Thermal Denaturation of Glycinin as a Function of Hydration[1]

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Hydration effects on the thermal stability of glycinin (soybean 11S protein) were examined by differential scanning calorimetry (DSC). In a model system with pure glycinin, the denaturation temperature (T_d) decreased with increasing moisture. Between 22 and 44% moisture, two endotherms were observed, where the lower-temperature endotherm became progressively reduced in magnitude with a concomitant increase in a higher-temperature transition. At 45.5% moisture, only a single endotherm was observed. The regression curves over the entire moisture range from 2 to 66% were derived as asymptote functions, where M equals the percentage total moisture. Equations were developed from the curves, and the relationship between T_d and moisture were: $T_d = 92.4 + 196.5e^{-0.068M}$ and, for the low-temperature endotherm, $82.4 + 144.3e^{-0.068M}$. By interaction of 11S protein with either ethanol, a neutral detergent (Triton X-100) or 40% sucrose, both one- and two-endotherm curves were generated. Such calorimetric behavior is indicative of nonequilibrium denaturation and supports the notion that structure reorganization during DSC is water content-dependent. Our findings suggest that either glycinin's acidic/basic subunits or a change in secondary protein structure may give rise to two endotherms.

KEY WORDS: Differential scanning calorimetry, glycinin, hydration effects, soybean, storage protein, thermal stability.

Water is a plasticizer for most natural and fabricated food ingredients and products. Levine and Slade (1) have developed a "food polymer science" approach to water relationships in foods, which has led to new perspectives on moisture management and innovative concepts of "water dynamics" and "glass dynamics". They explored the relationship between moisture and stability of proteins in the solid state. Their new concepts are focused on kinetically metastable, dynamically constrained states of water and its management in food systems rather than the more traditional equilibrium thermodynamic approach. Most proteins for use in food systems or protein-based industrial products are in a dehydrated state and require hydration to impart desired functional properties. Schnepf (2) reviewed the types of bonding between water and protein molecules, and how their interaction relates to these functional properties. Numerous methods for assessing protein hydration include dielectric studies (3), nuclear magnetic resonance (4–8) and calorimetry (9–13).

Thermal analysis is a valuable tool for studying the effects of heat processing. Differential scanning calorimetry (DSC) has been used extensively to study the thermodynamics and kinetic properties of protein denaturation, both in solution and solid state. The selection of processing conditions needed to impart desired functional properties requires hydration or rehydration of these proteins.

Thermal denaturation and coagulation of glycinin in dispersions have been the subject of numerous papers and reviews. Yet, little research has been performed on the thermal denaturation of pure 11S in dehydrated and rehydrated states. Kitabatake *et al.* (11) were the first to examine the denaturation of purified soybean 7S and 11S proteins under low-moisture conditions. Both soy 7S and 11S denaturation temperatures increased with the decrease in moisture. Sessa (9) used the regression curves from his model systems with pure soybean 7S and 11S, which had been thermally denatured under low to intermediate moisture conditions, to investigate hydration effects on the thermal stability of proteins in cracked soybeans and defatted soy flour. A biphasic endothermic event was observed only for the pure glycinin model system when moistened above 22%, moisture, which was attributed to the lack of moisture equilibrium because only one endothermic event appeared at 45% and higher moisture contents when equilibrated for four or more days. More extensive investigation of this biphasic endothermic event demonstrated that this occurrence is definitely not an artifact or an equilibration problem but is a real sequential event. Thermal denaturation of glycinin at various moisture concentrations is the primary objective of this investigation.

EXPERIMENTAL PROCEDURES

Materials. Dr. Walter J. Wolf of the National Center for Agricultural Utilization Research (Peoria, IL) kindly provided freeze-dried, electrophoretically pure glycinin prepared from Raiden variety soybeans (14). Triton X-100 (pfs) was purchased from Sigma Chemical Company (St. Louis, MO); sucrose, "Difco" certified from Difco Laboratories (Detroit, MI); and ethyl alcohol, 200-proof dehydrated alcohol, U.S.P. from Quantum Chemical Corporation (USI Division, Tuscola, IL).

Treatments. Moisture concentrations of weighed samples of purified glycinin were adjusted by micropipetting weighed amounts of distilled water onto the inner portion of the DSC aluminum pan cover prior to sealing and reweighing. Each sample was equilibrated at room temperature for a variety of times from 5 min to 14 d. To evaluate the effects of neutral detergents, sucrose and ethyl alcohol on pure glycinin, the protein samples were each wetted directly with weighed amounts of Triton X-100, 40% aqueous sucrose solution and 200-proof ethyl alcohol, prior to moistening the samples as described above. Percentage moisture in each sample at the time of analysis was then calculated on a wet-solids basis. For all samples, the moisture content of the original material, dry basis, was taken into account in calculating the total moisture content of each system. When aqueous 40% sucrose was added, the 60% water of the sucrose solution was considered in calculating total moisture content.

Analyses. Thermal denaturation of soy protein samples with various moisture levels and additives were each

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evaluated with a Perkin-Elmer DSC 7 equippped with a TAS 7 software package (The Perkin-Elmer Corporation, Oak Brook, IL) as previously reported (9). Glycinin samples in crimped aluminum pans capable of withstanding up to two atmospheres pressure and an empty crimped aluminum reference pan were scanned at heating rates of 1.0 to 50.0°C/min from 50 to 200°C to assess the effect of heating rate on thermal denaturation. Otherwise, a scan rate of 5.0°C/min was selected. Calibration of onset temperature and enthalpy of denaturation (ΔH) for the DSC cell were determined with a weighed amount of indium at the above scan rates over the same temperature range. Thermal denaturation gave endothermic event(s) where temperature, designated T_d, was used to signify the peak maximum. The enthalpy (ΔH) was calculated by fitting a straight line from peak onset to peak conclusion and recorded as joules per gram of dry protein. Where endothermic baselines could not be separated, a total enthalpy for the two transitions was evaluated.

RESULTS

Moisture effects. DSC thermal curves (Figs. 1 and 2) of pure glycinin at 11.5 and 14.7% moisture gave a single endotherm. Moisture values of 22.8, 26.8, 33.4 and 37.0% gave two endotherms, where the lower-temperature endotherm became progressively smaller with a concomitant development of a second, higher-temperature transition, and at 45.5% moisture only one endotherm was observed, with a shoulder preceding the main event. Each sample was equilibrated overnight at room temperature and scanned at a heating rate of 5°C/min. Figure 3 shows the entire sequence of events for glycinin thermally denatured at 2.5 to 67.0% moisture. The equations for the regression curves were derived as asymptote functions where M equals the percentage total moisture (9). Based on the 0.068 M factor for the respective T_d's of $92.4 + 196.5e^{-0.068M}$ and the less-stable $82.4 + 144.3e^{-0.068M}$, the two endotherms were parallel. Both equations, when fit with a nonlinear procedure in the Statistical Analysis Systems software package (SAS/STAT Guide for Personal Computers, Version 6 ed; SAS Institute, Cary, NC, 1987) had R^2 values of 0.99. The mean ΔH of 12.8 J/g with a standard error of 0.587, based on 30 observations, in Figure 3 resulted from data compiled from the three different batches of purified glycinin. The value of 11.7 J/g reported by Sessa (9) in an entirely different series was within the 95% confidence level. The endotherm previously published by Sessa (9), ($T_d = 95.0 + 138.2e^{-0.087M}$) will pass about midway through the values between 22 and 45% moisture. For example, at 37% moisture, T_d calculated on this regression curve (9) would be 100.5°C *vs.* a T_d of 101.2, which is about midway between the T_d's calculated from the two regression curves.

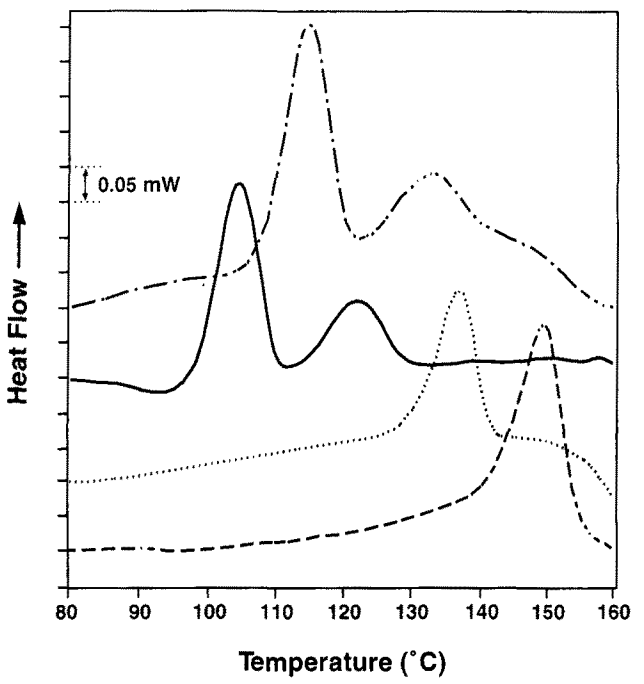

FIG. 1. Differential scanning calorimetry thermal curves of glycinin at moisture contents (wet basis) of (– – –) 11.5%, (···) 14.7%, (—) 22.8%, (– · –) 26.8%. Scan rate was 5°C/min.

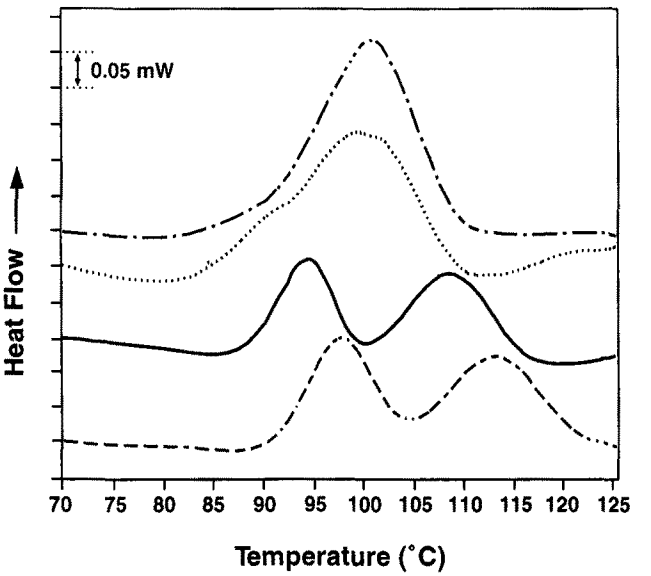

FIG. 2. Differential scanning calorimetry thermal curves of glycinin at moisture contents (wet basis) of (– – –) 33.4%, (—) 37.0%, (···) 45.5%, (– · –) 47.6%. Scan rate was 5°C/min.

According to Chou and Morr (15), the probable sequence for protein-water interaction starting with a dry powder is a multistage event with no clear-cut boundary between the individual stages. These authors suggest that water molecules will first be absorbed onto all available surface polar sites to form a monolayer coverage, followed by multilayer coverage upon further water absorption to result in liquid-water condensation from water-water interaction. In this state, the protein particles become swollen and solubilized if the protein is water-soluble. DSC measures a population of thermal events, with each heat input producing a thermogram of a predominating event, such as endotherm/exotherm, denaturation/renaturation.

The presence of two endotherms is unusual for a pure protein system. A literature search provided several incidences where a dual endothermic event occurred. Since the literature lacks adequate data and theoretical background to explain dual endotherms for pure proteins,

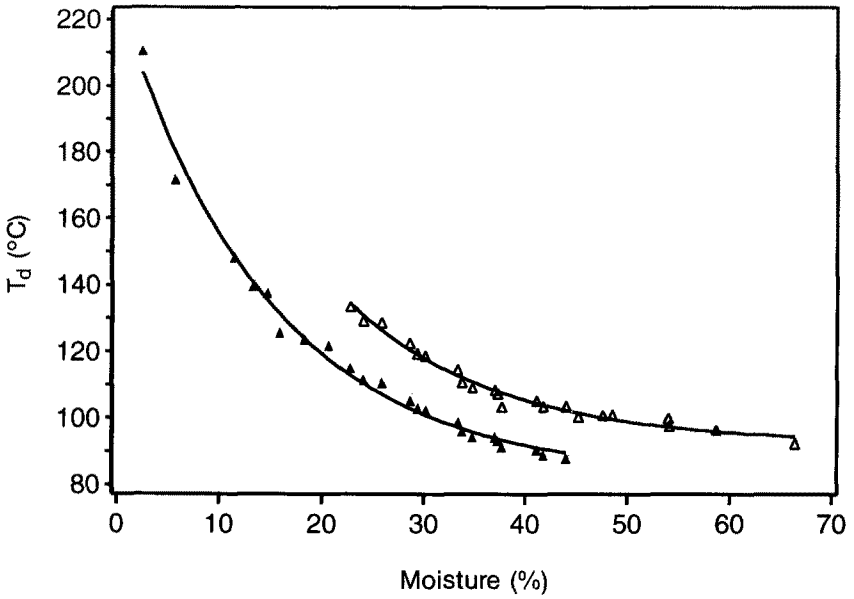

FIG. 3. Effect of moisture on the thermal denaturation of pure glycinin analyzed by differential scanning calorimetry at 5°C/min. Regression equation temperatures are: (△) (°C) $T_d = 92.4 + 196.5e^{-0.068M}$, (▲) (°C) $T_d = 82.4 + 144.3e^{-0.068M}$, where T_d = peak denaturation temperature and M = % total moisture in the sample. Both equations had $R^2 = 0.99$.

some analogous situations were taken with starch and polymers that generate dual endotherms. A biphasic endotherm can result from a purely mechanical effect (16,17), termed by Guenet (16) as a "constraint release", which results from the constraint of a sample such as gel on the bottom and the side of the pan. Mutin and Guenet (17) observed a dual enthalpic event in DSC thermograms of aged polyvinyl chloride gels in various solvents. They attributed the lower-temperature endotherm to a gel-pan contact effect where the magnitude of this endotherm was not affected by sample preparation or heating rate. In our thermal curves for glycinin (Figs. 1 and 2), we observed a definite sequence of events where the magnitude of the lower-temperature endotherm diminished with increased moisture content over a moisture range of 22 to about 44%, while magnitude of the higher-temperature endotherm increased with increased moisture content. At 45.5% moisture, one endotherm was generated with a shoulder preceding the major peak. Based on our sequence of events, the "constraint release" enthalpic artifact was not a likely explanation of the biphasic endothermic phenomenon.

The biphasic endothermic phenomenon may reflect two types of denaturation, where solids containing the least-stable associations denature first; on denaturation, the less-stable protein form can absorb water, thus making it unavailable for reaction with the remaining protein. This means that the effective water concentration was further reduced by repartitioning. Consequently, the more-stable protein crystallites melt at higher temperatures. This explanation is analogous to the explanation for the biphasic endothermic process for starch gelatinization (18,19).

Overall, such calorimetric behavior is indicative of non-equilibrium denaturation and supports the notion that structure reorganization during DSC is water content dependent. This type of multi-event endothermic phenomena is commonly observed with starch/aqueous systems (19). We may have observed a net effect of several opposing processes, such as partial denaturation, reorganization and final denaturation, or two different morphological states of glycinin.

Equilibration time. Equilibration times were varied from 0 to 2 wk at room temperature over the moisture range 13.3 to 66.4%; scan rate was 5.0°C/min; the T_d's for the two endotherms were compared with calculated values from the two regression curves (Table 1). All T_d values were within 2.7% of their calculated values. From our findings in Table 1, moistened samples of glycinin in sealed aluminum pans require no equilibration. Water vapor absorption by protein gives rise to "irregularities that reflect changes in the conformation and/or dynamic behavior of the biopolymer molecule" (20). Bryan (20) has suggested that, as water is added to a protein in the solid state, a small conformation change occurs, which increases the flexibility of the protein structures. In our sealed aluminum pan containing protein and a water droplet, a water-protein dynamic system results from application of heat where equilibration is essentially inconsequential.

Heating rate. Two endotherms were still observed when heating rates were varied from 1 to 50°C/min on glycinin samples moistened 31 to 42% (Table 2). However, at the slower heating rate of 1°C/min, the two endotherms were completely separated with a peak-to-peak spread of 18.0°C at 32.6% moisture and 16.6°C at 33.6%, while heating rates of 10 to 50°C/min caused merging of the two endotherms with peak-to-peak spread being less than 16.6. At the heating rate of 1°C/min, both the lower-temperature endotherm (#1) and the higher-temperature endotherm

TABLE 1

Effect of Equilibration Time on the Thermal Denaturation of Moisturized 11S Protein

Total moisture (%)	Equilibration time (h)	T_d #1 (°C) (calc. value)[a]	T_d #2 (°C) (calc. value)[b]
13.3	18	139.6 (140.8)	—
14.7	336	137.4 (135.5)	—
22.6	0	111.0 (113.4)	—
28.7	18	105.2 (102.9)	122.6 (120.3)
29.4	336	103.0 (101.9)	119.4 (118.9)
30.2	336	102.4 (100.9)	118.7 (117.6)
33.4	70	97.6 (97.3)	112.9 (113.2)
33.8	96	95.9 (96.8)	110.7 (112.1)
37.0	0.08	94.2 (94.1)	108.5 (108.3)
37.3	0	93.2 (93.8)	107.4 (107.9)
41.1	18	90.4 (91.2)	105.1 (104.4)
44.0	96	87.9 (89.6)	103.4 (102.2)
45.5	0.25	—	99.2 (101.2)
48.5	2	—	101.0 (99.7)
54.0	72	—	100.0 (97.3)
58.7	336	—	96.5 (96.1)
66.4	72	—	92.4 (94.6)

[a]Calculated from regression curve $T_d = 82.4 + 144.3e^{0.068M}$. T_d = denaturation temperature.
[b]Calculated from regression curve $T_d = 92.4 + 196.5e^{0.068M}$.

(#2) appeared at T_d's that are either equal to or slightly lower than the observed temperatures for samples heated at 5°C/min (values in parentheses). For samples heated 10 to 50°C/min, both endotherms (*i.e.*, #1 and #2) were higher than their respective T_d's heated at 5°C/min. The ΔH of 15.9 J/g for all observations, except those scanned at 1°C/min (ΔH = 13.2 J/g; data not shown) in Table 2, was significantly higher ($P \leq 0.05$) than 12.8 J/g obtained from data of all ΔH's in Figure 3. Such calorimetric behavior at the different scan rates is indicative of nonequilibrium denaturation where structure reorganization or conformation change during heating is water content dependent.

Interactions of glycinin with ethanol or Triton X-100. Various solvents and detergents have been used to study the nature of forces that stabilize native conformations of proteins. Alcohols progressively lower the temperature of denaturation and enthalpy of proteins as either alcohol concentration or as alkyl chainlength was increased (21). When we wetted glycinin, which has endogenous moisture of 11.5%, with 21 to 44% absolute ethanol, we observed a 75°C decrease in T_d. Yet, the ΔH of 10 J/g was well within the 95% confidence limit of 12.8 J/g, thus indicating that glycinin wetted with absolute ethanol did not cause denaturation. When the glycinin samples were first wetted with absolute ethanol, (*e.g.*, 21%) and then moistened with water to 37% moisture, only one endothermic event was observed with a T_d of 70.2°C and a significantly ($P \leq 0.05$) decreased ΔH of 1.9 J/g. Ethanol-water mixtures have a higher denaturing effect on soy proteins than that attained by the individual components alone (22). Alcohol not only weakens the hydrophobic interaction between nonpolar residues but also perturbs the water structure around the protein molecules. Only one endotherm was observed in the presence of ethanol.

The effect of neutral detergent, Triton X-100, directly added to glycinin, either at its endogenous moisture of 11.5% or when moistened to 33.6, 34.8 and 45.3%, caused a decrease in T_d (Table 3). The ΔH of 13.5 J/g for all samples wetted with Triton X-100 was not significantly different ($P \leq 0.05$) from 12.8 J/g obtained from data for all ΔH's in Figure 3. These results indicate that the

TABLE 2

Effect of Heating Rate on the Thermal Denaturation of Glycinin

Total moisture (%)	Heat rate (°C/min)	Endotherm #1 T_d (°C)[a]	Endotherm #2 T_d (°C)[a]
32.6	1	95.2 (98.1)	113.2 (114.0)
33.6	1	96.0 (97.1)	112.6 (112.4)
31.0	10	103.6 (100.0)	119.7 (116.4)
36.4	10	95.7 (94.5)	110.2 (109.1)
40.1	20	94.3 (91.8)	107.9 (105.2)
41.2	20	93.5 (91.2)	106.3 (104.4)
37.9	30	98.2 (93.3)	112.0 (107.5)
38.0	30	98.0 (93.4)	111.4 (107.3)
40.8	40	97.5 (91.5)	110.2 (104.8)
42.1	40	98.4 (90.6)	109.7 (103.6)
38.2	50	102.7 (93.2)	116.0 (107.1)

[a]Value in parentheses is the T_d for glycinin heated at 5°C/min. See Table 1 for regression equations and abbreviation.

TABLE 3

Effect of Neutral Detergent on the Thermal Denaturation of Glycinin[a]

Triton X-100 added (%)	Total moisture (%)	Endotherm #1 T_d (°C)	Endotherm #2 T_d (°C)
None	11.5	149.9	—
39.8	11.5	141.5	—
None	33.6	97.1	112.6
50.6	33.6	95.5	109.2
None	34.8	95.9	111.1
44.7	34.8	94.4	109.2
None	45.3	—	99.4
54.1	45.3	—	99.0

[a]See Table 1 for regression equations and abbreviation.

addition of Triton X-100 to moistened glycinin caused a slight destabilization and no denaturation, which did not affect the biphasic phenomenon.

The results with ethanol, ethanol-water and neutral detergent acting on glycinin fit the mechanism proposed by Fukushima (22). Glycinin, a globular protein, is surrounded by a hydrophilic shell and an outside hydration layer, while its hydrophobic side-chain residues are located toward the center. Detergents, such as Triton X-100, destroy the hydrophilic shell because of their hydrogen bond-forming ability and disrupt hydrophobic interactions among amino-acid side chains. Amphiphilic compounds, such as ethanol, disrupt the hydrophobic region portion of the molecule, particularly when water is present. To observe two endotherms, sufficient water (in this case about 22% total moisture) should be added to glycinin to give the protein structure flexibility, much like that needed to initiate the onset of enzyme activity of lysozyme powders (3). If the objective is to retain the biphasic event at low moisture concentrations, then one should not disrupt the hydrophobic bonding, which plays a dominant role in stabilizing the helical, globular conformation of the protein molecule in an aqueous environment in preference to the random coil form (15).

Glycinin/sucrose interaction. The addition of carbohydrates to proteins is known to increase protein's denaturation temperature. Back *et al.* (23) studied the effect of twenty different sugars and polyols on several egg white proteins. The extents of stabilization observed by the different sugars and polyols were due to their influence on the structure of the water present in the vicinity of the protein, which affected the hydrophobic interaction. In our study (Table 4), when 30.3, 44.1 and 48.7% sucrose solute was added and the total moisture concentration was adjusted to 29.7, 36.2 and 40.7%, only one endotherm was apparent, which possessed T_d's equivalent to the higher-temperature endotherm. The ΔH's of 13.2 J/g for 11S protein with added sucrose were not significantly different ($P \leq 0.05$) from those obtained from glycinin moistened with water alone or neutral detergent. Stabilization observed by added sucrose is due to the influence of carbohydrate on the structure of water present in the vicinity of the protein, which consequently affects the hydrophobic interaction. Upon concentrated sucrose solute addition to glycinin, water is redistributed from the protein. Excess water mobilized by sucrose thereby gives only one endotherm at the higher thermal stability. As moisture is increased (*i.e.*, 55.7%) the amount of water-protein perturbed by sucrose becomes negligible; hence, the protein stabilization effect becomes minimal.

TABLE 4

Effect of 40% Sucrose on the Thermal Denaturation of Glycinin[a]

Sucrose added (%)	Total moisture (%)	Endotherm#1 T_d (°C)	Endotherm #2 T_d (°C)
None	29.7	101.6	118.7
30.3	29.7	—	119.0
None	36.2	94.6	109.3
44.1	36.2	—	107.9
None	40.7	91.5	104.8
48.7	40.7	—	102.4
None	55.7	—	96.9
27.7	55.7	—	97.4

[a]See Table 1 for regression equations and abbreviation.

DISCUSSION

The presence of two endotherms that occur within water content levels of 22 to 44% may reflect cooperative water binding by two different structural domains of glycinin, such as its acidic and basic subunits or perhaps polypeptide chains that strongly differ in hydrophilicity. Hence, at 22–44% moisture, one class of polypeptides may be more stable and refractory to disruption by water and heat than the other, and its sequence of events should give two endotherms. The multiple peaks with water alone may also arise from partial denaturation, reorganization and subsequent denaturation. Ethanol can effectively disrupt and destabilize glycinin secondary structure, regardless of involvement of acidic or basic polypeptides. Sucrose addition may also restrict the extent of conformational disordering when there is competition between protein and sugars for water. Hence, addition of either ethanol or sucrose should give only one endotherm, which is consistent with our findings.

At moisture contents below 22%, the dependence of protein denaturation temperature may reflect the dependence of the glass transition temperature of globular proteins on moisture content (Tolstoguzov, V., personal communication). Their denaturation can occur at slightly higher temperature than the glass transition where molecular mobility arises. At intermediate moisture contents (*i.e.*, 22–46%), the temperature dependencies of the glass transition and denaturation on water content can be more sensitive to the differences of polypeptide composition of protein. After saturation of the strongly water-binding protein groups at low moisture level, further increase in water content is accompanied by a change in the dependence of the glass transition temperature of protein.

Grinberg *et al.* (24) proposed that the glycinin molecule, which is an ensemble of twelve thermodynamically cooperative domains when in dilute solution, unfolds according to a two-state model. These authors suggested that high helix content exists in the boundary region within the interior of the molecule. Such a model for glycinin in dilute solution should give rise to a single endotherm, which was what we observed when moisture content was 45% and higher.

Recently, Chen *et al.* (25) and Ker *et al.* (26) assessed the secondary structure changes of glycinin, caused by elevated temperature and shearing, as monitored by Fourier transform infrared spectroscopy and found that glycinin increased in a α-helix content from 7.8% in the native state to 28.1% when heated to 90°C. There was a corresponding decrease in random coil from 63.8% in the native state to 42.5% when heated. According to Chou and Morr (15), a change in protein conformation affects its ability to interact with water, whereby folding and aggregation may alter the hydrophilic group's ability to bind water. Under certain protein conformational states, the interior hydrophilic region of the protein or protein aggregate may be freely exposed so that these sites may now be available to interact with water.

Peptide-solvent interactions are the predominant characteristic of the random coil conformation, whereas peptide-peptide unit interactions are the predominant

factor for helix conformation. Therefore, the helix conformation would tend to be more heat stable than the random coil conformation. In essence, the biphasic event may arise from Random coil + $nW_1 \triangleq$ Helix + nW_2, where W_1 and W_2 represent different states of water. Conformational changes that have different degrees of interactions with water are believed to be responsible for the biphasic endothermic event when the amount of water is restricted for the 11S globulin. As stated earlier, sucrose addition may restrict the extent of conformational disordering when there is competition between protein and sugars for water. Ethanol, at the high concentrations used in our current study, perturbs hydrophobic interactions in the interior of the protein, thereby destabilizing and denaturing the protein (24). Hydrophobic bonding may play a dominant role in stabilizing the helical, globular conformation of the glycinin molecule in an aqueous environment in preference to the random coil form. Our findings suggest that the two endotherms at moisture of 22 to 44% may result either from glycinin's acidic and basic subunits or from changes in glycinin's secondary structure during heating.

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