152.4 \pm 15.3^{b,e}$
		0.6	$91.1 \pm 9.3^{c,e}$	$220.6 \pm 19.5^{a,e}$	$200.7 \pm 17.7^{a,e}$	$161.1 \pm 14.7^{b,e}$
Contact angle (°)	Top	0.2	$77.3 \pm 2.7^{a,g}$	$70.3 \pm 3.7^{a,e,f}$	$63.3 \pm 3.0^{b,f}$	$55.3 \pm 4.5^{c,f}$
		0.4	$85.0 \pm 2.1^{a,f}$	$75.0 \pm 4.6^{b,e}$	$70.1 \pm 3.9^{b,e}$	$65.9 \pm 3.2^{b,e}$
		0.6	$93.3 \pm 2.5^{a,e}$	$80.2 \pm 4.2^{b,e}$	$74.5 \pm 4.6^{c,e}$	$70.0 \pm 4.0^{c,e}$
	Bottom	0.2	$70.5 \pm 2.6^{a,g}$	$57.3 \pm 3.6^{b,f}$	$43.1 \pm 4.3^{c,f}$	$22.8 \pm 4.6^{d,g}$
		0.4	$79.0 \pm 2.2^{a,f}$	$65.3 \pm 3.9^{b,e}$	$50.6 \pm 4.2^{c,f}$	$35.7 \pm 3.2^{d,f}$
		0.6	$87.0 \pm 2.9^{a,e}$	$71.3 \pm 3.7^{b,e}$	$62.3 \pm 4.6^{c,e}$	$51.5 \pm 4.8^{d,e}$

The data for TS and EB are the means and standard deviations of more than six determinations, and that for contact angle the means and standard deviations of more than 10 determinations.
[a-d] Significant difference at $P < 0.05$ level among untreated and/or succinylated RPI samples.
[e-g] Significant different at $P < 0.05$ level among various glycerol levels, for any item (TS, EB, and contact angle) of a given cast film from the same protein.

12–35%). Furthermore, there were no significant differences in TS between the anhydride levels of 0.4 and 0.6 g g^{-1} (Table I). In contrast, the succinylation resulted in remarkable increase in ES of RPI films, with the extent of increase (2.1–3.4 times) depending on the anhydride level and glycerol level (Table I). At any test glycerol level, highest extent of increase was observed for succinylated RPI film at 0.2 and 0.4 g g^{-1} anhydride levels, and the EB at 0.6 g g^{-1}anhydride level was on the contrary significantly lower than that at 0.2 or 0.4 g g^{-1} anhydride levels (Table I). Moreover, the improvement of EB was more distinct at lower glycerol level (e.g., 0.2 g g^{-1}) than that at higher glycerol level (e.g., 0.6 g g^{-1}). These results indicated that the mechanical properties and EB in particular of RPI films were greatly improved by the succinylation treatment, especially at relatively low levels (e.g., 0.2–0.4 g g^{-1}). The improvements of mechanical properties by the succinylation seem to be associated with the protein-protein association or aggregation induced by the succinylation. However, it should be kept in mind that severe protein aggregation by the succinylation would be unfavorable for the improvement of mechanical properties.

To elucidate the succinylation-induced aggregation, we analyzed the molecular weight distribution of soluble proteins of untreated and succinylated RPI films in 50 m*M* phosphate buffer (pH 7.2) containing 50 m*M* NaCl using SEC technique, as shown in Figure 1. In the untreated RPI case, there were prominent elution peaks centered at around 17 min and a minor peak at 18 min, which might correspond to the 7S (major; vicilin) and 11S (minor) globulins, respectively. The succinylation resulted in progressive decrease in the magnitude of the peak at 18 min, and concomitant increase in the magnitude of the peak at 15 min (Fig. 1). In the succinylated RPI cases, an aggregate peak eluting almost at void time (about 8 min) was also distinctly observed. The phenomena clearly indicated the occurrence of succinylation-induced protein aggregation or association. On the other hand, it can be observed that the magnitude of the peaks eluted in the range 8–16 min progressively increased with increasing the anhydride level from 0.2 to 0.6 g g^{-1}, but the total integrated area under the elution profiles in various succinylated RPI films was similar (Fig. 1). This observation indicated gradual improvement of protein solubility by the

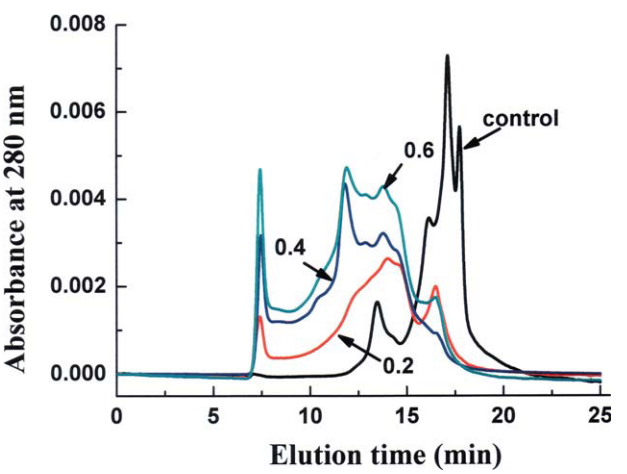

Figure 1 Typical SEC elution profiles of soluble proteins of untreated (control) and succinylated RPI films. The numbers (0.2, 0.4, and 0.6) indicate the level of applied succinic anhydrides (g g^{-1} of protein). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

succinylation, in an anhydride level dependent manner. The improvement of protein solubility by succinylation has also been observed for kidney protein isolate, using SEC analysis,[20] where the appearance of the aggregate peak was more attributed to the transformation of insoluble precipitate by the succinylation, than to succinylation-induced aggregation of the proteins. The present data clearly showed that the succinylation treatment led to both protein aggregation of the proteins and the improvement of protein solubility in the RPI cast films.

Surface hydrophobic properties

The contact angle of water upon the film surface by sessile drop method was applied to evaluate the surface hydrophobic properties of cast protein films. The contact angle was measured in a static manner at time 0 s when the water drop was just deposited onto the test film surface. Usually, a large contact angle represents a hydrophobic surface, whereas a small contact angle implies a hydrophilic surface. The quantitative definition of the relative terms "hydrophobic" and "hydrophilic" surfaces has been done respectively, for surfaces exhibiting a water contact angle $\theta > 65^\circ$ and $\theta < 65^\circ$.[11,26] Table I also displays the initial contact angle values with water for air (top) and support (bottom) sides of untreated (control) and succinylated RPI cast films, at three plasticizer levels of 0.2, 0.4, and 0.6 g g^{-1}, respectively. On the whole, the top side contact angle of any a tested film at a specific plasticizer level was significantly higher than the bottom side contact angle, especially in the succinylated RPI films (Table I). In our previous work, it was also observed that the contact angle on top side of cast films was higher than that on bottom side,[11] where the difference was attributed to the difference of re-orientation of hydrophobic clusters of proteins during film-forming process.

All the cast films exhibited different contact angle values (top or bottom) depending on the plasticizer level and the applied anhydride level (Table I). As for a given RPI cast film, the contact angle (top or bottom) progressively and significantly increased with the plasticizer level increasing from 0.2 to 0.6 g g^{-1}, indicating more exposure of hydrophobic clusters of the proteins at higher plasticizer levels. This may be a result of enhanced plasticizer-protein interactions, and concomitantly lessened protein-protein interactions (especially the hydrophobic interactions), when the plasticizer level is increased. On the other hand, at a given plasticizer level, the contact angle generally progressively decreased with the applied anhydride level from 0 to 0.6 g g^{-1} (Table I), indicating gradual decrease in surface hydrophobicity of the films by the succinylation. This is clearly associated with the increase in net negative charge of the proteins, due to gradual transformation of cationic ε-amino groups of the proteins into anionic succinyl moieties.[11] It can be observed that the extent of decrease in contact angle upon increase in anhydride level was much distinct for the bottom side (40.8–67.7%) than for the top side (22.5–28.5%). This further reflects that more hydrophobic groups of the proteins (that was to a less extent affected by the succinylation) would be located in the air-film interface than in the support-film interface.

Although the contact angle of cast films gradually decreased with increasing the anhydride level, the contact angle on top side was still more than 65°, with an exception for succinylated RPI films at plasticizer level of 0.2 g g^{-1} and at anhydride levels of 0.4 and 0.6 g g^{-1} (Table I). Thus, the surface hydrophobicity for top side of cast RPI films can be considered to be "hydrophobic." In contrast, the contact angle for bottom side of succinylated RPI films at anhydride levels 0.4–0.6 g g^{-1} and at all test plasticizer levels was less than 65°, and the support side of these films thus can be considered to be "hydrophilic."

MC and TSM

The MC and TSM data of untreated (control) and succinylated RPI cast films are shown in Table II. The MC data of untreated RPI and succinylated RPI films, determined after conditioning at 50% RH and 25°C for 2 days progressively increased with the glycerol level increasing from 0.2 to 0.5 g g^{-1}. This is because the glycerol molecules at higher levels have more hydroxyls to adsorb or trap more water molecules (at a given RH condition). At any applied plasticizer level, the MC of succinylated RPI films was significantly ($P < 0.05$) higher than that of untreated films (control), but the extent depending on the applied plasticizer level (Table II). Highest MC values were observed for succinylated RPI films at 0.2 g g^{-1}plasticizer level, and the MC of succinylated RPI films gradually decreased with increasing the plasticizer level from 0.2 to 0.6 g g^{-1}. The increase in MC by the succinylation could be attributed to enhanced charged groups, e.g., anionic succinyl moieties. The gradual decline in MC of succinylated RPI films upon the increase in applied anhydride level may reflect that there would be interactions between anionic succinyl moieties and the hydroxyls of glycerol, and the magnitude of the interactions seem to increase with the increase in applied anhydride level, and as a consequence, the relatively content of glycerol molecules (to adsorb or entrap water) decreases.

The TSM (about 25–26%) of untreated RPI films was independent of the applied plasticizer level (Table II). In contrast, all the succinylated RPI films were completely soluble in the water medium. The

TABLE II
MC, TSM, WVTR, and WVP for Cast Films from Untreated and Succinylated RPI Products

Items	Glycerol level (g g^{-1})	Untreated RPI films (control)	Succinylated RPI films (anhydride level, g g^{-1})		
			0.2	0.4	0.6
MC (%)	0.2	$19.3 \pm 1.2^{c,g}$	$22.7 \pm 0.6^{a,g}$	$21.9 \pm 0.7^{a,g}$	$20.9 \pm 0.7^{b,c,g}$
	0.4	$23.5 \pm 2.0^{d,f}$	$30.3 \pm 1.0^{a,f}$	$28.4 \pm 1.2^{b,f}$	$27.0 \pm 0.9^{b,c,f}$
	0.6	$30.1 \pm 2.2^{c,e}$	$41.1 \pm 1.3^{a,e}$	$39.4 \pm 1.3^{a,e}$	$36.3 \pm 1.1^{b,e}$
TSM (%)	0.2	26.4 ± 2.0	CS	CS	CS
	0.4	25.9 ± 2.2	CS	CS	CS
	0.6	25.5 ± 2.0	CS	CS	CS
WVTR (g/h.m^2)	0.2	$17.8 \pm 1.8^{b,g}$	$38.8 \pm 1.1^{a,e}$	$39.5 \pm 1.4^{a,e}$	$39.6 \pm 1.3^{a,e}$
	0.4	$22.9 \pm 2.1^{b,f}$	$40.1 \pm 1.1^{a,e}$	$40.2 \pm 1.3^{a,e}$	$40.2 \pm 1.3^{a,e}$
	0.6	$28.5 \pm 2.3^{b,e}$	$40.3 \pm 1.3^{a,e}$	$40.7 \pm 1.4^{a,e}$	$40.8 \pm 1.4^{a,e}$
WVP (g.mm/kPa.h. m^2)	0.2	$0.5 \pm 0.0^{b,g}$	$1.0 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$
	0.4	$0.6 \pm 0.1^{b,f}$	$1.1 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$
	0.6	$0.8 \pm 0.1^{b,e}$	$1.1 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$	$1.1 \pm 0.0^{a,e}$
Permeability coefficient of oil (PO) (g.mm/m^2.d)	0.2	0	$0.4 \pm 0.1^{c,f}$	$1.9 \pm 0.2^{b,f}$	$2.6 \pm 0.3^{a,f}$
	0.4	0	$1.0 \pm 0.1^{c,e}$	$3.2 \pm 0.2^{b,e}$	$4.4 \pm 0.3^{a,e}$
	0.6	0	$0.4 \pm 0.1^{c,f}$	$1.0 \pm 0.1^{b,g}$	$1.5 \pm 0.2^{a,g}$

The data for WVT, WVP, and lipid barrier property are the means and standard deviations of more than three determinations, while that for MS and TSM the means and standard deviations of more than four determinations.

[a-d] Significant difference at $P < 0.05$ level among various untreated and succinylated RPI products (at a given plasticizer level).

[e-g] Significant difference at $P < 0.05$ level among various glycerol levels, for any item (MC, TSM, WVTR and PO) of a given cast film from the same protein.

CS = completely soluble.

phenomenon is well in agreement with the SEC analysis (Fig. 1), confirming that the succinylation resulted in considerable increase in protein solubility, due to transformation of insoluble precipitate (initially present) into soluble protein components.

WVTR, WVP, and PO

The WVTR, WVP and PO of untreated (control) and succinylated RPI films were also evaluated, and the results presented in Table II. At any test plasticizer level, the succinylated RPI films exhibited significantly much higher WVTR and WVP values than the untreated RPI films, but there were no significant differences in WVTR and WVP among various succinylated RPI films (Table II). In the untreated RPI film case, the WVTR and WVP gradually increased with increasing the plasticizer level from 0.2 to 0.6 g g^{-1}, whereas in the succinylated RPI film cases, the WVTR and WVP were almost independent of the applied plasticizer level. The remarkable increase in WVTR and WVP by the succinylation (compared with the untreated RPI films) is possibly because the introduction of anionic succinic moieties would not only lead to enhanced charged negative groups, but also induce protein molecular structural unfolding (more hydrophilic groups would be exposed), which are favorable for water vapor transmission and permeability through the films.[27] However, the influence of the interactions between anionic succinic moieties and the glycerol molecules should be also considered. Thus, the no significant differences in WVTR and WVP among various succinylated RPI films may reflect the counterbalance of the contribution of the anionic succinic moieties (or charged groups) and the glycerol molecules.

The PO of untreated RPI films was nearly zero, irrespective of the applied plasticizer level (Table II), indicating excellent lipid barrier property. However, at any given plasticizer level, the PO progressively and significantly ($P < 0.05$) increased with the anhydride level increasing from 0.2 to 0.6 g g^{-1}, suggesting that the succinylation impaired the lipid barrier property. The decrease in lipid barrier property may be associated with more exposure of hydrophobic clusters to the exterior of the protein molecules, as a result of succinylation-induced structural unfolding, since the hydrophobic clusters would preferably interact with oil phase, through hydrophobic interactions. Interestingly, most distinct decrease in lipid barrier property was observed at the plasticizer level of 0.4 g g^{-1} (Table II). The underlying mechanism is not yet known, but it can be ensured that this phenomenon is associated with balanced interactions between hydrophobic groups of the proteins, and between the plasticizers and the hydrophobic groups of the proteins.

CONCLUSIONS

The succinylation greatly improved the mechanical properties of RPI cast films, especially elongation at

break (EB). The improvements were associated with succinylation-induced protein aggregation and enhanced protein solubility. However, the succinylation resulted in remarkable decreases in surface hydrophobic properties, and concomitant increases in MC, TSM, WVTP, WVP, and PO. The effects of succinylation on these properties of cast RPI films were also to a variable extent dependent upon the applied plasticizer level. It would be interesting work to apply combined succinylation and other treatments (like heat curing) on the properties of cast films from these proteins.

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