

Synergistic Effect of Oleic Acid and Glycerol on Zein Film Plasticization

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ABSTRACT: Oleic acid and glycerol are two different molecules with different plasticization mechanisms, and their effects on the mechanical properties of zein film were investigated. The mechanical parameters of tensile strength (TS) and elongation to break (*E*) were improved by either oleic acid or glycerol in a dose-dependent manner up to 20%. When a combination of the two plasticizers in different ratios was used to make zein films, the highest TS was observed at a ratio of 3:1 of oleic acid to glycerol, and a synergy between them was revealed through a statistical analysis. A significant decrease of glass transition temperature (T_g) and different microscopic patterns examined by scanning electronic microscopy (SEM) indicate the supramolecular structure was changed by the plasticizers. A combination of plasticizers with different plasticization mechanisms might be a better way to synergistically improve the mechanical properties of biopolymer-based films for practical applications.

KEYWORDS: zein film, glycerol, oleic acid, synergistic plasticization

■ INTRODUCTION

Zein is the major alcohol-soluble storage protein located in the endosperm of maize (*Zea mays* L.) grains in a form of protein body with a diameter of 1–2 μm .¹ Depending on its solubility in aqueous alcohol solutions, four distinct fractions of α -, β -, γ -, and δ -zein have been identified;^{2,3} α -zein accounts for 75–80% of the total zein protein. The amino acid composition analysis has shown that zein protein comprises a large amount of hydrophobic amino acid residues such as leucine, proline, alanine, and phenylalanine,⁴ which are the structural basis for its significant hydrophobic property. On the basis of the study of Argos et al.,⁵ α -zein has a molecular structure consisting of nine adjacent antiparallel helices that are clustered within a distorted cylinder, and the polar residues are located on the helical surface for intra- and intermolecular hydrogen bonding. Later investigation by Bugs et al.⁶ confirmed the α -helical segments as the basic structural component of zein protein, but revealed an opened and extended structure in alcohol solutions and the apolar property of the side of α -helical segments. This unique structure makes zein proteins readily self-assembled through hydrophobic interaction to form particles or films⁷ that can be used as biodegradable packaging materials or a biocompatible system for drug delivery and controlled release.⁸

The film-forming property of zein protein has long been recognized, and there have been extensive studies focusing on improving application-oriented properties such as mechanical flexibility and water or oxygen barrier property.^{9,10} Although zein protein can readily form films under appropriate conditions, the strong intermolecular forces between the zein protein molecules make the film too brittle for most practical applications. Thus, plasticizers are always used to improve zein film flexibility through modification of its supramolecular structure, decreased interactions between zein protein chains, or other ways to increase the mechanical properties of the zein film. The plasticizers need to have polar functional groups for effective zein plasticization, and a proper balance between polar

and nonpolar functional groups is also required for plasticization efficiency.³ Glycerol, oleic acid, polyethylene glycol, sugars, and fatty acid are the most often used plasticizers to improve the mechanical properties and other characteristics of zein film as reported in the available literature.^{11–13} Apparently, plasticizers with different chemical structures and physicochemical properties will produce different effects on the film properties^{14,15} and a combination of different plasticizers might be a better way to further improve the properties of zein films for practical applications. Indeed, Paramawati et al.¹² found improved mechanical property and water vapor permeability using combined plasticizers of polyethylene glycol (PEG) and lauric acid; Parris and Coffin¹⁶ studied the effect of the mixture of glycerol and poly(propylene glycol) on the tensile strength and elongation to break (*E*) and found that *E* was increased almost 50 times compared to glycerol alone. However, due to the complexity of zein film plasticization with different plasticizers, there are not many mechanistic studies regarding the effect of combination of different plasticizers on zein film plasticization. In the current investigation, glycerol and oleic acid were chosen as representatives of polar and amphiphilic plasticizers to study their combined effects on zein film plasticization to pave a new way for the application of zein proteins, such as antioxidant and antimicrobial packaging materials,^{17,18} that have been claimed as the 21st century industrial biopolymer.¹⁹

■ MATERIALS AND METHODS

Zein protein (soluble in 80–92% ethanol) extracted and purified from corn protein powder was obtained from Wujiang City Bache Pharmaceutical Adjuvant Factory (Wu Jiang, China) with a moisture

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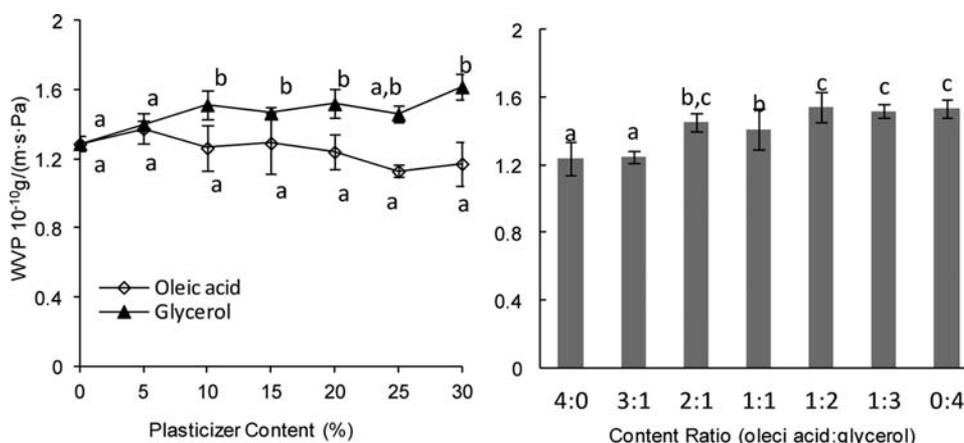


Figure 1. Water vapor permeability of zein film affected by plasticizer of glycerol, oleic acid, and their blend (final content is 20%). Different letters represent significant difference at $P < 0.05$.

content of ~8.0%. The Kjeldahl test showed a nitrogen content of 15.0% (dry weight basis), which is equal to a purity of ~94% converted by a factor of 6.25. Glycerol (ACS reagent, ≥99.5%) and oleic acid (≥99%, GC) were purchased from Sigma-Aldrich (Shanghai, China). The film casting plate with a dimension of 10 × 10 × 1 (length, width, and height in centimeters) was made in our laboratory using poly(methylmethacrylate)-based plexiglass.

Zein Film Casting. Zein protein (1.0 g) was first completely dissolved in 10 mL of 80% ethanol by continuous shaking in a water bath (60 °C), and then plasticizers with different contents (based on weight of zein protein) were added into the zein protein solution followed by further shaking for 30 min. The zein film was cast by slowly pouring zein protein solution into the casting device that was leveled and preheated in a drying oven set at 60 °C. After drying for 2 h in the oven, the zein film was pulled out and conditioned in a desiccator with 50% relative humidity maintained by a saturated salt solution of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ at 25 °C for 2 days before testing. The thickness of the film was measured by taking five points on the film to ensure the thickness difference was within 5 μm. The average of film thickness was around 100 μm.

Water Vapor Permeability (WVP) Measurement. The measurement of WVP was based on ASTM Standard Method E96-95²⁰ with slight modifications. Briefly, 10 g of dried silica was put into a weighing bottle, which was then sealed with a zein film slice of 40 mm × 25 mm by vacuum grease. After the initial weight was recorded, the sealed bottle was conditioned in a desiccator with 80% relative humidity maintained by a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ at 25 °C for 24 h, and then the new weight was recorded. The WVP was calculated according to the equation

$$\text{WVP} = \frac{\Delta m \times L}{A \times t \times \Delta p}$$

where WVP is given in g/(m·s·Pa), Δm is increased weight (g), L is the film thickness (m), A is the area for vapor permeation (m^2), t is the time for vapor permeation (24 h × 3600s/h), and Δp is the water vapor pressure difference across the film (Pa).

Determination of Tensile Strength (TS) and Elongation to Break (E). Five specimens of zein film cut into 20 mm × 70 mm slices were conditioned for 24 h in a desiccator with a relative humidity of 50% before measurement. TS and E were measured by a TA-XT Texture Analyzer (Stable Micro Systems, Surrey, UK) according to ASTM Standard Method D882-88.²¹ Initial grip separation was set at 30 mm, and the test was run at a cross-head speed of 500 mm/min. TS and E were calculated according to the equations

$$\text{TS} = \frac{F}{S} \quad \text{and} \quad E = \frac{\Delta L}{L_0}$$

where TS is given in MPa, F is the force at break (N), S is the area of the cross section of film (m^2), E is given in %, L_0 is the initial length (m), and ΔL is the elongated length (m).

Fourier Transform Infrared Spectrometry (FTIR). A transmission FTIR spectrometer (Nexus 470, Thermo Nicolet Corp., Madison, WI, USA) fitted with attenuated total reflection (ATR) accessory and equipped with a deuterated triglycine sulfate (DTGS) detector was used to scan the zein films from a wavelength of 650 to 4000 cm^{-1} . Triplicate scans were averaged for each sample, and the secondary structures were estimated by second-derivative and Gaussian curve fitting of the amide I band in the regions of 1600–1700 cm^{-1} using OMNIC 6.2 software (Thermo Nicolet Corp.) and PeakFit v 4.12 software (Systat Software Inc., San Jose, CA, USA). Generally, the Gaussian bands at 1652, 1675, 1691, and 1618 cm^{-1} were assigned to α -helical structure, β -turn, β -sheet, and random coil arising from amino acid side-chain vibrations.²² The percentage of secondary structure is calculated on the basis of the area under the curve of deconvoluted Gaussian bands.²³

Glass Transition Temperature Measurement. The glass transition temperature (T_g) of plasticized zein film was measured by a differential scanning calorimeter (DSC) Pyris1 (PerkinElmer Inc., Waltham, MA, USA). The zein film samples were first conditioned in a desiccator with 50% relative humidity for 2 days to balance the effect of moisture on the T_g measurement. Zein film sample (2.0 mg) was put into the aluminum pan, hermetically sealed, and scanned from 30 to 175 °C at a rate of 10 °C/min. The glass transition temperature was analyzed according to a change of heat capacity (C_p) using the PyrisTM software. Four duplications of measurement for each sample were performed.

As the water is a natural plasticizer, the water content of each sample was also determined by measuring the weight difference after drying for 24 h in an air oven set at 130 °C following American Association of Cereal Chemists (AACC) Method 44-15A.²⁴

Scanning Electronic Microscopy (SEM). To analyze the changes of the supramolecular structure of zein protein in the film, cross sections of dried zein films were used as samples for SEM analysis. The sample was first fixed by osmium tetroxide and sputter coated with platinum to a level of 250–500 nm. Scanning electron micrographs were then obtained with a Quanta 200 scanning electron microscope (FEI Co., Switzerland) under a vacuum of 13.33 Pa at an operating voltage of 20 kV.

Statistical Analysis. Multiple replications of measurement with means and standard variation were applied throughout the experiments. The significant difference at $P < 0.05$ was analyzed with an F test for variance provided by Microsoft Excel software, and two-way ANOVA analysis was carried out using SPSS software (version 17.0, IBM) to evaluate interactions of glycerol and oleic acid in zein film plasticization.

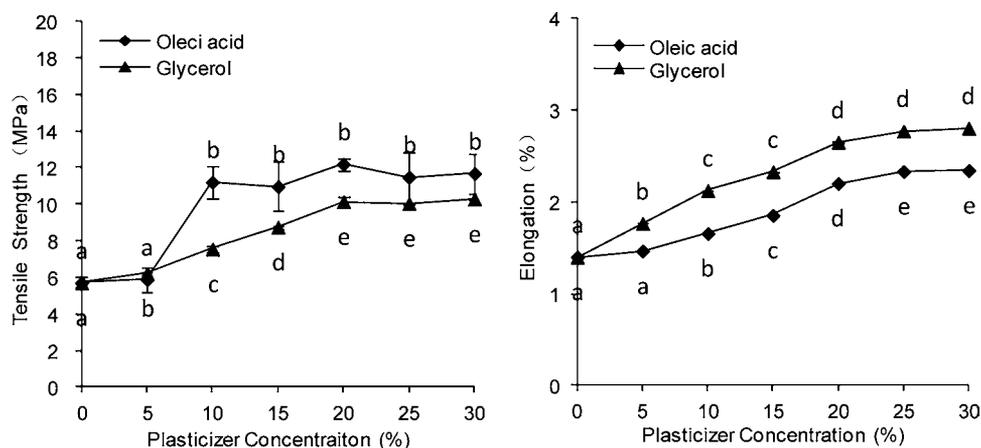


Figure 2. Tensile strength and elongation to break of zein film under different plasticizers with different concentrations. Different letters represent significant difference at $P < 0.05$.

RESULTS AND DISCUSSION

Water Vapor Permeability of Zein Film. The WVP of a film is an important attribute for practical applications, and distinct values of WVP were shown for zein film plasticized by glycerol and oleic acid (Figure 1, left). A dose-dependent increase of WVP was shown for glycerol, whereas the WVP of zein film plasticized by oleic acid showed only a slight decrease, and no statistical significance was detected in the used concentrations. A similar trend was also observed when oleic acid and glycerol were combined as a blended plasticizer in cast zein film (Figure 1, right). Thus, although both glycerol and oleic acid have long been used as plasticizers for zein film plasticization, their different effects on WVP indicate they are two different types of plasticizer in terms of plasticization effect. Actually, according to Hansen's classification system,²⁵ glycerol as a hydrophilic molecule belongs to the secondary plasticizer that has limited effect on zein when used alone due to its incompatibility with zein protein, which would lead to phase separation and increased WVP.^{16,26} In contrast, oleic acid as a hydrophobic molecule is a primary plasticizer, meaning it can effectively plasticize the zein film on its own with a low level of WVP, which is consistent with the literature.^{11,27}

Mechanical Properties of Plasticized Zein Film. Tensile strength and elongation to break are two parameters commonly used to describe the mechanical properties of a film, and the brittleness of nonplasticized zein film is associated with lower tensile strength and poor elongation to break. When glycerol or oleic acid was used as the plasticizer, both TS and E of zein film were increased (Figure 2) within a concentration from 0 to 20% (weight base of zein), and no further increase of TS and E was observed in samples with higher plasticizer contents. Additionally, oleic acid is more effective in improving the TS of the zein film, whereas glycerol is more effective in increasing the E of the film (Figure 2). When a combination of glycerol and oleic acid in different ratios with a total content of 20% was used as the blended plasticizer (Figure 3), the highest TS was shown for the ratio of 3:1 of oleic acid to glycerol, and the lowest TS was shown for glycerol. Thus, oleic acid and glycerol affect the mechanical property of plasticized zein film differently, which is likely associated with their molecular structure and polarity as glycerol has a high polarity, which is hydrophilic, and oleic acid has a lower polarity and is hydrophobic. This difference might result in different modes of interaction with zein protein in which hydrophobic

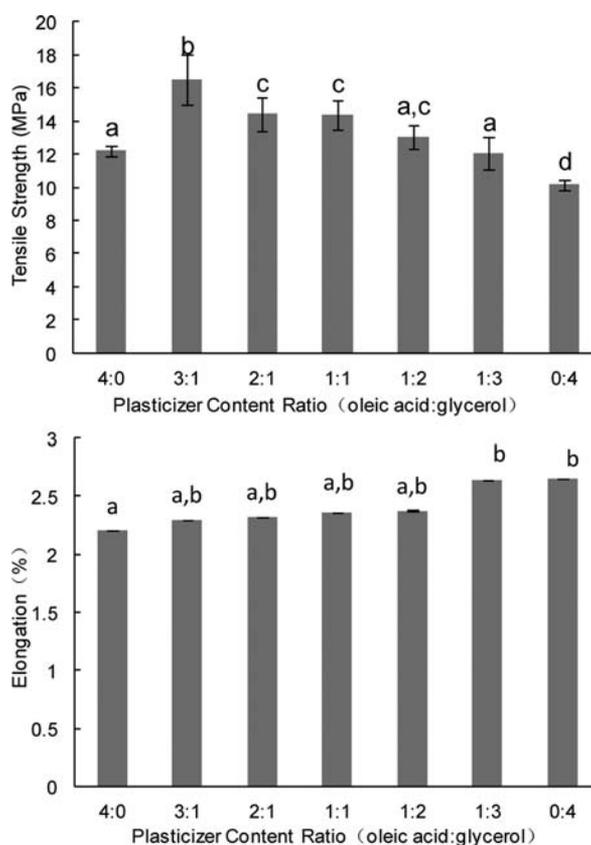


Figure 3. Mechanical property of zein films plasticized with a blend of oleic acid and glycerol at different ratios (final content is 20%). Different letters represent significant difference at $P < 0.05$.

interaction is mainly responsible for the plasticization effect of oleic acid,²⁸ whereas hydrogen bonds formed with amide groups are mainly responsible for the plasticization effect of glycerol.²⁹

It is notable that the parameters of the tensile strength and elongation to break showed similar trends (Figure 2), which is different from the common observations that a large E (in the presence of plasticizers) is generally accompanied by a decrease of TS.^{12,16} To explain this result, the structure of zein protein molecules in the prepared film might be one factor that needs to be considered. The mechanical property of zein film has

been reported to be affected by the drying conditions⁹ and highly ordered zein nanocomposites simultaneously have a high tensile strength and a high elongation to break.³⁰ Thus, the preparation procedure in our used conditions probably leads to a relatively ordered structure of zein protein in the prepared film that showed similar trends in TS and *E* when either oleic acid or glycerol was used as the plasticizer. However, the opposite trends of TS and *E* in the zein films with blended plasticizer of oleic acid and glycerol indicate the detailed mechanism still needs further investigation to achieve a good understanding of the relationship between preparation conditions, mechanical properties, and molecular structure of the zein protein in the film.

The fundamental reason for the application of blended plasticizers in film preparation is to achieve a better result in terms of the mechanical properties of the prepared films, and a synergy between individual plasticizers on the mechanical properties of the film is likely an important factor for the expected outcomes. Indeed, when the effects of oleic acid and glycerol on the mechanical properties were analyzed using a statistical two-way ANOVA with an experimental design of 2 × 2 (0 and 10% for glycerol and oleic acid in the blended plasticizers), a significant interaction was found between oleic acid and glycerol in TS improvement (Tables 1 and 2). Thus,

Table 1. Tensile Strength (TS) and Elongation to Break (*E*) of Plasticized Zein Film^a

plasticizer	TS (MPa)	<i>E</i> (%)
control	5.70 ± 0.33	1.40 ± 0.000690
oleic acid	11.20 ± 0.89	1.66 ± 0.000215
glycerol	7.55 ± 0.18	2.12 ± 0.000949
oleic acid + glycerol	14.37 ± 0.91	2.36 ± 0.002400

^aThe plasticizer blend of oleic acid and glycerol was in a ratio of 10%:10%.

Table 2. Variance Analysis (ANOVA) on the Effects of Oleic Acid and Glycerol on TS and *E*

plasticizer	<i>F</i> or <i>P</i> value	TS	<i>E</i>
oleic acid (10%)	<i>F</i>	818.711	6.104
	<i>P</i>	0.000	0.025
glycerol (10%)	<i>F</i>	134.778	195.053
	<i>P</i>	0.000	0.000
oleic acid (10%) + glycerol (10%)	<i>F</i>	9.345	3.961
	<i>P</i>	0.008	0.064

the highest TS with a ratio of 3:1 of oleic acid to glycerol observed previously (Figure 3) was likely due to a synergistic interaction between these two plasticizers. With regard to their interaction contributing to *E* of the film, the statistical analysis did not achieve a significant level, which suggests there is not a

synergistic effect on *E* of the film or the statistical power is not high enough to achieve a statistical significance. Thus, this analysis indicates that TS and *E* are likely independent variables in the evaluation of the mechanical properties of a film. Similarly, Parris and Coffin's study on zein film plasticization by blended plasticizers of poly(propylene glycol) and glycerol also showed a strong synergy in increasing the *E*,¹⁶ but not TS. Thus, the molecular mechanisms for strong TS and high *E* still need further investigation so that appropriate plasticizers can be applied to simultaneously improve both the TS and *E* of zein films.

Glass Transition Temperature of the Plasticized Zein Film. Glass transition refers to the change from a hard, noncrystalline, glass-like material to a rubbery solid, and the glass transition temperature (T_g) is the point at which this transition occurs with a change of heat capacity (C_p). To examine the effect of oleic acid and glycerol on the T_g of zein film, the effect of water, which is the most common natural plasticizer, needs first to be evaluated. A similar level of water content (Table 3) in all of the samples suggests the plasticization effect of water on zein film could be leveled off, and different values of T_g of zein films are mainly due to the effect of oleic acid or glycerol. When the T_g was measured by a differential scanning calorimeter (Table 3), the nonplasticized zein film (control) showed the highest value of 112.6 °C, which is consistent with the literature.¹⁰ Comparatively, oleic acid is more effective than glycerol in reducing the T_g of zein film, and when blended plasticizers with a ratio of 3:1 of oleic acid to glycerol were used to plasticize the zein film, the T_g was decreased to the lowest value of 93.4 °C, which is likely consistent with the synergistic interactions between oleic acid and glycerol in zein film plasticization.

The glass transition temperature of polymer reflects the tightness of molecular packing³¹ or supramolecular structure³² that is mainly governed by noncovalent interactions such as hydrogen bonding, van der Waals, and hydrophobic interaction.³³ For amorphous zein protein in the cast films, a layered structure²⁸ of zein proteins has been reported in the presence of hydrophobic oleic acid, and this structure makes the zein protein chains have high mobility, less friction, and so a decreased T_g .²⁷ For glycerol, the hydrogen bonding with zein protein²⁹ would increase the number of end groups and therefore the free volume of zein protein, leading to a reduction of T_g . This explanation is supported by a literature report of large zein fibers (large free volume) formed with the increase of glycerol content in plasticized zein films.¹³

Fourier Transform Infrared Spectrometry Examination of Zein Film. On the basis of the literature,²⁹ glycerol has the ability to increase α -helix of zein protein by affecting the hydrogen environment shown by a diminished FTIR band intensity of 1620 and 1680 cm^{-1} that are β -sheet secondary structure. Similarly, Gao et al.³⁴ also reported that the presence of glycerol increased the intensity ratio of 1650 to 1630 cm^{-1} in

Table 3. Effects of Different Plasticizers (20%) on T_g of Zein Films Measured after Equilibrium under 50% Relative Humidity

plasticizer	water content (%)	T_g (°C)	control	oleic acid	glycerol
control	5.2 ± 0.29	112.6 ± 0.57			
oleic acid	4.7 ± 0.32	99.03 ± 0.04	0.000752 ^a		
glycerol	7.9 ± 0.49	110.07 ± 0.22	0.075577	0.012431 ^a	
blend ^b	5.4 ± 0.15	93.35 ± 0.10	0.010235 ^a	0.08958	0.131162

^aStatistical significance at $P < 0.05$ using the *F* test. ^bCombined: oleic acid/glycerol = 3:1.

the amide I band, suggesting the change of the conformation of the protein with much more helical forms at the expense of β -sheets. Thus, to explain how oleic acid and glycerol synergistically affect the TS of zein film at a molecular level, the secondary structure of the zein protein was examined by a FTIR experiment.

The FTIR spectra of zein film plasticized by different plasticizers are shown in Figure 4. The peptide bond is

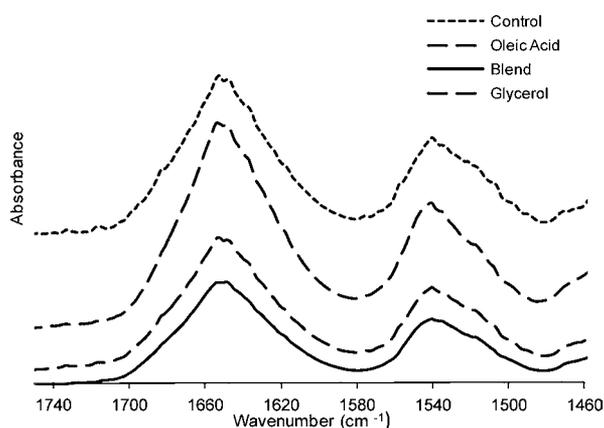


Figure 4. FTIR spectra of zein films plasticized by different plasticizers at a final content of 20% (weight basis of zein). Zein films nonplasticized, plasticized by glycerol or oleic acid, and their blend are shown from top to bottom in the graph.

normally represented by C=O stretching vibration at amide I band ($1600\text{--}1700\text{ cm}^{-1}$) and N–H bending vibration and C–N stretching vibration at amide II band ($1500\text{--}1600\text{ cm}^{-1}$).³⁵ Generally, the amide I band indicates secondary structure such as α -helix and β -sheet of the protein, whereas the amide II band represents the environment for hydrogen bonding.³⁶ Although oleic acid has a substantial impact on the mechanical property and T_g of zein film, FTIR analysis did not show a noticeable difference compared to the nonplasticized zein film, but a reduced intensity of the amide II band peak around 1540 cm^{-1} was shown for both glycerol and the blended plasticizer, indicating the hydrogen environment was changed by plasticizer–amide interaction. Similarly, slightly reduced band intensities at 1620 and 1680 cm^{-1} for glycerol and the blended plasticizer indicate β -sheet-like structures are reduced, which is consistent with the literature.²⁹

Although the FTIR data showed consistent results with the literature, no significant difference in the percentage of different types of protein secondary structure was shown among zein films plasticized with different plasticizers (Table 4). In fact, an increase of β -sheet (at the expense of α -helices) of zein protein can make the protein more flexible³⁷ and easily elongated. Therefore, zein film plasticization by glycerol and oleic acid is likely accomplished by modifying the supramolecular structure of zein protein molecules through noncovalent forces of

Table 4. Percentage of Secondary Structure Type of Zein Protein in the Plasticized Films

plasticizer	α -helix	β -sheet	β -turn	random coil
control	31.84	16.47	16.66	35.03
oleic acid	31.89	16.43	16.51	35.17
glycerol	31.69	16.66	16.52	35.13
oleic acid + glycerol	32.28	16.88	15.68	35.16

hydrophobic and hydrophilic interactions, and the secondary structure of zein protein is not likely significantly affected by the used plasticizers of glycerol and oleic acid.

Scanning Electronic Microscopy. To observe the supramolecular structure of zein film plasticized by oleic acid and glycerol as well as their blend, SEM examination on the cross section of the zein film was performed (Figure 5). There are pores or empty spaces in the oleic acid plasticized zein film, whereas glycerol does not cause substantial changes of the film. When their blend was used, the cross section became very smooth, indicating the plasticizer's effect on the zein film is realized through modification of the supramolecular structure of the protein chains inside the film. Although globular protein structure on the surface of zein film plasticized by oleic acid has been shown in the literature,¹⁰ our result from the cross section (Figure 5B) of zein film did show some fiber or sheet-like structures, which are likely formed by confining the orientation-correlated zein molecules in the presence of oleic acid. This molecular confinement was also likely the reason for the enhanced TS of the film, which is indirectly supported by the exceptional mechanical property of polymer nanofiber due to a confinement of the supramolecular structure.³² The confinement of zein protein chains might also be related to the empty spaces formed within the oleic acid-plasticized zein film under a quick evaporation of solvent in film casting. Actually, the literature²⁷ does mention the pores in zein film being filled by linoleic acid polymerization as one of the reasons for its decreased WVP.

The structural differences shown in the cross section of the films not only reflect different effects on the structure of plasticized film but also suggest that the plasticization mechanisms are different. For oleic acid, the study of Wang et al.²⁸ showed that zein film is formed by the initial absorption of oleic acid onto the zein protein surface through hydrophilic interaction followed by hydrophobic interaction to form layered structures, so oleic acid is located between the chains of zein protein, which can be called internal plasticization to increase the main chain movement leading to a decreased T_g . For glycerol, which is a hydrophilic molecule, there is less possibility to interact with the hydrophobic region of zein protein, and it is deposited on the hydrophilic surface of zein protein,³⁴ which will cause an increase of the number of side chains of zein protein through hydrogen bonding.²⁹ Therefore, glycerol would not dramatically change the structure of zein protein arrangement in the film, and so the reduction of T_g is not so effective as that of oleic acid.

Synergistic Interaction between Glycerol and Oleic Acid in Zein Film Plasticization. Glycerol and oleic acid are representatives of polar and amphiphilic plasticizers for zein plasticization. Their physicochemical differences are likely the fundamental reason for their different behaviors in zein film plasticization. The increase in WVP for glycerol-plasticized film is probably related to glycerol's hydrophilicity-induced phase separation,^{16,26} whereas oleic acid is embedded in the protein matrix and so gives no significant contribution to WVP. Apparently, of the methods to improve film flexibility (decrease of T_g), internal plasticization is the way used by oleic acid, whereas an increased number of side chains is the way for glycerol. Actually, the mechanism for oleic acid is called molecular plasticization and that for glycerol, structural plasticization.³⁸ These two different plasticization mechanisms provide the possibility for their interactions during zein film formation.

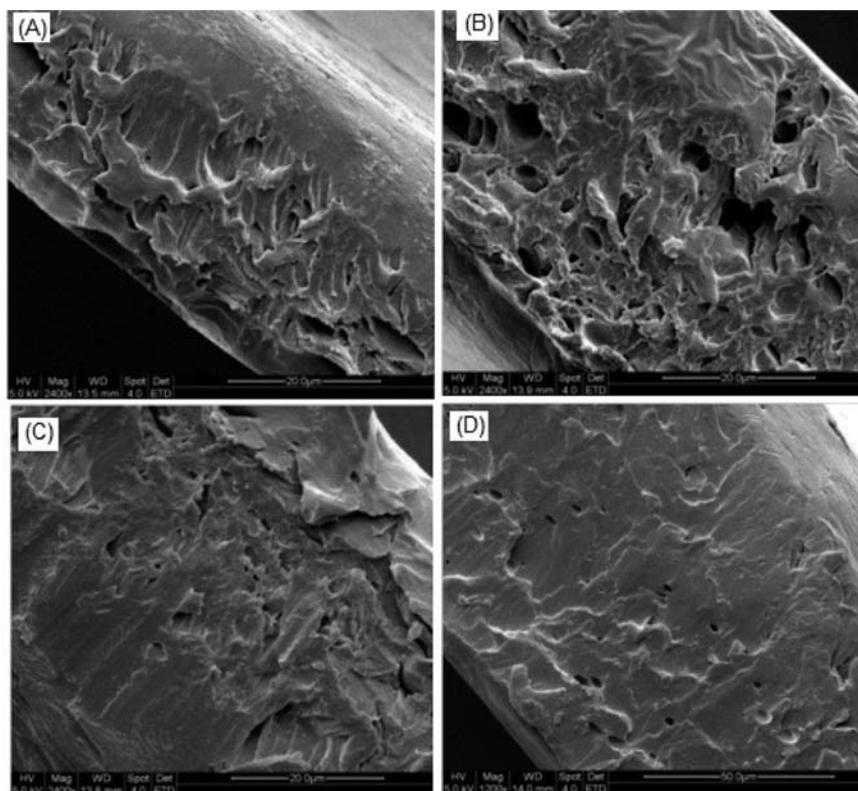


Figure 5. SEM of cross section of zein films containing different plasticizers at a content of 20%: (A) control; (B) oleic acid; (C) glycerol; (D) oleic acid + glycerol (at a ratio of 3:1).

During the zein film casting process, solubilized zein protein, glycerol, and oleic acid coexisted in the aqueous alcohol solution. With the evaporation of alcohol and water, the hydrophobic interaction between zein protein and oleic acid, as the predominant event, occurred, and glycerol would remain in solution for a longer period with its high polarity, until there is not enough solution for glycerol to stay. During this period, the glycerol would interact with zein protein through hydrophilic interaction to become part of the zein film. When the layered structure is formed between zein protein and oleic acid,²⁸ more hydrophilic side chains of amino acid residues might be exposed, which would result in more interactions between glycerol and zein protein, leading to a synergistic interaction in zein film plasticization. However, the detailed process of their synergistic interaction still needs further investigation from different perspectives.

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Notes

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