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Effects of Water Activity and Lipid Addition on Secondary Structure of Zein in Powder Systems

YUKIKO MIZUTANI,*,† YASUKI MATSUMURA,† KOREYOSHI IMAMURA,‡ KAZUHIRO NAKANISHI,‡ AND TOMOHIKO MORI†

Laboratory of Quality Analysis and Assessment, Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University (Uji Campus), Gokasho, Uji, Kyoto 611-0011, Japan, and Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushima-naka, Okayama 700-8530, Japan

The effects of water activity (A_w) and lipid addition on the secondary structure of powdery zein were investigated using Fourier transform infrared spectroscopy. Two fatty acid esters, i.e., the linolenic and eicosapentaenoic acid ethyl esters (LAE and EPE), were mixed with the zein powder. The powders were stored in the "dry" state (with silica gel) and the "humid" state ($A_w = 0.9$). The powdery zein without the lipids was shown to have a high content of the intermolecular hydrogen-bonded β-sheet in the "dry" state, indicating the presence of protein aggregates. An increase in A_w induced a decrease in this β-sheet, concomitant with increases in the α-helix and β-turn structures. The addition of LAE caused decreases in the α-helix and intermolecular hydrogen-bonded β-sheet of zein when the powder was stored in the "humid" state, suggesting the strong interaction of LAE and zein molecules. However, LAE did not affect the secondary structure of zein in the "dry" state. The addition of EPE hardly influenced the secondary structure of zein, irrespective of A_w. These results are discussed in relation to the antioxidative activity of zein in the powder system, which had studied previously.

KEYWORDS: Zein; secondary structure; powder system; water activity; LAE; EPE; FT-IR spectroscopy

INTRODUCTION

The powdery lipid systems established by microencapsulation techniques are available to retard lipid peroxidation (*1*). Cereal prolamins are expected to be new useful encapsulants for food oils. In fact, mixed powders of prolamins and unsaturated fatty acids have been tested as ingredients of breads (*2*), cookies (*3*), and noodles (*4*). Zein, which is a prolamin of maize, has also been used as an encapsulant for fish oil (*5*).

Previous reports have shown the high antioxidative activity of cereal prolamins in the model powder systems (*6*−*11*). These reports suggest that such a high antioxidative activity is due to the physical shielding of the lipid molecules from oxygen by the prolamin matrix. However, no experimental evidence clearly supporting this speculation has been available so far. The detection of subtle conformational changes of prolamins induced by mixing with lipids is necessary to prove the physical interactions of the two components in the powdery lipid systems.

Fourier transform infrared (FT-IR) spectroscopy is a vibrational spectroscopic technique that can be used to monitor the secondary structures of proteins (*12*, *13*). FT-IR spectroscopy has been used to study the conformation of various food proteins (*14*−*16*) under the influence of various environmental factors (*14*) because the technique can be applied to liquid, semisolid, and solid samples (*13*, *17*).

In this study, the effects of lipid addition on the secondary structure of zein in the powder system were studied by FT-IR spectroscopy. Two lipids with different unsaturated degrees, i.e., linolenic and eicosapentaenoic acid ethyl esters (LAE and EPE), were used. In previous studies (*8*−*10*), the strong antioxidative effects of zein could be observed only when the powdery lipids were stored under a high water activity (A_w), whereas the lowering of A_w dramatically decreased the stability of the lipids against peroxidation. This suggests the important role of the water content in the interaction of zein and lipid molecules in the powdery systems. Therefore, the experiments were done under the high (humid) and low A_w (dry) conditions.

MATERIALS AND METHODS

Materials. Zein was purchased from Nacalai Tesque Inc. (Kyoto, Japan). LAE, lysozyme, and β-casein were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). EPE was supplied by Nippon Suisan Co. (Tokyo, Japan). All the other reagents of analytical grade were purchased from Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd.

Preparation of Powder and Solution Systems. Commercial zein was washed with ethyl acetate to remove potential antioxidants before the preparation (*11*). LAE or EPE was dissolved in *n*-hexane and added to the zein powder. The mixing ratio of the lipid and zein was 1:9 or 1:4 (w/w). The sample powders were stored in a desiccator with silica

* To whom correspondence should be addressed. Telephone: +81-774-38-3750. Fax: +81-774-38-3749. E-mail: yukiko@food2.food.kyoto-u.ac.jp.
† Kyoto University.
‡ Okayama University.

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gel or with 22% (w/w) sulfuric acid ($A_w = 0.9$) for 7 days. We called these storage conditions "dry" and "humid", respectively. The zein solution (5% concentrated) was prepared by dissolving the zein powder in 70% ethanol.

Measurement of Water Content. The sample powders (50 mg) prepared in the previous section were dried at 100 °C for 60 min. The water content in the powders was determined from the weight difference of the samples before and after the drying.

FT-IR Measurement. The FT-IR transmission spectra were recorded on a FT-IR-480 Plus spectrophotometer (Jasco Co., Tokyo, Japan). The spectra of the zein solution and the LAE and EPE liquids were measured using a cell equipped with two CaF_2 windows. Zein and the zein−lipid powders were measured with KBr. After the powders and KBr were homogenized with an agate mortar and pestle, the mixed powders were pressed into pellets by using an MT-1 Micro KBr Die Kit and an MP-1 minipress (Jasco Co.). For each spectrum, a total of 50 scans were collected at 4 cm^{-1} resolution.

For the secondary structure analysis of the zein, deconvolution of each spectrum was performed using Jasco FT-IR software according to the methods of Fourier self-deconvolution (FSD) (*18*) and the finite impulse response operator (FIRO) (*19*). The spectra were analyzed by second derivatization (*20*, *21*) and Gaussian curve fitting (*22*, *23*) in the amide I region (1600−1700 cm^{-1}) using the software. The secondary structural content was calculated from the relative areas of the individual assigned bands in the amide I region. The assignment of individual components to the secondary structural elements was done according to refs *16*, *24*−*36*. The assignment of the major bands in the lipid region (1000−3100 cm^{-1}) was based on the data of the previous FT-IR analyses of lipid molecules (*37*, *38*).

RESULTS AND DISCUSSION

Secondary Structure of Zein in Powdery States and Solution. The FT-IR technique has not been applied to the analysis of the zein structure. Therefore, before examination of the effects of the lipid addition, the FT-IR spectra of the zein without lipids were measured in the powdery state and in solution. As described in the Materials and Methods section, the zein powder was stored under both "dry" and "humid" conditions. The measured spectra in the amide I region were carefully deconvoluted according to the procedure described in the Materials and Methods section. Band fitting with Gaussian band shapes was performed on the deconvoluted spectra to estimate the content of the various secondary structures. The original and Fourier-deconvoluted amide I bands, curve-fitted with sums of Gaussian bands of zein powder in the "humid" state, are shown in **Figure 1a** and **b**, respectively. **Figure 2** shows the relative areas of the bands which were assigned to the structural components. The positions of the peaks were slightly variable according to the samples.

Conformation of Zein in Solution. Zein in the solution state has been known to be relatively rich in α-helix compared to the other prolamins. Thus, we assign the major band at 1652−1657 cm^{-1} of zein in 70% ethanol to the α-helix, though some contribution of random coil is also possible (*16*, *27*−*29*). Our FT-IR data revealed that the α-helix content of zein in 70% ethanol was 34% (**Figure 2**). This value corresponds to approximately 40% of the total secondary structure if we exclude the relative amount of the NH_2 side chain (the band near 1612 cm^{-1}). The content of the α-helix thus obtained is lower than that estimated by the circular dichroic (CD) analysis (44−59%) or predicted from the primary structure (60%) by Argos et al. (*39*). We also calculated the α-helix content of the zein in solution at about 50% using our own CD measurement results (data not shown). FT-IR spectroscopy has been shown to be a suitable method for monitoring changes in the β-sheet, whereas CD spectroscopy is more applicable to the correct estimation of the α-helix content (*40*). Therefore, our results from FT-IR

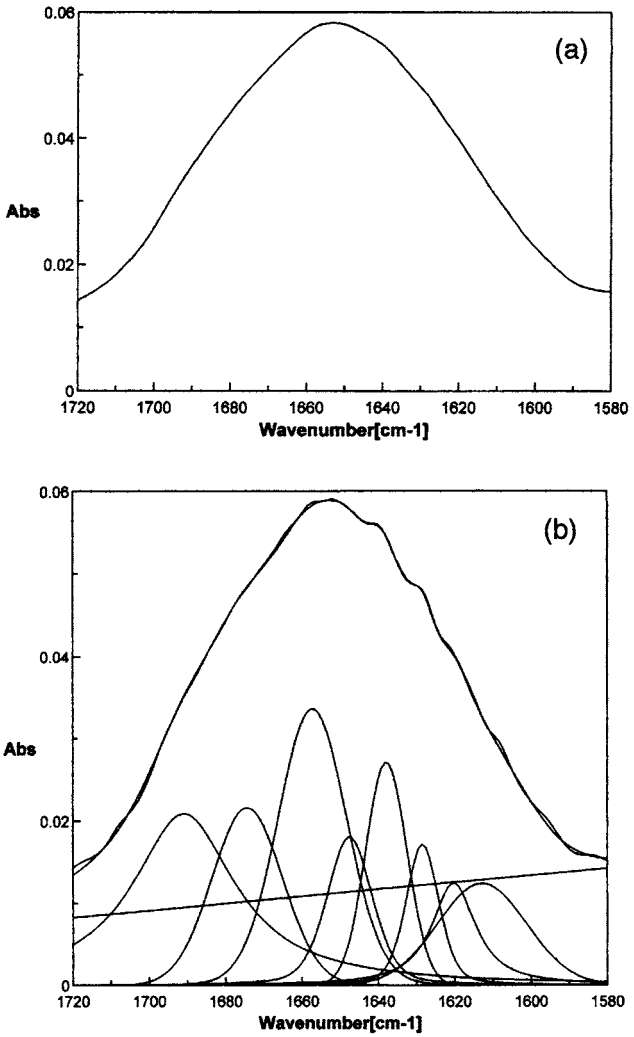

Figure 1. FT-IR spectra of zein powder in the "humid" state. (a) Original spectrum in amide I region, (b) Deconvoluted spectrum and reconstituted spectrum after curve fitting. The reconstituted spectrum was drawn as sums of fitted Gaussian bands. Abs, absorbance.

measurement might tend to show lower α-helix but higher β-sheet contents compared to the data from the CD analysis. Considering this point, the α-helix content of the zein estimated by FT-IR seems to be consistent with the previous data, i.e., the α-helix propensity of the zein. On the other hand, the β-sheet content of the zein in the solution state (main β-sheet band near 1635 cm^{-1}; 15%) was low in comparison with the α-helix content (**Figure 2**). This result is in agreement with a possible structural model for zein which displays low probability of the β-sheet (*39*). The band near 1680 cm^{-1} can possibly be assigned to both the β-turn and β-sheet, but the resolution has not yet been clarified. In accordance with previous results, the 1620 cm^{-1} band may arise from intermolecular β-sheets (*31*). Finally, we can also point out the relatively high content of the β-turn (27%), the band of which appeared near 1671 cm^{-1}.

Conformation of Zein in the Powder State: Comparison with the Solution State. For the "dry" powder state, the α-helix and β-turn (near 1671 cm^{-1}) bands of the zein drastically decreased (from 34% to 10% and from 27% to 6%, respectively), concomitant with a disappearance of the β-sheet band around 1635 cm^{-1} (**Figure 2**). On the other hand, the band around 1690 cm^{-1}, which was not observed in the solution, appeared with the high content (35%) (**Figure 2**). Many authors previously assigned the 1690 cm^{-1} band to the antiparallel intermolecular

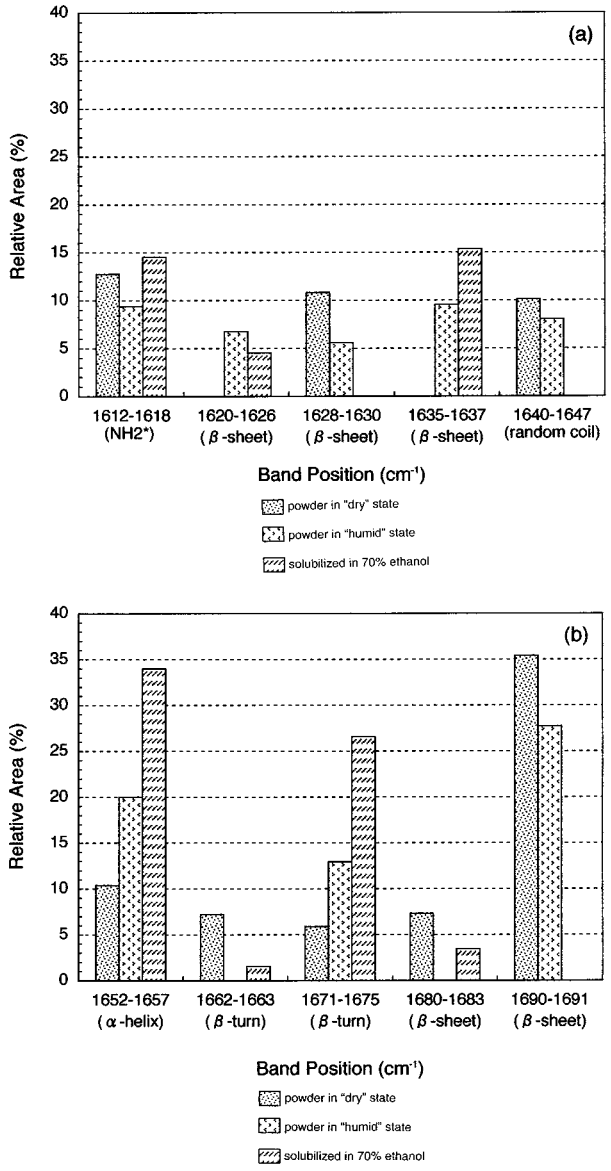

Figure 2. Positions and relative areas of the bands fitted to the Fourier-deconvoluted spectra of zein. Each band was assigned to the component of the secondary structure according to the previous data. Panels a and b depict the low- and high-wavenumber regions, respectively. *NH_2, NH_2 scissoring from the glutamine side chains.

hydrogen-bonded β-sheets belonging to protein aggregates (*16*, *29*, *33*). Moreover, they insisted that the combination of bands at 1620 and 1690 cm^{-1} was important (*41*), while we found that the 1690 cm^{-1} band was combined with the band at 1628 cm^{-1} rather than at 1620 cm^{-1}. The new random coil (1640 cm^{-1}) band occurred in the "dry" powder state (**Figure 2**). On the whole, the secondary structure of the zein in solution, which was characterized by high contents of the α-helix and the β-turn, was drastically rearranged to the new structure, rich in the intermolecular hydrogen-bonded β-sheet in the powder state. The zein powder in the "dry" state was shown to include only 3% water, based on the water content measurement. Under such low water environments of the dry powder, zein molecules may be in close contact with the neighboring molecules, and the formation of the intermolecular hydrogen-bonded β-sheets should thereby be enhanced. A similar phenomenon, i.e., a decrease in the α-helix concomitant with an increase in the intermolecular hydrogen-bonded β-sheets, was also observed in the case of the lysozyme powder (data not shown). Griebenow has reported that the lyophilization substantially increases the intermolecular hydrogen-bonded β-sheet content and lowers the α-helix content of various proteins (*42*). For the wheat gliadin, it has been noted that the drying step from the solution to the film consequently induces the formation of intermolecular hydrogen-bonded β-sheets (*41*). Therefore, the conformational changes in the zein, which were found in the drying process of the powder in this study, should be common for the other proteins.

Influence of Water Activity on the Conformation of Zein in the Powder State. The relative areas of the α-helix band (20%) and the β-turn band at 1671 cm^{-1} (13%) in the "humid" state ($A_w = 0.9$) were intermediate between those of the solution and the "dry" powder states (**Figure 2b**). The β-sheet bands at 1635 and 1620 cm^{-1} appeared again in the "humid" state as in the solution, though they were not observed in the "dry" state (**Figure 2a**). In contrast, the 1690 cm^{-1} band, which was assigned to the antiparallel intermolecular hydrogen-bonded β-sheet, decreased with the increase in A_w, as did the β-sheet band at 1628 cm^{-1}. The random coil structure also decreased in the "humid" state in comparison with the "dry" state. The 1662−1663 (β-turns) and 1680−1683 cm^{-1} (β-sheet) bands were observed in the "dry" state but disappeared in the "humid" state. These results indicate that an increase in A_w induced the partial "refolding" of the zein in the powder from the deformed structure rich in the antiparallel β-sheet to the "native" conformation including the α-helix, β-turn, and β-sheet in the solution. In the "humid" state, the water content was elevated to approximately 8%. Such water molecules introduced into the zein powder may break the intermolecular hydrogen bonds, stabilizing the antiparallel β-sheet and causing the "refolding" of the hydrated parts in the zein molecules.

Influence of Lipid Addition on Secondary Structure of Zein. It was shown in a previous paper (*11*) that LAE was very stable when it was stored with the zein powder in the "humid" state, but EPE was not protected from oxidation. This suggests that LAE can be well encapusulated by the zein and be protected from oxidation, whereas the interaction of EPE and the zein is not satisfactory. To test this speculation, the changes in the FT-IR spectra of the zein and lipid molecules were investigated when the two components were stored in the powdery system.

LAE Addition. Zein powder was mixed with LAE at two different ratios [9:1 and 4:1 (w/w)] and stored in the "dry" and "humid" ($A_w = 0.9$) states. The contents of the secondary structural components of the zein were calculated from the FT-IR spectra of these samples. As shown in the previous section, the α-helix is the major component of the secondary structure of zein. Therefore, first, the change in the α-helix content induced by the addition of LAE was compared (**Figure 3**). The α-helix content consistently decreased with an increase in the ratio of LAE in the "humid" state, suggesting the interaction of LAE and the zein, whereas the decrease was not obvious in the "dry" state.

The effects of LAE addition on other structural components were also observed when the powder was stored in the "humid" state (**Figure 4**), although no significant changes were detected in the case of the "dry" condition (data not shown). Like the α-helix, the contents of the intermolecular hydrogen-bonded β-sheets at 1690−1691 cm^{-1} and the NH_2 scissoring band at 1608 cm^{-1} (originating from the glutamine side chains) decreased with an increase in LAE. The decrease in the intermolecular hydrogen-bonded β-sheets suggests that the LAE molecules penetrate the sites in which neighboring molecules

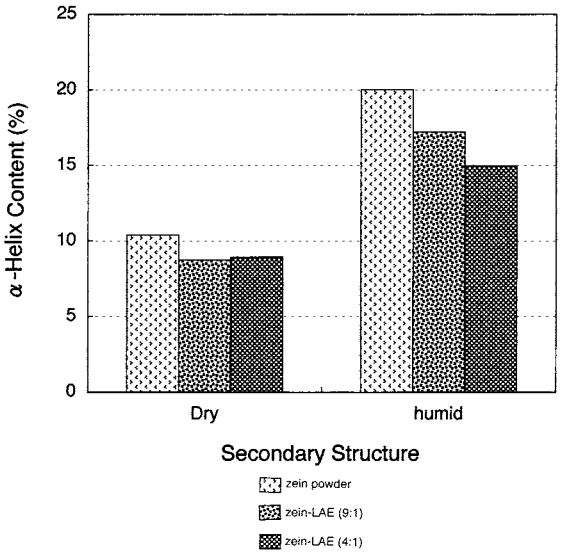

Figure 3. Effects of LAE addition on the α-helix content of zein in the "dry" state and in the "humid" state.

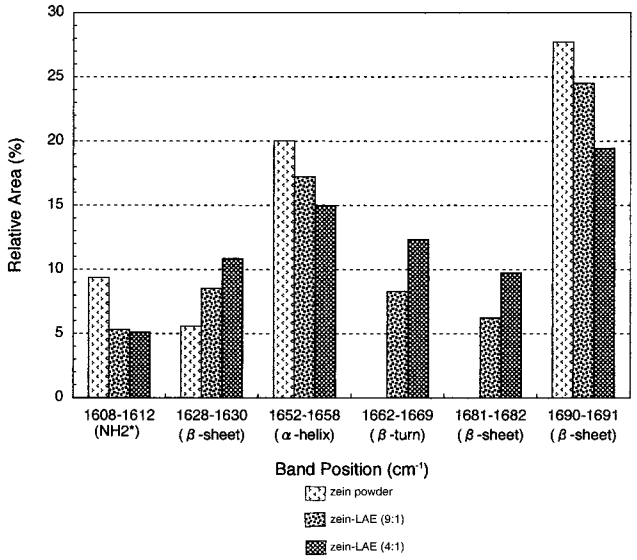

Figure 4. Positions and relative areas of the bands fitted to the Fourier-deconvoluted spectra of zein. Each band was assigned to the component of the secondary structure according to the previous data. The mixed powders of zein and LAE were stored at A_w (0.9) for 1 week before the measurements. *NH_2, NH_2 scissoring from the glutamine side chains.

tightly associate via antiparallel β-sheets. The contact plane may involve glutamine side-chain residues because of a decrease in the NH_2 scissoring band at 1608 cm^{-1}. On the other hand, the decrease in the α-helix resulting from the lipid addition suggests that the LAE molecules should interact with a part of the amino acid sequence which tends to fold into the α-helix. The model reported by Argos et al. indicated that the repetitive sequence of the zein-forming α-helix is highly hydrophobic, i.e., rich in leucine, and also includes phenylalanine and tyrosine (*39*). Thus, it is reasonable to speculate that such a hydrophobic α-helix region in the zein has a high affinity for lipid molecules. Compensating the decreases in the intermolecular hydrogen-bonded β-sheets and α-helix, the β-sheet band at 1628 cm^{-1} increased with an increase in LAE (**Figure 4**). The β-turn (1662 cm^{-1}) and the β-sheet (1681 cm^{-1}) bands appeared again with the addition of LAE, although they disappeared when the zein powder was stored in the "humid" state (**Figures 2** and **4**). The

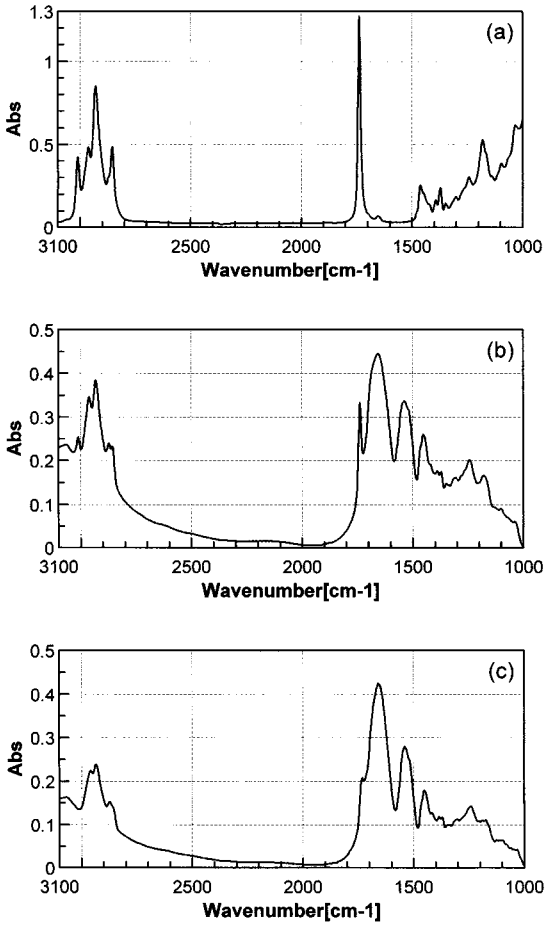

Figure 5. FT-IR spectra of LAE (a) and zein-LAE (4:1) powder in the "dry" state (b) and in the "humid" state (c). Abs, absorbance.

mechanism whereby LAE causes the increases in the β-turn and β-sheet structure is not clear. However, it is possible that the amphiphilic β-sheet structure was formed at the boundary with the lipid phase. This structure has recently been found in the transmembrane regions of the membrane-bound proteins (*43*, *44*).

Figure 5 shows the FT-IR spectra of LAE in the presence and in the absence of the zein. In the absence of the zein (**Figure 5a**), the bands at 3010 and 2960, 2930, 2850, and 1730 cm^{-1} are clearly separated and can be assigned to the bands of the asymmetric CH_3 stretching, the antisymmetric CH_2 stretching, the symmetric CH_2 stretching, and the ester C═O double bond stretching, respectively. These bands were also observed in the FT-IR spectrum of the zein-LAE (4:1) mixture in the "dry" state (**Figure 5b**). However, the pattern of these peaks became obscure, and the peaks at 3010 and 1730 cm^{-1} disappeared or drastically diminished in the "humid" state (**Figure 5c**). N−H stretching of the proteins as well as O−H stretching of the proteins in the case of the "humid" state gave rise to an important signal in the 3000−2800 cm^{-1} region, which may affect the form and position of the LAE bands. To confirm this point, we compared the FT-IR spectra in the "dry" and "humid" states in this region. The three bands at 2960 and 2930, 2870 cm^{-1}, which might originate from N−H and O−H stretching of the protein, were observed, but the bands at 3010 and 1730 cm^{-1} were not observed in the FT-IR spectra of zein powder, irrespective of water activity (data not shown).

Therefore, the possibility that the original LAE bands overlapped with protein signal may be low. Since it is unlikely that the vibration of the chemical bonds stopped even when

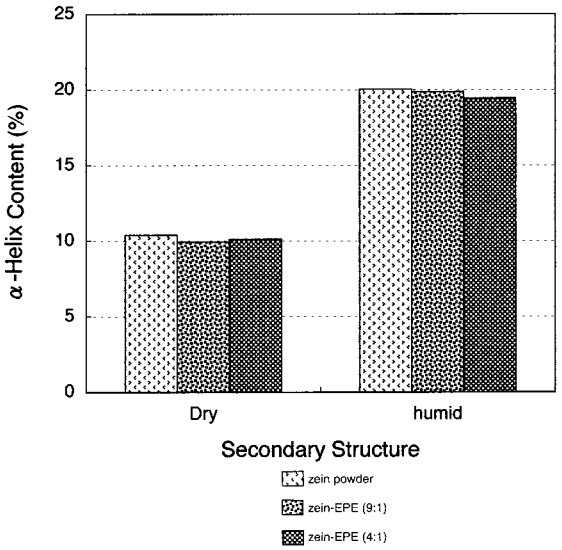

Figure 6. Effects of EPE addition on the α-helix content of zein in the "dry" state and in the "humid" state.

the lipid molecules were encapsulated with the zein powder, this change in the FT-IR pattern may be due to the peak shifts to the overlapping region. Such peak shifts are indicative of the strong interaction of the zein and LAE in the "humid" powder.

On the basis of the results shown in **Figures 4** and **5**, we can conclude that the zein strongly interacts with LAE when the mixed powder is stored in the "humid" condition. In contrast, the "dry" condition did not allow such an interaction in the powder during storage. As already shown in **Figure 2**, the water affected the secondary structure and aggregate formation of the zein in the powder form. The dissociation of zein aggregates by water in the "humid" condition should increase the probability of incorporating lipids into the zein matrix. The partial "refolding" to the "native-like" structure of the zein by water may also be important for the strong interaction with LAE molecules. As mentioned in the Introduction, the previous reports demonstrated that the strong antioxidative effects of the zein could be observed only when the mixed powder of LAE and zein was stored under high water activity ($A_w = 0.9$). Therefore, the interaction between the zein and LAE, which was demonstrated only for the "humid" state in the present study, should be closely related to the antioxidative activity of the zein in the powder system.

EPE Addition. The analysis of the FT-IR spectra was also performed for the zein−EPE [9:1 and 4:1 (w/w)] mixed powder. No significant change was observed in the α-helix content of the zein with the addition of EPE, irrespective of water activity (**Figure 6**). Similarly, no changes in the contents of the other secondary structural components were detected (data not shown). For the EPE molecules, the bands at 3010 and 2960, 2930, 2870, and 1730 cm^{-1} (the asymmetric CH_3, the antisymmetric CH_2, the symmetric CH_2, and the ester C═O stretchings, respectively) appeared in the FT-IR spectrum, as observed for LAE (**Figure 7a**). No significant difference was observed between the "dry" and "humid" states, and the four bands characteristic of lipid molecules were clearly separated (**Figure 7b**,**c**). These results indicate that there was no interaction between zein and EPE, even in the "humid" state. This may explain the previous finding, in which EPE could not be protected from oxidation when the zein−EPE powder was stored under a high A_w condition (*11*).

It remains unclear why EPE cannot be efficiently incorporated into the zein matrix even in the "humid" condition, unlike LAE.

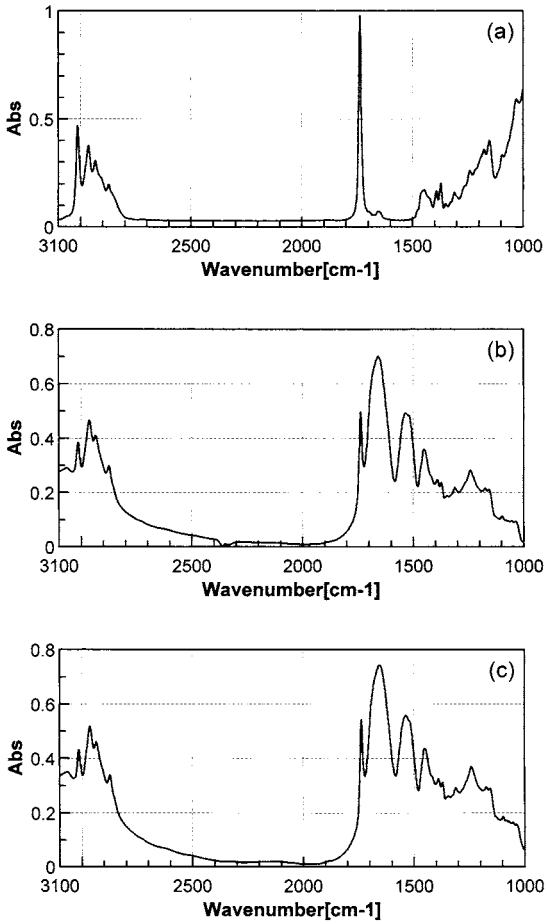

Figure 7. FT-IR spectra of EPE (a) and zein-EPE (4:1) powder in the "dry" state (b) and in the "humid" state (c). Abs, absorbance.

The factors possibly affecting the interaction with the zein are the chain length and the number and position of the double bonds in the two lipids. EPE is a longer fatty acid ester with a more zigzag structure compared to LAE. To understand which factor is responsible for the reduced interaction of EPE with the zein matrix, precise FT-IR measurements using EPE with a specific position labeled by deuterium are necessary. The fluorescence technique should also be useful to clarify this point. Alternatively, systematic approaches should be made using several kinds of fatty acid esters, varying in size and number/position of the double bonds.

Conclusion. This study demonstrated, using the FT-IR spectroscopy technique, that zein in the powder state strongly interacted with LAE at a high A_w, but not in the "dry" state. This physical interaction may be responsible for the antioxidative activity of zein in the powder system which was found in previous studies (*6*−*11*). Probably, the intermolecular packing of zein molecules in the "dry" state is so strong that not enough space is available for the incorporation of lipids. EPE was difficult to incorporate into the zein matrix, even in the "humid" condition, possibly because of its larger and more zigzag structures compared to LAE. Such a significantly lower interaction can explain the poor antioxidative activity of zein in the powder system including EPE (*11*). Since it was shown that the interaction of zein and lipid molecules could be a good indicator of lipid stability against oxidation in the mixed powder system, further studies using the FT-IR technique are promising toward understanding the ability of cereal prolamins as encapusulants of food lipids.

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