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Chemistry and Physical Properties of Melt-Processed and Solution-Cross-Linked Corn Zein

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Corn zein was cross-linked with glutaraldehyde (GDA) using glacial acetic acid (HAc) as catalyst. The objectives are to evaluate the swelling characteristics of GDA cross-linked zein gels in water, ethanol, and their combinations. Similar formulations, upon solvent evaporation, form films. The mechanical properties of the films are compared to compression molded tensile bars from GDA melt-processed zein as a second objective. Chemistry of the cross-linking reaction was based on the aldehyde binding characteristics defined by use of fluorescence spectroscopy; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) to demonstrate the cross-linking reaction; FTIR to observe absorption differences of the cross-linked product; differential scanning calorimetry, dynamic mechanical analysis and thermogravimetric analysis to assess thermal properties; and the use of Instron Universal Testing Machine to evaluate mechanical properties. A reaction mechanism for acid catalyzed GDA cross-linking of zein is proposed. Thermal and mechanical properties of tensile bars cut from either film or formed by compression molding were similar, where both showed increased tensile strengths, ductility and stiffness when compared with unmodified controls. Samples that were reacted with 8% GDA by weight based on weight of zein from either process retained their integrity when tensile bars from each were subjected to boiling water for 10 min or soaking in either water or HAc for 24 h. The melt-processed, cross-linked zein is a more environmentally friendly method that would eliminate the need for HAc recovery.

KEYWORDS: Cross-linking; proteins; reaction chemistry; mechanical properties; thermal properties

INTRODUCTION

Maize α-zeins belong to a class of hydrophobic proteins known as prolamins. They are a group of major storage proteins in corn as well as in corn gluten meal, a coproduct of the ethanol industry generated from wet-milled corn. These proteins are made up of two microheterogenous groupings within apparent molecular weights of 23.8 and 26.7 kDa, respectively (*1*). Aqueous ethanol and aqueous acetone or acetonyl acetone are generally used to dissolve zein for preparing films and coatings from zein because zein is insoluble in water. This solubility characteristic is attributed to its high content of nonpolar amino acids (*2*).

Recently, Sessa et al. (*3*) published research on the properties of films from a Japanese white zein reacted with glutaraldehyde (GDA). In that publication, those investigators demonstrated the positive attributes of using glacial acetic acid (HAc) as an excellent solvent for generating their poured films, where HAc also was used to catalyze the GDA cross-linking zein. The focus of that investigation was to assess the structure property correlations of their GDA-modified zein films by thermal and rheological measurements. In that investigation a concentration of 4% or higher GDA, based on weight of zein, was found to generate films as well as organo-gels that were insoluble in solvents common for zein. Based on these findings, a criterion for assessing GDA cross-linked zein will be its insolubility in HAc.

In order to tailor zein proteins modified with GDA for potentially new uses in the pharmaceutical, medical, plastics, and coatings industries, a basic understanding of the chemistry of the reaction is needed. One objective is to evaluate the swelling characteristics of GDA cross-linked zein gels formed in a hermetically sealed system. Similar formulations, open to the air, form films. Because a wet process based on solvent/carrier evaporation of dispersed or solubilized proteins poses environmental, health, and safety concerns with the use of HAc as well as the need to recover solvent, a dry process based on the thermoplastic properties of zein will be evaluated for the cross-linking of zein with GDA as a second objective. Mechanical properties and thermal proper-

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ties of GDA cross-linked films and melt-processed zein will be compared.

MATERIALS AND METHODS

Materials. The commercial zein used in this investigation was Freeman yellow zein FC4000 (Freeman Industries Inc., Tuckahoe, NY). This zein possessed crude protein 89.02% (Dumas N x 6.25), crude fat 5.03%, crude fiber 0.04%, ash 0.05% and moisture 4.37%. Chemicals and electrophoresis materials were purchased as follows: ortho phthaldialdehyde reagent, labeled OPA; ethylamine, 70% aqueous solution; glutaraldehyde, designated GDA, grade 1, 50% in water; Coomassie Brilliant Blue R-250 all from Sigma, St. Louis, MO. Broad range molecular weight standards from Bio-Rad Laboratories, Hercules, CA; NuPAGE, Novex high-performance, precast 4−12% gradient bis-tris gels from Invitrogen, Carlsbad, CA. Glacial acetic acid, reagent grade, designated HAc, was obtained from EM Science, Gibbstown, NJ. All other chemicals and reagents used in this study were of reagent grade.

Derivitization of Available -NH_2 Sites. Primary amines, including the α-NH_2 group of N-terminal site, react with OPA in alkaline media in the presence of β-mercaptoethanol, to give strongly fluorescent derivatives (*4*). Because zein is insoluble in aqueous borate buffer, both the ethylamine, as standard, and zein were each dissolved in a combination of 5 mM NaOH, adjusted to pH10 and added to absolute ethanol to make an 80% basic ethanol solution (w/w). Fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Walnut Creek, CA) with slit width 5 nm, excitation wavelength 335 nm and emission wavelength 435 nm at 25 °C.

The experimental details of the procedure are (1) an OPA solution, prepared in a darkened environment, consisted of dissolving 7.5 mg of OPA in 750 μL of 80% ethanol (w/w) prior to blending with 45 mL of the basic ethanol solution. While this solution is stirring, 750 μL of β-mercaptoethanol is added; (2) purchased ethylamine solution is 10.87 M in 70% ethanol; 500 μL of this solution is blended with 500 μL of 80% basic ethanol and stirred for about 5 min in a covered beaker; a 1:1000 dilution of that solution was then prepared (i.e., 10 μL of basic ethylamine diluted with 990 μL of 80% basic ethanol; (3) a 10 mM stock solution of zein, with an assumed molecular weight of 23.7 kDa was prepared by dissolving 1.185 g of zein in 5 mL of 80% basic ethanol; this solution was constantly stirred to prevent gelation. Stock solutions of ethylamine and zein were then each diluted further to equalize molarities of zein to ethylamine. Those diluted samples were each blended with OPA solution, vortexed individually for 8 s, then analyzed on a spectrofluorometer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed with NuPAGE, 4−12% gradient bis-tri gels according to the method of Sessa et al. (*5*). Gels were scanned with UN-SCAN-IT gel, version 5.1, Automated Digitizing System (Silk Scientific Corp., Orem, UT). Protein stained bands were scanned along with the molecular weight standards, where the mobility of the standards were used to estimate the molecular weight of the zein control and GDA-modified zein products. A timed reaction sequence from 0 to 24 h consisted of 7.5 g of zein dissolved in 50 mL of HAc to which solution was added 4% GDA (w/w zein) while stirring with a magnetic stirrer at room temperature. Ten mL aliquots were taken at the designated times of 0, 1, 2, 4, 24 h where each aliquot was quenched with 750 mg dithiothreitol. To achieve a 0 time for the zein reaction with GDA, dithiothreitol, as a quenching agent, had to be added to the zein solution in HAc prior to the addition of GDA. The quenched reaction solutions, while stirring, were each neutralized to pH 7 with 5N NaOH and then dialyzed against water for 2 days in 1000 molecular weight cutoff dialysis casing (Spectra/Por Biotech Cellulose Ester Dialysis Membrane, Spectrum Laboratories, Inc., Rancho Dominguez, CA). Dialyzates were then freeze-dried. Each dialyzed sample was dispersed in 0.055 M TRIS buffer containing 2.0% SDS, 7.0% glycerol, 4.3% mercaptoethanol, as a reducing agent, and 5 M urea, then, centrifuged at 12,000 × g for 5 min with an Eppendorf model 5415 centrifuge to remove undissolved material.

Gel and Film Preparations. Gels were prepared from 25% solid solutions of zein in HAc (i.e., 25 g of zein/100 mL of HAc) to which 0.5, 1, 2, 3, 4, or 8% GDA, based on weight of zein, was added. After being stirred for 2 h, solutions were each sonicated with a Branson 2510 (Process Equipment and Supply, Cleveland, OH) for 30 min. The degassed solutions were poured into molds consisting of 3 mm thick silicon rubber gasket sandwiched between two Teflon coated glass plates. The molds, hermetically sealed, remained clamped shut for one week at room temperature to allow for a completed cross-linking reaction.

Zein formulations for films were similar to the gel formulations except that the 3% GDA modification was not included. These degassed solutions, along with a zein control with no added GDA, were poured within the 3 mm silicon gasket stuck onto a Teflon coated plate with a glue stick. The plates were placed in a hood at room temperature to evaporate solvent.

The air-dried films, while still attached to the gasket on the glass plate, were twice washed with 500 mL batches of absolute ethanol to remove HAc residues. Upon removal of the films from the gaskets, they were air-dried to remove ethanol residues. Test strips were cut from the resulting films for dynamic mechanical analysis, thermal analyses, FTIR analysis and tensile bars for physical property testing using an Instron. Test strips of ZF cross-linked with 8% GDA were subjected to boiling water for 10 min or soaked for 24 h. These tensile bars were blotted dry prior to mechanical testing with the Instron. Data were based on the average of two replicates.

Swelling of Zein Gels. The swelling response of GDA-modified zein gels were tested on four different solvents: absolute ethanol, 80% ethanol, 60% ethanol, water, with an as-is control with no solvent. Only the 4% and 8% GDA-modified zeins gelled while the other reaction products were either liquid or partially gelled. The zein cross-linked with 8% GDA was cut into 2 cm square pieces using a razor blade. Each piece was weighed, placed into a Petri dish and covered with approximately 60 mL of one of the four above-mentioned solvents and the dish covered to prevent evaporation of solvent. The solvent was changed every 24 to 72 h for six changes in 18 days to leach out any unreacted species and entrained HAc. The gel pieces were assumed to have reached a swollen equilibrium state 24 h after the final solvent change. Each gel piece was then blotted dry and weighed, giving its swollen mass. After the swollen mass was measured, the gel pieces were first air-dried and then dried for 7 days in a hot air oven set at 105 °C to remove remaining solvent. The mass of the dried gel pieces was taken as the dry mass. The percent swelling of each piece was calculated where % swelling = (swollen mass-dry mass)/dry mass. Two replicates were run for each evaluation and data averaged.

FTIR of Zein Films. A single bounce diamond Durascope from Sens IR in a Thermo Nicolet Avatar 370 Fourier Transfer Infra Red spectrometer was used to obtain a midrange ATR spectrum of zein and GDA-modified zein films under high pressure.

Torque Rheometry/Compression Molding. Blends of zein with various percentages of GDA from 0 to 8%, HAc as catalyst, and water, as plasticizer, were stirred together with a spatula in a mixing bowl at room temperature to provide a crude mixture. Specifically, an addition of water to the endogenous moisture in zein was equivalent to 10% total moisture; 0.25% HAc was added to each blend as catalyst; blends were made with 0, 0.5, 1, 2, 4, 8% GDA added, taking into account that GDA is 50% in water. Sixty g of each blend were used to fill the chamber of a Haake Fisons, Rheocord 90, using the 600 series mixing bowl (Thermo Electron Corp., Madison, WI). The rheometer was equipped with high shear roller rotors. The bowl temperature was kept at 90 °C and rotor speed set at 50 rpm to process each blend for 4 min.

The taffy like blend of zein was pulled from the roller rotors, cooled to room temperature, snipped into small pieces, then frozen with liquid nitrogen. The frozen mass was ground in a Wiley mill. The ground material was sieved through a 2 mm mesh screen to remove fines. That matter retained on the sieve was compression molded 20 min with tensile bar specifications D638 type V on a Carver Press at a pressure of 12500 psi and temperature of 99 °C,

Thermal Analyses. Differential scanning calorimetry (DSC) measurements of heat flow into and from a sample were performed either on powdered zein samples or film segments packed into either aluminum pans or stainless steel pressure pans that were hermetically sealed, using a DSC 2920 (TA Instruments, New Castle, DE) that was

calibrated against an indium standard. Sample pans and empty aluminum or stainless steel reference pans were heated from 15 °C up to 220 °C at a scan rate of 5 °C/min for two cycles. The glass transition temperature, T_g, representing the midpoint between the onset temperature (T_i) and the final temperature (T_f), was measured on the second heating cycle.

Thermogravimetric analysis (TGA) was used to investigate the change in mass of zein films due to decomposition or evolution of volatiles when films or ground materials were subjected to heating. TGA experiments were performed on a 2050 TGA (TA Instruments, New Castle, DE). Samples were run at a heating rate of 10 °C/min from 25 to 800 °C. All samples were run in a nitrogen atmosphere with a flow rate of 90 mL/min.

For TGA kinetics, each sample was run at three different heating rates: 5 °C, 10 °C, 15 °C/min. The data was then analyzed for data kinetics using the TA Specialty Library software (Version 1.4, TA Instruments, New Castle, DE). This analysis used the TGA Kinetics Analysis feature of the software, which is based on ASTMS Standard E1641 "Decomposition Kinetics by TGA."

Dynamic Mechanical Analysis (DMA). Rectangular test strips with dimensions 15 mm long, 9 mm wide, 0.8 mm thick, cut from air-dried films or rectangular sections cut from compression molded tensile bars were evaluated by DMA. The magnitude and temperature dependence of the storage modulus (G'), loss modules (G") and loss tangent (tan δ) were measured with an ARES LS2 controlled strain rheometer (TA Instruments, New Castle, DE) equipped with a torsion rectangular fixture. Measurements of G', G", and tan δ were conducted at a frequency of 1.0 rad/s and a strain of 0.05%. Each sample was heated twice with a forced air oven at a rate of 2 °C/min. The samples were first heated from 20 to 100 °C for the control sample or to 160 °C for the GDA-modified sample, and held at that temperature for 1 h. The samples were cooled to 20 °C and then reheated until bubbles formed in film samples or the sample swelled in the compression molded bars where each sample became distorted.

Mechanical Property Measurements. Films from zein formulations either with or without GDA modification were cut with a die to produce dumb-bell shaped test specimens with a gauge length of 7.62 mm and a width of 3.18 mm. Compression molded tensile bars had specifications D638, type V. Two or more replicate films or compression molded bars with five test specimens for each treatment were exposed to a relative humidity of 50% for seven days. Five thickness measurements across the gauge length of each specimen were taken using a Minitest 2500 (Electro Physik, Germany) and averaged. Testing was performed with an Instron Universal Testing Machine (Model 4201, Canton, MA) in a constant relative humidity room (50%) at 23 °C where series 1X software handled data recording and manipulation. For those specimens, a cross-head speed of 10 mm/min was used with a grip distance set at 50.00 mm. Tensile strength (MPa), elongation % and Young's modulus (MPa) were compared between GDA-modified zein films and melt-processed tensile bars versus control films and melt-processed bars with no GDA.

Moisture evaluations were calculated by taking the difference in weight of test strips before and after heating in a hot air over at 105 °C for 2 h and dividing that difference by the wet weight. Tensile bars from either 8% GDA-modified zein films or melt-processed pieces were subjected to either soaking in water at room temperature for 24 h or boiled for 10 min then blotted dry with filter paper prior to physical testing on an Instron.

RESULTS AND DISCUSSION

Quantitation of Available -NH_2 Sites. OPA in the presence of β-mercaptoethanol will react with primary amines in addition to amino acids and proteins. The chemical structure of the adduct formed is an isoindole where the -NH_2 nitrogen becomes part of a five proton aromatic ring system based on proton NMR spectral data (*6*). Five dilutions of equimolar amounts of ethylamine and zein analyzed spectrofluorometrically after their reactions with OPA in the presence of β-mercaptoethanol shown in **Table 1** yielded an average ratio of 0.98. Therefore, the only primary amine for zein reacted with OPA is the α-NH_2 group on N-terminal. That reaction was immediate when performed at room temperature of 23.5 °C.

Table 1. Spectrofluorometric Quantitation of Available -NH_2 Sites on Zein (Z) Compared with Ethylamine (EA) Reacted with *o*-Phthaldialdehyde (OPA)

	intensity (a.u.)[b]		ratio
dilution[a]	Z-OPA	EA-OPA	Z-OPA / EA-OPA
1:450	233.02 (61.90)	221.98 (26.52)	1.05
1:475	237.95 (62.73)	230.89 (42.90)	1.03
1:500	235.71 (61.39)	236.98 (39.09)	1.00
1:525	234.22 (52.43)	248.21 (48.47)	0.94
1:550	242.94 (57.95)	282.37 (56.11)	0.86

[a] Dilutions analyzed were based on equimolar amounts of Z and EA. [b] Fluorescence intensity in absorbance units averaged for three replicates (standard deviation).

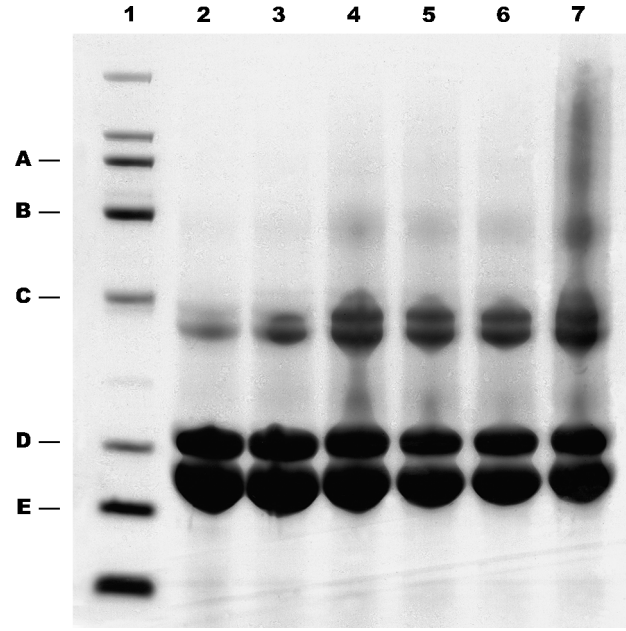

Figure 1. SDS−PAGE patterns of zein (lane 2) and zeins incubated with glutaraldehyde at 22 °C from 0 time (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 24 h (lane 7); lane 1 is molecular weight standards with A, 97 kDa; B, 66kDa; C 45 kDa; D, 22 kDa; E, 14 kDa.

SDS−PAGE of GDA-Modified Zein. SDS−PAGE analysis of the dialyzed GDA-modified zein samples was used to follow a timed sequence. Except for the control and 0 time samples, all others possessed precipitates. Apparent on our protein stained gel, shown in **Figure 1**, the control and 0 time samples in lanes 2 and 3 respectively were identical, consisting mainly of the monomeric α and α' species along with their respective dimeric forms. Cross-linking of the α and α' species of zein with a bifunctional agent, GDA, can give rise to a combination of intra- as well as intermolecular products. Apparent on our gel in **Figure 1** both intra- and intermolecular reactions have occurred. To define the intramolecular GDA reaction, ten replicates of unmodified zein, analyzed by SDS−PAGE with subsequent gel scans of the α and α' zein monomers, yielded respective molecular weights with standard deviations in parenthesis of 20,660 (234) Da and 16,072 (511) Da when compared with molecular weight standard in lane 1. Consistent with those means of the zein control samples and the 0 time sample, the molecular weights of those monomers showed increases in molecular weights when reacted with GDA in the 1 h to 24 h

samples where the sample reacted for 24 h with GDA gave increased molecular weights of 1908 and 1367 Da respectively for the α and α' species. The intramolecular GDA reaction most likely occurred with the α-NH_2 group of the respective N-terminal amino acids as exhibited by the binding of that group with the fluorescent probe, OPA, discussed in the previous section. That reaction generated a chain lengthening of the α and α' species. Whether or not the attached GDA self-oligomerized to the extent needed to raise the molecular weights above the monomeric α zein species in the controls is questionable. GDA modification of the N- terminal group should bind less SDS per unit mass (*7*). This would lead to slower migration of those GDA-modified species due to decreased charge-to-mass ratio. That anomalous behavior is visually apparent on SDS−PAGE in the monomers of ribonuclease that were reacted with glyceraldehyde in a timed sequence (*8*). In that investigation the reaction of ribonuclease with GDA gave an immediate aggregate formation with molecular weight greater than 205 kDA when subjected to SDS−PAGE, whereas, in our investigation on the cross-linking of zein with GDA the timed sequence gel pattern showed increases in molecular weights of the monomeric species as well as formations of molecular weight species higher than the dimeric forms.

Intermolecular reaction of GDA with these species would give rise to a series of cross-linked products including 1:1 conjugates, polyconjugates and polymers of each of the reactant proteins some of which were insoluble in our TRIS/area buffer system. The higher molecular weight aggregates observed in all the GDA reacted samples above 0 time represent only the soluble portions, whereas, the precipitated materials represent the higher molecular weight species. Because the components of the insoluble precipitates could not be subjected to SDS-PAGE analysis, we defined intermolecular binding with GDA based on differences observed in the amino acid contents of GDA-modified zein compared with untreated zein. GDA is known to react with the ε-amino group of lysine and other nucleophiles including cysteine, the imidazole ring of histidine, the guanidine group of arginine, the phenolic group of tyrosine (*9*). Cross-linking of cottonseed proteins by formaldehyde, GDA and glyoxal gave rise to acid hydrolysis resistant compounds (*10*). Based on an amino acid composition analysis of zein and 8% GDA-modified zein performed by the Experimental Station Chem., Laboratories, Univ. of Missouri, Columbia MO using a 24 h acid hydrolysis with 6N HCl under nitrogen with amino acids compared with NIST standards, changes in those amino acids that gave rise to acid resistant species, when normalized for protein (based on %weight/100 g protein) were losses of 5.7% for cysteine (0.70 to 0.66), 4.7% for histidine (1.28 to 1.22), 4.9% for arginine (1.42 to 1.35), 7.3% for tyrosine (4.94 to 4.58), and 80% for lysine (0.10 to 0.02). Based on these results, GDA cross-linked zein gives rise to acid resistant materials.

Swelling of Gels from GDA-Modified Zein. Gels from the unmodified zein and zeins modified with 0.5, 1, and 2% GDA remained fluid, the system with 3% GDA was partially solidified, whereas those systems modified with 4 or 8% GDA gelled completely. Those gels were insoluble in solvents common for zein including aqueous ethanols of 60 and 80% (w/v), HAc, dimethyl formamide and methylcellosolve. Gel formation indicated that a cross-linking reaction occurred that led to the formation of an insoluble polymer where the chains are joined together to form a three-dimensional network structure. All the HAc becomes part of the gel network. Swelling characteristics of 8% GDA-modified zein gels are given in **Table 2**. These gels were subjected to five different treatments. The recorded data is the means of two replicates. Those gels can contain imbibed HAc, residual moisture, unreacted zein and unreacted residual GDA. The gel soaked in water turned white and hardened because water dilutes the HAc within the gel matrix. The GDA-modified zein, both water and aqueous HAc insoluble, precipitates as a white, hardened mass. Unreacted zein was leached out into the first three washings with either 60 or 80% aqueous ethanol because those washings became milky upon addition of water. After three washings, no further milkiness was observed in those washings. Because we did not measure volume changes of the gels in the individual solvents, an equilibrium state is based on a constant sample volume. An assumption was made that a swelling equilibrium state of gel in contact with excess solvent was achieved within 18 days of soaking.

Table 2. Swelling of 8%[a] GDA-Modified Freeman Zein Gels

treatment	original mass (g)	swollen mass (g)	oven-dried mass (g)[b]	% swelling (dry basis)
absolute ethanol	1.2	0.46	0.25	84
80% aqueous ethanol	1.3	1.23	0.21	486
60% aqueous ethanol	1.3	0.99	0.23	330
water	1.5	0.52	0.28	86
none	1.3	0.38	0.30	27

[a] w/w % based on protein. [b] Hot air oven-dried at 105 °C for one week.

Our findings demonstrate that the swelling characteristics for gels soaked either in water or absolute ethanol are similar; swelling of gels in 80% aqueous ethanol is about 5.7-fold higher than gels soaked in either absolute ethanol or water and 1.5 fold higher than gels soaked in 60% aqueous ethanol. Commercial zeins are insoluble in both water and absolute ethanol, moderately soluble in 60% aqueous ethanol and very soluble in 80% aqueous ethanol. The swelling of the 8% GDA-modified zein gels is greatest in 80% aqueous ethanol, less in 60% aqueous ethanol and least in either water or absolute ethanol. Our findings demonstrate that gel swelling is highest in the aqueous ethanol solvent system where zein is most soluble.

On the basis of our tabulated data for air-dried masses, comparison of those values with our no solvent control sample, unreacted zein and possibly unreacted GDA leached out into the wash solvent. A constant loss in weight due to moisture was achieved within seven days when gels were desiccated in a vacuum desiccator; the non solvent processed gel attained a constant weight when heated in a hot air oven at 105 °C within seven days where heating was needed to remove HAc from the gel matrix. This gave greatest shrinkage when % swelling (shrinkage) was calculated on original mass loss. If we assume that the non solvent processed gel, when dried for one week in a hot air oven at 105 °C, that gel should possess monomeric zein, GDA polymerized zein, oxidized GDA with no residual water or HAc. A calculated 77% loss in mass from the original mass was observed. The GDA-modified zein gel, soaked in several changes of water for 18 days should have leached residual HAc as well as oxidized and/or unoxidized GDA. When those gels were oven-dried for one week, an 81% loss in mass was observed. The 4% difference indicates the loss of unoxidized and oxidized GDA. GDA-modified gels soaked with several changes in 80% aqueous ethanol for 18 days yielded an 84% loss in mass. Because 80% aqueous ethanol will dissolve monomeric zein as well as unoxidized and oxidized GDA, the difference of 3% between water soaked gels and 80% aqueous ethanol soaked gels indicates that an estimated 3% monomeric form of zein may still exist in those gels. This finding would explain the observed milkiness in the waste solvent of the 80%

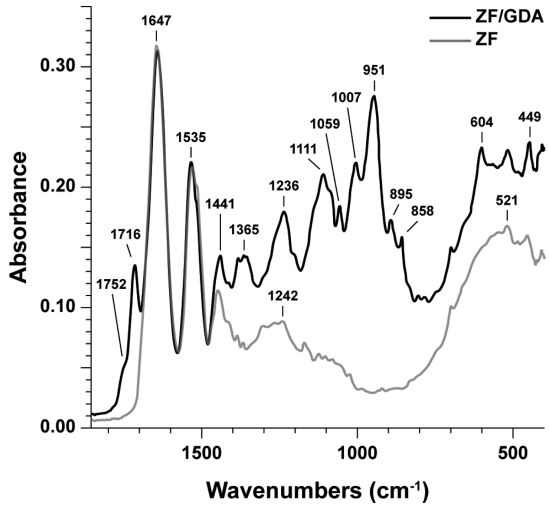

Figure 2. IR spectra of zein film (ZF) and film from zein cross-linked with 16% (wt/wt % based on protein) glutaraldehyde (ZF/GDA).

aqueous ethanol soak water when water was added for the first three changes in solvent. The findings in this section suggest that the dried films are collapsed, insoluble organo-gels.

FTIR of Zein and 16% GDA-modified Zein Films. A 16% GDA-modified zein film was used for this analysis to enhance the FTIR absorbances attributed to the GDA cross-linking. The FTIR spectrum over wavenumbers 440−1800 cm^{-1} as shown in **Figure 2**, normalized with regard to the amide 1 bond for zein at 1647 cm^{-1}, showed the prominent C═O stretching and for protein amide at that wavelength, the amide 2 band at 1535 cm^{-1} and the amide 3 bands at 1242 cm^{-1} involving C−N stretching and N−H in plane deformation respectively. The amide 3 bands of zein with a prominent band at 1242 cm^{-1} show evidence of a triplet which upon modification with GDA caused a denaturation and modification of the amide 3 band to give a more intense band at 1236 cm^{-1}. The amide 3 bands in the region of 1220−1350 cm^{-1} are very sensitive to structural changes involving C−N stretching and N−H in place deformation (*11, 12*). The increased absorption at 1441 cm^{-1} for GDA reacted zein arises from CH_2 stretching from GDA. Also, the increased absorption in the region 1420−1330 cm^{-1} is due to O−H in plane bending with C−H wagging vibrations.

Carboxylic acids undergo strong intermolecular hydrogen bonding interactions in condensed phases where the 1716 cm^{-1} absorption may be due to the hydrogen bonded C═O groups of such a chain structure, while the apparent shoulder at 1752 cm^{-1} represents non-hydrogen bonded or weakly bonded groups (*13, 14*). Those absorptions at 1716 cm^{-1} and 1752 cm^{-1} are due to HAc entrapped in the matrix of the GDA-modified zein. Other observations pertinent to our FTIR spectral interpretations of GDA-modified zein include the possibility of an ester, R-CO^a-O^b-R, where band "a" resides at about 1250 cm^{-1} and band "b" in the vicinity of 1100 cm^{-1} (*15*). The minor band at 1059 cm^{-1} appears to be the C−O stretch band of an alcohol. Conceivably the HAc used to dissolve zein and catalyze the GDA reaction may have generated an acetate ester upon oxidation of the GDA.

The predominant absorption band at 951 cm^{-1} in the GDA-modified zein film shown in **Figure 2** may be due to out of phase C−O−C stretching frequency of an oxygenated five member carbon heterocyclic structure (*16*). The combination of absorption bands at 858, 895 and 951 represent C−C skeletal vibrations from the heterocyclic structure. The increased absorption for GDA-modified zein at 1441 cm^{-1} is due to the bending vibration of CH_2 groups contributed by GDA. Aqueous GDA solutions are known to be mixtures primarily of the cyclic hemiacetal in equilibrium with the dihydrate, hemihydrate and free dialdehyde forms (*17*). The cyclic hemiacetal form has the tendency to self-oligomerize (*18*). Based on these FTIR spectral interpretations, along with the rapid reaction of OPA with the N-terminal group of zein, and the increased molecular weights, observed by SDS-PAGE, of the α and α' zeins reacted with GDA, we propose the formation of a series of linear polymeric cross-links consisting of dihydroxy-piperidine zein complex covalently bound to self-oligomerized GDA cyclic hemiacetals as shown in **Figure 3** (*19–21*). An organo-gel is generated from zein cross-linked (**Figure 3**) with 4% and higher concentrations of GDA that is insoluble in solvents known to dissolve zein. This finding indicates that the linear polymeric cross-links are cross-linked with GDA to generate acid resistant bonds (*9*).

Rheometric and Thermal Analyses of Melt-Processed Zein (MPZ) and Zein Films (ZF) from GDA-Modified Zeins. The results for the storage modulus, loss modulus and loss tangent for ZF/GDA and MTZ/GDA over the temperature ramp from 25 to 180 °C are shown in **Figure 4**. In a previous investigation on the mechanical properties of films from Japanese white zein modified with GDA (*3*), we found that an annealing step of holding the zein sample for 1 h at 160 °C was essential to stabilize the measurements. Therefore, we applied the same treatment in our current investigation. After the ZF/GDA sample was held at that elevated temperature followed by cooling and reheating, the G' value in the second scan remained high until the sample was heated above 130 °C. Beyond that temperature the modulus dropped, indicative of softening of the sample. A peak for the loss tangent and G" occurred at 143.9 °C. Because the ZF/GDA test sample bubbled in the grips at 180 °C measurements beyond that temperature were not performed. The MPZ/GDA test sample, likewise held at 160 °C for 1 h, cooled and reheated, showed two transitions in tan δ and no changes in G' and G" upon second heating cycle. The MPZ/GDA sample, subjected to DMA, differed from the ZF/GDA sample, whereby, the MPZ/GDA test strip swelled upon heating above 160 °C. The annealing step of heating the test strip for 1 h at 160 °C showed no changes in G', G" or tan δ from that of the original scan. A test strip from the MPZ control with no GDA modification (data not shown) that was heated and held for 1 h as previously reported (*3*) likewise showed two transitions for the loss tangent with one at 93.1 °C versus 93.5 °C for the MPZ/GDA test strip and 131.4 °C versus 134.0 °C. The swelling characteristic was also observed upon heating that test strip. Because of the swelling in test strips from either the MPZ control or MPZ/GDA and the evidence of a biphasic phenomenon for both strips based on the two transitions in the loss tangent, the lower temperature transitions that resulted in a decrease in G' for MPZ/GDA or the MPZ control at about 93 °C may either reflect a relaxation due to loss of water from the test strips or may be attributed to a phase separation resulting from heterogeneity of the sample. The sample heterogeneity may be due to the physical nature of the test strip where the ground particles from MPZ/GDA represent a composite of reacted and unreacted species adhering to each other as a result of compression molding. The higher temperature transition represents the glass transition.

DSC scans of MPZ and MPZ/GDA represent the finely ground powders generated from torque rheometry after subjecting them to vacuum drying at 70 °C to constant weight, whereas ZF and ZF/GDA scans were performed on vaccuum-dried pieces of the original films. The glass transitions, T_g and changes in

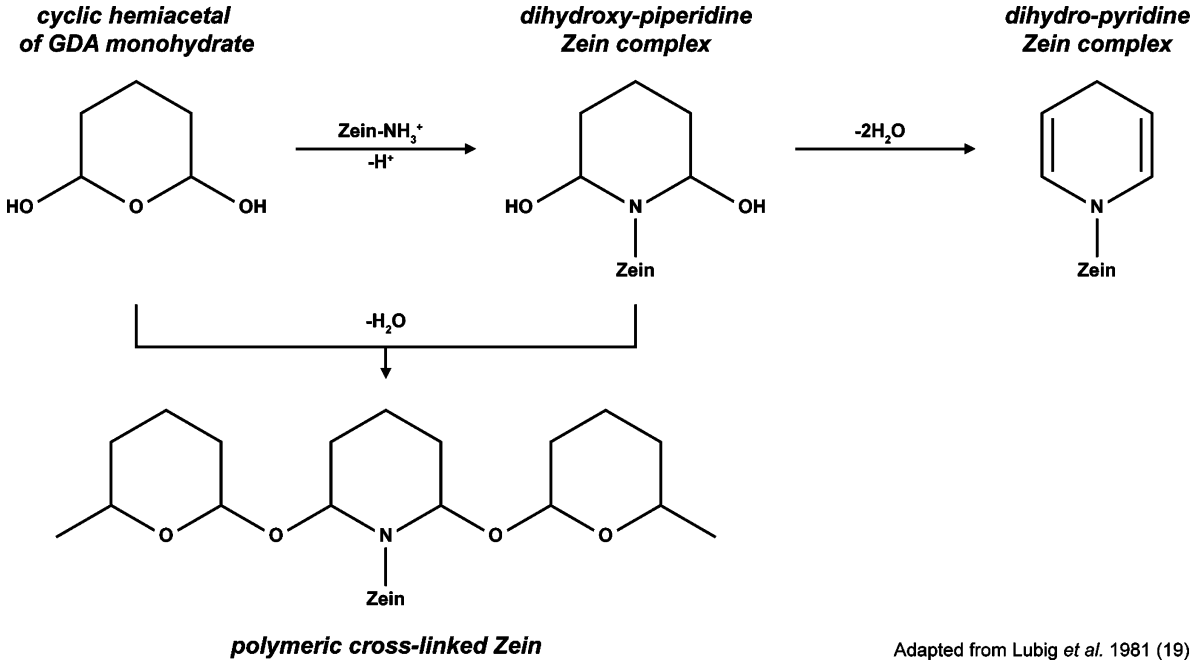

Figure 3. Proposed mechanism for intramolecular bonding of zein with glutaraldehyde catalyzed with acid.

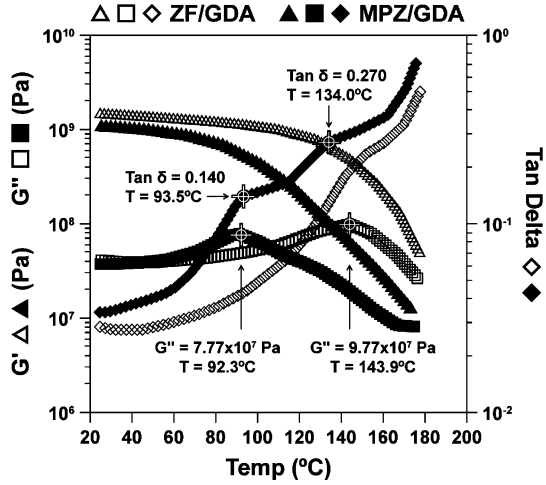

Figure 4. Comparison of mechanical properties of melt-processed zein modified with glutaraldhyde (MPZ/GDA) and film from zein modified with GDA (ZF/GDA). Measurements recorded resulted from second heating cycle after holding each sample at 160 °C for 1 h.

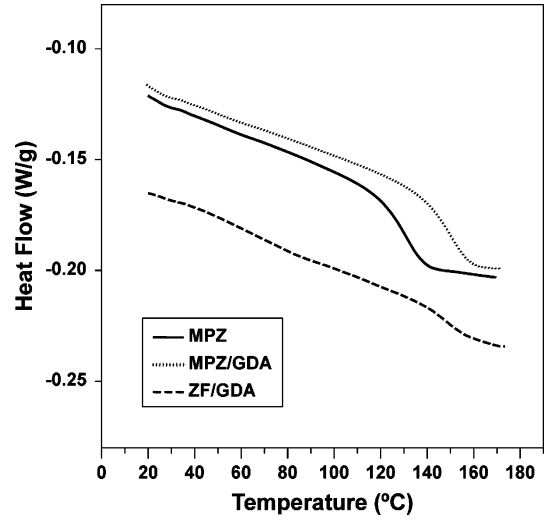

Figure 5. DSC scans of melt-processed zein (MPZ), MPZ modified with glutaraldehyde (MPZ/GDA), and zein film from GDA-modified zein (ZF/GDA). Data for each sample resulted from second heating cycle from 15 to 200 °C at a heating rate of 5 °C/min.

Table 3. Glass Transition (T_g) via DSC of Melt-Processed Zein (MPZ) and Zein Films (ZF) Modified with Glutaraldehyde (GDA)

sample[a]	T_g (°C)	ΔC_p (J/g °C)
MPZ	129.8	0.41
MPZ/GDA	148.6	0.37
ZF	143.7	0.28
ZF/GDA	148.5	0.28

[a] Data for each sample resulted from second heating cycle from 20 to 180 °C heated at 5 °C/min.

specific heat, ΔC_p, which represents changes in the slope at T_g for all four samples are given in **Table 3** with respective scans of MPZ, MPZ/GDA and ZF/GDA shown in **Figure 5**. Our MPZ T_g was 13.9 °C lower than ZF, which difference indicates that some moisture may have been retained in that sample despite vacuum drying. Madeka and Kokini (*22*) reported a T_g of a commercial yellow zein with a protein content of 95% at 139 °C for a "bone dry" sample. Based on their glass transition curve as a function of moisture content, our MPZ sample has an estimated 0.5% moisture. This finding complements our findings with DMA where the lower loss tangent peaks at 93.1 °C was attributed to a relaxation due to loss of water from the test strip. The second loss tangent peak for MPZ was at 131.4 °C which temperature is similar to 129.8 °C, given in **Table 3**, when we take into consideration differences in scan rate of 5 °C/min for DSC versus 2 °C/min for DMA. The T_gs of MPZ/GDA and ZF/GDA were identical, where both values were higher than T_gs of Japanese white zein film controls that were compared with films from GDA-modified Japanese white zein (*3*). Those investigators attributed the increase in T_g to a lower chain mobility caused by cross-linking. The ΔC_p values of all four samples (i.e., 0.28−0.41J/g°C) in **Table 3** are of the same order of magnitude for zein data as well as data for wheat gluten, glutenin, and gliadins reported in the literature (*23*).

Thermogravimetric analysis (TGA) was used to evaluate and compare thermal stabilities and decomposition kinetics of MPZ,

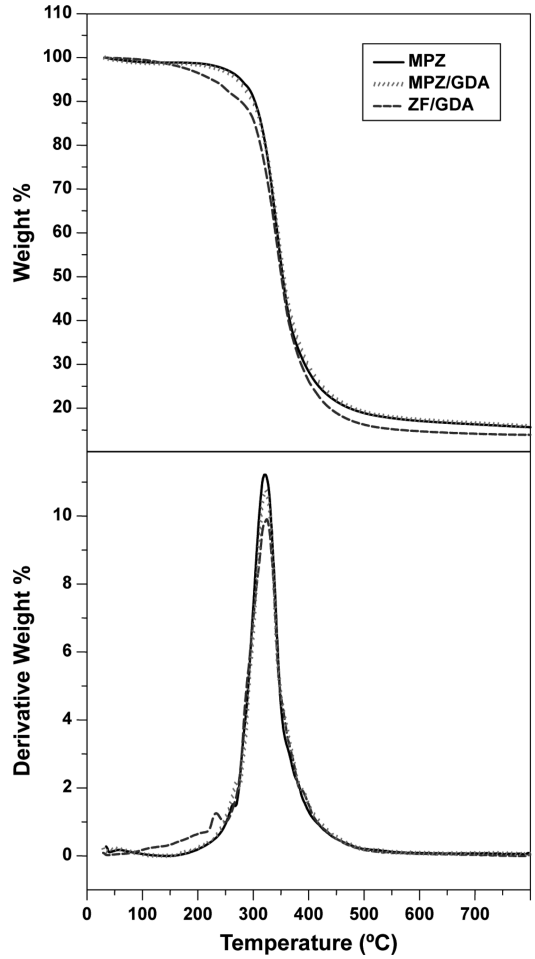

Figure 6. TGA thermograms of melt-processed zein (MPZ), MPZ modified with glutaraldehyde (MPZ/GDA), and zein films from GDA-modified zein (ZF/GDA) and their respective first derivatives. Data were recorded for each sample at a heating of 10 °C.

MPZ/GDA, ZF and ZF/GDA. The TGA curves and respective first derivatives for MPZ, MPZ/GDA and ZF/GDA are shown in **Figure 6**. ZF/GDA shows loss of mass from about 75 to 250 °C, which is attributed to loss of HAc entrapped within the film matrix. This loss in mass occurred during the annealing of the ZF/GDA film strip subjected to heating for 1 h at 160 °C for DMA discussed previously. The thermal degradation of MPZ, MPZ/GDA and ZF/GDA occurred at 322.3 °C, 323.0 and 324.5 °C respectively. Magoshi et al. (*24*) reported that zein thermally degrades at about 320 °C which value is similar to our finding with MPZ. The GDA modifications slightly increase thermal stability. This finding, along with the minimal changes observed in the glass transitions via DSC analyses, confirms our results from chemical analyses that the degree of cross-linking is minor.

Kinetic studies can be used to characterize thermally induced events. Its application in this investigation will be used in a limited way to compare differences in activation energy (E_a) and pre-exponential factor (Z) for a series of similar materials because we do not have a well-defined reaction. Differences can occur simultaneously based on sample composition, microstructure features, branching and steric effects all of which impact on the reaction rate at a given temperature (*25*). The software program used to determine E_a and Z is based on methodology developed by Ozawa (*26*). For the determination of E_a two points of the same conversion are chosen on three T_g curves obtained at different heating rates to generate a master curve. In that publication Ozawa (*26*) assumes the reaction order to be constant and calculates E_a - Z data pairs on the derived master curve. Thermal degradation kinetics with TGA showed increases in the Arrhenius activation energy (E_a) and log Z MPZ at 50% conversion upon modification with GDA (see data in **Table 4**). Also observed is an increase, though not as great, for E_a and log Z for GDA-modified ZF compared with its respective control. The increases in log Z with increasing E_a demonstrates a kinetic compensation effect that may be a consequence of the use of the Arrhenius equation. Our findings suggest that the dried, collapsed organo-gels generated by GDA modifications of zein possess a higher network density than respective control samples, but, because of the complexity of thermal degradation investigations further research is needed to explain those observed increases in E_a and log Z.

Table 4. Kinetics for Zein Samples at 50% Conversion

sample[a]	E_a (kJ/mol)	log Z (1/min)
MPZ	198.8	17.09
MPZ/GDA	278.1	24.03
ZF	189.6	16.4
ZF/GDA	205.1	17.94

[a] All samples were run at 5, 10, 15 °C/min where MPZ = melt-processed zein, ZF = zein film either with or without modification with glutaraldehyde (GDA).

Mechanical Properties. Data for the mechanical property measurements of MPZ, MPZ/GDA, ZF and ZF/GDA, where the zeins were modified with 1% to 8% GDA are shown in **Table 5**. All test samples were equilibrated at 50% relative humidity for 1 week. Comparison of the compression molded MPZ data with that of ZF demonstrated that compression molding imparted higher tensile strength, lower elongation % and significantly higher Young's modulus. Modifications of both MPZ and ZF with 1% and 2% GDA showed improvements in tensile strength, elongation %, where Young's modulus of the MPZ sample bars showed little improvement, whereas, the ZF samples increased significantly. HAc residues in the ZF and ZF/GDA tensile bars are acting as a plasticizer, thereby, lowering tensile strength and Young's modulus and increasing elongation % when compared with respective MPZ and MPZ/GDA tensile bars. The improvement in elongation % for both MPZ/GDA and ZF/GDA indicates that the intramolecular reactions of GDA with zein, as discussed previously, causes linear chain formations with the GDA that increases flexibility of the tensile bars. Those tensile bars prepared from either MPZ or ZF modified with 4% or 8% GDA yielded significantly higher tensile strength than their respective controls, no change in elongation % for MPZ/GDA sample bars and slightly diminished elongation % for the ZF sample bars, small to no improvement, of Young's modulus for MPZ/GDA, but, significant improvement of Young's modulus for the ZF/GDA sample bars. The sample bars from either MPZ/GDA or ZF/GDA, each reacted with 8% GDA, were insoluble in HAc indicative of a chemical cross-linking (*3*). Tensile strengths, elongation % and Young's modulus for these samples were of the same order of magnitude.

Sample bars of MPZ and ZF cross-linked with 8% GDA were each subjected to boiling for 10 min or soaking for 24 h where these water processed bars were each blotted dry prior to mechanical testing with the Instron. Films and melt-processed bars, so treated, swelled with consequent weakening of tensile strengths and Young's modulus along with respective increases in elongation percent (**Table 6**). The ZF/8%GDA tensile bars retained much more water than did respective MPZ/8% GDA tensile bars that were boiled or soaked in water. Conceivably, the HAc residues in the film samples made these films more

Table 5. Mechanical Properties[a] of Melt-Processed Zein (MPZ) and Zein Films (ZF) Modified with Glutaraldehyde (GDA) When Stored at 50% Relative Humidity[b]

%GDA w/w% based on zein	tensile strength (MPa)		elongation %		Young's modulus (MPa)	
	MPZ	ZF	MPZ	ZF	MPZ	ZF
0	25.3 (3.8)	15.5 (2.8)	8.5 (1.1)	14.2 (2.1)	438.6 (30.6)	179.4 (26.4)
1	33.8 (3.4)	23.2 (1.1)	9.5 (0.6)	30.7 (3.7)	457.6 (24.1)	292.5 (6.1)
2	31.4 (3.7)	34.8 (5.5)	10.6 (1.7)	26.8 (6.0)	437.3 (31.8)	290.6 (33.0)
4	46.3 (4.8)	39.1 (3.7)	13.2 (1.3)	21.5 (9.0)	457.1 (25.4)	423.3 (63.0)
8	42.6 (1.0)	42.5 (1.7)	11.4 (1.3)	19.8 (2.3)	479.9 (33.8)	408.0 (38.7)

[a] Data sets of mechanical properties are the average of three replicates with five tensile bars for each replicate with standard deviations in parentheses. [b] Storage at 50% relative humidity for one week.

Table 6. Mechanical Properties[a] of Melt-Processed Zein (MPZ) and Zein Films (ZF) Modified with 8% Glutaraldehyde (GDA) and Subjected to Water Treatments

process[b]	sample	moisture (%)	tensile strength (MPa)	elongation %	Young's modulus (MPa)
none	ZF	6.7	42.5 (1.7)	19.8 (2.3)	408.0 (38.7)
	MPZ	4.1	42.6 (1.0)	11.4 (1.3)	479.9 (33.8)
boiled	ZF	27.1	10.1 (1.3)	57.6 (10.5)	78.4 (6.1)
	MPZ	8.3	25.2 (3.1)	39.0 (12.7)	253.7 (22.1)
soaked	ZF	20.7	6.5 (1.9)	77.5 (7.4)	47.0 (11.9)
	MPZ	14.1	11.4 (2.4)	41.7 (10.8)	135.7 (16.1)

[a] Data sets are the means of three or more replicates with the standard deviation given in parentheses. [b] None = stored at 50% relative humidity overnight; soaked = 24 h in water at room temperature prior to testing; boiled = 10 min in boiling water prior to testing.

hydrophilic, thereby, increasing their capability to swell in water. Despite the water processing treatments, MPZ/GDA and ZF/GDA maintained their integrity where the MPZ/GDA samples retained a higher tensile strength, lower elongation percent, and higher Young's modulus than the ZF/GDA samples. Based on comparisons of MPZ and ZF samples formed by cross-linking zein with GDA, the GDA reaction can be successfully achieved via melt-processing techniques, a prerequisite for extrusion processing. This process not only would limit solvent usage as well as the necessity for solvent recovery but also is amenable to industrial scale operations.

SAFETY

Glutaraldehyde (GDA), as a 50% (by wt) solution, is highly toxic. Precautions for handling include use of chemical-resistant gloves and chemical safety goggles. All experiments were performed in a safety hood; a plexiglass vented hood was constructed for enclosing and venting the torque rheometer.

ABBREVIATIONS USED

GDA, glutaraldehyde; HAc, glacial acetic acid; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OPA, ortho phthaldialdehyde; MPZ, melt-processed zein; ZF, zein film.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

LITERATURE CITED

(1) Hamaker, B. R.; Mohamed, A. A.; Habben, J. E.; Huang, C. P.; Larkens, B. A. Efficient Procedure for Extracting Maize and Sorghum Kernal Proteins Reveals Higher Prolamin Contents than the Conventional Method. *Cereal Chem.* **1995**, *72*, 583–588.

(2) Gennadios, A.; Weller, C. L. Edible films and Coatings from Wheat and Corn Proteins. *Food Technol.* **1990**, *44* (10), 63–69.

(3) Sessa, D. J.; Mohamed, A.; Byars, J. A.; Hamaker, S. A. H.; Selling, G. W. Properties of Films from Corn Zein Reacted with Glutaraldehyde. *J. Appl. Polym. Sci.* **2007**, *105*, 2877–2883.

(4) Roth, M. Fluorescence Reaction for Amino Acids. *Anal. Chem.* **1971**, *43*, 880–882.

(5) Sessa, D. J.; Selling, G. W.; Willett, J. L. Viscosity Control of Zein Processing with Sodium Dodecyl Sulfate. *Ind. Crops Prod.* **2006**, *23*, 15–22.

(6) Simons, S. S., Jr.; Johnson, D. F. The Structure of the Fluorescent Adduct Formed in the Reaction of the O-Phthalaldehyde and Thiols with Amines. *J. Am. Chem. Soc.* **1976**, *98*, 7098–7099.

(7) Steele, J., Jr.; C., H.; Nielsen, T. B. Evaluation of Cross-linked Polypeptides in SDS Gel Electrophoresis. *Anal. Biochem.* **1978**, *84*, 218–224.

(8) Gerrard, J. A.; Brown, P. K.; Fayle, S. E. Maillard Crosslinking of Food Proteins. I: The Reaction of Glutaraldehyde, Formaldehyde and Glyceraldehyde with Ribonuclease. *Food Chem.* **2002**, *79*, 343–349.

(9) Habeeb, A. F. S. A.; Hiramoto, R. Reaction of Proteins with Glutaraldehyde. *Arch. Biochem. Biophys.* **1968**, *126*, 16–26.

(10) Marquié, C. Chemical Reactions in Cottonseed Protein Cross-linking by Formaldehyde, Glutaraldehyde, and Glyoxal for the Formation of Protein Films with Enhanced Mechanical Properties. *J. Agric. Food Chem.* **2001**, *4*, 4676–4681.

(11) Forato, L. A.; Bicudo, T. DeC.; Colnago, L. A. Conformation of α Zeins in Solid State by Fourier Transform IR. *Biopolym. (Biospectroscopy)* **2003**, *72*, 421–426.

(12) Kretschmer, C. B. Infrared Spectroscopy and Optical Rotary Dispersion of Zein, Wheat Gluten and Gliadin. *J. Phys. Chem.* **1957**, *61*, 1627–1631.

(13) Nuzzo, R. G.; L. H. Dubois, L. H.; D. L. Allara, D. L. Fundamental Studies of Microscopic Wetting on Organic Surfaces, 1. Formation and Structural Characterization of a Self-Consistent Series of

Polyfunctional Organic Monolayers. *J. Am. Chem. Soc.* **1990**, *112*, 558–569.
(14) Kepley, L. J.; Cooks, R. M. Selective Surface Acoustic Wave-Based Organophosphonate Chemical Sensor Employing a Self-Assembled Composite Monolayer: A New Paradigm for Sensor Design. *Anal. Chem.* **1992**, *64*, 3191–3193.
(15) Silverstein, R. M.; Bassler, G. C. In *Spectrometric Identification of Organic Compounds*, 2nd ed.; John Wiley & Sons Inc.: New York; 1967; Chapter 3, pp 64−109.
(16) Colthup, N. B. Interpretation of Infrared Spectra, In *An ACS Short Course on Film*; American Chemical Society: Washington, DC, 1967; p 27.
(17) Whipple, E. B.; Ruta, M. Structure of Aqueous Glutaraldehyde. *J. Org. Chem.* **1974**, *39*, 1666–1668.
(18) Korn, A. H.; Feairheller, S. H.; Filachione, E. M. Glutaraldehyde: Nature of the Reagent. *J. Mol. Biol.* **1972**, *65*, 525–529.
(19) Lubig, R.; Kusch, P.; Röper, K.; Zahn, H. Zum Reaction mechanismus von Glutaraldehyd mit Proteinen. *Monatsh. Chem.* **1981**, *112*, 1313–1323.
(20) Monsan, P.; Puzo, G.; Mazargui, H. Étude du mécanisme d'établissement des liasons glutaraldéhye-protéines. *Biochimie* **1975**, *57*, 1281–1292.
(21) Saleh, A. M.; El-Khordagui, L. K.; Robb, D. A.; Florence, A. T. Effect of Some Drugs and Additives on the Cross-linking of Bavine Serum Albumin by Glutaraldehyde. *Int. J. Pharm.* **1989**, *5*, 205–210.
(22) Madeka, H.; J.L. Kokini, J. L. Effect of Glass Transition and Cross-Linking on Rheological Properties of Zein: Development of a Preliminary State Diagram. *Cereal Chem.* **1996**, *73*, 433–438.
(23) L. DiGioia, L.; Cuq, B.; Guilbert, S. Thermal Properties of Corn Gluten Meal and Its Proteic Components. *Int. J. Biol. Macromol* **1999**, *24*, 341–350.
(24) Magoshi, J.; Nakamura, S.; Murakami, K.-I. Structure and Physical Properties of Seed Proteins. 1. Glass Transition and Crystallization of Zein Protein from Corn. *J. Appl. Polym. Sci.* **1992**, *45*, 2043–2048.
(25) Behnisch, J.; Zimmermann, H. Thermal Analysis and Kinetic Concepts of Solid-State Reactions. *J. Therm. Anal.* **1988**, *33*, 127–1268.
(26) Ozawa, T. A New Method of Analyzing Thermogravimetric Data. *Bull. Chem. Soc. Jpn.* **1965**, *38*, 1881–1886.

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