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Molecular basis of film formation from a soybean protein: comparison between the conformation of glycinin in aqueous solution and in films

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Abstract

Fourier transform infrared spectroscopy has been used to investigate the conformational changes of glycinin, a major storage protein of soybean seeds, upon film-forming. The results show that the secondary structure of glycinin is mainly composed of a β-sheet (48%) and unordered (49%) structures. The amide I band of glycinin in film-forming conditions, i.e. in alkaline media and in the presence of plasticizing agent, reveals the conversion of 18% of the secondary structure of the protein from the β-sheet (6%) and random coil (12%) to the α-helical conformation due to the helicogenic effect of the ethylene glycol used as the plasticizing agent. Conformational changes also occur upon the film-forming process leading to the formation of intermolecular hydrogen-bonded β-sheet structures. Results obtained from other plant families indicate that, whatever the origin and conformation of protein, formation of films leads to the appearance of intermolecular hydrogen-bonded β-sheet structures, suggesting that this type of structure might be essential for the network formation in films. Thus, it is hypothesized that, in the film state, intermolecular hydrogen bonding between segments of β-sheet may act as junction zones in the film network. This study reveals for the first time that there is a close relationship between the conformation of proteins and the mechanical properties of films.

Keywords: Film; Glycinin; Structure; Fourier transform infrared spectroscopy; Vegetable proteins; Structure–function relationships

1. Introduction

During the last decade, the loss of landfile space and a change in the public perception of acceptable waste, waste reduction and waste elimination, have increased interest in biodegradable plastics. Recent research efforts to develop polymeric alternatives to petroleum-based products have centered on biopolymers as starting materials. Several substances derived from biological materials, such as proteins and starch, have drawn attention for their film-forming ability making them good candidates as ingredients in package products and edible films [1–5].

Glycinin is one of the major globulins of the soya representative of 11S-type seed proteins that are largely distributed in plants. It is made of six subunits, each consisting of a basic polypeptide (β-polypeptide) and an acidic polypeptide (α-polypeptide), which are connected by a single disulfide bond forming the $\alpha\beta$ subunit [6,7]. It has

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been suggested that in the glycinin molecule, six subunits are packed in two stacked hexagonal rings [6] or in two identical trigonal antiprisms stacked on top of each other and held together by hydrophilic forces, such as electrostatic and hydrogen bonds [8,9], although neither model can account very well for the observed results [10]. A great deal of work has been done on functional properties of glycinin showing that it is a major functional ingredient commonly used as a gel, emulsifiant and foaming agent in technological processes [11,12]. Recently, it has been shown that glycinin is able to form films, making it a good candidate as an ingredient in package products and edible films [13–16]. These studies have shown that several factors, such as protein concentration, pH, ionic strength, heating temperature and plasticizers affect the formation of a film from soy proteins. Therefore, it has been suggested that the glycinin film network is formed via hydrogen bonds, hydrophobic and electrostatic interactions between polypeptides. However, the structural basis of the film-forming process is, at present, not understood. No attempts have been made to determine the relationship between the extent of formation of the film network and the structure of the protein in the film and nothing is known about the molecular basis which leads to the formation of films.

The formation of films from soy globulins and other globular proteins has been described as a two-step process involving the heat denaturation of the proteins followed by surface dehydration [17]. Heating alters the three-dimensional structure of proteins, exposing functional groups—such as CO and NH of peptidic bonds, side chain amine groups and hydrophobic groups—engaged in intramolecular hydrogen bonding and electrostatic interactions in the native state which become available for intermolecular interactions [18,19]. Upon drying, the unfolded proteins approach each other and become linked through intermolecular interactions (through disulfide and hydrophobic interaction), leading to the formation of a network that acts as the matrix for entrapping film components such as plasticizing agents [17]. Since the formation of film occurs in denaturated conditions (alkaline conditions and heating above the denaturation temperature of the protein), it is assumed that the protein remains in the fully denaturated state in the film. However, it is quite possible that the denatured protein may undergo partial refolding, thus regaining some secondary structure during the film process. It is conceivable that the extent of such refolding affects the number of functional groups available for intermolecular interactions and thus the formation and stability of the film network.

In order to elucidate the molecular basis of the film formation process, we have studied, for the first time, the conformational changes of glycinin during film formation. Only a few physical methods can provide information on the structural changes of proteins that take place during the formation of precipitates or gel assemblies. Recently, circular dichroism (CD) has been used to obtain insight into the conformation of globular proteins in heat-induced gels [19]. However, the necessity of using transparent samples for CD measurements [20] limits the range of application of this technique. In this work, we have used infrared spectroscopy to obtain detailed molecular information on the conformational behavior of glycinin during the film-formation process. It is now well established that infrared spectroscopy is a powerful method for the investigation of the secondary structures of proteins in solution [21–24] or in complex systems, such as biological [25–29] or food [30–32] systems.

2. Materials and methods

2.1. Purification of glycinin

Glycinin was purified from soy seeds as previously described [15]. Briefly, defatted soybean flour was stirred in a buffer ($Na_2S_2O_5$, NaOH, pH 8, 20°C) for 1 h and centrifuged at $10000 \times g$ for 20 min. Then the pH of the supernatant was adjusted to 5.5 with HCl and centrifuged again. The precipitate, enriched in 11S fraction (approximately 80%), was freeze dried and ground.

2.2. Preparation of the protein films

Protein films were prepared as described by Kokelaar et al. [16]. Briefly, the protein solution (13% w/w) was dispersed with a Polytron homogenizer (20 000 rpm) during 1 min in a glycine/sodium hydroxide solution (pH 10). The plasticizer was then added (w/w plasticizer/protein ratio of 1) and the solution was dispersed again with the homogenizer during 30 s and centrifuged

at low speed ($1500 \times g$) for 30 min to remove entrapped air bubbles. Then, the aerated supernatant was discarded and the film-forming solution was spread on a glass plate. After a drying period of 1 h at 70°C, the protein was cooled down and carefully peeled off the glass plate and stored in a box with controlled relative humidity (60% RH) and temprature (22°C) for 3 days.

2.3. Infrared spectroscopy

Infrared spectra were recorded with a Nicolet Magna 550 Fourier transform infrared spectrometer with a liquid nitrogen cooled mercury–cadmium–telluride detector. The instrument was continuously purged with dry air. For transmission measurements, protein solution (8%, w/v) was placed in a thermostated home-made closed cell consisting of two BaF_2 windows separated by a 6 μm mylar spacer. For each spectrum, a total of 250 scans were collected at 2 cm^{-1} resolution. The algorithm of Dousseau et al. [33] was used to substract the spectrum of the aqueous buffer from the corresponding spectrum of the protein solution. Spectra of films were obtained by attenuated total reflection (ATR) using a single reflection accessory (Harrick Scientific, USA) fitted with a zinc selenide prism. All data manipulations were performed with the Spectra Calc sofware (Galactic Industries, Salem, NH). Fourier deconvolution was carried out using the method of Griffiths and Pariente [34] with a narrowing parameter, γ, of 4.5 and an apodization filter of 0.19. These parameters were chosen in order to obtain optimum resolution without introducing significant sidelobes in the 1690–1720 cm^{-1} region where there is no protein band.

3. Results and discussion

3.1. Conformational behavior of glycinin in film-forming conditions

Fig. 1 displays the original (Fig. 1A) and deconvolved (Fig. 1B) infrared spectra of glycinin in the amide I band region (1590–1720 cm^{-1}) as a function of the film-forming conditions, i.e in alkaline conditions and in the presence of the ethylene glycol. This region, which is mainly due to the C=O stretching vibration and to a small extent to C–N stretching vibration of the peptide bonds, is sensitive to the secondary structure of proteins [21]. Fig. 1 shows the infrared spectrum of the soy protein recorded at room temperature and at pH 7 after correction for the spectral contribution of the water bending vibration according to the method of Dousseau et al. [33]. In the original spectrum, the amide I band appears as a broad band with an absorbance maximum around 1648 cm^{-1}. This band is composed of several overlapping components due to various protein segments with different secondary structures [35,36]. Deconvolution of the amide bands reveals that the amide I band is composed of at least five components at 1615, 1638, 1655 and 1687 cm^{-1}, and a shoulder one at 1670 cm^{-1}. The assignment of these bands, based on previous studies of proteins by vibrational spectroscopy, can be summarized as follows: the two bands at 1638 and 1687 cm^{-1} are highly characteristic of amide groups involved in the extended β-sheet structure [21–23,37] while the band at 1655 cm^{-1} results from either the α-helix or random coil structures. The 1670 cm^{-1} component can be assigned to the presence of β-turns [30] and the weak shoulder at 1615 cm^{-1} arises

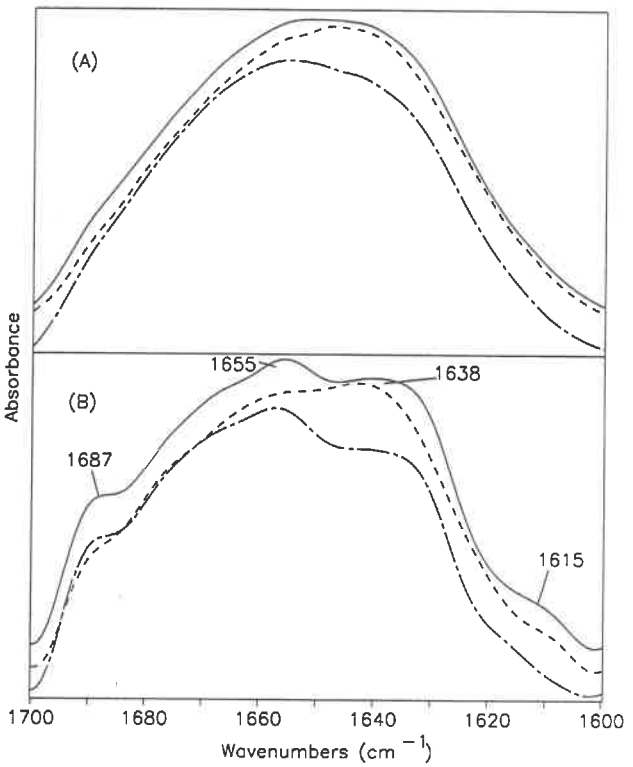

Fig. 1. Original (A) and deconvolved (B) infrared spectra of soy glycinin in (——) aqueous solution (pH 7), (- - -) alkaline conditions (pH 10) and (– – – – – –) film-forming conditions (pH 10 and presence of ethylene glycol).

from intermolecular β-sheets due to protein aggregation [38]. This qualitative analysis is in good agreement with previous data [39].

The various methods for the quantitative determination of the secondary structure of proteins in solution and their respective merits and drawbacks have recently been reviewed [22,23]. In this work, we have used the method of Dousseau and Pézolet [40] to quantify the secondary structure content of glycinin in aqueous solution since this method provides low prediction errors. The results obtained reveal that the native protein is composed of 48% β-sheets, 49% unordered structure or β-turns, and of only 3% α-helices. These results are in good agreement with those obtained previously by CD [19,41] revealing that glycinin belongs to the class of β-sheet proteins. This is a common feature of 11S globulins isolated from other legume seeds [42,24]. Our results therefore confirm that the conformation of glycinin is mainly composed of β-sheet structure. Moreover, they also clearly demonstrate that glycinin contains other secondary structures, such as turns, and provide the first direct identification of the different components.

Since film formation occured in alkaline conditions, we have recorded the infrared spectrum of glycinin at pH 10. It has been known for some time that glycinin undergoes several conformational and/or structural changes as a function of pH due to aggregation and disaggregation processes [12]. In order to detect and characterize these conformational changes, the infrared spectrum of glycinin solution at pH 10 has been recorded and is shown in Fig. 1. As seen in this figure, alkalination of glycinin solution has a marked effect on the infrared spectrum of the protein. The absobance maximum of the original amide I band is shifted from 1648 cm^{-1} in the spectrum recorded at pH 7 to 1641 cm^{-1} in the spectrum obtained at pH 10. These changes are more clearly observed in the deconvolved spectra. The spectrum shows a shift in the band observed at 1638 cm^{-1} at pH 7 to 1641 cm^{-1} at pH 10. This band cannot be assigned unambiguously to β-sheet structures [43] since it has previously been assigned to 'open loops' [44] and random coil structure [25]. However, since the increase in intensity of the band at 1641 cm^{-1} is accompanied by a concomittant decrease of the bands at 1637 and 1687 cm^{-1} due to the β-sheet and that at 1655 cm^{-1} characteristic of the α-helical conformation, this suggests that the band located at 1641 cm^{-1} is probably due to unordered structure. This is confirmed by quantitative analysis using the method of Dousseau and Pézolet [40] which reveals an increase of the unordered structure content of about 5%. These results are in agreement with those obtained by nuclear magnetic resonance (NMR) spectroscopy [45] showing that alkaline conditions lead to the partial denaturation of glycinin. This can explain the fact that even at pH 12 some of the polypeptide chains of the protein may still maintain a hydrophobic core [46], preventing some of the disulfide bonds from being cleaved [47]. Furthermore, it has been shown previously that alkaline conditions break step by step the oligomeric structure $(\alpha\beta)_6$ of glycinin into the $(\alpha\beta)_3$ intermediate and its $(\alpha\beta)$ subunits, which sediment around 7S and 3S, respectively [11]. From the infrared spectroscopy results, it is clear that the disruption of the quaternary structure of glycinin is accompanied by partial denaturation of the protein.

In addition to the film-forming protein, a major component of films is the plasticizer. The addition of a plasticizing agent to the film is required to overcome film brittleness caused by extensive intermolecular interactions [48]. Plasticizers reduce these forces and increase the mobility of protein chains, thereby improving flexibility and extensibility of the film [2]. Polyols, such as glycerol, ethylene glycol and sorbitol, are good plasticizers due to their ability to reduce internal hydrogen bonding while increasing intermolecular spacing. Because of their ability to affect the conformation of glycinin, the effect of plasticizer on the secondary structure of glycinin has been examined. Fig. 1 shows the infrared spectrum of an alkaline solution of glycinin in the presence of ethylene glycol used as the plasticizing agent. This spectrum displays a shift in the band observed at 1641 cm^{-1} in the absence of the plasticizing agent to 1638 cm^{-1} in the presence of ethylene glycol. Moreover, this spectrum shows a marked increase in the 1655 cm^{-1} band relative to that located at 1638 cm^{-1}. This result suggest that the plasticizing agent leads to an increase of the α-helical content of the protein. This is due to the conversion of 18% of the secondary structure contents of the protein from the β-sheet (6%) and random coil (12%) to the α-helical conformation. This helicogenic effect is a comon feature of most alcohols used as solvents for proteins [49]. It has been

shown for some time that hydroalcoholic mixtures, generally used to study proteins at low temperatures [50] or to mimic the membrane environment [51], induce helical secondary structures within polypeptide chains. This effect has been attributed to a combination of two factors: the high dipole moment of alcohols induces the disruption of the internal hydrogen bonds of the peptide groups by competition of the alcohol OH group and the peptide N–H group to hydrogen bond to the amide C=O and the low dielectric constant of alcohols that is able to perturb the structure of protein by reducing the hydrophobic effect. This last effect promotes unfolding of the protein and refolding into an α-helical conformation which is the most energetically stable conformation even in the case of proteins known to have a non-helical native structure, such as β-lactoglobulin, concanavalin A or β-casein [51]. Compared to most alcohols, ethylene glycol has a high enough dipole moment (2.2 Debye) to disrupt intramolecular hydrogen bonds and a low enough dielectric constant (38.6, which is about half that of water) to reduce the hydrophobic effect and to induce the formation of α-helices in glycinin.

In conclusion, our infrared results show that film-forming conditions, i.e. alkaline conditions and the presence of plasticizers, induce glycinin structural changes consistent with a reorganization of the secondary structure of the protein in which the α-helical content is more important. Moreover, they clearly demonstrate the potential of Fourier transform infrared (FTIR) spectroscopy to determine conformational changes of a globular protein as a function of environmental conditions.

3.2. Conformational behavior of glycinin during the film-forming process

Having characterized the structural change induced by alkaline conditions and the presence of plasticizer, we have also addressed the question of film process-induced conformational changes of glycinin. Since the first step of the film process corresponds to the gelation of protein, we examined conformational changes of glycinin upon heating the protein solution above the denaturation temperature of the protein, then upon cooling the solution to 20°C.

Fig. 2 shows the deconvolved infrared spectrum of the amide I region for a filmogen solution of glycinin obtained after a heating time of 1 h at a

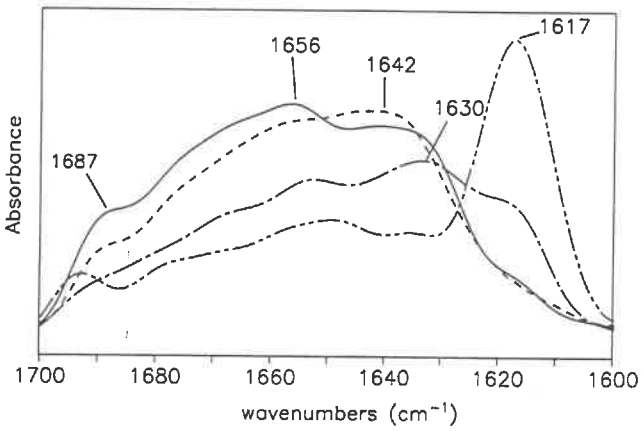

Fig. 2. Deconvolved infrared spectra of glycinin in (——) the film-forming conditions (pH 10 and ethylene glycol), (- - -) after heating for 1 h at 70°C, (– – – – – – –) after cooling to 20°C and (– – – – – – – –) in the film state.

temperature of 70°C. A comparison of this spectrum with that recorded at 20°C before thermal treatment (Fig. 2) indicates that the temperature has an important effect on the secondary structure of glycinin. On heat treatment, the band observed at 1638 cm^{-1} shifts to 1642 cm^{-1} and appears to become broader at 70°C, suggesting that thermal treatment results in extensive denaturation of the secondary structure of soy 11S. This unfolding is accompanied by a decrease in the intensity of the band located at 1655 cm^{-1}, indicating a decrease in α-helices content. Moreover, a striking feature of this spectrum is that it is practically identical with the spectrum recorded at pH 10 (in the absence of plasticizer) and at room temperature (see Fig. 1B). Since pH 10 represents the first stage of the alkaline denaturation and 70°C the first stage of the thermal denaturation [11], our results suggest that the thermal denaturation proceeds via the same intermediate state as the alkaline denaturation.

The heat-induced structural changes of glycinin observed in this study are consistent with previous NMR results [52] which show that heat treatment induces an increase of glycinin molecular motion and protein unfolding, and with CD data [19] which show a decrease of the α-helical and β-sheet structures at the expense of the random coil structure. Although similar results have been reported in the case of β-conglycinin, another globular protein from soy [19], most studies reported so far on the thermal denaturation of globular proteins such as β-lactoglobulin [53], bovine serum albumin [54], ovalbumin [55] or α-lactalbumin, reveal

the appearence of new β-sheets during heat denaturation. In the case of glycinin, new β-sheets appear only upon cooling the solution to 20°C as shown from Fig. 2. The main spectral effect is a marked increase in intensity of the β-sheet band at 1630 cm^{-1}. The frequency of this band (1630 cm^{-1}) is much lower than that we assigned to the β-sheet conformation (1637–1639 cm^{-1}), suggesting an increase in hydrogen-bond strength upon cooling the solution. This treatment also induces a decrease of the intensity of the 1655 cm^{-1} band due to the α-helix structure and to the appearance of a new band at 1617 cm^{-1}. The latter is assigned to the formation of an intermolecular hydrogen-bonded β-sheet structure since it occurs readily upon aggregation of proteins [56] or polypeptides [38]. These infrared results show that soy 11S globulin undergoes partial refolding during the cooling regime of the gelation process. Previous CD results [57] have also shown that glycinin regains secondary structures during the cooling phase of the thermal gelation. These authors showed that the extent of such refolding plays a critical role in the formation and stability of the gel network by modifying the number of functional groups available for intermolecular interactions. They suggested that, by controlling the extent of refolding of the protein during the cooling regime, it is possible to improve the gelation of glycinin [57]. Our results show that the mechanism of thermal gelation of glycinin in the film-forming conditions is comparable to that proposed for other globular proteins [58] and can be summerized in a two-stage sequential process: the first phase involves heat-induced conformational changes in the protein with unfolding of some polypeptide segments followed, upon cooling, by a subsequent phase of protein–protein interactions resulting in a buildup of a network structure.

The last step of the film process consists of surface dehydration. The spectral changes which follow this phenomenom are shown in Fig. 2. As can be seen, all the amide I bands lose intensity upon dehydratation of the film excepted for the band located at 1617 cm^{-1} which becomes strong and sharp. These results suggest a marked reduction of α-helical, β-sheet and unordered structures at the expense of intermolecular hydrogen-bonded β-sheet structure. Although it has not been possible to obtain quantitative results with low prediction errors on the conformation of the 11S protein in the film state since the currently available methods have been developed for proteins in solution [40] or for ATR measurements using deuterated protein films [25], it is clear that the protein in the film state displays a much higher content of intermolecular β-sheets and a lower content of α-helices, β-sheets and unordered conformation compared to the protein in solution.

The formation of intermolecular β-sheets during the film formation is not unique to glycinin and seems to be a common behavior of vegetable proteins whatever their origin [59]. In Fig. 3, the infrared spectrum of glycinin in the film state is compared to those obtained from legumin, a major globulin from pea, and wheat gluten proteins. A striking characteristic of this figure is that all spectra show the band characteristic of intermolecular β-structures located at 1618 cm^{-1}. These similarities could be due to similar secondary structures of these protein molecules. However, if legumin belongs to the same family of glycinin and contains mainly β-sheet and aperiodic structures in the native state [24], whole gluten proteins exhibit a different secondary structure [30] and contain approximately equal amounts of α-helices (31%), β-sheets (28%) and β-turns (27%). These results suggest that β-sheet structures might be essential for protein–protein interactions and network formation in films formed from vegetable proteins, whatever their origin and conformation. These results support the idea that intermolecular hydrogen-bonding interactions between β-sheets may act as junction zones and thus stabilize the film network. However, it should be pointed out that the spectrum of wheat proteins presents more regular secondary structure than those of the other

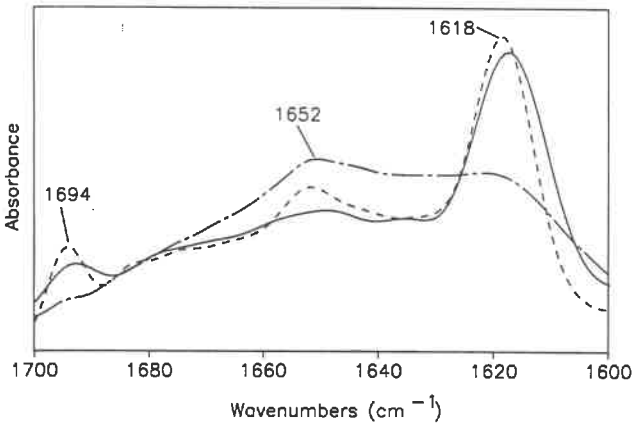

Fig. 3. Deconvolved infrared spectra of (——) soy glycinin, (- - -) pea legumin and (– – – – – –) wheat gluten proteins, in the film state.

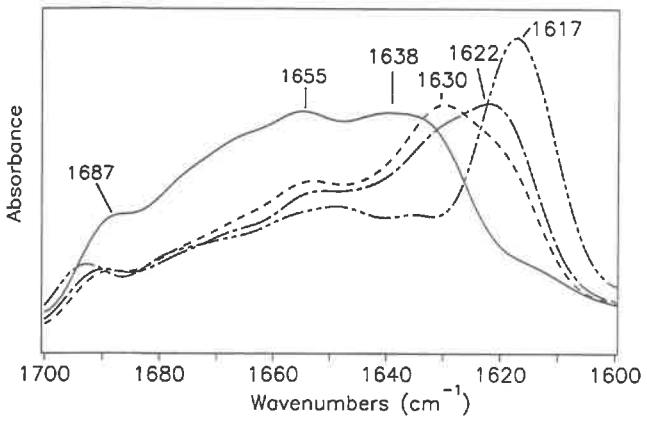

Fig. 4. Infrared spectra of glycinin in (——) aqueous solution and in the films prepared from (- - -) glycerol, (– – – – – – –) diethylene glycol and (– – – – – – – –) ethylene glycol.

proteins. It therefore seems that the α-helices are partially conserved in films prepared from wheat gluten proteins.

3.3. Effect of plasticizers on the network structure of films

Previous studies on films of wheat gluten [48], soybean globulins [14] and whey proteins [60] have shown that the nature of plasticizers used in film formation has a great effect on their mechanical properties. Since proteins may undergo structural changes in the presence of plasticizers, the extent of denaturation and the exposure of functional groups may affect the mechanical properties of films. However, the relationship between mechanical properties of protein films and conformational changes of proteins have not yet been studied. To understand the relationship between the conformation of glycinin and the mechanical properties of films of this protein, the infrared spectra of films of glycinin obtained from different plasticizers such as glycerol, diethylene glycol and ethylene glycol, were recorded. As seen in Fig. 4, the spectra obtained show strong differences compared to the spectrum of the native protein. The main spectral difference is a sharp increase in intensity of the band due to the intermolecular β-sheet at the expense of the lower frequency bonds assigned to other strutures. In addition, there is a shift of the position of the β-sheet band with the plasticizer used. The β-sheet band observed at 1638 cm^{-1} in the spectrum of the protein in solution shifts to 1630, 1622 and 1618 cm^{-1} in the film state in the presence of glycerol, diethylene glycol and ethylene glycol, respectively. A shift of this band to lower wavenumbers can be attributed to a strengthening of the hydrogen bonds between the peptide groups [61]. A previous study on the physical properties of glycinin films [16] reveals a plasticizer dependence of mechanical properties of films and shows that the young modulus of glycinin films decreases in the order ethylene glycol > diethylene glycol > glycerol. Close comparison of this behavior with corresponding changes in position of the infrared amide I band suggests for the first time that a close relationship exists between conformation of the protein in the film and the mechanical properties of these films.

On the other hand, the mechanical properties of macromolecules are not strongly influenced only by the structure of the material but also by molecular orientation induced by the forming process. In order to check for any preferred alignment of the β-sheet structure with respect to the film, polarized ATR–FTIR spectra of

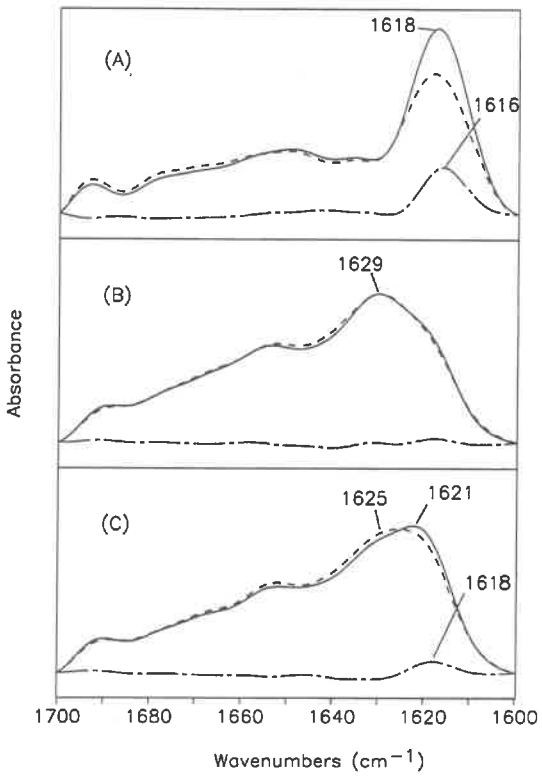

Fig. 5. ATR infrared spectra recorded with the incident radiation polarized parallel (——) and perpendicular (- - -) to the films prepared from (A) ethylene glycol, (B) diethylene glycol and (C) glycerol. The difference between the parallel and perpendicular spectra is also shown (– – – – – – –).

glycinin films have been recorded. Fig. 5 shows the difference spectra between the spectra recorded using the infrared radiation polarized parallel and perpendicular as a function of the plasticizer used for the film preparation. These spectra show clearly that the amide I band is strongly polarized in the case of the film obtained from ethylene glycol as opposed to those obtained from glycerol and diethylene glycol, suggesting that the β-sheets are highly oriented in the plane of the film obtained from ethylene glycol. One can suppose that this orientation is favored by ethylene glycol which can act as a bridge between the polypeptide chain increasing the strength of bonds and shifting the β-sheet band position to lower wavenumbers. Ethylene glycol is a relatively small hydrophilic molecule that could be easily inserted between protein chains and established hydrogen bonds with functional groups of glycinin. On the other hand, this behavior may be due to a more complete unfolding of the protein upon the film process in the presence of ethylene glycol which allows a closer alignment of polypeptide chains with the formation of much stronger hydrogen bonds. In the case of other plasticizers, the presence of residual secondary structures (indicated by the bands at around 1650 cm^{-1}) prevents such close alignment.

4. Conclusion

FTIR spectroscopy has been used in the present study to investigate the change in the conformation of glycinin during the film-forming process. As expected, this technique provides detailed information about the molecular structure of glycinin and can offer new insight into the protein–protein network. The results suggest that the hydrogen-bonded intermolecular β-sheet structure is essential for protein–protein interactions and network formation in the film obtained from vegetable proteins. Close comparison between the frequency of β-bands and mechanical properties of films reveals a relationship between conformation of the protein in the films and mechanical properties of the films. The mechanical properties are shown to not only be influenced by the structure of the protein but also by their molecular orientation.

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