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ARTICLE

Thermal Behavior of Soy Protein Fractions Depending on Their Preparation Methods, Individual Interactions, and Storage Conditions

Pablo A. Sobral,[†] Gonzalo G. Palazolo,[§,#] and Jorge R. Wagner*[,§,#]

[†]Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA-CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 116 (B1900AJJ) La Plata, Buenos Aires, Argentina, [§]Laboratorio de Propiedades Funcionales de Alimentos, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352 (B1876BXD) Bernal, Buenos Aires, Argentina, and [#]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Different soy protein isolates (SPI) and whey soy protein (WSP) samples were obtained from fresh and stored soybean flour. Some samples were subjected to a long, cold storage. DSC thermograms of SPI showed the two characteristic endotherms, corresponding to denaturation of β-conglycinin and glycinin. Low value of denaturation enthalpy and high glycinin denaturation temperature were related to a reduction of protein solubility of SPI. DSC thermograms of WSP also showed two characteristic endotherms, corresponding to Kunitz trypsin inhibitor and lectin. The methods and conditions of preparation and storage of WSP samples were factors that modified their thermal behavior. Some SPI−WSP mixtures (1:1) exhibited more complex thermograms and higher denaturation temperatures. Thermograms of SPI-denatured WSP mixtures showed that the thermal stabilization of soybean storage proteins was attributed to protein−protein interactions. The differences in the thermal behavior of single or mixed SPI and WSP could not be explained on the basis of mineral content.

KEYWORDS: Thermal behavior; DSC; isolates; whey; soybean; proteins

INTRODUCTION

Storage and whey proteins are the two major groups of proteins in soybean (*1*, *2*). Storage globulins are generally obtained from isoelectric precipitation by acidifying (pH 4.5−4.8) an aqueous extract of defatted soy flour and further solubilization and neutralization of the precipitate. The soybean protein isolate (SPI) is composed mainly by β-conglycinin (7S fraction) and glycinin (11S fraction) (*3*). To achieve high performance, these protein isolates are obtained from defatted soybean flour not thermally treated to avoid protein denaturation and, hence, with high protein solubility. The commercial SPI contain fully or partially denatured proteins, due to different treatments after the isoelectric precipitation. However, in the laboratory, it is possible to prepare SPI with low denaturation degree of its constitutive proteins and high protein solubility in water, so that they are designated "native soy isolates" (*2*). The thermal behavior of the SPI was fully studied by differential scanning calorimetry (DSC) (*4*−*6*). The temperature of protein denaturation in soy isolates may be affected by pH (*7*), increased significantly with the increase of ionic strength (*2*) and diminished when the salt content decreases by dialysis prior to drying (*5*). Moreover, the calcium salts exert a stabilizing effect on 11S globulin, resulting in an increase of its denaturation temperature with calcium content (*8*). In addition, the phenolic components can also alter the thermal stability of the storage globulins in SPI: the strong interaction between vegetable proteins and phenolic compounds has been previously reported (*9*). The influence of phenol content on the temperature and enthalpy of denaturation of SPI has been studied (*10*). A higher thermal stability of 11S globulin was observed when this protein forms complexes with phenolic compounds and flavonoids (*11*).

Soy whey (SW), the waste industrial liquid from the manufacture of SPI and tofu, contains mainly oligosaccharides and biologically active proteins, the latter being called whey soy proteins (WSP) (*2*, *12*). SW also contains minor components, such as phytates, salts, pigments, polyphenols, and isoflavones, such as genistein, daidzein, and their conjugated β-glucosides which can be quantified by means of high-performance liquid cromatography (*13*, *14*).

WSP represent nearly 30% of the total protein of soybean flour, and they are very soluble in a wide range of pH. These proteins were prepared by precipitation with ammonium sulfate and subsequently dialyzed and freeze-dried. An electrophoretic analysis (SDS-PAGE) revealed the presence of lipoxygenase, β-amylase, trypsin inhibitors (Kunitz and Bowman−Birk), and lectin (*1*). In a previous work, a comparative analysis of thermal behavior of WSP and native soy isolates has been performed (*2*). DSC thermograms of aqueous dispersions of WSP, similarly to those of SPI, show two endothermic transitions. In the case of WSP these endotherms correspond to the thermal denaturation of KTI and L (70.4 ± 0.3 and 90.4 ± 0.8 °C, respectively) (*2*), whereas for SPI the transitions are attributed to denaturation of 7S and 11S fractions (77.6 ± 1.2 and 90.5 ± 1.6 °C, respectively) (*2*, *4*). Unlike SPI samples, the thermal behavior of WSP is hardly affected by ionic strength.

*Corresponding author (phone +54-11-43657116; fax +54-11-43657132; e-mail jwagner@unq.edu.ar).

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Table 1. Conditions of Preparation of SPI (**A**) and WSP (**B**) Samples

(**A**) **SPI Samples**

		changes in standard procedure	
SPI sample	raw material	in step b: maintenance of the isoelectric precipitate at	in step e: pH adjustment before freeze-drying
SPI-1	fresh flour A	no	8.0
SPI-2	fresh flour B	no	8.0
SPI-3	flour A, stored for 1 year at 4 °C	no	8.0
SPI-4		4 weeks at 10 °C	8.0
SPI-5		4 weeks at −20 °C	8.0
SPI-6	flour A, stored for 8 years at 4 °C	no	8.0
SPI-7	fresh flour A	no	10.0

(**B**) **WSP Samples**

			treatment on SW	
sample type	WSP sample	raw material	cold storage of acid SW	pH adjustement before precipitation
WSP-I (precipitated with ammonium sulfate)	WSP-1	fresh flour A	no	8.0
	WSP-2		4 weeks at −20 °C	8.0
	WSP-3		1 week at 10 °C	8.0
	WSP-4		4 weeks at 10 °C	8.0
	WSP-5	fresh flour B	no	8.0
	WSP-6		no	4.5
WSP-II (precipitated with cold acetone)	WSP-7	fresh flour A	no	8.0
	WSP-8	flour A, stored for 2 years	no	8.0
	WSP-9	flour A, stored for 8 years	no	8.0
	WSP-10	fresh flour B	no	4.5
	WSP-11	flour B, stored for 2 years	no	4.5
	WSP-12		no	8.0
	WSP-13	tofu whey	no	6.5

Our research group has been studying the thermal behavior of soy proteins for over 10 years. Differences in denaturation temperatures that merit further analysis have been detected. The aim of this study was to analyze the factors influencing the thermal behavior of soy proteins, including the preparation methods and storage time. In addition, mixtures of SPI and WSP samples have been evaluated to analyze the potential interactions between their components, which could affect the thermal behavior.

MATERIALS AND METHODS

Materials. This work included results obtained from 1999 to date. Defatted soy flours A (from 1999 to 2007) and B (from 2007) were provided by Santista (SP, Brazil) and Solae (SP, Brazil), respectively. The tofu whey (TW) was supplied by a local industry (Soyana S.H. Argentina). Distilled water was always used, and all other chemicals were analytical grade reagents.

Preparation of Soy Protein Isolates (SPI). SPI samples were obtained by following the basic experimental procedure (*2*), which can be summarized as follows: (a) aqueous solubilization of flour at pH 8.0 for 2 h under magnetic stirring at room temperature using a 1:10 w/v flour/water ratio; (b) separation of the aqueous extract by centrifugation (10400*g*, 15 min, 20 °C); (c) isoelectric precipitation of storage globulins by acidification of the extract to pH 4.5 with 1 M HCl and maintenance at 4 °C for 2 h; (d) separation of soy whey (SW) by centrifugation (10400*g*, 20 min, 20 °C); (e) resuspension of the precipitate with water (up to 40 mg/mL), adjustment to pH 8.0 with 2 M NaOH, and subsequent freeze-drying. Isolates obtained by using this standard procedure from flours A and B were called SPI-1 and SPI-2, respectively. SPI-3−SPI-7 were obtained by modification in some steps of the mentioned procedure, as shown in **Table 1A**.

Preparation of Glycinin (G). The enriched 11S fraction was prepared from defatted soy flour A according to the method of Thanh and Shibasaki (*15*) with slight modifications (*16*), giving sample G-1. Deffated soybean flour was extracted with 0.03 M Tris-HCl buffer, pH 8.0, containing 2 mM β-mercaptoethanol at 24 °C. The extract was adjusted to pH 6.4 with 1 M HCl, and the glycinin precipitate was washed with Tris-HCl buffer, pH 6.4, and then solubilized in the same buffer at pH 8.0, dialyzed against ammoniacal water, pH 8.0, and subsequently freeze-dried. A glycinin sample identified as G-2 was obtained following the same procedure but from long-stored flour A (8 years at 4 °C).

Preparation of Whey Soy Proteins (WSP). According to the method of precipitation used, two types of WSP samples were obtained from SW with or without previous adjustment to pH 8.0 with 1 M NaOH. WSP-I samples (WSP-1−WSP-6) were precipitated with ammonium sulfate to 90% saturation, dialyzed against water containing sodium azide (0.03% w/v), and subsequently freeze-dried (*1*, *2*), whereas WSP-II samples (WSP-7−WSP-13) were precipitated with cold acetone (SW/acetone 1:1 v/v, 0 °C), washed with water, and freeze-dried (*14*). Different WSP samples were obtained according the raw material and conditions of pH and cold storage of SW (**Table 1B**).

Differential Scanning Calorimetry (DSC). Sample dispersions (30% w/w in water) were hermetically sealed in standard aluminum pans. Thermograms were obtained at a 10 °C/min heating rate in a range of 30−130 °C using DSC Polymer Laboratories equipment (Rheometric Scientific, U.K.). A double empty pan was used as reference. The peak temperature (T_p, °C) and total denaturation enthalpy (ΔH, J/g dry matter) were obtained from thermogram analysis. All experiments were performed at least in triplicate.

SPI/WSP and G/WSP mixtures were also analyzed. Samples were mixed in a 1:1 w/w ratio, water was added to form a dispersion (30% w/w), and then the samples were kept at 4 °C for 30 min to ensure total hydration. In other series of DSC experiments, SPIs were mixed with thermally denatured WSP samples or ash (calcination at 550 °C) of WSP samples. SPI mixtures with denatured WSP were carried out as follows: an aqueous dispersion of WSP (15% w/w) was thermally treated (20 min, 100 °C) and subsequently cooled to room temperature. Then, the SPI sample was added (to obtain a total concentration of 30% w/w), mixed, and kept at 4 °C for 30 min. In addition, SPI mixtures with ash from WSP were carried out as follows: ash from 50 mg of WSP was mixed with 50 mg of SPI, and water was added to obtain an aqueous dispersion (30% w/w), which was finally kept at 4 °C prior to DSC measurement.

UV−Visible Spectroscopy. Absorption spectra of aqueous dispersions (0.05% w/v in pH 7.0, 10 mM phosphate buffer) of 11S fractions and whey soy proteins were obtained in a Beckman DU 600 spectrophotometer between 200 and 800 nm.

Determination of Mineral Content. The determination of total minerals was carried out by calcination of samples at 550 °C, expressing the result as ash (mg/g of sample). Ash was dissolved in 0.14 M nitric acid and 0.5% lanthanum and filtered through a membrane (0.45 μm pore size). Calcium and magnesium concentrations were determined on the resulting solution by atomic absorption spectroscopy using a Shimadzu AA-6650 spectrophotometer at 422.7 and 285.2 nm, respectively, and expressed as milligrams per gram of sample. Electrical conductivity (EC) was determined on aqueous dispersions of SPI and WSP samples (0.05% w/v double-distilled water) at 25.0 ± 0.5 °C (ASTM D 1125-64) using an YSI conductimeter model 35 with a YSI 3401 cell (cell constant = 1.084 ± 0.067/cm). With standard NaCl solutions, a correlation between EC and NaCl concentration (in mol/L) was determined: EC (μS) = 5.266 + 96379.19 × [NaCl] (r^2 = 0.99). Using this correlation, the salt concentration expressed in grams of NaCl per gram of sample was calculated.

Protein Solubility. Aqueous dispersions (1% w/v) were prepared by dispersing the samples by gentle magnetic stirring (2 h, 25 °C) and subsequently centrifuged (9300*g*, 20 °C, 20 min). Protein concentration was determined in supernatants by using the Kjeldahl method (N × 6.25). Protein solubility (% w/w) was expressed as PS = (mg of soluble protein/mg of total protein) × 100.

Statistical Analysis. Data were analyzed by ANOVA, and significant difference between the Fisher's test was determined (Systat, 5.0; Systat, Point Richmand, CA). An α level of 0.05 was used to determine significance. Each value reported was the mean of at least three determinations and expressed as such with the standard deviation (mean ± SD).

RESULTS AND DISCUSSION

Thermal Behavior of Soy Protein Isolates. According to previous papers (*1*, *17*), electrophoretic profiles of SPI samples showed the presence of β-conglycinin subunits (α, α′, and β) and glycinin polypeptides (A and B). The protein (N × 6.25) and total carbohydrate contents of SPI samples, expressed on a percent dry basis, were 90.7 ± 2.5 and 5.3 ± 2.0, respectively. All SPI samples exhibited typical DSC thermograms of soy isolates in which the endothermic transitions I and II correspond to the denaturation of β-conglycinin (7S) and glycinin (11S), respectively (*2*). In previous works, other authors reported T_p I and T_p II values in the ranges of 74−79 and 90−98 °C, respectively, and a significant variability in ΔH values (*5*, *6*, *8*, *18*, *19*). In the present study, we also observed variations in the thermal behavior of 7S and 11S globulins between different SPI samples. For instance, **Figure 1** shows a remarkable difference between the thermal behavior of SPI-1 and SPI-5. ΔH and T_p values of SPI samples are summarized in **Table 2**. It can be observed that samples SPI-1−SPI-4 exhibit T_p I and T_p II values comparable to those of SPI obtained by other authors, using a similar experimental procedure (*2*, *5*, *8*). However, only SPI-1 and SPI-2 gave ΔH values comparable to those corresponding to native soy isolates (*2*). These samples were prepared from fresh flours (flour A or B stored for < 1 month) and exhibited a PS value of > 95% (**Tables 1A** and **2**). In contrast, SPI-3 and SPI-4, with a PS in the range 85−91%, exhibited ΔH values significantly less (18−30%) than those of SPI-1. The slight protein denaturation degree observed in SPI-3 could be attributed to the fact that this sample was obtained from long-stored flour (1 year at 4 °C). Besides, the higher protein denaturation degree in sample SPI-4 could be attributed to the storage of isoelectric precipitate at 10 °C for 4 weeks. A similar degree of protein denaturation was observed for SPI-3 after a long storage at 4 °C (9 years), which also evidenced a significant loss of PS (~15%), or in sample SPI-6, which was prepared from a long-stored soy flour. These samples also showed an increase in their thermal stability, mainly in the 11S fraction: a shift of T_p toward higher temperatures (2−3 and 4−7 °C for T_p I and II, respectively) was effectively

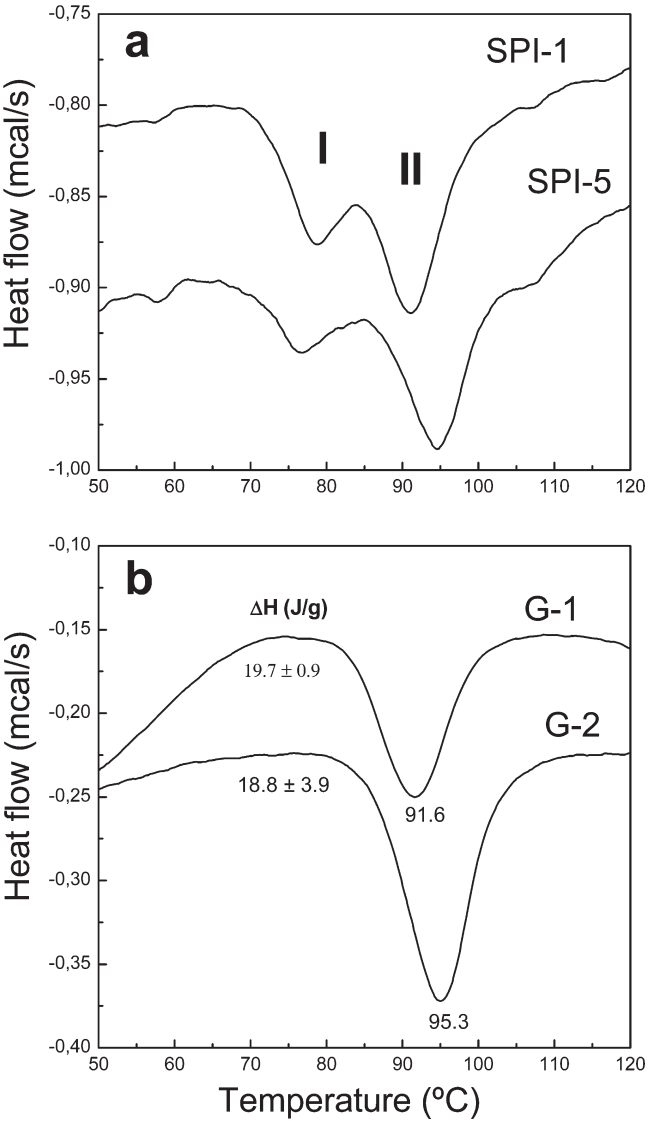

Figure 1. DSC thermograms of aqueous dispersions (30% w/w) of (**a**) SPI and (**b**) G samples.

observed. In this context, abnormally high values of denaturation temperature for 7S and 11S globulins (T_p = 82.5 and 98.9 °C, respectively) were reported for a control isolate prepared from soybean seeds previously stored at −20 °C (*18*). This sample exhibited very low values of ΔH and PS (< 70%), indicating that 7S and 11S globulins were partially denatured and aggregated. These results can be understood if one considers that the freezing of soybeans produces an increase in the surface hydrophobicity of proteins, which favors protein aggregation (*20*).

SPI-5 and SPI-7 exhibited a loss of PS and were also partially denatured (30−40% with respect to SPI-1). The contribution of 7S fraction to the total denaturation enthalpy of SPI samples was expressed by the I/(I + II) area ratio (**Table 2**). According to this parameter, the 7S globulin was largely affected (**Table 2**,; **Figure 1a**). These changes would be attributed to preparation procedures of protein isolates: (i) for SPI-5, the negative effect of freezing of the isoelectric precipitate or (ii) the destabilizing effect of strong alkaline conditions (adjustment to pH 10.0) in SPI-7 (**Table 1A**). These soy isolates (similarly to SPI-6 and stored SPI-3) were also characterized by a higher value of T_p II, which indicates an increase in the thermal stability of glycinin (**Table 2**). This result could be due to the formation of protein aggregates, which was reflected in the low PS values. The formation of aggregates is one of the factors that determine the

Table 2. Protein Solubility and Thermal Behavior of SPI Samples

	thermal behavior				
SPI sample	T_p I (°C)	T_p II (°C)	ΔH (J/g)	(I + II) area ratio (%)	protein solubility (%, w/w)
SPI-1	77.5 ± 0.5 a[a]	91.0 ± 0.4 a	17.1 ± 0.8 a	≈40	98.0 ± 2.0 a
SPI-2	77.6 ± 0.5 a	92.4 ± 1.0 a	13.0 ± 2.0 b		
SPI-3	78.2 ± 0.4 a	91.5 ± 0.4 a	13.8 ± 1.1 b	≈40	90.5 ± 3.0 a
stored SPI-3[b]	81.4 ± 0.5 b	98.6 ± 1.5 b	11.9 ± 0.5 c	≈30	78.5 ± 3.5 b
SPI-4	77.2 ± 0.6 a	91.9 ± 0.9 a	11.8 ± 0.2 c	≈30	∼85
SPI-5	80.2 ± 1.2 b	96.6 ± 0.4 b	11.3 ± 1.4 c	10−20	∼85
SPI-6	80.0 ± 0.5 b	95.5 ± 0.5 b	10.3 ± 0.7 c		nd[c]
SPI-7	77.0 ± 0.2 a	94.2 ± 0.2 b	11.6 ± 0.6 c		85.0 ± 3.5 c

[a] Means within the same column followed by different letters are significantly different ($p < 0.05$). [b] Stored SPI-3: sample SPI-3 located within a closed bottle in a chamber at 4 °C for 9 years. [c] nd, not determined.

higher thermal stability of some proteins (*10*, *11*). Protein aggregation would be induced by various mechanisms: first, a long storage of soy flour, prior to SPI preparation; second, a long storage of SPI samples; third, a short frozen storage of the isoelectric precipitate. In the latter mechanism, 11S aggregation was induced by the known effect of cold and/or the cryoconcentration in the unfrozen aqueous phase (*21*).

A difference between the values of denaturation temperature of glycinin samples was also observed (**Figure 1b**). It can be seen that the endotherm corresponding to G-2 has a T_p of 95.3 ± 0.5 °C, which is 3.7 °C higher than that corresponding to G-1 (91.6 ± 0.1 °C). It is important to note that G-2, SPI-5, SPI-6, SPI-7, and long-stored SPI-3 had a slightly brownish color, which would suggest the formation of colored polymers derived from Maillard reactions or oxidation of polyphenols.

It is known that soybeans have oligosaccharides and phenolic compounds that may be present in SPIs in variable quantities (*10*, *22*, *23*). Moreover, the Maillard reaction during the storage of protein isolates was also reported (*24*). Although the presence of phenolic compounds was observed in soy isolates, a relationship between phenol content and temperature of protein denaturation was not clearly detected (*10*). The formation of glycinin derivates by interaction at pH 9.0 with phenolic acids and flavonoids was studied by Rawel et al. (*11*). The denaturation temperature and total denaturation enthalpy for nonmodified soy glycinin were 93.1 °C and ΔH = 5.6 J/g of protein, respectively. The DSC data for the derivates of glycinin with phenolic acids (chlorogenic, caffeic, and gallic), as well as with the flavonoids myricetin and quercitin, showed a significant increase (3−7 °C) of the denaturation temperature. SDS-PAGE profiles of the mentioned 11S derivates reveal the formation of protein aggregates by new inter- and intramolecular interactions (*11*). In this context, G-2 exhibits a brownish appearance; hence, an aqueous dispersion of G-2 was analyzed by UV−visible spectroscopy. This glycinin sample, which was prepared from a long-stored soy flour, showed a wide adsorption peak between 360 and 500 nm. According to Rawel et al. (*11*), the absorption below 400 nm can be attributed to the presence of polyphenols. In contrast, absorption values higher than 400 nm are attributed to turbidity as a consequence of the presence of the protein aggregates. It should be noted that PS of G-2 was <80%, unlike the sample G-1, which was completely soluble.

Phenolic compounds are susceptible to both enzymatic and nonenzymatic oxidation in the presence of oxygen. In alkaline pH, an *o*- or *p*-diphenol is converted to the corresponding *o*- or *p*-quinone, which can react with the nucleophilic groups of the protein molecules (*25*, *26*). Interaction with phenolic compounds is favored at alkaline pH, which would explain the brownish appearance and greater thermal stability of glycinin in sample SPI-7.

In a previous work, it was been reported that the gelling properties of soy protein are affected by many factors, one of which is the oxidation of protein structure (*24*). Those authors reported the thermal behavior of three native soy isolates; one of them with high protein aggregation exhibited the lowest value of T_p for glycinin (88.9 °C). Due to the off-flavor of soybean flour, these authors suggested that protein aggregation was induced by reaction with lipid peroxides present in the isolate. This result allows us to discard this type of aggregation reaction in our SPI samples, which exhibited T_p II values of >90 °C.

Thermal Behavior of Whey Soy Proteins. According previous papers (*2*, *12*), WSP-5 and WSP-7, representative samples of WSP-I and WSP-II, are composed by lectin (L) and Kunitz trypsin inhibitor (KTI) as major components. SDS-PAGE revealed that WSP-13 was mainly constituted by L and Bowman−Birk trypsin inhibitor (BBTI) and a low content of subunits of globulins 7S and 11S. The crude protein (N × 6.25) and total carbohydrate contents of WSP-I samples (expressed in percent dry matter) were 96.2 ± 1.3 and 3.1 ± 1.2%, respectively. These parameters for all WSP-II were in the ranges of 56−62 and 20−25%, respectively. As reported previously, the DSC thermogram of WSP presents two transitions, I and II, corresponding to the denaturation of KTI and L, respectively (*2*). In the present study we registered differences in the thermal behavior between WSP-I and WSP-II attributed to the isolation procedures (salting-out and precipitation with acetone, respectively, **Figure 2**). T_p, ΔH, and PS values for WSP samples are shown in **Table 3**. In addition, the contribution of KTI thermal denaturation to total denaturation enthalpy was expressed through the I/(I + II) area ratio parameter. For the WSP-I group, WSP-1 and WSP-5 samples were taken as control samples because they were prepared from fresh soybean flour and exhibited a high PS (>98%, **Table 3**). Nevertheless, their values of ΔH were lower and T_p I and T_p II values were slightly higher than those of WSP samples previously reported in other works (70.4 and 90.4 °C, respectively) (*2*).

WSP-2, WSP-3, and WSP-4 samples were prepared from the same flour used to prepare WSP-1. For WSP-2, the frozen storage of acid SW caused no changes in the thermal behavior of KTI and L. In contrast, the chilling of acid SW during the preparation of WSP-3 and WSP-4 (1 and 4 weeks, respectively) caused a noticeable reduction in ΔH values, although without significant changes in T_p values ($p < 0.05$). On the other hand, the long storage (9 years at 4 °C) of WSP-2 and WSP-3 samples caused not only a remarkable reduction of the PS and ΔH but also a significant increase of denaturation temperature of KTI (T_p I) and a concomitant decrease of the I/(I + II) area ratio ($p < 0.05$). On the basis of these results, for WSP-I samples, KTI was largely affected by protein aggregation and denaturation processes as a consequence of prolonged storage at 4 °C.

WSP-5 and WSP-6 samples were prepared from the same defatted soy flour, but with a different pH value of SW during the salting-out. For WSP-6, precipitated at pH 4.5, there was an

increase of T_p I and a noticeable decrease in T_p II, as well as a reduction of ΔH ($p < 0.05$). The relatively high value of the I/(I + II) area ratio (~60%, **Table 3**) suggests that the lectin is the more denatured protein. The effect of the pH of SW was also reflected in the protein solubility: for WSP-6, PS was significantly less than the value corresponding to sample WSP-5 ($p < 0.05$, **Table 2**). On the basis of the overall results, the type of proteins and the different factors inducing the aggregation have a noticeable influence on denaturation temperature.

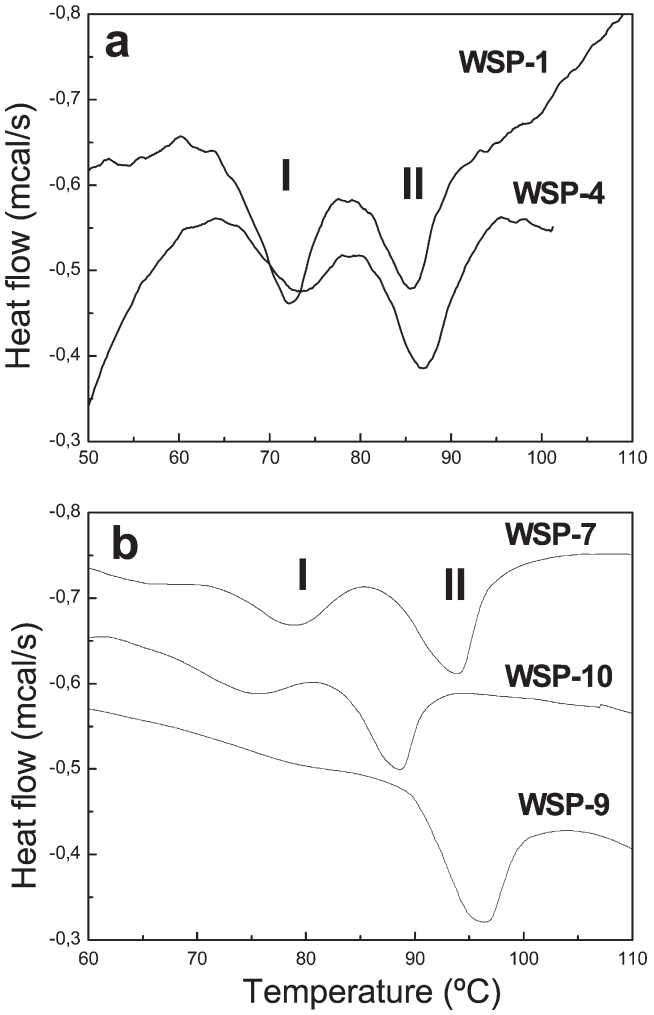

Figure 2. DSC thermograms of aqueous dispersions (30% w/w) of (**a**) WSP-1 and (**b**) WSP-2 samples.

On the other hand, for the group of samples obtained by precipitation with acetone (WSP-II), WSP-7 was taken as control because this sample has been obtained from a fresh flour and precipitated at pH 8.0. **Table 3** shows that the ΔH value of WSP-7 is lower than that of WSP-5 (WSP control sample with the highest ΔH value) and has higher values of T_p (**Table 3**). Taking into account that the protein content in this sample was 60%, the ΔH value was 12.7 J/g of protein, which is higher than that of WSP-5 (9.9 J/g of protein). However, PS of WSP-7 was lower than that of WSP-I control sample (**Table 3**). These results show that precipitation with cold acetone led to preparation of WSP samples with a lower degree of denaturation, but at the same time, with a higher degree of aggregation. For the preparation of these protein samples, it is most important that temperature is kept low; otherwise, denaturation effects become substantial.

On the other hand, WSP-8 and WSP-9, which were prepared from the same flour but with different periods of chilling (2 and 8 years, respectively), exhibited a correlative reduction in the ΔH value (**Table 3**). The lower area of the peak I indicates the denaturation of KTI, whereas the increase of T_p II could be interpreted as a increase of thermal stability of L. The higher is the storage time, the greater is the sample denaturation degree. As was observed in the WSP-I group, in the samples WSP-II also was observed an effect of pH of SW (comparison between WSP-7 and WSP-10 and between WSP-11 and WSP-12): T_p II values were lower when the pH of precipitation was 4.5. For WSP-II obtained at pH 4.5, the effects of soy flour (comparison between WSP-10 and WSP-11) and sample aging (comparison between fresh WSP-10 and WSP-10 stored for 2 years) were also observed. It is remarkable that WSP-7−WSP-12 samples showed very similar PS (84−87%), indicating that the effect of the solvent is more important than the pH of the SW prior to precipitation.

Tofu whey (TW) is a waste residual liquid obtained from thermally treated soy milk. WSP-13 was obtained from TW by precipitation with cold acetone (**Table 1B**). This sample contains almost totally denatured KTI and L, which is in accord with a

Table 3. Protein Solubility and Thermal Behavior of WSP Samples

WSP sample		thermal behavior				protein solubility (%, w/w)
		T_p I (°C)	T_p II (°C)	ΔH (J/g)	(I/I + II) area ratio (%)	
WSP-I	WSP-1	76.0 ± 0.2 a[a]	91.4 ± 0.2 a	8.5 ± 1.0 a	~50	98.0 ± 1.9 a
	WSP-2	75.4 ± 0.6 a	91.8 ± 0.9 a	9.1 ± 0.8 a		90.4 ± 1.4 b
	stored WSP-2[b]	78.0 ± 1.2 b	91.5 ± 0.5 a	7.0 ± 0.1 b		~82
	WSP-3	76.4 ± 0.6 a	92.3 ± 0.6 a	7.9 ± 0.3 b	35−40	89.1 ± 1.8 b
	stored WSP-3[b]	77.7 ± 0.1 b	91.6 ± 0.2 a	6.6 ± 0.8 b		~76
	WSP-4	76.0 ± 0.2 a	92.2 ± 0.4 a	3.6 ± 0.2 c		88.8 ± 1.6 b
	WSP-5	76.5 ± 0.2 a	92.3 ± 0.5 a	9.5 ± 0.7 a	~50	98.5 ± 0.9 a
	WSP-6	77.7 ± 1.2 b	85.1 ± 0.4 b	5.7 ± 1.1 d	~60	86.4 ± 1.6 c
WSP-II	WSP-7	76.2 ± 0.6 a	93.0 ± 0.3 a	7.6 ± 0.2 b	40−50	87.4 ± 2.0 c
	WSP-8	77.8 ± 0.1 b	95.7 ± 0.4 c	5.8 ± 0.3 d	20−30	85.5 ± 1.7 c
	WSP-9	78.4 ± 0.1 b	95.6 ± 0.3 c	3.7 ± 0.2 c	~10	83.7 ± 2.5 d
	WSP-10	74.4 ± 0.3 c	88.5 ± 0.2 d	7.0 ± 0.2 b	~50	86.5 ± 1.6 c
	stored WSP-10[c]	74.3 ± 0.5 c	88.8 ± 0.8 d	3.3 ± 0.3 c	~40	nd[d]
	WSP-11	80.3 ± 1.0 b	90.1 ± 0.5 e	4.3 ± 0.3 c	~40	nd
	WSP-12	78.8 ± 0.4 b	93.5 ± 0.5 a	6.0 ± 0.2 d	~35	nd
	WSP-13	76.9 ± 0.2 a	94.5 ± 0.3 c	<0.1	nd	79.9 ± 1.3 e

[a] Means within the same column followed by different letters are significantly different ($p < 0.05$). [b] Samples stored within closed bottles in a chamber at 4 °C for 9 years. [c] Samples stored within closed bottles in a chamber at 4 °C for 1 year. [d] nd, not determined.

Table 4. Data of Mineral Content of Different SPI and WSP and Electrical Conductivity (EC) of Aqueous Dispersions (0.05% w/v) of These Samples in Double-Distilled Water

sample	EC (μS)	ash (mg/g)	Ca (mg/g)	Mg (mg/g)
SPI-1	38.5 ± 1.5 a[a]	31 ± 1 a	0.63 ± 0.01 a	0.18 ± 0.02 a
SPI-3	41.9 ± 1.1 a	44 ± 1 b	1.90 ± 0.05 b	0.9 ± 0.03 b
SPI-4	43.6 ± 1.4 b	35.0 ± 1.1 b	2.80 ± 0.05 c	0.60 ± 0.04 c
SPI-5	43.5 ± 1.8 b	46 ± 9 b	nd[b]	nd
SPI-7	46.0 ± 1.3 c	51 ± 8 b	1.4 ± 0.05 d	0.18 ± 0.05 a
WSP-1 and -5	21.9 ± 1.6 d	19 ± 2 c	2.20 ± 0.10 b	0.20 ± 0.02 a
WSP-2	8.1 ± 0.5 e	4.0 ± 0.5 d	0.64 ± 0.03 e	0.20 ± 0.01 a
WSP-3 and -4	35.8 ± 5.4 f	30 ± 2 a	1.40 ± 0.05 d	0.18 ± 0.02 a
WSP-8	129.5 ± 5.9 g	174 ± 9 f	27.10 ± 0.10 g	9.90 ± 0.04 d
WSP-12	96.2 ± 3.5 g	112 ± 8 e	10.90 ± 0.12 f	7.30 ± 0.03 e
WSP-13	95.6 ± 3.7 h	163 ± 10 f	69.60 ± 0.15 h	3.60 ± 0.02 e

[a] Means within the same column followed by different letters are significantly different ($p < 0.05$). [b] nd, not determined.

residual antitryptic activity of <20% due to presence of active BBTI (*27*). Moreover, WSP-13 showed the lowest PS value (**Table 3**).

Some WSP (WSP-3, WSP-4, WSP-8, and WSP-9) showed a brownish appearance. UV−visible spectra of aqueous dispersions of these samples revealed high values of absorbance at 400−450 nm. This result could be related to the presence of phenolic substances, although Maillard glycosylation cannot be ruled out (*24*, *26*). These reactions are possible if one takes into account that SW, which is the raw material for the preparation of WSP samples, contains various amounts of oligosaccharides and polyphenols (*12*, *14*). During the preparation of WSP samples, some interactions between oligosaccharides and phenolic compounds with KTI and L could occur. Maillard reactions would be particularly possible in WSP-II samples, which contain high levels of carbohydrates (>20%). According to Rawel et al. (*11*), KTI complexes with polyphenols at relatively low temperatures (4 °C). On the basis of this observation this kind of reaction would be possible in samples WSP-3 and WSP-4, as a consequence of cold storage of SW during the process of preparation. In addition, for WSP-8 and WSP-9, these interactions could be the result of cold storage of the soy flour used in its preparation.

Relationship between Thermal Behavior and Salt Content. The differences in thermal behavior of SPI and WSP samples, mainly in peak II of SPI (11S globulin), could also be attributed to the presence of salts. **Table 4** shows the values of conductivity and ash, calcium, and magnesium contents for some representative samples of SPI and WSP. A linear relationship between the mineral content, determined as ash, and electrical conductivity (EC) was effectively observed (ash $= -2.95 + 0.20 \times$ EC, $r^2 = 0.87$). This linear relationship leads implies that a high proportion of salts present in the samples is dissociable. Moreover, these salts are not strongly linked to proteins because they significantly contribute to the conductivity. In contrast, the correlation between EC and calcium content is not linear, which reflects the low contribution of total calcium minerals (except in sample WSP-13 prepared from TW), presumably due to the linkage of calcium with proteins. Samples SPI-3, SPI-4, SPI-5, and SPI-7 exhibited a salt content significantly higher than in SPI-1 and SPI-2. In the first three samples, the increase in salinity is attributed to the storage of flour or isoelectric precipitate, inducing a greater interaction of proteins with salts of the flour. For SPI-7, the highest salt content can be attributed to the additional amount of NaOH necessary to reach a pH 10.0 during alkaline neutralization. A previous assay carried out in SPI by Sorgentini and Wagner (*2*) revealed a noticeable shift of T_p for 11S globulin toward high temperatures (from 3.2 to 20.3 °C) when the NaCl content was increased from 0.02 to 0.2 g/g of isolate. As was mentioned above (see Materials and Methods), a correlation between EC and NaCl content was effectively obtained. According to this relationship, the salt concentration for SPI-5 and SPI-7 samples was <0.011 g/g, which would be insufficient to explain the significant increase (>3 °C) in T_p II values. This lack of correlation between salt content and T_p for 11S globulin is reinforced by evaluating the thermal behavior of long-stored SPI-3 sample: a noticeable increase of T_p (>7 °C) with respect to fresh SPI-3 sample was obtained.

In other work, the differences in thermal behavior of soy protein isolates with various concentrations of calcium were analyzed (*8*). These authors have found that the influence of calcium also has an impact mainly on the thermal stability of 11S. The values of T_p for 11S globulin and calcium content respond to a linear correlation ($\Delta T_p = -0.32 + 0.89 \times$ [Ca], $r^2 = 0.97$), where ΔT_p is the increase of T_p with respect to a control SPI, without addition of calcium, and [Ca] is the calcium content, expressed as milligrams of calcium per gram of sample. According to this correlation, an increase of 3 °C in the T_p for 11S globulins requires an increase in calcium content of at least 3 mg/g of sample. On the basis of this result, the increase in the T_p II in thermograms of SPI cannot be explained solely on the basis of high calcium contents.

On the other hand, the WSP-I group (WSP-1−WSP-5, obtained by salting-out) exhibited values of EC and salt content in the ranges of 8−36 μS and 4−30 mg/g, respectively. However, these values were lower than those of WSP-II samples (WSP-8, WSP-12, and WSP-13, obtained by precipitation with organic solvent) (**Table 4**). Particularly, WSP-8 showed the highest salt content and WSP-13, the highest calcium content because it comes from TW, in which $CaCl_2$ is commonly used for coagulation of soy milk. These results suggest that the precipitation of KTI and L with cold acetone induces a strong interaction of these proteins with salts, which could contribute to enhance protein aggregation. This may explain, in part, the high values of T_p for KTI and L in the WSP obtained by cold acetone precipitation.

Thermal Behavior of SPI/WSP Mixtures. In this section, the thermal behavior of mixtures in equal parts of 11S or SPI with WSP is examined. The aim of this study was to evaluate by DSC the existence of interactions between these samples through changes in thermal behavior of their constitutive proteins. In this study, G-1, SPI-1, and SPI-2, which exhibit low values of T_p (**Table 2**), and various WSP samples were employed. **Figure 3a** shows that the thermogram of the G-1/WSP-3 mixture exhibited a value of T_p II higher than those observed for the G-1/WSP-2 mixture. This effect of WSP on T_p of 11S was not observed when the enriched 7S fraction/WSP mixture was analyzed by DSC in the same condition (data not shown). Similarly, a shift of peak II to a higher temperature in the mixture of SPI-1 with WSP-3 can be seen (**Figure 3b**). In contrast, G-1/WSP-2 and SPI-1/WSP-2 mixtures gave T_p II values practically similar to those corresponding to thermal denaturation of separate samples. A more pronounced shift of T_p II was observed when SPI-2 was mixed with WSP-2 or WSP-3 previously stored for a long time (**Figure 3c**). It is remarkable to note that these three samples showed thermal transitions (I and II) with T_p values practically coincident (**Tables 2** and **3**). However, when they are mixed, a more complex thermogram was effectively obtained with at least three transitions, named peaks I, I′, and II, respectively (**Figure 3**). We hypothesized that peak I is superimposed on both samples, peak II corresponds to the denaturation of the 11S affected by the presence of WSP, and peak I′ would be attributed to L, where its

thermal behavior seems to be less affected by external conditions with respect to that observed for 11S globulin.

Table 5 summarizes the results from these experiments with SPI−WSP mixtures. It can be seen that the increase in T_p II (ΔTp II) for SPI samples was >3 °C when the other component of the mixture was a WSP sample that exhibited some denaturation degree of KTI or L (**Table 3**). When long-stored WSP-2 and WSP-3 samples were used, the shift of T_p II was significantly higher (2- or 3-fold) than those obtained with fresh samples.

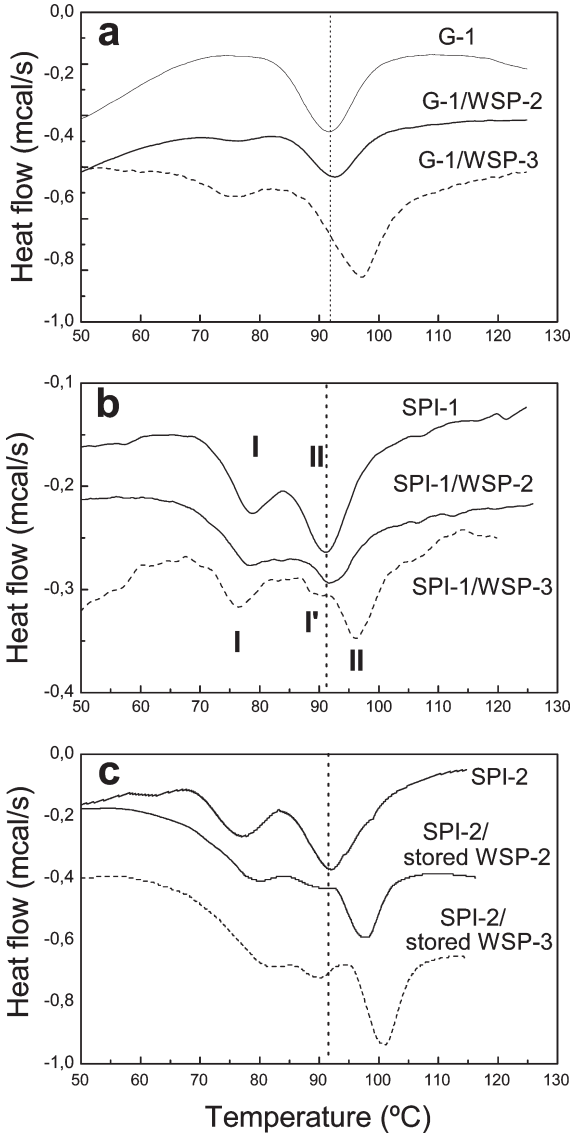

Figure 3. DSC thermograms of aqueous dispersions (30% w/w) of (**a**) G and G−WSP mixture, (**b**) NSI and NSI−WSP mixture, and (**c**) NSI and NSI-stored−WSP mixture.

As was mentioned above, sample WSP-10 exhibited relatively low values of T_p I and T_p II (**Table 3**). When this sample was mixed with SPI-2, an increase of the T_p II value was observed (**Table 5**). Remarkably, WSP-9 and WSP-13 samples, with low values of ΔH, induced not only a noticeable shift of T_p II toward higher temperatures (ΔT_p II = 9−10 °C) but also a significant increase of T_p I. In all mixtures ΔH values showed no trend with respect to sample characteristics, and these values are comparable to those derived from the theoretical calculation of the denaturation enthalpies for the samples included in the mixture.

Evidently, the DSC assays on SPI−WSP mixtures showed an increase of T_p II. These results would be consistent with the presence of interactions between some components present in G-1 or SPI and WSP samples, which generate an increase of thermal stability of constitutive proteins. This behavior could be attributed to different causes: First, the ionic strength and/or calcium content would have an influence on the thermal behavior of proteins. Second, some interactions are established between SPI and WSP proteins when the samples are mixed. The WSP proteins can be either in native state or denatured and aggregated.

To evaluate if salt content could explain the modification of thermal behavior in the SPI−WSP mixtures, we examined first previous results that evaluate the influence of NaCl and calcium content on the denaturation temperature of glycinin (*1*, *7*). From the EC measurements and calcium content of separate samples, the total salt and calcium contents in SPI−WSP mixtures (expressed as g of NaCl/g of sample and mg of Ca/g of sample, respectively) were estimated. According to this, only the mixtures of SPI-2 with WSP-9, WSP-10, and WSP-13 (WSP-II group) exhibited relatively high NaCl and Ca levels (at least 0.05 g/g and 2 mg/g, respectively). These values would be sufficient to produce a noticeable increase (> 5 °C) of T_p II (**Table 5**). However, when SPI-1 and SPI-2 were mixed with WSP-I samples, the NaCl and Ca levels were very low (< 0.01 g of NaCl/g and < 2 mg of Ca/g). This result suggests clearly that the shift of T_p II toward higher temperatures observed when SPIs are mixed with WSP-I samples cannot be explained by an increase in ionic strength or in the calcium content. Moreover, a partial denaturation of proteins, although without an appreciable shift of T_p values for KTI and L, was effectively observed in long-stored WSP samples, regardless of the raw material and preparation method (**Table 3**). However, when they were mixed with SPI-2, a strong influence on the thermal stability of proteins was observed (**Table 5**).

So far, the results of the present study correspond to assays carried out with mixtures containing SPI and WSP proteins, native or partially denatured. These mixtures exhibited a more complex DSC thermogram with respect to separate samples

Table 5. Denaturation Enthalpy (ΔH) and Peak Temperature (T_p) Values of Endothermic Transitions Corresponding to DSC Thermograms of SPI−WSP Mixtures (1:1 w/w)[a]

mixture		ΔH (J/g)	T_p I (°C)	T_p I′ (°C)	T_p II (°C)	ΔT_p I	ΔT_p II
SPI-1 +	WSP-1	12.2 ± 0.5 a[b]	78.4 ± 0.3 a		91.6 ± 0.2 a	0.2	0.1
	WSP-2	12.3 ± 1.0 a	77.0 ± 0.4 a		92.1 ± 0.5 a	−1.2	0.6
	WSP-3	11.5 ± 0.9 b	77.4 ± 0.6 a	90−91	95.7 ± 0.4 b	−0.8	4.7
	WSP-4	nd[c]	77.2 ± 0.2 a	91−92	95.0 ± 0.8 b	−1.0	4.0
SPI-2 +	stored WSP-2	11.4 ± 0.5 b	78.8 ± 0.6 a	89.3 ± 0.6 a	97.6 ± 0.7 b	1.2	5.2
	stored WSP-3	11.7 ± 0.7 b	80.4 ± 1.4 a	90.3 ± 0.8 a	100.6 ± 0.4 c	2.8	8.2
	WSP-9	9.3 ± 0.3 c	88.9 ± 0.6 c	93.4 ± 0.8 b	101.6 ± 0.6 c	11.3	9.2
	WSP-10	12.3 ± 0.2 a	78.6 ± 0.1 a	92.6 ± 0.3 b	97.5 ± 0.3 b	0.8	5.1
	WSP-13	13.8 ± 0.1 d	83.3 ± 1.0 b	94.8 ± 0.7 b	102.4 ± 1.1 c	5.7	10.0

[a] Values of T_p and ΔH of SPI and WSP are shown in **Tables 2** and **3**. ΔT_p was calculated as $T_{p,mixture} - T_{p,SPI}$. [b] Means within the same column followed by different letters are significantly different ($p < 0.05$). [c] nd, not determined.

Table 6. Peak Temperature (T_p) Values of Endothermic Transitions Corresponding to DSC Thermograms of Mixtures of SPI-Teated WSP (1:1 w/w) and Mixtures of SPI with Ash from WSP[a]

mixture		T_p I (°C)	ΔT_p I (°C) with respect to SPI	ΔT_p I (°C) with respect to mixture SPI−WSP	T_p II (°C)	ΔT_p II (°C) with respect to SPI	ΔT_p II (°C) with respect to mixture SPI−WSP
SPI-2 +	WSP-10	78.6 ± 0.1 a[b]	0.8		97.5 ± 0.3 a	5.1	
	heated WSP-10	83.5 ± 0.2 b	5.9	4.9	102.3 ± 0.1 b	9.9	4.8
	ash from WSP-10	77.2 ± 0.8 a	−0.4	−1.4	92.9 ± 0.3 c	0.5	−4.6
SPI-2 +	WSP-9	88.9 ± 0.6 c	11.3		101.6 ± 0.6 b	9.2	
	heated WSP-9	86.7 ± 1.2 c	9.1	−2.2	104.7 ± 0.5 d	12.3	3.1
	ash from WSP-9	78.1 ± 0.7 a	0.5	−10.8	92.5 ± 0.3 c	0.1	−9.1
SPI-2 +	WSP-13	83.3 ± 1.0 b	5.7		102.4 ± 1.1 b	10.0	
	heated WSP-13	81.0 ± 0.2 b	3.4	−2.3	100.8 ± 1.1 b	8.4	−1.6
	ash from WSP-13	99.7 ± 2.4 d	22.1	16.4	124.5 ± 1.2 e	32.1	22.1

[a] Mixture control: SPI with WSP not previously heated. Values of T_p of SPI and WSP are shown in **Tables 2** and **3**, respectively. ΔT_p (with respect to SPI) was calculated as $T_{p,\text{mixture}} - T_{p,\text{SPI}}$; ΔT_p (with respect to mixture SPI−WSP) was calculated as $T_{p,\text{mixture}} - T_{p,\text{mixture control}}$. [b] Means within the same column followed by different letters are significantly different ($p < 0.05$).

(**Figures 1**−**3**). As was previously stated, we hypothesized that I, I′, and II endothermic transitions would be attributed to 7S-KTI, L, and 11S, respectively. Indeed, the results shown above cannot certainly determine whether these transitions correspond to mentioned SPI and WSP proteins. To answer these questions and to better understand the thermal behavior of SPI−WSP mixtures, two additional series of DSC experiments were carried out. First, SPI-2 was mixed with thermally denatured WSP samples. Then, SPI was mixed with minerals (ash) from calcination of WSP samples. To perform both experiments, three WSP-II samples were used: WSP-9, WSP-10, and WSP-13. Results of these assays are shown in **Table 6**. It should be remembered that all mixtures in which WSP thermally denatured or WSP ashes were used, the thermal transitions in DSC thermograms were exclusively attributed to 7S and 11S globulins.

Table 6 shows that thermally denatured WSP-10 induced a higher shift of T_p I and II with respect of those corresponding to unheated sample. This result is in accordance with a high tendency of denatured KTI and L to interact and stabilize 7S and 11S globulins and would be related with a noticeable increase of surface hydrophobicity, as was stated in a previous work (*28*). As was mentioned in **Table 1**, WSP-9 was obtained from a long-stored soy flour, which favors protein aggregation. When WSP-9 is heated, more reactive denatured aggregates are formed, which enhance the shift of T_p I and T_p II values toward high temperatures. SPI-2−WSP-13 mixtures showed similar values of T_p I and II, regardless of the thermal treatment ($p < 0.05$). This result was attributed to the particular characteristics of WSP-13, which was prepared from TW and contains almost totally denatured KTI and L.

In addition, an interesting result was observed when sample SPI-2 was mixed with ash obtained from calcination of WSP samples. With regard to the effect of ash from WSP-9 and WSP-10, it can be seen that they are unable to cause the shift of endothermic transitions, which was effectively observed in SPI−WSP mixtures (**Table 6**). These results would indicate that the minerals present in WSP-9 and WSP-10 have no significant effect on the thermal stability of 11S. A different result was observed with the ash from calcination of WSP-13. According to **Table 4**, the calcium content of this sample was significantly higher with respect to those reported for other WSP samples. After the calcination process, the calcium is in a free state and, hence, is highly reactive with 7S and 11S globulins. This fact suggests that calcium is strongly bound to proteins in WSP-13; otherwise, it should have a greater effect on T_p I and T_p II in SPI-2−WSP-13 mixtures.

Finally, we can remark that thermally denatured KTI and L have a high tendency to interact and increase the thermal stability of storage globulins, especially 11S globulin. In a recent study, Ren et al. (*29*) have reported a coprecipitation of whey proteins and storage globulins at pH 4.5 after thermal treatment of soy milk. According to the overall results, the influence of other minor components on the thermal stability of proteins in SPI−WSP mixtures cannot be discarded and, hence, will require further investigation.

Conclusions. This study showed that the thermal behavior of soy protein isolates and whey soy proteins can be modified by many factors, among those being the storage processes of flour, intermediate materials, and final product. The denaturation and protein aggregation cause an increase of thermal stability. This study also confirms that the thermal behavior of the soybean protein isolates is influenced by the presence of whey soy proteins. The thermal stability of soy storage proteins, mainly glycinin, can be increased by their interaction with whey soy proteins, even more if they are previously denatured and aggregated. The results suggest that storage or thermal denaturation of the soy whey protein leads to more reactive species with 11S globulin. Perhaps partially denatured KTI and/or L (regardless of the protein aggregation) have a high thermal reactivity and specificity for glycinin. It is not excluded that this reactivity is enhanced by the presence of calcium. Although both soy protein isolates and whey soy proteins are highly enriched products, they cannot be regarded as pure systems. Then, in these products, the interaction between soy storage proteins and whey soy proteins can not only affect their thermal behavior but also may produce changes desirable or not in their functional properties, which requires further studies.

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