X-ray Diffraction Characterization of the Structure of Zein–Oleic Acid Films

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ABSTRACT: Wide-angle (WAXS) and small-angle X-ray scattering (SAXS) studies of dry granular zein, zein fibers, zein–oleic acid resin, and zein–oleic acid films are reported. WAXS patterns showed two diffuse rings for these samples indicative of noncrystalline structures. Measured d-spacings of ~ 4.6 Å and ~ 10.5 Å were found for zein–oleic acid resins and films, consistent with the presence of α-helical segments. The granular zein and zein fibers had ~ 4.6-Å and ~ 9.5-Å spacings. Neither the films nor the fibers showed evidence of orientation of the molecular axes. SAXS studies of zein–oleic acid films indicated that the structure of the films was affected by preparation method. Biaxially drawn resin films showed periodicities of ~ 170 Å along the film surface direction and ~ 135 Å in the thickness direction, while the cast films had weaker intensity periodicities of ca. 80 Å for all beam directions; a weak, diffuse 45-Å spacing was also observed for both samples. The 170-Å periodicity was present in the resin before deformation and following uniaxial deformation. No SAXS periodicity was observed for the granular zein or zein fibers. Several structural models are presented for the resin films that are consistent with reports in the literature that zein, in solution, consist of prism-like particles consisting of four or more molecules. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 71: 1267–1281, 1999

Keywords: zein; oleic acid; protein films; small-angle X-ray scattering, wide-angle X-ray scattering

INTRODUCTION

Interest in nonfood uses of cereal starches and proteins is increasing driven by environmental concerns and agricultural economics. Cornstarch utilization for fuel ethanol and bulk chemicals is expected to continue growing. However, starch separation leaves behind a protein-rich residue for which new industrial uses and markets are sought. The total protein content of the corn kernel is about 10%, chiefly distributed in germ and endosperm. Based on the solubility, these proteins (corn gluten) can be grouped into four categories: albumins (water soluble), globulins (saline soluble), prolamines (soluble in alcoholic solutions), and glutelins (soluble in dilute alkali solutions).[1] Zein is the prolamine in corn. It is abundant in corn gluten meal and corn gluten feed, byproducts from corn wet milling, which are currently chiefly used as ingredients for animal feeds despite deficiencies in several amino acids. Corn gluten meal contains about 70% protein (dry base) with zein amounting to 60% of that protein.[1,2] At present, zein has very limited commercial applications, the only current industrial use being as a coating agent in the pharmaceutical

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and food industries. For new agricultural and consumer uses it has the additional potential of being renewable and biodegradable. Thus, increased value-added utilization of zein in any form is of economical importance to both the corn-refining industry and farmers, while its use as a bioplastic material could be of plastics industry and environmental interest.

Zein is separated from the gluten meal by solvent extraction, usually with isopropanol. After centrifugation, zein is precipitated by cooling the supernatant to −15°C and then dried to a powder.[1] Zein occurs as aggregates linked by disulfide bonds in whole corn. After extraction, zein loses some of its disulfide bonds.[1] According to differences in solubility, zein consists of three families of proteins: α-zein, β-zein, and γ-zein.[3] α-Zein accounts for 75 to 85% of the total zein, and is dominated by two groups of proteins, Z19 and Z22 according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[4] Z19 refers to the faster band with apparent molecular weight around 19,000 and Z22 is the slower band with apparent molecular weight around 22,000. However, according to the complete amino acid sequences of α-zeins, the true molecular weights of Z19 and Z22 are about 23,000–24,000 and 26,000–27,000, respectively.[5,6]

The zein amino acid sequence has been derived from chromatography on zein itself and determination of the genes that code the protein. Z19 and Z22 consist of 210 and 245 amino acids, respectively.[5–7] Z19 and Z22 have sequence homology: the N-terminals of their sequence contains 35 to 37 amino acids, the C-terminals have 8 amino acids, and the central domain consists of 9 (for Z19) or 10 (for Z22) repetitive domains. These repetitive domains contain blocks of 14 to 25 amino acid residues, with an average length of 19 and 20 amino acids for Z19 and Z22, respectively.[6,7] Early studies on the conformation of zein in alcoholic solutions utilized ultracentrifugation, birefringence, and dielectric experiments.[8–11] These studies were interpreted as indicating that zein particles in alcoholic solutions have an asymmetric ellipsoid or rod shape with 7 : 1 to 28 : 1 axial ratios, depending on the authors and characterization techniques utilized. Subsequently, the secondary structure of zein in solution was investigated by spectrometry: optical rotation, optical rotary dispersion, and circular dichroism.[4,12] These studies suggest that the average α-helical proportion of zein amounts to 50 to 60%, the proportion of β-sheets is about 15%, and the remainder of the molecule is aperiodic. Also, by employing the sequence prediction method of Chou and Fasman,[13] it was determined that the α-helices predominate in the central domains of zein.[11] On the other hand, the predictions for reverse turns and β-sheet suggest that the zein contain little of these structures.[11] Based on the hydration potential, polarity, and secondary structure properties of the residue, a possible 9- or 10-helix zein structure was proposed by Argos et al.[4] In their model, the nine successive helical segments are arranged in an antiparallel ring fashion stabilized by intramolecular hydrogen bonds. However, no direct structural evidence was given for the model.

More recently, small-angle X-ray scattering (SAXS) has been used to study the dimensions and shape of zein in alcoholic solutions.[11,14] Tatham et al.[11] obtained values of R_g (radius of gyration) of 44.1 ± 2.2 Å and R_c (radius of gyration of the cross-section) of 2.45 ± 0.12 Å in aqueous 70% methanol at a concentration of 2.5 and 8 mg/mL (the data were said to be identical in the angular range examined). These data were interpreted in terms of the zein particles being either a prolate ellipsoid of length 196 Å and cross-section diameter 7 Å or a rod of length 153 Å, diameter 13.8 Å. Viscometric measurements yielded values of 238 and 18 Å for the length and diameter, regardless of the shape. All of these sets of dimensions are too small for a single zein molecule. They suggest the (50%) α-helical segments alone would have a length of > 200 Å and a diameter, including side chains, of 15 Å. Neither their suggestion of a folded-back α-helix bundle or distortion to shorten the length would seem to overcome the problem.

Matsushima et al.[14] have also reported SAXS measurements of R_g and R_c of zein, in 70% aqueous ethanol, over a range of concentrations from 2–40 mg/mL. They report values of R_g = 40.0 ± 0.3 Å and R_c = 13.9 ± 0.5 Å for reduced α-zein (for solutions containing 0.1 or 2% v/v β-mercaptoethanol); i.e., a similar value to that of Tatham et al.[11] for R_g but a considerably larger value for R_c. For the nonreduced α-zein, which is what Tatham et al.[11] also used, they obtained values of 49.8 ± 0.4 and 19.0 ± 0.4 Å. The larger R_c is probably the result of using the larger range of concentrations, the R_c data being obtained at large angles where the intensity is low. Contrary to Tatham et al.'s[11] results of a particle size smaller than the molecular size, Matsushima et al.[14] concluded the reduced α-zein particle in so-

lution consists of an aggregate of four zein molecules; the unreduced form obviously consists of more than four molecules, but no model was presented. Their model and its relation to our results for SAXS from zein in the solid state are considered further below, along with other related WAXS results for wheat gluten. As pointed out by both Tatham et al.[11] and Matsushima et al.,[14] the SAXS results are incompatible with the ring model proposed by Argos et al.[4]

To our knowledge, only one wide-angle X-ray scattering (WAXS) study has been conducted on zein. Yang et al.[15] studied dry-spun zein fibers by WAXS, and observed two diffuse rings in the pattern; however, the d-spacings were not reported. They found no orientation in the fibers as spun and only slight orientation, by sonic velocity measurements, when the molecules were crosslinked before being drawn. In a more recent article by Zhang et al,[16] the properties of wet spin zein (and zein–soy protein blend) fibers were described; however, no X-ray data was presented.

Among cereal proteins, wheat protein is the most studied, due to its bread-making properties. Arndt and Riley[17] have shown that the patterns obtained from a wide range of animal proteins and plant proteins fall into three distinct classes: α, β, and γ. The first group contains the largest number and consists of globular proteins such as egg albumin, ribonuclease, and hemoglobin. They consist of folded polypeptide chains in the α-helix configuration of Pauling and Cory.[18] Traub et al.[19] observed two strong diffuse rings in the X-ray diffraction pattern of wheat grain in the region of 5 and 10 Å. The X-ray scattering patterns of the dry gliadin, glutenin, as well as wheat flour and wheat grain, all showed two diffuse rings in this range. These patterns most nearly agree with the α-group proposed by Arndt and Riley.[17] Wheat protein (gluten) consists of many heterogeneous proteins. Gliadin and glutenin, the main proteins in wheat, contain considerable proportions of α-helix conformation in their structure. Gliadin is a prolamine, similar to zein, while glutenin is a glutelin. However, a recent report on high molecular weight wheat gluten, which forms about 1% of gluten, suggests it consists basically of the β-turn structure.[20]

Hess[21] showed that the native protein in the wheat grain could be physically separated into wedge (between starch grains) protein and bound (to the starch grains) protein. Two SAXS spacings were obtained from both of these fractions, one representing a spacing of d ~45–47 Å, and the other d ~90–100 Å. Traub[19] later examined sections of the endosperm of wheat, rye, corn, rice, and barley on a low-angle focusing camera. His results indicated that the ~47-Å spacing was due to the phospholipid bound in wheat protein, presumably bilayers in a phospholipid domain. In his experiment, the free fat, which is extractable with cold petroleum ether, showed a broad diffuse ring (40–80 Å) as well as the 90–100-Å spacing. Part of the bound fat could then be further extracted with cold acetone; this showed a strong, sharp spacing at 47 Å. The extracted flour still showed a strong 47-Å spacing and gave a good bread with excellent texture; presumably not all of the phospholipid was extracted. Lipoidal materials extracted by acetone from the whole meal of corn and barley did not show the 47-Å spacing; however, the spacing was observed in the acetone extract from rye, a cereal with limited baking properties.

Based on electron micrographs and X-ray studies, Grosskreutz[22,23] proposed that gluten forms platelet structures ~ 70-Å thick, independent of the presence of lipids. However, although he found the 45-Å (and 62-Å) spacings attributable to the lipid in dry and moist gluten, no additional periodicities attributable to the protein or protein–lipid complexes were observed. The lipid comprises ca. 7% by weight of the gluten; the presence of the peaks indicates domains consisting of at least several bilayers. His minimum scattering angle was 8 milliradians, restricting resolution to ca. 170 Å. The 70-Å platelet structure was derived from the shape of the central, diffuse scattering, intensity curves from moist gluten, and a 6% gluten dispersion in acetic acid, the 70 Å being the maximum thickness whose scattering curve would be similar to that observed. Stretched, freeze-dried gluten gave elliptical, diffuse SAXS patterns when the beam was parallel to the film surface, elongated along the film thickness direction. Although the shape of the pattern would be in agreement with plate-like structures, Grosskruetz[22,23] indicated the intensity distribution along the film thickness direction, similar to that in dry, powdered gluten, suggested aggregation of the platelets into thicker structures. With the beam normal to the film a circular (unoriented) diffuse scattering was observed with a weak, oriented, diffuse scattering superimposed; he suggested some of the platelets are rotated with respect to the plane of stretch, by folding.

Grosskruetz[23] also examined related (stretched, freeze-dried) films by transmission electron microscopy. Micrographs from cross-sections of these films, which had been OsO_4 fixed and stained, were interpreted in terms of sheets a few hundred to several thousand Å thick; most of the visible sheets in the micrograph published were 2–5000 Å thick, separated by unstained domains of similar thickness. Because the OsO_4 will stain both the lipid and the protein, the origin of the unstained domains is not clear; possibly they were an artifact of the sample preparation accompanying the aggregation of the platelets. He proposed a model, which seems generally accepted, in which stretched gluten consists of sheets predominantly parallel to the surface, each made up of platelets that have H-bonded together through an interstitial aqueous phase under the action of hydration and mechanical working. The phospholipid bilayers were proposed to be dispersed, individually, between some of the platelets within a sheet. The latter act as slip planes within (and presumably between) the sheets.

Also of concern relative to our results is the structure of oleic acid at room temperature. For pure oleic acid a quasi-smectic mesophase was proposed for temperatures between 13 and 30°C[24] with three possible crystal structures (α, β, and γ) below 13°C. As discussed further below, a 17.3-Å spacing is observed for the mesomorphic state and 41–43-Å spacings for the crystalline state.

In previous articles, it was reported that the tensile, thermal, and permeability properties of zein–oleic acid films varied with the film-forming processes employed.[25–28] Films cast from zein-plasticizer solutions, formed by evaporation of the solvent, were stiff and brittle, while films drawn from plasticized resin showed more flexibility and toughness. DSC studies indicated phase separation in the cast films during the heating and cooling cycles, which was not observed for resin films. Scanning electron micrographs also showed a degree of structure development for resin films that was not apparent in cast films. It was suggested that the ability of zein to form films might be explained by alignment of its rods or ellipsoids. However, most studies on zein conformation have been on its form in solution.[11,14] No SAXS or WAXS studies on zein in a solid film form have been reported. In this article, we investigate the structure of zein films by X-ray scattering to help gain an understanding of the structure–function relationship of zein-based films.

EXPERIMENTAL

Zein, regular grade, was obtained from Freeman Industries Inc. (Tuckahoe, NY) Oleic acid, 90% was from Aldrich (Milwaukee, WI). Ethyl alcohol (190 proof) was from Midwest Grain Products Co. (Pekin, IL).

Cast films were obtained by dissolving zein to 16% (w/v) in warm (75–80°C) aqueous ethanol (1 : 3). Oleic acid was added to the warm solution at 0.5 g oleic acid/g zein and stirred. Cast films were prepared by allowing the emulsion to cool to room temperature and then casting on a Teflon-coated surface (Teflon is a registered trademark of E. I. du Pont de Nemours & Co., Inc.). Films were formed by solvent evaporation and peeled off when dry. Resins were obtained from zein–oleic acid emulsions prepared as described above, precipitated by the addition of cold water. Resins were collected as soft solids and kneaded in a Brabender Farinograph (C. W. Brabender Co., Hackensack, NJ) to form a resin dough from which biaxially stretched resin–oleic acid films were drawn by hand. These films were also allowed to dry at room temperature and ambient relative humidity. Uniaxially stretched resin films were obtained by passing the zein–oleic acid resin dough through a roller; elongations of 3400% were obtained. Zein fiber was obtained by comelting zein powder with water in a microwave oven (1500 Watt Power, Sears, Chicago, IL) and stretching the resulting mass by hand into a long fiber.

WAXS data were obtained with a Philips X-ray power supply (Philips Electronic Instrument, Inc., Mount Vernon, NY) operated at 20 mA, 35 kV, and a Statton camera, with a camera length of 5.5 cm, using Ni filtered CuK_α radiation and 2–4 h exposures. Films were scanned with a Relysis 9624 scanner using Adobe Photoshop-ArtScan software at 600 dpi resolution. NIH Image software was used to prepare intensity plots of rectangular sections of the pattern as well as to measure distances between the resulting peaks.

SAXS data were initially obtained using the same Statton camera (17-cm sample to film distance) followed by use of a Siemens M18HF[22] Rotating Anode power supply with a 0.3 × 3 mm filament and Cu target operated at 80 mA, 40 kV. A Siemens Anton Parr small-angle camera was used with a Siemens HiStar 2D position sensitive detector. In addition to shorter exposures, the latter permitted obtaining azimuthally (ξ) integrated intensities either over a 360 or a 20° range

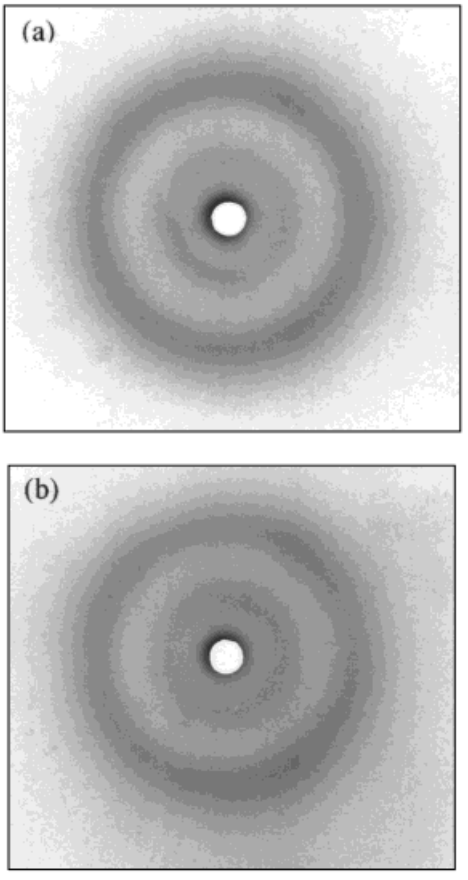

Figure 1 WAXS patterns of zein–oleic acid resin films prepared by biaxial stretching of resin dough. (a) Beam normal to film; (b) beam parallel; thickness direction vertical.

centered at 0 and 90°. 0° is vertical on the patterns below, corresponding to the thickness direction when the beam is parallel to the film surface and the draw direction when the beam is normal to uniaxially drawn films. Peaks were calculated and spacings determined using the Siemens software. The patterns obtained on the two instruments were similar.

RESULTS AND DISCUSSION

WAXS Patterns

Diffraction patterns from zein–oleic acid resin biaxially stretched films are shown in Figure 1(a) and (b), with beam directions normal and parallel to the film surface, respectively. The uniform intensity distribution around the ring indicates no preferred orientation either along the film surface or through its thickness. Although the pattern obtained with the beam parallel to the surface is circular, with spacings of 4.6 and 10.9 Å, with the beam normal to the film both rings were slightly elliptical, giving spacing of 4.6 and 10.4 Å at 0° and 4.8 and 10.9 Å at 90° (Table I). The spacings between 10 and 11 Å are thought to be the spacings of the interhelix packing or the mean distance of approach of neighboring helices. The shorter d-spacings of 4.6 and 4.8 Å might be related to the average backbone distance within the helices.

Traub et al. reported[19] that the 5-Å ring was weakened when wheat gluten was denatured, and that a sharp 4.65-Å ring appeared. They speculated the 4.65-Å ring was due to the transformation of the α-helical structure to a β-sheet structure. Although the β-sheet structure, either from the preexisting β-structure in zein or transformed from the α-structure due to heat and mechanical treatment, may contribute to our 4.6-Å spacing, we are still inclined to believe that the 4.6 and 4.8-Å spacings are mainly contributed by the α-helical structure in zein. The various values of α-helix spacings in this range found in nature vary from 4 to 5 Å.[17] Schulz and Schirmer[29] suggested that the typical α-helix (both right handed and left handed) has a helix diameter of about 4.6 Å. The α-helix polypeptide chain is held in its configuration by intramolecular H-bonds. Thus, it is very stable; it is the most abundant protein structure. Many synthetic polypeptides also contain α-helical structures. Geil et al.[30] and Tajima et al.[31–33] synthesized copolypeptides composed of γ-benzyl L-glutamate, L-phenylalanine, L-valine, and L-alanine, and found these copolypeptides preferentially form α-helix structure (note that zein also contains high proportions of the last three amino acids). From WAXS, they observed ca. 4.6 and 10.3-Å spacings, both spacings changing slightly with the solvent used for casting. Apparently, the ca. 5-Å d-spacing values of the α-helix are affected by the amino acid sequence; i.e. the side group's type and packing. However, the origin of the difference in spacings observed with the beam normal to the surface presumably is not due to a difference in sequence, but rather to some distortion of the helix packing in the plane of the film (see below).

In the present study, the interchain spacing between the α-helices is about 10.5 Å. Assuming the zein molecule subunits in neighboring molecules are packed in a hexagonal array (considered to be a reasonable packing because zein is a storage protein), this corresponds to a diameter of the

Table I Values of d-Spacing for Zein-Based Films

Samples	d-Spacing (Å)	
	WAXS	SAXS
Resin film, beam normal (biaxially deformed)	10.4, 10.9; 4.6, 4.8	165, ~ 45
Resin film, beam parallel	10.6; 4.9	135 (0°); 170 (90°)
Dried zein–oleic acid resin	10.5; 4.9	165; ~ 45 (?)[a]
Resin film (uniaxially deformed with a roller)	10.7; 4.9	170
Cast film, normal beam	10.6; 4.9	80; ~ 45 (?)
Cast film, beam parallel	10.2; 4.9	65 (0°); 80 (90°)
Granular zein	10.1; 4.7	—[b]
Zein fiber	9.9; 4.7	—[b]

[a] (?), presence of peak is questionable due to breadth and low intensity.
[b] No apparent peak.

zein helix of 12.1 Å. This value is in good agreement with the 12-Å zein α-helix diameter assumed by Matsushima et al.[14] for construction of their model, with values of 10–14 Å being considered reasonable depending on the side-chain lengths.

WAXS patterns were also obtained from granular zein, zein fiber, dried zein–oleic acid resin, cast zein–oleic acid film, and zein–oleic acid resin film uniaxially stretched with the roller. These results indicated that granular zein and zein fiber had significantly smaller d-spacings than the other samples (Table I). As expected, the cast film patterns were circular (not shown), as were the patterns from all of the other samples. Although the uniform intensity distribution around the rings indicates no molecular axis orientation was present in any of the samples, the lateral spacing of the helices in the granular zein and fibers, in particular, appears to be different from that of the other samples. We can suggest that zein, in the absence of oleic acid, has a smaller helix diameter. Neither the granular zein nor zein fiber had been dissolved in aqueous ethanol, nor had fatty acids been added. The increased d-spacing after treatment could be a residual effect due to the dissolution of zein in ethanol, or it could be due to hydrophobic interactions of the side chains with the oleic acid. Also, it was noted that granular zein and zein fiber, for equivalent exposure time and sample thickness, had a much weaker interhelix ring than that of the other samples. Based on the low 9.5-Å ring intensity in the granular zein and zein fiber, one might speculate that heating and dissolution permits rearrangement of the zein molecules and enhances packing of the α-helix. More important, the results suggest that the basic α-helix structure in zein is stable and is not easily changed by the heating, dissolution, and mechanical treatment employed in our film preparation, even though the side-chain packing may change.

Despite the breadth of the WAXS rings, the differences in spacings listed are believed correct to at least 0.1 Å. Thus, both the difference in spacings for the two reflections at 0 and 90° in the normal beam direction resin film samples and the difference in the interhelix spacings in the cast film are believed real. The difference in the interhelix spacing in the cast film may be due to the shrinkage in thickness occurring during drying, the lateral dimensions of the films being restrained by the substrate. A related effect may also explain the results for the biaxial film. If the sample was taken from the center of the film, no difference with direction in the plane of the film would be expected; simultaneous expansion in all directions occurring when stretched, as at the end of a balloon. On the other hand, at the edge of the film, deformation in the radial and tangential directions would differ, being greater in the tangential direction. The interhelix spacing in the side-view pattern, taken from the same set of strips of film, is intermediate between the values for the normal view.

Of particular interest is the observation that WAXS patterns from the "melt"-drawn fiber and the rolled films, although both samples were highly elongated, show no sign of either molecular orientation or asymmetry in interhelix spacing (side-view patterns from the rolled film were not obtained). If the small interhelix spacing in the fibers was due to better lateral packing of molecules oriented in the draw direction, one would

expect an elliptical pattern, those molecules normal to the draw direction being "pulled" apart. A similar effect was observed by Yang et al.[15] for dry-spun zein fibers; no orientation was observed as spun or after stretching, even when partially crosslinked before being drawn. In the precrosslinked fibers, even though the tenacity increased significantly with the degree of stretch, which the authors attributed to the orientation of unfolded molecules, the X-ray patterns were said to still show no apparent orientation. This was attributed to intramolecular crosslinks preventing full unfolding of the zein molecules at the same time intermolecular slip was being reduced by intermolecular crosslinks. They suggested the intramolecular forces in the folded noncrosslinked zein molecules are sufficiently stronger than the intermolecular forces that the molecules slip during deformation. The addition of fatty acids could only enhance the potential for molecular, or larger structural unit, slip. If the structural units, the individual folded molecules, are asymmetric, as generally proposed, and are independent, they should align during deformation. For instance, if the molecular axis is normal to the long axis of elliptical or rod-like molecules, the molecular axis would be expected to align normal to the draw direction, but probably random about the draw direction. This has been observed for the needle-like crystals in drawn PVC.[34] The observation that no orientation is seen suggests little asymmetry in the deformation units, either because they are not asymmetric or because they cluster into larger, nearly symmetric units.

SAXS Patterns

SAXS patterns of cast and drawn resin zein–oleic acid films, with beam direction normal and parallel to the film surface, are shown in Figure 2. Patterns with the beam direction normal to the film surface have diffuse rings [Figs. 2(a) and (c)], with the cast films having patterns of lower intensity. Spacings are described below based on the integrated patterns. However, when the beam direction was parallel to the surface of the drawn resin film, the pattern is elliptical, with intense arcs at both ends [Fig. 2(b)]. This is attributed to a layered structure parallel to the surface of the films. In addition, there is an intense vertical streak. Patterns from cast zein–oleic acid films with the beam direction parallel to the film surface have an elongated central diamond and a slightly elliptical, low-intensity, diffuse ring with no arc [Fig. 2(d)]. The elongated streaks parallel to the thickness direction in both samples are attributed to voids elongated parallel to the surface; i.e., plate-like or needle-like.

Figure 3 shows azimuthally (χ) integrated (360°) intensity plots of the resin and cast films with the beam direction normal to the film thickness, corresponding to Figures 2(a) and (c). The resin films have a strong and sharp peak at $2\theta = 0.52°$ (170 Å) and a weak and broad peak at around $2\theta = 2.0°$ (45 Å). The cast films, however, show a very broad peak, nearly 2° 2θ wide, centered at ca. 1.2° (80 Å). The difference in patterns and plots of the resin and cast films confirms differences in morphological structure, as suggested by Lai and Padua.[26] In their study, resin films were found to have better tensile properties than those of cast films (refer to Table II).

The integrated intensity plots for the resin and cast films with the beam parallel to the film surface are shown in Figure 4. When integrated from −10 to 10° [Figs. 4(b) and (d)], e.g., along the arc of Figure 2(b), Figure 4(b), in particular, shows the effect of the meridional streak; the peak, with ca. 135-Å spacing, is only a shoulder on the strong central diffuse scattering. For the cast film [Fig. 4(d)], the streak is considerably weaker at the 2θ of the peak and a broad, low-intensity peak similar to that in Figure 3(b) is seen. On the other hand, if integrated from 80 to 100° the resin films [Fig. 4(a)] show a peak similar in shape and spacing (170 Å) to that in Figure 3(a); in cast films the peak remains weak and diffuse, with the same spacing (ca. 80 Å) as in Figures 3(b) and 4(c).

In summary, the resin films have a well-defined 170 Å periodicity, randomly oriented in the plane of the film, as expected for a simultaneously, biaxially drawn film. This is in contrast to the difference in WAXS spacings at 90° angles, even though the SAXS sample was the same stack of films used for the WAXS patterns. In the side view pattern, the same periodicity was seen parallel to the surface, while there is an even more intense, 135 Å periodicity normal to the film surface. The breadth of the SAXS peak from the cast films suggests a distribution of periodicities (possibly paracrystalline in nature[37]) centered at ca. 80 Å. This pattern is observed as a uniform, low-intensity circle for both beam directions. In the side view of the resin film the 135-Å arc appears to be superimposed on the 170 Å ring.

The breadth of the peak for the cast films indicates a very broad distribution of spacings and/or a small crystal size, i.e., only a few repeats of the

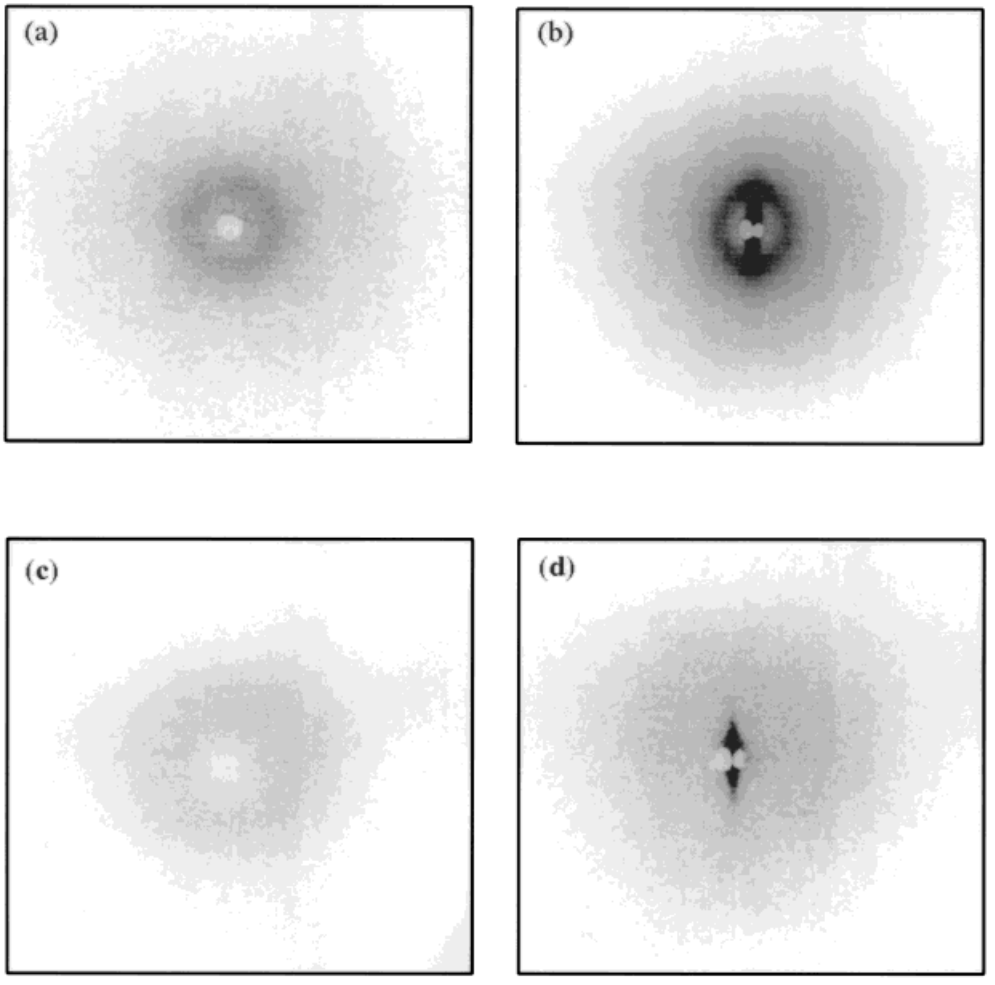

Figure 2 SAXS patterns of cast and biaxially drawn zein–oleic acid films. (a) Resin films, beam normal to film; (b) resin films, beam parallel to film; thickness direction vertical; (c) cast films, beam normal to film; (d) cast films, beam parallel to film; thickness direction vertical.

periodic structure giving rise to the SAXS peak. The photograph patterns suggest both films have elongated voids oriented parallel to the film surface. The fact there is no central diffuse scatter in the beam normal patterns suggests the voids are planar, parallel to the surface, rather than needle-like as in drawn synthetic polymer fibers and films.[38] We note the intensities of the various peaks of the patterns in Figures 3 and 4 are not directly comparable because both samples of different thickness and different scale factors were used for the plots.

SAXS studies were also conducted on zein granules, zein fibers, dried zein–oleic acid resin, and resin film uniaxially oriented by rolling. The dried zein–oleic acid resin and the uniaxially oriented resin film (beam normal) have similar pat-

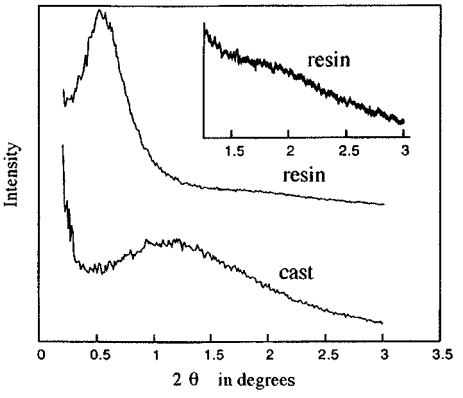

Figure 3 Azimuthally (χ) integrated (360°) intensity plots of biaxially drawn resin (a) and cast, (b) zein–oleic acid films. The inserted plot is a magnified portion of (a) from $2\theta = 1.25$ to 3.0.

Table II Tensile Properties of Solvent Cast and Biaxially Drawn Resin Zein–Oleic Acid Films[a]

Preparation Method	Tensile Strength (MPa)	Strain to Break (%)	Elastic Modulus (MPa)	Toughness (MPa)
Cast	6.81	3.18	317.12	0.081
Resin	8.68	11.89	267.19	0.829

[a] Data adapted from Lai and Padua (1997).[25]

terns to that of the biaxially oriented resin films in Figure 3(a), with strong and sharp peaks around the same 2θ positions (0.52°, 170 Å) (Fig. 5); this implies that they have similar structure. However, both granular zein and zein fibers had no apparent peaks or void scatter (plot not shown). This suggests that oleic acid plays an important role in the formation of both the SAXS periodicity and WAXS spacing in the resin and resulting films. In addition to the fact that zein granules and fibers, neither of which contained oleic acid, did not show observable SAXS peaks, the WAXS observations described above (see Table I) indicate that these samples have smaller interchain d-spacings than the oleic acid-containing samples.

The above results are in general agreement with numerous studies on protein–lipid interactions in wheat gluten. The proposed structure and effect of dough formation for wheat gluten, with close association between the protein and phospholipid components (we note phospholipids are essentially absent in zein), provide points of reference for understanding the observation that the physical properties of zein films depend on the method of preparation.[25–27] Apparently, the formation of zein–fatty acid dough through hydration and mechanical treatment allows closer association or interaction between zein and fatty

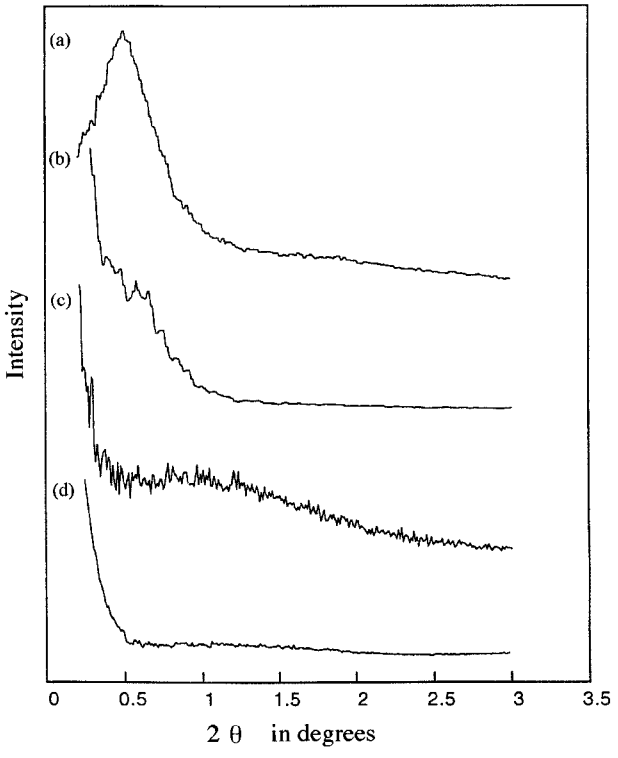

Figure 4 Azimuthally (χ) integrated (360°) intensity plots of biaxially drawn resin and cast zein–oleic acid films with beam parallel to surface, thickness direction = 0°. (a) Resin films integrated 80 to 100°; (b) resin films integrated −10 to 10°; (c) cast films integrated 80 to 100°; and (d) cast films integrated −10 to 10°.

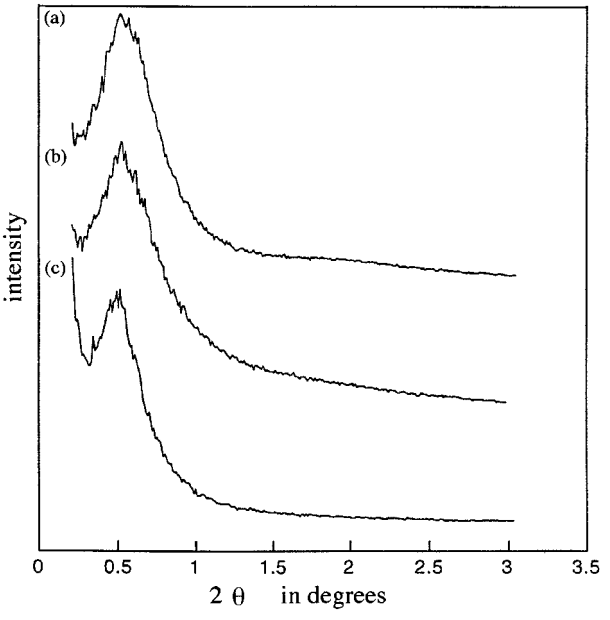

Figure 5 Aximuthally (χ) integrated (360°) intensity plots of (a) zein–oleic acid resin dough; (b) resin film prepared by stretching zein–oleic acid resin dough biaxially [same as in Fig. 3(a)]; (c) resin film prepared by uniaxially deforming zein–oleic acid resin dough with a roller.

acid, thus decreasing the observed phase separation in cast films.[26] This closer association of zein and fatty acid also resulted in the development of the periodic structure revealed by SAXS. For the case of wheat, McCaig and McCalla[35] suggested that a protein–lipid complex might be formed when dough is made. In addition, it was also proposed that mere wetting of flour causes binding of a considerable part of the native lipid.[36] As discussed above, X-ray studies of phospholipids were interpreted as suggesting that lipids in dough are in the form of bimolecular leaflets, giving rise to the 47-Å spacing;[19] its presence as a periodicity indicates clusters or domains of the leaflets in addition to those presumed dispersed individually within and between the relatively thick (ca. 300–5000 Å) protein sheets.[22,23]

It is very likely that the interaction mode between gluten and phospholipid also exists in the case of zein and oleic acid in the resin films. Oleic acid and zein associate during wetting and doughing processes. The difference in structure between resin and cast films implies that this interaction mode is engineerable. Simple evaporation of zein–oleic acid alcoholic solutions yielded a smaller, much less well-defined periodicity. The presence of the well-defined, larger periodicities, larger then can be attributed to domains of oleic acid, in the zein resin films and their apparent absence in gluten films indicates significant differences in structure between zein and gluten. Although the differences may, in part, be due to the difference in concentration of the lipid ($\sim$ 7%) in gluten and oleic acid ($\sim$ 33%) in zein–oleic acid resins, we suggest there is also a significant difference in the interaction; lipid domains would be even more likely for the higher concentration and are not observed.

Proposed Model for the Structure of Zein–Oleic Acid Resin Films

The models proposed here are based on the wide-angle and small-angle X-ray diffraction patterns obtained in the present study, Matsushima et al's.[14] SAXS studies of zein in solution, and the conformational analysis of zein in alcoholic solutions presented by Tatham et al.[11] and Argos et al.[4] In the present study, the interhelix d-spacings of zein–oleic resin film were about 10.5 Å. If the zein segments in neighboring zein molecules are assumed to pack in a hexagonal array, which is a reasonable assumption because zein is a storage protein, the diameter of the zein molecule would be $10.5 \cdot \frac{2}{\sqrt{3}} = 12.1$ Å, essentially the same as the 12-å α-helix diameter assumed by Matsushima et al.[14]

SAXS determinations of the size of reduced α-zein (aggregates disrupted by β-mercaptoethanol) and nonreduced α-zein were also reported by Matsushima et al.[14] Reduced α-zein had a radii of gyration $R_g = 40$ and $R_c = 1.39$Å, where R_g corresponds to the overall radius of gyration and R_c to the cross-sectional radius of gyration. They proposed the reduced α-zein structural unit has a rectangular prism shape with edge lengths a, b, and c, where a represents the longest edge, and b and c are lateral dimensions. If $a >> b$ and c, $a^2 = 12\,(R_g{}^2 - R_c{}^2)$, $R_c{}^2 = 1/12\,(b^2 + c^2)$; this yields $a = 130$ Å. Matsushima et al.[14] assumed a model related to that of Argos et al.,[4] consisting of 9 (Z19) or 10 (Z22) folded, antiparallel, helical segments, but aligned to form a ribbon instead of a ring, with an additional helical segment at the end of the ribbon contributed by a portion of the N-terminus. Assuming $b = 12$ Å = the diameter of the α-helix, then R_c yields c = the lateral width of the ribbon = 47 Å. This is approximately 1-1/2 times the length of the α-helix (30 Å for a 1.5-Å pitch) corresponding to the average 19–20 residues/tandem repeat (19 for Z19, 20 for Z22). On the other hand, assuming $c = 30$ Å, the fold period of the helices, yields $b = 42$ Å. With hexagonal close packing of the helices in neighboring zein molecules, they suggested this corresponds to a tetramer aggregate consisting of four ribbon-like zein molecules lying face to face. The 130-Å length (value of a) is appropriate for 10–11 segments. No account is taken, however, of the 10 C-terminus and 16–17 N-terminus residues not in the motif.

If, on the other hand, one assumes the zein molecules in the film correspond to the nonreduced molecules in solution then, for the measured values of $R_g = 49.8$ Å and $R_c = 19.0$ Å, a =160 Å; under these circumstances for $b = c$, each = 46 Å; for $c = 35$ Å, $b = 53$ Å, and for $b = 12$ Å, $c = 65$ Å. The larger value of a was presumed to be due to the presence of some larger zein molecules, presumably due to S-bridges between two of the Z19 and/or Z22 molecules; this, however, would not necessarily increase the length of the molecule. These aggregates would correspond to more than four molecules; no overall aggregate model was proposed.

A relatively simple model is proposed here based on the 160 × 46 × 46-Å aggregate. However, according to Matsushima et al.,[14] only a few of the molecules are dimerized and, furthermore, the SO_2 used in the steeping stage of corn wet milling partially disrupts the disulfide bonds.[2] Thus, our use of the reduced values for purposes of constructing a model also seems appropriate, although there still remains, as discussed below, a choice between the various values of *b* and *c*. Transmission electron microscopy and further X-ray studies, complemented by other structural characterization techniques, are in progress to permit determination of the "correct" model.

For all of the models there is also a choice to be made for the packing of the oleic acid. Iwahashi et al.[24,39] showed that pure oleic acid at room temperature has a sharp 17.3-Å spacing and, in addition, a sharp 4.58 intermolecular spacing. They suggest oleic acid, in the temperature range from ca. 13 to 30°C, has a quasi-smectic, mesomorphic structure, being isotropic above 30°C. Because oleic acid readily dimerizes, by H-bonding between the carboxyl heads, simple bilayer packing should lead to at least double this spacing; they, thus, suggest interpenetration of the tails. We note even this is insufficient; using Cerius[2] (Molecular Simulations, Inc.) to model the molecule, an extended oleic acid molecule, including the *cis* "kink" at the double bond, has a length of at least 22 Å; thus the molecules must also be tilted at a substantial angle to the plane of the heads. Although we question whether there is sufficient space in the "tail region" for both molecular tilt and full interpenetration with, simultaneously, near close lateral packing of the heads, the 17.3-Å spacing should be representative of the expected oleic acid structure in the zein films if H-bonded, dimerized bilayers are present. A double layer of interpenetrating molecules, heads on the surfaces to permit H-bonding to the associated protein and at the center (as dimers, i.e., four molecules, in total, through the thickness) would give an oleic acid thickness of 35 Å. On the other hand, if there is a hydrophobic protein–oleic acid interaction, a single, noninterpenetrating bilayer could have a thickness similar to that in the oleic acid in the solid state (below 13°C), i.e., ca. 42 Å.[24,39]

If the zein structural unit in the resin films has the same dimensions as the nonreduced α-zein aggregate, the model shown in Figure 6 is proposed. Here we assume stacking of pairs of the aggregates, alternating with hydrophobically bound oleic acid bilayers, forming platelets 135 Å thick. Within the plane of the platelet the aggregates are presumed to be aligned in rows two aggregates thick, with the aggregates in neighboring rows packed side by side, either aligned or staggered. The platelets are presumed to stack, with a common alignment of the aggregate rows, to form sheets of alternating aggregate pairs and oleic acid bilayers parallel to the surfaces giving rise to the 135-Å arcs. The sheets would be randomly aligned in the plane of the film in the biaxially oriented resin films. These sheets are similar to those proposed for gluten-lipid in wheat dough.[22,23] However, in gluten, no periodicity has been observed related to a protein–lipid layer structure. The 170-Å reflection, observed as a uniform ring with the beam normal to the surface and near the equator with the beam parallel to the surface, is attributed to the spacing along the prism axis; i.e., the 160-Å length of the rectangular prism model. The broad 45-Å spacing, seen primarily with the beam normal to the resin film and parallel to the surface in the side view, is attributed primarily to the aggregate diameter and, possibly, the third order of the 135-Å spacing. As sug-

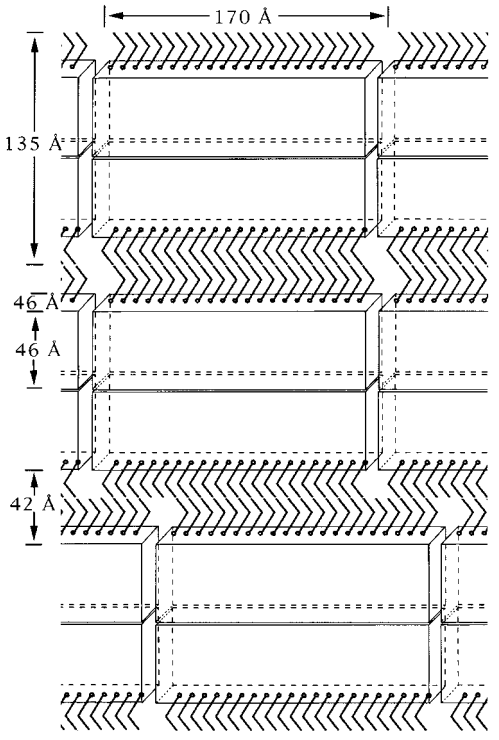

Figure 6 Proposed structure model of zein–oleic acid resin films based on the dimensions of nonreduced α-zein. The oleic acid bilayer corresponds to the type of packing expected in the solid state; i.e., below 13°C.[38,39] The thickness is vertical.

gested by the model, presumably slip between the platelets can easily occur in the oleic acid bilayers. The uniform distribution of intensity in the 10.5-Å interhelix spacing WAXS rings implies that there is no preferred orientation of the α-helical segments in either the aggregates or sample. Although this model can explain the SAXS and WAXS patterns, we know of no reason for the interaction of pairs of the aggregates with the oleic acid in preference to either more than two or to individual aggregates. However, similar interactions are required for the models based on reduced zein.

For the models based on the reduced α-zein dimensions, we estimate the value of c based on the residual consensus repeat motif proposed by Argos et al.,[4] with glutamine residues said to be in the folds, i.e., on the ends of the motif. The Z19 and Z22 zein supposedly consist of 9 or 10 tandem repeats averaging 19–20 residues. With at least two residues in the fold (to span the 12-Å intermolecular spacing), this leaves an average helix length of 25–27 Å, and a fold surface on the order of 6–8-Å thick. We conclude the average fold period is around 30–35 Å, choosing this value for c. We, thus, choose the tetramer model of four stacked ribbons as the basic structural unit with average dimensions of 130 × 42 × 32 Å, admittedly with no known reason why a tetramer should be a stable unit. With these dimensions there is no dimensional reason for a particular orientation of b and c during deformation.

As for the model in Figure 6, the strong 135-Å arcs [Fig. 2(b)] suggest that in the resin films there is a platelet-like structure parallel to the surface with a spacing of 135 Å. Considering the morphology of the reduced α-zein and the oleic acid molecular packing, three possible models can be suggested. In the first model the 135-Å spacing is from the long axis of the zein aggregate. Thus, the zein tetramers would be stacked vertically along the film thickness with the α-helical segments parallel to the surface. The fatty acid does not contribute to this spacing. The 45-Å reflection could come from a random lateral packing of aggregates and oleic acid bilayers, with no reasonable explanation for the 170-Å reflection. We also know of no physical reason for the long axis to be normal to the film surface and draw direction and, thus, reject this model. The second possibility is that zein prisms are stacked in the direction of the film thickness with oleic acid layers in between. Assuming, as above, that the average fold period in the tetramer zein aggregate is

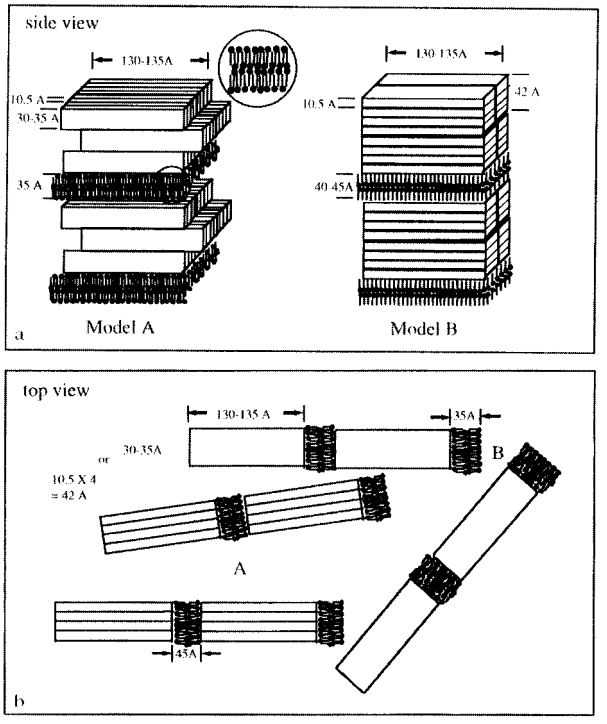

Figure 7 Proposed structure models of zein–oleic acid resin films based on the dimensions of reduced α-zein. The interdigitated, 35-Å-thick oleic acid layer in Figure 7(a). Model A corresponds to the structure proposed by Iwahashi et al.[38,39] based on their observation of a sharp 17.3-Å spacing at room temperature. It is assumed that, in the top view [Fig. 7(b)], additional tetramers will aggregate laterally [as in Fig. 7(a)] and longitudinally to form platelets.

30–35 Å, the 135-Å spacing could result from the alternation of three protein layers with one double layer of H-bonded oleic acid. The H-bonds would be between the carboxyl heads of the oleic acid and the glutamine residues at the folds, as well as dimers, at the midplane of the double layer. This is shown in Figure 7(a) as Model A. Presumably, the tetramers can be translated laterally and longitudinally in the trilayers, with hydrophobic bonds between the planes of helices both within and between the tetramers parallel to the plane of the film, but H-bonding between the glutamine fold residues in the thickness direction. This would permit easy shear primarily in the oleic acid layers (as depicted in Fig. 6). As for the pairs of aggregates in the model in Figure 6, however, we know of no obvious reason for the trilayer because we expect the fold surfaces and ribbons to be identical. The broad 45-Å spacing corresponding to the tetramer width would be observed as a ring in the beam normal pattern

and on the equator for the pattern with the beam parallel to the surface.

The third possibility [Fig. 7(a), Model B] incorporating reduced zein tetramers has two of the tetramers aggregate with ribbon planes parallel (possibly forming an octamer) due to hydrophobic interaction of the lateral faces of the helices. Two tetramers, oriented as shown, plus one oleic acid hydrophobically bound bilayer would also yield a 135-Å spacing similar to the structure shown in Figure 6. As in Figure 6, here also we assume the hydrophobic interaction is sufficient that the oleic acid retains the packing in the crystalline state, i.e., has a bilayer thickness of ca. 42 Å. In this case the ~ 45-Å spacing would be expected in the film thickness direction only, as the third order of the 135-Å spacing.

For both Models A and B, the 170 Å periodicity is attributed to double layers of oleic acid, similar to those in Model A, with H-bonding to the ends of the tetramers, as shown in Figure 7(b). Thus, the 170-Å spacing would be expected to be seen, as it is, randomly oriented in the plane of the film for patterns with the beam normal to the surface and oriented parallel to the surface for the side view. We also note the 170-Å peak, despite being well defined in Figure 4(b), has a considerably lower intensity than the 135-Å peak. This also is as expected, because any orientation of the 170-Å periodicity parallel to the film surface will give the 135-Å platelet spacing peak. With hydrophobic (Model A) or H-bonding (Model B) between neighboring tetramers parallel to the surface, the result would be platelets in which the tetramers have a common orientation within a given platelet, stacked to form sheets similar to those described relative to Figure 6. The tetramers could be aligned in rows, either arranged side by side between rows, or staggered, in a single platelet. The former would result in rows of alternating oleic acid and tetramers, the latter to isolated small islands of oleic acid in the platelet. For the models in both Figures 6 and 7, the sharpness of the 170-Å reflection suggests it is a periodicity rather than being related to the form factor of the structural unit, and thus, the side-by-side packing is to be preferred. We note our suggestion for Figure 7 of the 170-Å periodicity arising from oleic acid double layers at the ends of the molecules is unexpected; we know of no other biological system for which such a structure has been proposed.

Neighboring sheets are again expected to be randomly oriented in and parallel to the plane of the film. The 10.5-Å interhelix reflections would have a random orientation in the plane of the film (beam normal pattern) and on the equator for the beam parallel pattern for Model A, while for Model B it would be random for the beam normal pattern and at both 0 and 90° for the parallel beam pattern. The difference in the intermolecular spacing observed may be due to residual strain in the films giving rise to variations in side-chain packing in different directions.

Both of the models in Figure 7 based on reduced α-zein seem to reasonably well, but not perfectly, explain the SAXS and WAXS reflections and their orientation, as does the nonreduced α-zein model in Figure 6. The exterior surfaces of the helical segments plane are rather hydrophobic;[14] thus, individual zein molecules may easily attract each other through hydrophobic interaction. This interaction permits folding of the zein molecules to form ribbons, their stacking to form tetramers, and further aggregation of the tetramers both parallel and perpendicular to the film surface to form the sheets depicted in Models A and B. However, as already pointed out, we know of no chemical or physical reason as to why the ribbons of laterally aggregated, folded helices should form stacks limited to tetramers, or why these should interact further to form the postulated protein bi- or trilayer platelets. In the proposed models the oleic acid forms liquid–like interlayers. During deformation the resulting platelets would be expected to align parallel to the surface, but with no preferred orientation in the surface unless they are sufficiently anisotropic in the plane and the sample is deformed uniaxially.

For the cast films we suggest the broad peak centered at 80 Å, but extending from about 170 to 45 Å, corresponds to more or less random stacking of oleic acid layers (of either form) and mono-, bi-, or trilayers of zein tetramers, even mixtures of the models in Figures 6 and 7. The oleic acid would be inserted randomly between the tetramers based on composition alone.

Further examination, by SAXS, WAXS and TEM, particularly of uniaxially rolled resin films and as a function of plasticizer composition, is in progress to further clarify the molecular and morphological structure and orientation. In the case of synthetic polymers the use of deformation by rolling permitted clarification of the deformation mechanism of the crystalline synthetic polymer, polyoxymethylene, a mechanism generic to lamellar, folded chain crystalline polymers.[40] Present results, we suggest, indicate the zein–oleic acid

association is induced in the resin forming (doughing) process and aligned in the film forming process. As proposed by Yang et al.,[15] the resulting structure is presumed primarily due to sliding of the protein sheets or platelets (of laterally and longitudinally aggregated tetramer stacks), with little or no molecular unfolding, let alone uncoiling of the α-helices to form, for instance, β-sheets. The improved toughness of the resin films would be due to this organization into platelets, with the low elongation at failure due to the weak interaction between platelets. The presence of planar voids parallel to the platelets, possibly due to evaporation of residual moisture and/or solvent following processing, would also contribute to lower elongation. Presumably, as also proposed by Yang et al.,[15] still further improvement in properties should result if the molecules can be unfolded (and uncoiled) and aligned in the directions in which stress is to be applied, mimicking the structure of synthetic polymer fibers and films.[38]

CONCLUSIONS

The structure of granular zein, zein fibers, zein–oleic acid resin, and zein–oleic acid films was investigated by WAXS and SAXS. The WAXS results indicate zein–oleic acid resin and films have similar d-spacings, i.e., $\sim$ 4.6 and $\sim$ 10.5 Å, corresponding to the α-helix backbone distance along the chain and the interchain spacing between helices, respectively. Granular zein and zein fibers have smaller interchain spacings, i.e., $\sim$ 9.5 Å. The increase of the interhelix spacing in the presence of oleic acid is believed related to the resin preparation process. The diffuse rings in the WAXS patterns indicate that these samples do not contain three-dimensional crystallites; localized parallel packing of molecular segments is possible. The film forming processes, resin deformation or solution casting, did not change the basic internal structure of the α-helix, nor, apparently, its relative proportion.

The SAXS results, however, showed that the forming processes affected the film morphology. Resin films have a periodicity in the plane of the film of about 170 Å. This periodicity was still observable when the X-ray beam was parallel to the surface (side view). Parallel to the thickness direction, however, a strong 135 Å periodicity was observed. The results are interpreted in terms of a platelet structure in the resin films, developed during the doughing process and aligned during film formation. Cast films did not show as clear a periodicity. In both types of films planar voids are oriented parallel to the surface, possibly resulting from evaporation of residual moisture and/or solvent from between some of the platelets. The oleic acid appears to play an important role in the formation of the platelet structures.[25,26] Contrary to the phospholipids in the wheat gluten films examined by Grosskruetz,[22,23] it was present in sufficient concentration to develop a periodic protein–fatty acid-layered complex throughout the film. Granular zein and zein fiber, both without oleic acid, did not show evidence of an SAXS periodicity.

Several possible structural models of zein–oleic acid resin films are proposed. It is suggested the basic zein structural units are composed of ribbons of folded (antiparallel) α-helical segments with a fold period, including the folds, of ca. 35 Å that are then stacked face to face in tetramers, with possible further aggregation in nonreduced zein. Two types of association of the protein tetramers and the oleic acid are proposed, consisting of either two or three reduced zein tetramers stacked in layers alternating with an oleic acid bilayer or double layer, depending on the tetramer surface (folds or helix sides) exposed to the oleic acid; the nonreduced zein model similarly consists of two layers of zein aggregates alternating with an oleic acid double layer. Even though we can offer no chemical or physical reason for the postulated protein–protein associations, the resulting models reasonably well explain our WAXS and SAXS observations.

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