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Alteration of Kafirin and Kafirin Film Structure by Heating with Microwave Energy and Tannin Complexation

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Heating with microwave energy and tannin complexation of kafirin both increase the tensile strength of cast kafirin bioplastic films. The effects of these treatments on the molecular structure of kafirin and of kafirin in the film were investigated. SDS−PAGE of heated wet kafirin showed an increase in kafirin oligomers. Disulfide groups increased in heated kafirin and in films made from the heated kafirin. Fourier transform infrared (FTIR) spectroscopy of heated kafirin and films made from the heated kafirin indicated an increase in β-sheet conformation. In contrast, kafirin complexation with tannic acid (TA) and sorghum condensed tannin (SCT) resulted in a slight decrease in β-sheet conformation in the kafirin and a larger decrease in the kafirin in the films. Raman spectroscopy showed that, with TA, there was a shift in peak from 1710 to 1728 cm^{-1} for kafirin−tannic acid complexes, indicating kafirin and tannic acid interaction. The protein conformational changes presumably facilitated cross-linking between kafirin molecules and/or between kafirin and the tannins. Thus, although both heating with microwave energy and tannin complexation cause cross-linking of kafirin to increase film tensile strength, their effects on kafirin structure appear to be different.

KEYWORDS: Kafirin; bioplastic films; microwave energy; tannins; SDS−PAGE; protein secondary structure; FTIR; Raman; disulfide bonds

INTRODUCTION

Kafirin is the prolamin protein of sorghum grain. It can be made into bioplastic films (*1*−*3*), which are an environmentally friendly alternative to synthetic plastics.

In an effort to modify the functional properties of kafirin films, heating with microwave energy and tannin complexation have been investigated. Tannins can bind with kafirin to form insoluble complexes, which can, for example, be measured as haze (*4*). Both heating with microwave energy (*3*), and tannin complexation (*2*) of kafirin were found to increase the tensile strength of cast kafirin films, probably through cross-linking. However, the effects of heating with microwave energy on the molecular structure of kafirin and kafirin films are not known. Similarly, although tannins are known to interact with kafirin to form insoluble complexes (*4*), the effects of this interaction on the structure of kafirin have not been elucidated.

In this study, the effects of heating with microwave energy and tannin complexation on kafirin and kafirin film structure were investigated by electrophoresis and vibrational spectroscopy with the aim of elucidating the molecular causes of the changes in tensile properties of the films.

MATERIALS AND METHODS

Materials. Kafirin was isolated from a mixture of two condensed tannin-free white sorghum cultivars, PANNAR PEX 202 and 606. Two procedures were used:

(a) Extraction with Aqueous Tertiary Butanol (60% w/w) from Decorticated Sorghum Flour at 25 °C (5). The extracted kafirin was freeze-dried and defatted with hexane. The protein content of the kafirin was 81.2% (dry basis, db, N × 6.25). This kafirin was used for the heat treatment study because there is evidence that extraction at ambient temperature would result in less thermally induced alteration in the kafirin structure (*6*).

(b) Extraction with Aqueous Ethanol (70% w/w) from Decorticated Sorghum Flour at 70 °C (4). The protein content of the freeze-dried and defatted kafirin was 97.0% (db). This kafirin was used for complexation with tannins and to make films modified with tannins. The conditions of extraction are similar to those for commercial zein (*7*), which is the prolamin of maize and very similar to kafirin (*8*).

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Commercial tannic acid (TA) (Merck, Darmstadt, Germany) and sorghum condensed tannin (SCT) powder extracted from red tannin sorghum (Nola GH 91) (*4*) were used to complex kafirin.

Heating of Kafirin. A 2 kW Sairem GMP 20KSM microwave generator (Sairem, Neyron, France) fitted with a power regulator was used to heat wetted kafirin powder (containing 1.44 g of protein and 6 g of distilled water) (*3*). The wet kafirin was heated at 80 W to 96 °C in a glass test tube and held at this temperature for 1, 2, 3, 4, or 10 min. The temperature was measured with a Thermalert MID infrared thermometer (Raytek, Santa Cruz, CA). Wetted kafirin that received no heat treatment was used as the control.

Tannin Complexation of Kafirin. TA and SCT preparation at 0%, 5%, 10%, 15%, and 20% (as-is basis) to kafirin were added to the kafirin for complex formation. The tannin−kafirin complexation was performed as in ref *4*. In brief, the tannins were added to a solution of kafirin in aqueous ethanol, and the mixture was incubated at 55 °C for 1 h and then chilled overnight at 4 °C to promote haze and precipitation. The next day the mixture was centrifuged and the pellet was freeze-dried and then ground into a fine powder. The pellet represented the insoluble kafirin−tannin complex. The freeze-dried pellets were identified as uncomplexed kafirin (control), kafirin−TA complex, or kafirin−SCT complex.

Preparation of Kafirin Films. In all cases, kafirin powder (containing 1.44 g of protein) was used in casting the films. Heat-treated kafirin was mixed with 0.6 g of plasticizer [a mixture of equal weights of lactic acid, poly(ethylene glycol) 400, and glycerol] in 9 g of glacial acetic acid (98%) at 25 °C and cast into films (*3*). Glacial acetic acid at 25 °C was used as the film casting solvent for the heat-treated kafirin to minimize heating effects, and aqueous ethanol at 70 °C was used to promote tannin−kafirin complexation. The kafirin with the tannins at 0%, 5%, 10%, 15%, and 20% modification levels were mixed with 0.6 g of plasticizer in 70% (w/w) aqueous alcohol at 70 °C and the films were cast (*2*).

Analyses: *SDS−PAGE*. This was performed under nonreducing and reducing conditions with a discontinuous Tris-HCl/glycine buffer in a Bio-Rad vertical electrophoresis system (Protean II xi Cell, Bio-Rad Laboratories, Hercules, CA). The separating gel was 12% with regard to acrylamide (acrylamide:bisacrylamide ratio 19:1) of 12.5 cm × 14 cm and 0.75 mm thickness.

Sample loading was 40 μg of protein of the heat-treated kafirin and films made from it per well. Premixed protein molecular markers (low range marker, catalog no. 1 495 984, Roche Molecular Biochemicals, Indianapolis, IN) were used. Electrophoresis was conducted at constant current of 50 mA. The gels were stained with Coomassie blue R-250, destained, and scanned.

Disulfide Bond Assay. A solid-state direct colorimetric method (*9*) was used to measure the disulfide (SS) bond content of heat-treated kafirin and films made from the heat-treated kafirin samples. The SS group content was calculated as the difference between SH group content before and after reduction of SS bonds with sodium sulfite and expressed as micromoles of SH/gram of protein. Total cysteine content was calculated as (−SH) + 2(−S−S−).

FTIR and Raman Spectroscopy. All samples were stored in a desiccator containing silica gel for at least 48 h at room temperature to ensure minimal moisture content before spectroscopic analysis. The following samples were examined: heat-treated kafirin; films made from heat-treated kafirin; ethanol-extracted kafirin; dissolved ethanol-extracted kafirin in aqueous ethanol, TA, or SCT; uncomplexed and tannin-complexed kafirins; and unmodified and tannin-modified kafirin films. An FTS 175 spectrometer (Bio-Rad, Hemel Hempstead, U.K.) with a Golden Gate diamond horizontal attenuated total reflectance (ATR) system (Specac, Hemel Hempstead, U.K.) was used. Kafirin, TA, and SCT samples (2 mg) were spread, or kafirin film sample (10 mm × 10 mm) was placed, on the ATR crystal to cover the crystal surface area. The sample was gently squeezed by a screw to promote contact with the crystal. The spectra (128 scans at 2 cm^{-1} resolution) were collected with the frequency range of 4000−800 cm^{-1}. The angle of incidence for the ATR crystal was 45°. The empty crystal was used as background. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 1.5 and a bandwidth of 15 cm^{-1}.

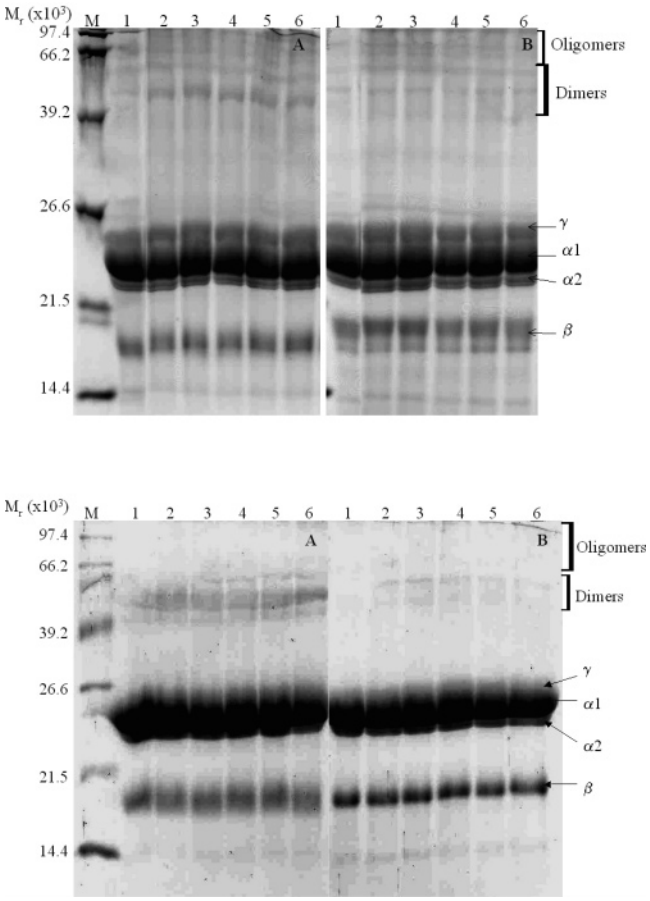

Figure 1. SDS–PAGE of (**top panels**) aqueous tertiary butanol-extracted kafirin heated at 96 °C for different times and (**bottom panels**) films made from the heated kafirin under (A) nonreducing or (B) reducing conditions: (**M**) molecular weight markers; (**1**) control (untreated kafirin); (**2**) 1 min heated kafirin, (**3**) 2 min, (**4**) 3 min, (**5**) 4 min, (**6**) 10 min.

The Raman spectra of kafirin, SCT, and TA samples were measured with a FT-Raman spectrometer (Bio-Rad, Hemel Hempstead, U.K.). About 2 mg was placed in the cavity in a circular aluminum stub. The sample was aligned to the incoming laser. A near-infrared Nd:YAG laser (106 nm, 300−500 mW power) was used for excitation. The number of recorded spectra was 256, and resolution was 8 cm^{-1}. Light scattered at 180° was collected. The Raman spectra of heat-treated kafirin, films obtained from heat-treated kafirin, and tannin-modified kafirin films are not reported as they were very weak light scatterers.

Statistical Analysis. Mean values from the disulfide bond assay and the FTIR data of estimated ratio of α-helix to β-sheet were obtained from three independent heating and tannin binding experiments, respectively. One-way analysis of variance (ANOVA) was performed on the data to determine the effect of all the different heat treatments tannin binding on the individual kafirin and kafirin film properties. The least significance difference test was performed at $p < 0.05$.

RESULTS AND DISCUSSION

SDS−PAGE. SDS−PAGE of aqueous tertiary butanol-extracted kafirin [**Figure 1 (top and bottom), lanes 1**] showed major bands with M_r of approximately 26K, 24K, 22K, and 18K. These bands can be identified as γ-, α_1-, α_2- and β-kafirin polypeptides (*10*). There were also bands of M_r 41 and larger (**Figure 1**). Kafirin bands of similar M_r have been described as oligomers (*10*).

Figure 1 (A, top panel) shows that, under nonreducing conditions, heat-treated kafirin samples (lanes 2−6) had a higher intensity of oligomer bands as compared to the control (lane 1). The same trend was observed in the films made from the

Table 1. Sulfhydryl, Disulfide Group, and Cysteine Content[a] of Kafirin and Kafirin Films[b]

	heating time					
	0 (control)	1 min	2 min	3 min	4 min	10 min
			Kafirin			
free SH groups	21.7 a[c] (0.5)[d]	16.3 b (0.4)	16.9 b (0.6)	16.5 b (0.6)	15.8 b (0.5)	14.7 b (0.7)
SH groups after reduction	27.9 a (0.2)	26.1 b (0.7)	24.8 b (0.3)	24.9 b (0.3)	23.9 b (0.6)	23.4 b (0.5)
SS groups	6.2 a (0.1)	9.8 b (0.7)	7.9 b (0.5)	8.3 b (0.6)	8.1 b (0.4)	8.7 b (0.5)
total cysteine	34.1 a (0.5)	35.8 a (0.8)	32.6 b (0.5)	33.2 b (0.5)	32.1 b (0.5)	32.1 b (0.6)
			Kafirin Films			
free SH groups	22.0 a (0.5)	8.9 b (0.4)	8.6 b (0.6)	8.2 b (0.7)	7.9 b (0.8)	8.4 b (0.7)
SH groups after reduction	23.9 a (0.5)	12.7 b (0.7)	11.7 b (0.5)	12.1 b (0.3)	11.3 b (0.6)	12.1 b (0.5)
SS groups	1.9 a (0.4)	3.8 b (0.5)	3.2 b (0.2)	3.9 b (0.5)	3.4 b (0.4)	3.6 b (0.4)
total cysteine	26.7 a (0.5)	16.6 b (0.7)	14.9 b (0.6)	15.0 b (0.5)	14.7 b (0.6)	15.7 b (0.5)

[a] Sulfhydryl (SH), disulfide (SS), and cysteine contents are given as micromoles of SH per gram of protein. [b] Kafirin was heated at 96 °C and held at 96 °C for different time periods. [c] Values in the same row with different letters are significantly different ($p < 0.05$). [d] Values in parentheses are standard deviation of means.

heat-treated kafirin [**Figure 1 (A, bottom panel)**] under nonreducing conditions. However, there was no clear dose response with heating time in the pattern of kafirin oligomers. The lower intensity of oligomer bands in the non-heat-treated kafirin indicates less cross-linking of the kafirin. The increase in oligomer band intensity after heating is consistent with the high oligomer band intensity reported in pepsin digestion residues of films made from heat-treated kafirin (*3*). Heat-induced protein polymerization has also been reported in rapeseed protein (*11*) and beef liver protein (*12*). Kafirin polymerization due to wet cooking has also been reported (*13*, *14*).

The intensity of the oligomer bands under reducing conditions in the heated kafirin samples [**Figure 1 (B, top panel)**] and films made from the heated kafirin [**Figure 1 (B, top panel)**] was lower than under nonreducing conditions [**Figure 1 (A, top and bottom panels)**]. In the controls (lanes 1), the intensity of oligomer bands was generally lower as compared to the heat-treated samples. The decrease in oligomer band intensity after reduction indicates the involvement of SS cross-links in the formation of oligomers. However, the fact that some oligomers remained after the addition of a reducing agent in the heat-treated kafirin and films made from heated kafirin indicates that not all the cross-links could be cleaved by the mercaptoethanol. This was possibly because of the presence of hidden SS cross-links or non-SS covalent cross-links. Duodu et al. (*15*) suggested that wet cooking of sorghum can cause both intermolecular SS and non-SS covalent cross-links between kafirin molecules and that some of the SS bonds may be inaccessible to reducing agents such as mercaptoethanol.

Complexation of tannin with kafirin (results not shown) showed the occurrence of a high M_r material for the kafirin−SCT complex sample that did not fully enter the separating gel, unlike the uncomplexed kafirin. Sarni-Manchado et al. (*16*) applied SDS−PAGE under reducing conditions to salivary protein complexed with grape seed tannins and also found a band that did not enter the separating gel. The material that did not fully enter the separating gel is presumed to be a very high M_r kafirin−SCT complex.

SS Bond Assay. Table 1 shows a significant decrease in free SH groups of heat-treated kafirin and films made from the heat-treated kafirin compared to the controls. In all cases, the decrease in free SH groups was accompanied by an increase in SS groups. This indicates that microwaving induced the formation of SS bonds, as suggested by SDS−PAGE data. This is consistent with research that showed significant decrease in the free SH group content with concomitant increases in the SS group content after wet heat treatment of film-forming solutions of whey protein (*17*). Similar results were reported in heated soy proteins (*18*) and whey proteins (*19*).

It is apparent that there was no dose response of free SH groups and SS bonds with increased heating time of kafirin and films made from heated kafirin (**Table 1**). This lack of a dose response is in agreement with the SDS−PAGE results (**Figure 1**). Similarly, Li-Chan (*20*) found that there was no significant difference in the free SH groups and SS bonds content of whey proteins subjected to increasing temperature. The lack of a dose response in the SS bonds of heated kafirin and films made from heated kafirin was possibly because most or all of the available free SH groups had reacted. Additional reactions could have involved the exchange between SH and SS groups as reported in whey (*21*) and/or the conversion of intramolecular SS bonds to intermolecular SS bonds, as suggested by Morel et al. (*22*) in gluten films. Such reactions would cause a zero net effect on the free SH groups and SS bond content with increasing exposure to microwave energy.

Heating decreased the total SH groups after reduction in both the kafirin and the films (**Table 1**). This decrease was possibly because of inaccessibility of some SH and SS groups caused by conformational changes as a result of kafirin polymerization. This supposition is corroborated by the SDS−PAGE data (**Figure 1**) in which reduction-resistant oligomers were found. As stated earlier, heat-induced resistant oligomers upon reduction are presumably a result of hidden SS and/or non-SS bonds in kafirin (*13*, *14*).

It is apparent that the measured content of SS groups and total cysteine in the films made from heated kafirin was very low as compared to that of the heated kafirin (**Table 1**). This could also be attributed to heat-induced changes in protein structure, which can limit access of the reducing agents to all SS groups (*23*). According to Wedemeyer et al. (*24*), access to such hidden SS groups requires global unfolding of the protein to expose them.

FTIR and Raman Spectroscopy. The spectra of the aqueous tertiary butanol-extracted kafirin [**Figure 2 (top), spectrum a**] and the aqueous ethanol-extracted kafirin (**Figure 3, spectrum a**) had two broad bands, one between 1680 and 1600 cm^{-1} and another between 1550 and 1500 cm^{-1}. The bands appearing between 1680 and 1600 cm^{-1} and between 1550−1500 cm^{-1} are termed amide I and amide II, respectively (*25*). The amide I vibration corresponds mostly to C═O stretching, coupled with some contribution from CN stretch, CCN deformation, and in-plane NH bending modes of groups in the polypeptide chain (*26*). The amide II vibration is believed to be an out-of-phase combination of CN stretch and in-plane NH deformation mode of groups in the polypeptide chain (*26*). Each band has two

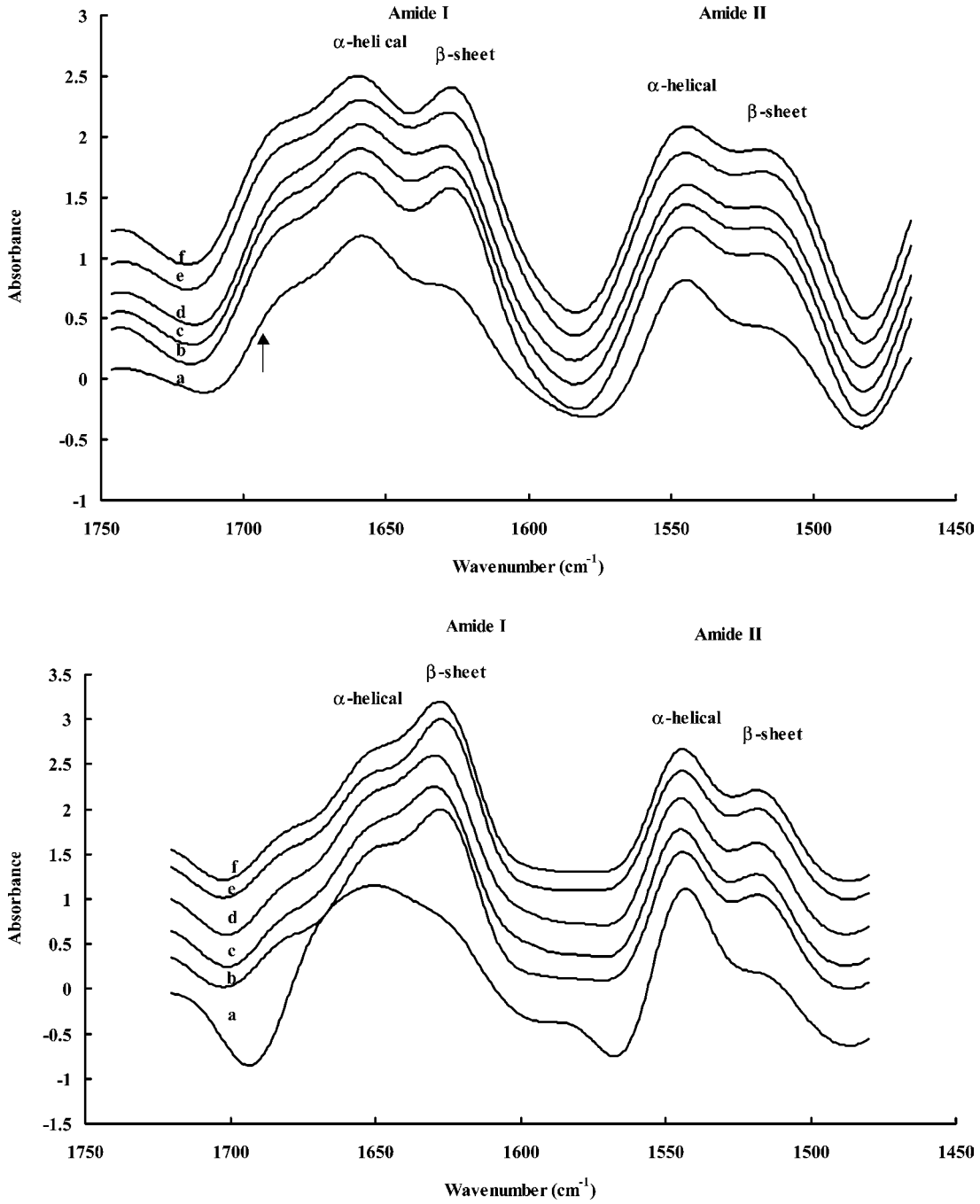

Figure 2. FTIR spectra of (**top panel**) aqueous tertiary butanol-extracted kafirin heated at 96 °C for different times and (**bottom panel**) films made from the heated kafirin: (**a**) control, (**b**) 1 min, (**c**) 2 min, (**d**) 3 min, (**e**) 4 min, (**f**) 10 min.

peaks. In the amide I region, the peak in the frequency range of 1650−1658 cm^{-1} can be assigned to protein α-helices (*25*, *27*). However, random coils can also contribute to intensity in this region (*28*). The frequency range 1620−1640 cm^{-1} has been assigned to the *β*-sheet structures (*26*, *27*). In the amide II region, α-helices can be assigned to 1545−1547 cm^{-1} and *β*-sheets to about 1524 cm^{-1} (*25*−*27*). In addition to the above peaks, the spectra of the aqueous tertiary butanol-extracted kafirin [**Figure 2 (top), spectrum a**] and the aqueous ethanol-extracted kafirin (**Figure 3, spectrum a**) had a shoulder at 1670−1690 cm^{-1} (indicated as an arrow on each figure). Peaks at this wavenumber can be assigned to *β*-sheets or *β*-turns (*29*).

The spectra for kafirin extracted by aqueous tertiary butanol [**Figure 2 (top), spectrum a**] and aqueous ethanol-extracted kafirin (**Figure 3, spectrum a**) showed differences in the ratios of intensities in the 1620−1658 cm^{-1} region. The ratio is calculated from the maximum peak intensities assigned to α-helix and *β*-sheet conformation. It is an indication of the changes in relative peak intensites, which are indicative of the relative amounts of the respective conformations in the protein (*27*). The ratios have been worked out in both the amide I and II regions. However, due to interference from tyrosine at about 1515 cm^{-1} (*30*), the relative amouts of the different conformations in this work are based on ratios in the amide I region. The ratio of intensities at wavenumbers 1658 and 1620 for the aqueous tertiary butanol-extracted kafirin was 1.49 in the amide I region (**Table 2**), whereas the ratio for the aqueous ethanol-extracted kafirin was 0.96 (calculated from **Figure 3, spectrum a**). Duodu et al. (*27*) studied sorghum protein bodies and found that kafirin is primarily α-helical in conformation. The rather

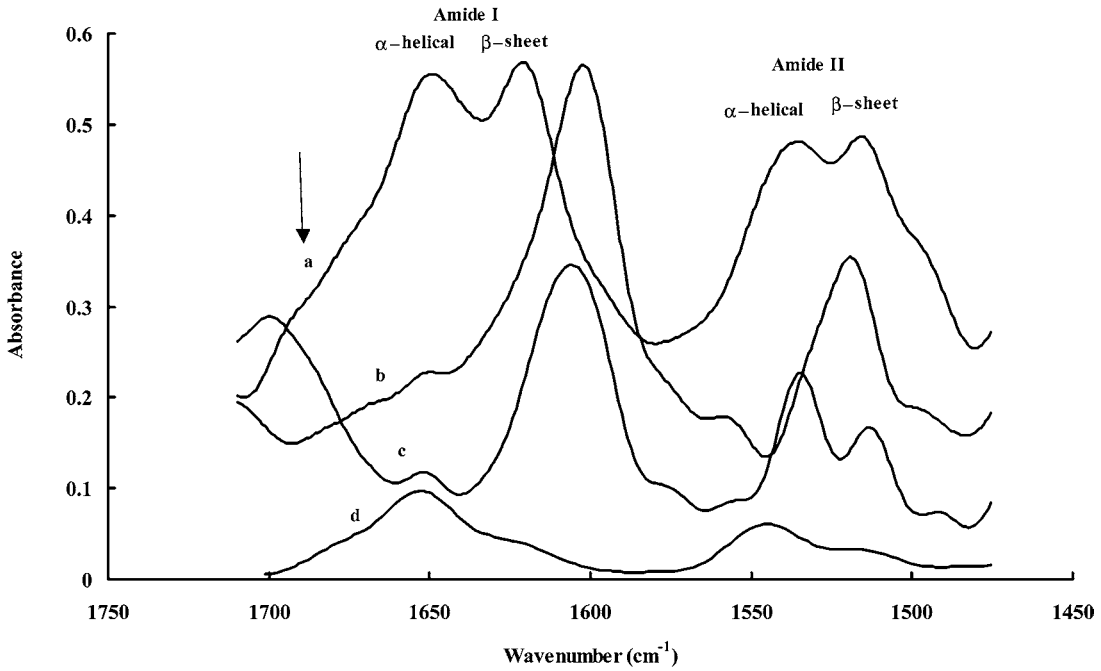

Figure 3. FTIR spectra of aqueous ethanol-extracted kafirin and tannins: (**a**) kafirin; (**b**) sorghum condensed tannins; (**c**) tannic acid; (**d**) kafirin in solution.

Table 2. Estimated Ratio of α-Helical to Intermolecular β-Sheet Conformation of Kafirin and Kafirin Films[a]

	ratio of α-helices to β-sheets	
heating time (min)	amide I[b]	amide II[c]
	Kafirin	
0 (control)	1.49 b[d]	1.92 c
1	1.10 a	1.22 b
2	1.10 a	1.12 ab
3	1.10 a	1.12 ab
4	1.04 a	1.10 a
10	1.04 a	1.10 ab
	Kafirin Films	
0 (control)	1.33 c	6.14 b
1	0.75 ab	1.38 a
2	0.79 b	1.27 a
3	0.75 ab	1.17 a
4	0.79 b	1.17 a
10	0.69 a	1.17 a

[a] Heated at 96 °C. [b] Amide I: α-helices (1650 cm^{-1})/β-sheets (1620 cm^{-1}). [c] Amide II: α-helices (1545 cm^{-1})/β-sheets (1516 cm^{-1}). [d] Values for amide I and amide II with respect to kafirin and kafirin films with different letters are significantly different ($p < 0.05$)

higher proportion of β-sheet structures of kafirin extracted with aqueous ethanol at 70 °C suggests that the protein secondary structure was affected by the extraction procedure. This is in agreement with the results of Gao et al. (*6*), who found that the high-temperature (70 °C) extraction of kafirin with aqueous ethanol promoted more β-sheet structures than extraction with aqueous tertiary butanol at room temperature.

The intensity of the peaks at 1650 cm^{-1} (assigned to α-helices) and 1625 cm^{-1} (assigned to β-sheet) in the kafirin and kafirin films changed as a result of heat treatment (**Figure 2, top and bottom panels**, respectively). When wet kafirin was heated, the estimated ratio in the amide I region significantly ($p < 0.05$) decreased from 1.49 in the untreated kafirin (control) to 1.04−1.10 in treated kafirins (**Table 2**). Similarly, in the films, the ratio of α-helices to β-sheet conformation in the amide I region significantly ($p < 0.05$) decreased from 1.33 in the control to 0.69−0.79 in those made from treated kafirin. This indicates that heating induced an increase in β-sheet formation accompanied by a decrease in α-helical conformation. This is consistent with earlier research in which wet cooking of sorghum was reported to increase β-sheet with concomitant decrease in α-helical conformation of kafirin (*27*). The increase in β-sheet structure is consistent with heat-induced kafirin polymerization shown by SDS−PAGE (**Figure 1**), as β-sheet formation can be indicative of protein aggregation (*31*, *32*).

It can be seen from **Figure 2** and **Table 2** that the heated kafirin was predominantly α-helical in conformation while the protein in the films made from heated kafirin was predominantly of β-sheet structure. Similarly, Subirade et al. (*33*) found that the secondary structure of soy protein changed from a mixture of β-sheet and unordered structures in the native state to β-sheet conformation in the film state. The predominance of β-sheet structure in the kafirin films could be the cause of their apparently lower SS group content as compared to the protein from which they were made (**Table 1**). As stated, β-sheet formation is indicative of protein aggregation; thus predominance of this structure could mean folding of the protein in a manner that limited the accessibility of reducing agents to SS groups and 2-nitro-5-thiosulfobenzoate to all SH groups. The β-sheet structure probably arises from partial unfolding of the α-helices and subsequent aggregation.

The FTIR spectra of SCT and TA (**Figure 3, spectra b and c**) showed several peaks, with a major one at about 1605 cm^{-1}. The other peaks for TA were at about 1700, 1535, and 1515 cm^{-1}. In addition to the peak at 1605 cm^{-1}, SCT gave a high-intensity peak with maximum absorbance at 1520 cm^{-1}. Chemically, TA has a glucose core surrounded by several galloyl groups, while SCT is a polymer of flavanoids such as anthocyanidins (*34*). Kemp (*35*) and Rao and Rao (*36*) reported that aromatic rings absorb at about 1605 cm^{-1} in the infrared region. Thus, the main peak at about 1605 cm^{-1} for TA and SCT can be assigned to aromatic rings. The peak for TA at about 1700 cm^{-1} has been assigned to C═O vibration from the galloyl group of phenolic compounds (*37*). The peaks at 1605 and 1700

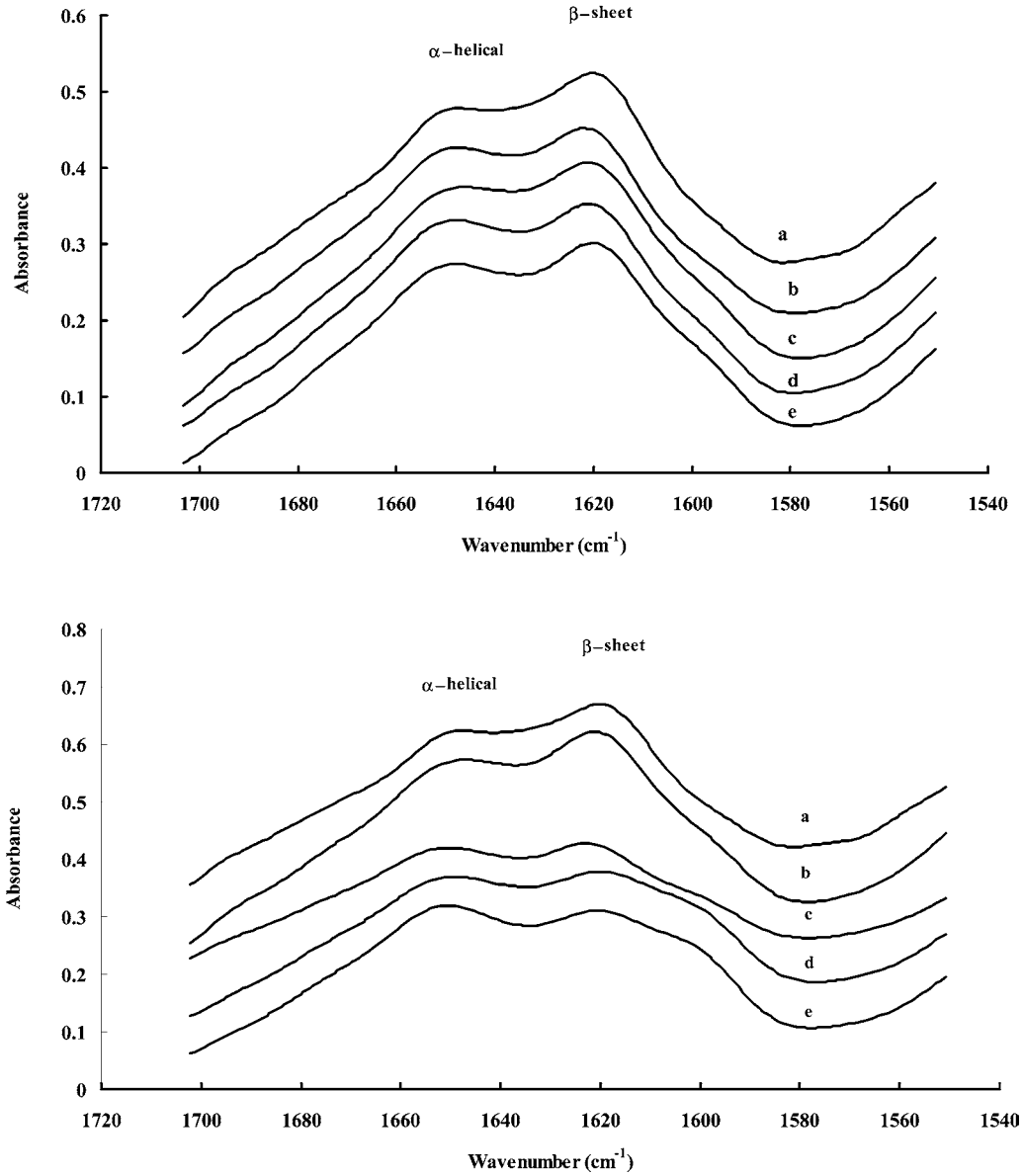

Figure 4. FTIR spectra of aqueous ethanol-extracted kafirin complexes with tannins: (**top panel**) tannic acid complexes; (**bottom panel**) sorghum condensed tannin complexes. (**a**) Uncomplexed kafirin; (**b**) 5% level of complexation with tannin, (**c**) 10%, (**d**) 15%, (**e**) 20%.

cm^{-1} for TA, and at 1605 cm^{-1} for TA and SCT, did not completely overlap with the peaks at 1650 and 1620 cm^{-1} in the amide I region of the kafirin. However, the TA peaks at 1535 and 1515 cm^{-1} and the SCT peak at 1520 cm^{-1} overlapped with the kafirin peaks at 1540 and 1517 cm^{-1} in the amide II region. Therefore, only the amide I region was taken into consideration when the effects of TA and SCT on kafirin secondary structure were assessed.

When TA and SCT were complexed with kafirin (**Figure 4**), there was a slight decrease in the absorbance of the peak at about 1620 cm^{-1} compared to the peak at 1650 cm^{-1}. The ratio of the peak intensity of the 1650 cm^{-1} to 1620 cm^{-1} increased nonsignificantly ($p > 0.05$) from 0.91 for the control to a maximum of 0.94 for TA−kafirin complexation (**Table 3**). There was no change in the ratio when kafirin was complexed at 5% with SCT compared to the control, but the ratio significantly ($p < 0.05$) increased to 1.03 at a 20% level of complexation. The decrease in the peak at 1620 cm^{-1} was, however, much greater for the tannin-modified films (**Figure 5**). The ratio of intensities at 1650 to 1620 cm^{-1} increased progressively and significantly ($p < 0.05$) from 1.23 for the control to 1.50 and 1.86 for films modified with 20% SCT and TA, respectively.

Table 3. Estimated Ratio of α-Helical to β-Sheet Conformation in the Amide I Region of the Spectra of Modified Kafirin and Kafirin Films[a]

	ratio of α-helices to β-sheets[b]	
treatments[a]	kafirin	kafirin films
control	0.91 a[c]	1.23 a
5% TA	0.94 ab	1.31 b
10% TA	0.92 a	1.47 bc
15% TA	0.94 ab	1.60 d
20%TA	0.92 a	1.86 e
5% SCT	0.92 a	1.41 b
10% SCT	0.97 bc	1.42 b
15% SCT	0.98 bc	1.46 b
20% SCT	1.03 c	1.59 cd

[a] Control, aqueous ethanol-extracted kafirin and kafirin film; TA, modified with tannic acid; SCT, modified with sorghum condensed tannin. [b] Amide I: α-helices (1650 cm^{-1})/β-sheets (1620 cm^{-1}). [c] Values in the same column with different letters are significantly different ($p < 0.05$).

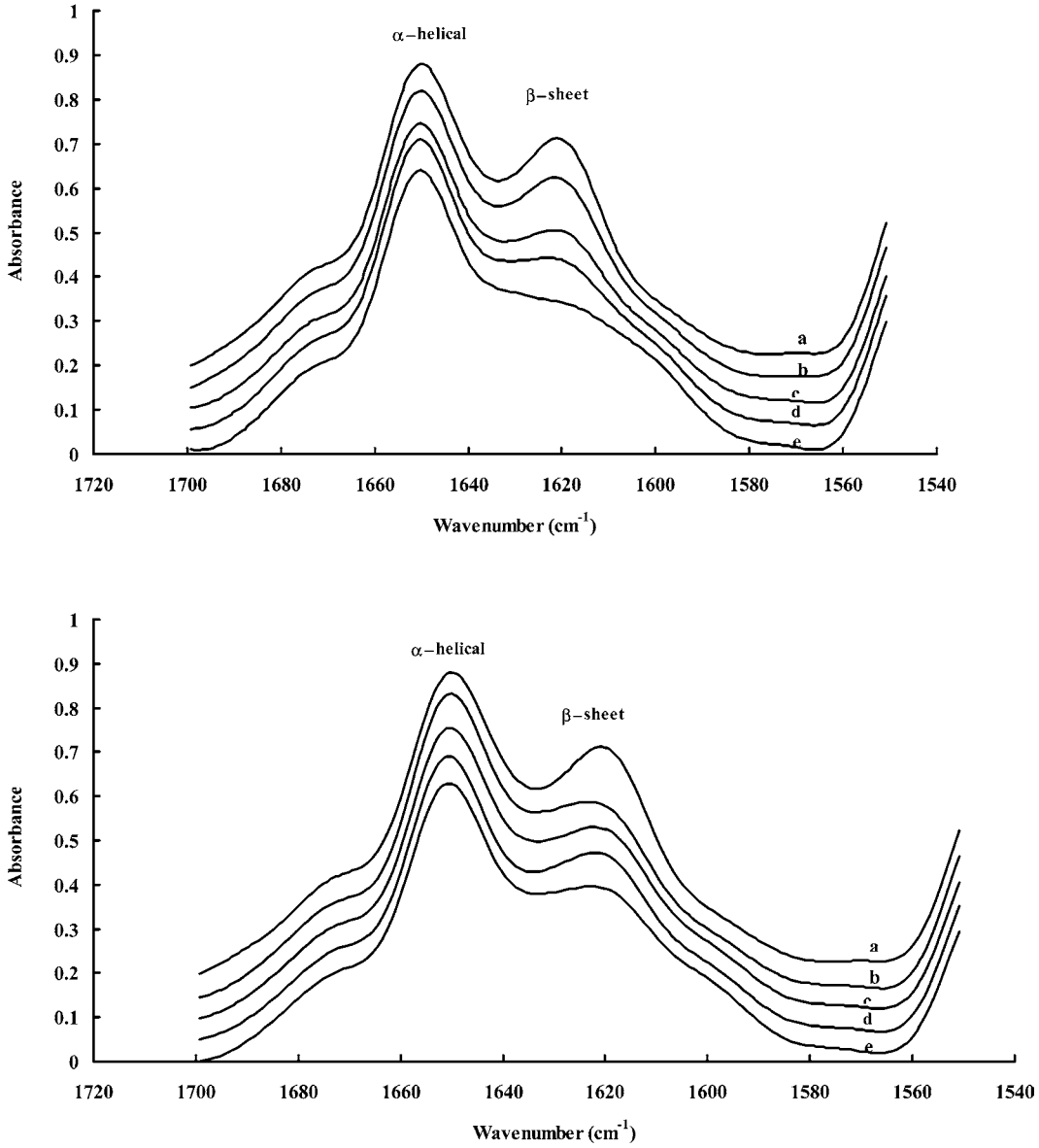

Figure 5. FTIR spectra of aqueous ethanol-extracted kafirin films modified with tannins: (**top panel**) tannic acid modification; (**bottom panel**) sorghum condensed tannin modification. (**a**) Unmodified kafirin; (**b**) 5% level of modification with tannin, (**c**) 10%, (**d**) 15%, (**e**) 20%.

As the peak at 1620 cm^{-1} is associated with a β-sheet conformation (*26*, *27*), it seems that the interaction of TA and SCT with kafirin slightly decreased the level of β-sheet structures relative to the α-helical structures at 1650 cm^{-1} in the kafirin complexed with tannin and much more in tannin-modified kafirin films. Production of the kafirin complex with tannins and tannin-modified films involved the dissolution of the kafirin at high temperatures (65 °C with vigorous stirring) and then incubation at 55 °C with the tannins. Duodu et al. (*27*) suggested that heat can unravel some α-helical chain structures of kafirin by breaking hydrogen bonds, and then these unravelled chains can realign and reorganize into β-sheet conformation. It can be hypothesized that, during application of heat during the dissolution process and the subsequent incubation, hydrogen bonds of α-helical structures and β-sheets may be broken, leading to random coils. The random coils could provide complexation sites for tannins to interact. The complexation sites are probably the carbonyl groups of the polypeptide, which hydrogen-bond with the hydroxyl groups of the tannins (*38*). During cooling of the kafirin solution to 4 °C and during solvent evaporation for film formation, tannins can possibly prevent interaction of the polypeptides and thus prevent reorganization into β-sheet structures.

To test the above hypothesis, the spectrum of kafirin in solution before modification with the tannins was measured. Kafirin in aqueous ethanol solution showed a peak at about 1650 cm^{-1} and a weak peak at 1620 cm^{-1} (**Figure 4, spectrum d**). The intensity at 1650 cm^{-1} could be α-helices or random coils (*28*). The low relative intensity in the β-sheet region suggests that the hydrogen bonds between the β-sheets in kafirin were broken down, either to form random coil structures or possibly to re-form α-helices in solution. However the formation of random coils seems more likely since refolding is entropically unfavorable. The hydroxyl groups of tannins could then complex these random coils by hydrogen-bonding with the carbonyl groups of the polypeptide chains. Thus the carbonyl groups are less available to form β-sheet structures during cooling of kafirin and kafirin film formation. The decrease in β-sheet structures was greater for tannin-modified kafirin film than tannin-complexed kafirin (**Table 3**). This difference showed tannins to interact more with kafirin in the film than in powder form.

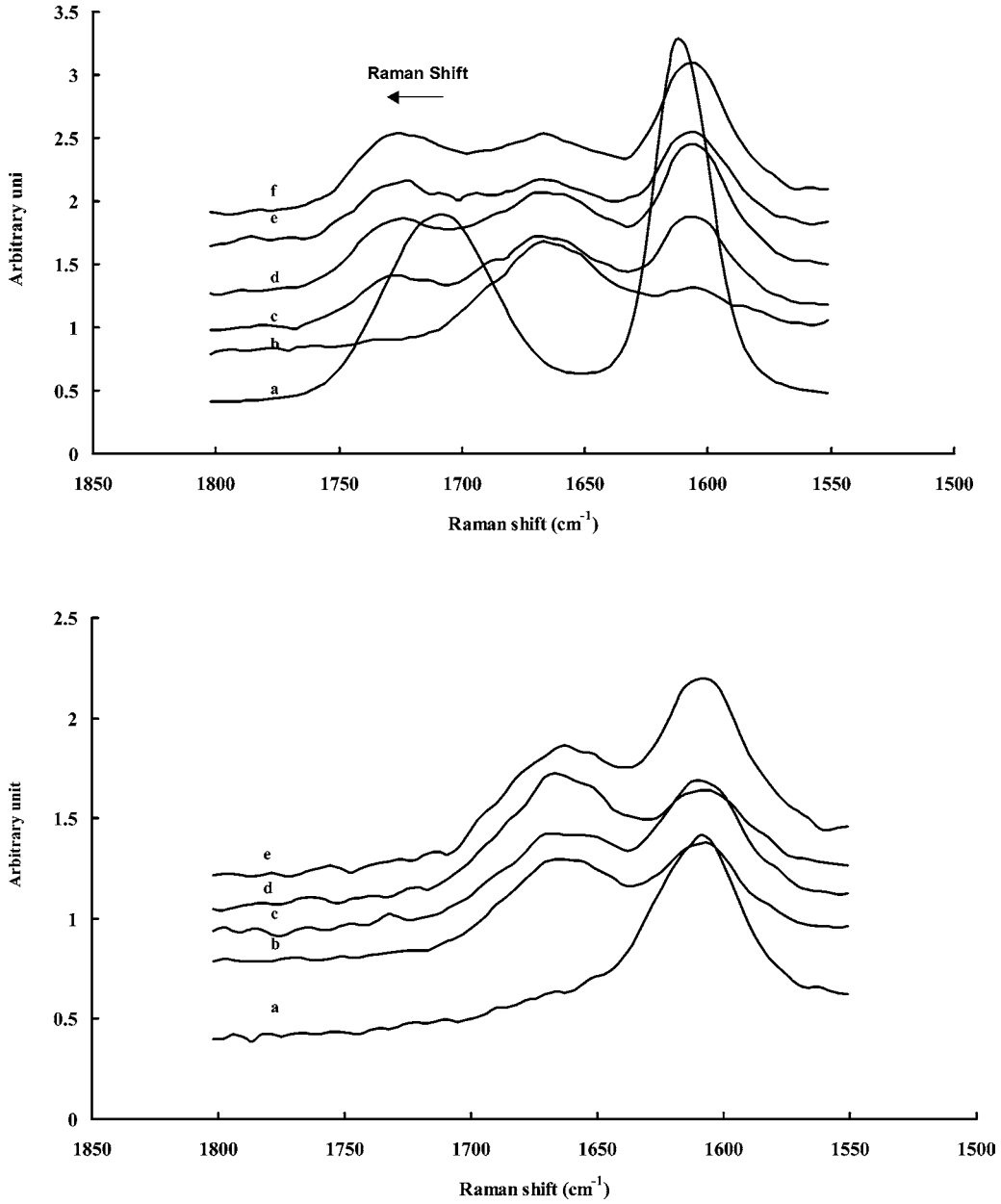

Figure 6. Raman spectra of kafirin–tannic acid (**top panel**) and kafirin–sorghum condensed tannin complexes (**bottom panel**): (a) tannic acid; (**b**) uncomplexed kafirin; (**c**) 5% level of complexation with tannic acid, (**d**) 10%, (**e**) 15%, (**f**) 20%.

The Raman spectra of TA showed peaks at 1613 and 1710 cm^{-1} [**Figure 6 (top), spectrum a**]. The Raman spectra of SCT also gave a peak at about 1613 cm^{-1} [**Figure 6 (bottom), spectrum a**]. The peak at 1613 cm^{-1} has been assigned to aromatic rings with hydroxyl groups (*36*). The peak at about 1710 cm^{-1} has been assigned to carbonyl groups (*37*). This peak can thus be assigned to the carbonyl groups of galloyl rings in TA. There was no peak at 1710 cm^{-1} for SCT because chemically SCT does not have any carbonyl group (*34*). The Raman spectra of kafirin protein in the region of 1700−1630 cm^{-1} showed a broad band with a peak at about 1665 cm^{-1} [**Figure 6 (top and bottom), spectra b−e**]. This region has been assigned to the amide I region (*25*). The Raman spectra of the kafirin−TA complex showed an increase in intensity of the peak at 1613 cm^{-1} with increased level of tannin complexation [**Figure 6 (top), spectra c−f**]. This indicates the presence of an increasing concentration of tannic acid in the kafirin−TA complex. Similarly, an increase in the intensity of the Raman spectrum was observed at about 1613 cm^{-1} for the kafirin−SCT complex compared to uncomplexed kafirin [**Figure 6 (bottom), spectra c−f**]. The kafirin−TA complex also showed a peak at about 1710−1728 cm^{-1} compared to no peak for the uncomplexed kafirin [**Figure 6 (top), spectra c−f**]. In addition to the increased intensity, there was a Raman shift of the peak from 1710 to about 1728 cm^{-1} when kafirin was complexed with TA [**Figure 6 (top)**]. The Raman shift increased with increasing level of TA addition. This shift might indicate interaction of tannic acid with kafirin at the molecular level, possibly as a result of hydrogen bonding of the kafirin to the carbonyl group of TA.

The FTIR data suggest that tannins can hydrogen-bond with carbonyl groups of kafirin polypeptide chains. The Raman data suggest that the carbonyl group of TA can possibly hydrogen-bond with kafirin. This is in agreement with what is known about other proline-rich proteins. Hydrogen bonding is believed to occur between salivary proline-rich protein (PRP) and pentagalloyl glucose tannin (*38*). It has also been proposed that tannins complex with several polypeptide chains of salivary PRP

(*39*). This multiple complexation can cross-link the salivary PRP polypeptide chains to form large M_r complexes that precipitate out (*40*). It is likely, therefore, that the observed haze formation that occurs when kafirin interacts with TA and SCT (*6*) and the possible occurrence of the very high M_r kafirin−tannin complex result from the same mechanism.

In conclusion, heating with microwave energy induces SS cross-linking of kafirin and changes the secondary structure from α-helical to β-sheet conformation to form oligomers of kafirin. Although tannin complexation with kafirin seems to promote formation of high M_r complexes, tannin complexation with kafirin and films modified with kafirin reduced the formation of β-sheets. Thus, although heating and the presence of tannins both cross-link kafirin to change film functional properties, their modes of cross-linking and effects on kafirin secondary structure are different. It appears that heat-induced cross-linking is by disulfide bond formation, whereas tannin cross-linking is probably by hydrogen bonding.

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