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Effect of Water Content on Thermal Behavior of Freeze-Dried Soy Whey and Their Isolated Proteins

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ABSTRACT: Thermal behavior of lyophilized soy whey (LSW) and whey soy proteins (WSP) at different water contents (WC) was studied by DSC. In anhydrous condition, Kunitz trypsin inhibitor (KTI) and lectin (L) were more heat stable for WSP with respect to LSW sample. The increase of WC destabilized both proteins but differently depending on the sample analyzed. Thermal stability inversion of KTI and L was observed for WSP and LSW at 50.0% and 17.0% WC, respectively, which correspond to the same water−protein content mass ratio (W/P ≈ 1.9). At W/P < 1.9, KTI was more heat stable than L. Before the inversion point, WC strongly modified the peak temperatures (T_p) of KTI and L for WSP, whereas this behavior was not observed for LSW. The high sugar content was responsible for the thermal behavior of KTI and L in LSW under anhydrous condition and low WC. These results have important implications for the soy whey processing and inactivation of antinutritional factors.

KEYWORDS: Soy whey, thermal behavior, DSC, water content, whey soy proteins

INTRODUCTION

During the preparation of soy protein isolates, a residual liquid called "soy whey" is generated during isoelectric precipitation of storage globulins.[1,2] The main components of soy whey (SW), all soluble in a wide pH range, are the Kunitz and Bowman−Birk trypsin inhibitors (KTI and BBTI, respectively), the lectin (L), various carbohydrates (sucrose, stachyose, raffinose, traces of glucose, other oligosaccharides, and soluble fiber), and other minor components as enzymes, minerals, phytates, and phytoestrogens.[3,4] KTI is the main antinutritional factor of soybean with a molecular weight of 20.1 kDa and the presence of two disulfide bridges. This protein has a relatively high thermal stability in low moisture conditions and is totally stable over a wide pH range from 3.0 to 10.0.[5] BBTI, with a low molecular weight of about 8.0 kDa has seven disulfide bridges, so that it possesses a high thermal stability.[6−8] Lectin or soybean hemagglutinin is a tetramer composed of identical subunits (30 kDa) lacking disulfide bridges and having two carbohydrate binding sites; this allows the complex formation with sugars.[9] Although the activity of the lectin has little incidence in the nutritional value of soybean foods, the heat treatments carried out to inactivate the antitryptic factors also inactivate the lectin.

The use of SW as a byproduct of soybean processing is economically feasible if it is subjected to concentration and drying process, which reduces transportation costs. Moreover, for its use in human food or animal requires a heat treatment until there are adequate levels of antritryptic factors inactivation. Heating of SW can be directly performed on the liquid state or on previously concentrated or dried whey, so as to ensure the lowest cost and maximum inactivation. Therefore, it is necessary to know the thermal behavior of the whey soy proteins under different conditions, as well as the moisture content variation and the presence of sugars and salts.

The effects of hydration on the thermal stability of ovalbumin, lysozyme, and sunflower globulins were measured by differential scanning calorimetry (DSC).[10−13] The hydration effect on the thermal stability of soy proteins has been also studied by DSC in cracked soybeans and defatted soy flour, soy isolates, and storage globulins (7S and 11S fractions).[14−17] The authors agree that the smaller is the amount of water, the greater is the thermal stability of these soy proteins. Sessa[14] has included the study of thermal stability of KTI, showing that in the anhydrous state, KTI exhibited a thermal transition near 200 °C similarly to soybean storage globulins. The thermal behavior of whey soy proteins (WSP, mainly composed by KTI and L) in dilute aqueous dispersions was studied by DSC in comparison with native soy isolates, NSI, composed of 7S and 11S fractions.[2] The thermogram of aqueous dispersions of WSP showed two endothermic peaks (T_p I, 74.0 ± 0.5 °C; T_p II, 90.4 ± 0.8 °C) attributed to thermal denaturation of KTI and L, respectively. In the same condition, a similar thermogram was obtained for NSI, as a consequence of the denaturation of 7S and 11S globulins. Recently, a DSC study on lyophilized soy whey was carried out by Sobral and Wagner.[4] In that work, it was shown that the denaturation of the antitryptic factors and lectin occurs at temperatures above 120 °C, simultaneously with aggregation−glycosylation reactions. Unlike what happens in the presence of water, KTI seems to be more stable than L in the dehydrated soy whey. A total denaturation of KTI was reached when the heat

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treatment of the lyophilized soy whey was carried out at 150 °C. These results suggest that the thermal behavior of KTI in anhydrous conditions is not the same in soy whey (the presence of sugars and other minor components) as in the isolate state.

The aim of this work was to study by DSC the thermal transitions of whey soy proteins (mainly KTI and L) in the lyophilized soy whey without and with addition of variable contents of water, and to compare them to the behavior of the same isolated proteins. This Article seeks also to analyze the possible inversion of the thermal susceptibility of KTI and L by the effect of water content and the effect of sugars on the thermal behavior of both proteins in the soy whey.

MATERIALS AND METHODS

Materials. Defatted soy flour (Prosam R), not thermally inactived to avoid protein denaturation, was provided by Solae Latin America (SP, Brazil). The soy flour contains 95 g of dry solids per 100 g of powder, and the chemical composition (in dry basis, w/w, as was given by the producer) was 56% crude protein (N × 6.25), 7.0% ash, 3.5% total lipids (0.8% triglycerides), and 14.0% dietary fiber. The Kunitz trypsin inhibitor (KTI, Type I-S, freeze-dried powder) and lectin (L) were provided by Sigma-Aldrich (MO). Distilled water was always used in all assays, and all other chemicals were analytical grade reagents.

Preparation of Lyophilized Soy Whey (LSW). The soy whey was obtained as the supernatant of the isoelectric precipitation at pH 4.5 of storage globulins from a fiber free-aqueous extract of defatted soybean flour. The experimental procedure reported by Sorgentini and Wagner[2] was followed. The soy whey was adjusted to pH 8.0 (0.5 M NaOH) and then freeze-dried, resulting in the sample LSW. LSW was ground and stored at −80 °C. Because of its high tendency to hydration, LSW was always maintained and manipulated at room temperature at zero relative humidity (P_2O_5) during all assays. The chemical composition of LSW (w/w) was 17.60 ± 0.03% w/w crude protein (N × 6.25), 54.57 ± 2.86% w/w total carbohydrate by phenol-sulfuric method,[18] and 13.4 ± 0.30% ash (calcination at 550 °C for 5 h). Besides, the Lowry method[19] was used to determine the real protein content of the sample, resulting in a value of 10.73 ± 0.09% w/w. This assay was performed on sugar-free precipitates obtained with 12.0% w/v trichloroacetic acid and washed at least three times with the same acid solution. The ratio between the amount of lectin (L) and Kunitz trypsin inhibitor (KTI), estimated by SDS-PAGE in reducing conditions, was 3:2.

Preparation of Whey Soy Proteins (WSP). Whey soy proteins (WSP) were precipitated from an aqueous solution of LSW (10% w/v) at 0 °C by addition of acetone (serum:solvent ratio 1:1 v/v) under gentle stirring for 10−15 min. The proteins were then separated by centrifugation (10 400*g*, 20 min, 4 °C), frozen, and subsequently freeze-dried. The chemical composition of WSP sample (w/w) was 54.25 ± 2.13% crude protein (N × 6.25), 49.63 ± 1.80% protein,[19] 11.1 ± 0.6% total carbohydrates,[18] and 11.2 ± 0.4% ash (calcination at 550 °C for 5 h). The mineral fraction is composed by 9.7% w/w calcium y 6.5% w/w magnesium. The ratio between the amount of lectin (L) and Kunitz trypsin inhibitor (KTI), estimated by SDS-PAGE in reducing conditions, was 3:2. In addition, a WSP−sucrose mixture was also prepared. Briefly, sucrose was added to an aqueous dispersion of WSP, and the liquid mixture was freeze-dried and then used in further assays. The sucrose content of this mixture was similar to the total carbohydrate content in the LSW sample.

Thermal Behavior of LSW and WSP. The thermal behavior of the samples (10−20 mg) without or with variable amount of water was determined in hermetically sealed aluminum pans, using a differential scanning calorimeter Q-200 (TA Instruments, U.S.). Heating rates of 5 or 10 °C/min were always selected. To ensure the complete dehydration (0% water) in assays carried out in the absence of water, an open pan with the sample (LSW or WSP) was placed in an atmosphere of zero relative humidity (P_2O_5) before sealing. For studies with LSW in the presence of water, samples were kept in open pans to ensure the hydration at room temperature and relative humidity 60−70%. The water content in the sample was calculated by weight difference before and after hydration. The effect of water content (w/w %) on the thermal behavior of WSP was studied by adding different amounts of distilled water to the sample inside the pan. From DSC thermograms, the peak temperature (T_p) and the enthalpy (ΔH) of the transitions were determined. ΔH values were always expressed in J/g protein, using the data of total sample into the pan and crude protein percentage in the sample.

In other series of experiments, modulated DSC technology (MDSC, TA Instruments, U.S.) was employed to determine the glass transition temperature (T_g) for WSP and sucrose−WSP mixtures in anhydrous condition, and to measure the freezable water (FW) for LSW at different water contents. In both types of assays, the temperature modulation amplitude and period were set at ±0.5 °C and 50 s, respectively. FW was measured as follows: The samples were placed in aluminum pans and rapidly cooled to −50 °C. Next, they were isothermically kept at −50 °C for 20 min and subsequently heated to −20 at 10 °C/min. This cycle was repeated at least once to ensure water crystallization. Heating to 30 °C was then performed at 10 °C/min. The same calorimetric measurements were carried out for pure water. The melting enthalpy of pure water was obtained and used as the reference enthalpy.[15] FW values were expressed as (g freezable water/total water in the sample) × 100. To analyze the glass transition, the anhydrous samples were heated from 20 to 200 °C at 5 °C/min. The T_g values were obtained from reversing heat flow thermograms or the heat capacity component of total heat flow. All assays were conducted at least in triplicate.

Statistical Analysis. Data were analyzed by analysis of variance (ANOVA) and differences between mean values by Fisher's least significant differences (LSD). Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint Inc. 2005, U.S.). Significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Thermal Behavior of Lyophilized Soybean Whey (LSW) at Different Water Contents. For LSW, the DSC thermogram, from subzero temperatures up to 200 °C, was analyzed in a previous work.[4] At temperatures below 100 °C, a second-order transition corresponding to the glass transition of the system (T_g = −2 to −3.3 °C) and an endotherm attributed to melting of sugar crystals were observable, the latter only in the stored sample. Figure 1a shows the thermograms of LSW at different water contents. As previously mentioned, the endotherms of whey protein denaturation in anhydrous LSW appear at temperatures above 100 °C.[4] The endothermic peaks in the range 111−138 and 144−155 °C, with peak temperatures (T_p) of 120.5 ± 3.0 and 142.3 °C ± 2.7 °C, were attributed to the thermal denaturation of major whey protein fractions of soybean, lectin (L), and Kunitz trypsin inhibitor (KTI), respectively.[4] The location of the first peak (corresponding to L) and the second peak (attributed to KTI) was mainly based on the following: heating to 150 °C was required to achieve the inactivation of antitriptic activity. The antitrypsin activity in the heated LSW was reduced only by 15% when it reaches 132 °C. At this temperature, L was totally denatured. Moreover, total denaturation of KTI was just achieved when the whey was heated to 150 °C. Under these conditions, the sample evidenced a residual antitryptic activity (<15%) due to the presence of the Bowman−Birk trypsin inhibitor (BBTI).[4] In the thermogram corresponding to anhydrous

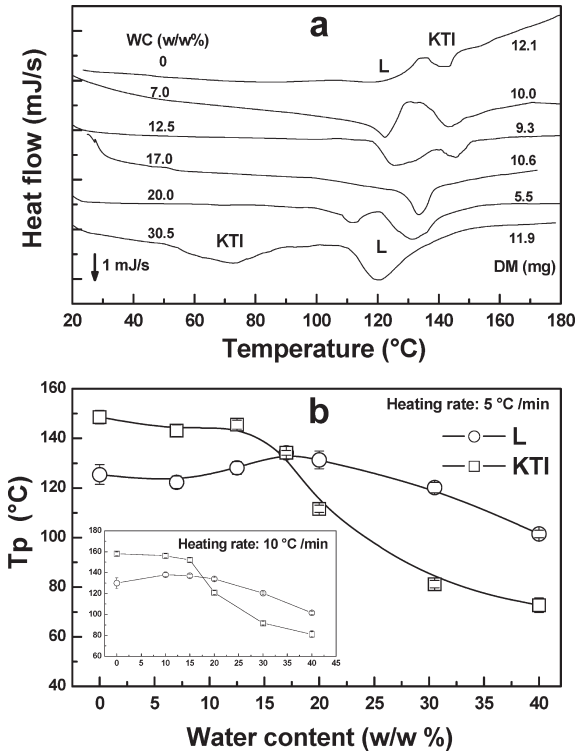

Figure 1. (a) DSC thermograms of lyophilized soy whey (LSW) in anhydrous conditions and at different water contents (WC, 7.0−30.5 w/w %). Heating rate: 5 °C/min. DM: dry mass. (b) Effect of water content (w/w %) on peak temperatures (T_p, °C) of Kunitz trypsin inhibitor (KTI) and lectin (L) for LSW sample. Heating rate was set at 5 (main figure) and 10 °C/min (inseted figure).

LSW, the small peak at 155 °C could be attributed to thermal denaturation of this thermostable trypsin inhibitor.

The thermograms obtained with 7.0% and 12.5% water exhibited more defined peaks than those observed in the absence of water (Figure 1a). At 17.0% water, thermal denaturation of both whey protein fractions generated almost a single endotherm. At higher water contents (≥20% w/w), thermograms showed an inversion in the area ratio between the first and second peak, which suggests that in these conditions KTI is denatured at lower temperatures with respect to L. This suggestion is reinforced by the ratio of areas between the endothermic peaks of LSW, which was similar to the relationship between the denaturation endotherms of KTI and L reported for an aqueous solution of soybean whey proteins at 30% w/w water.[2] Moreover, the T_p value of the first peak of high hydrated LSW is comparable to that reported by the same authors for an aqueous dispersion of 30% w/w KTI (T_p = 75.7 °C). On the basis of this evidence and the thermograms of anhydrous and hydrated LSW samples, the effect of water content on T_p values of KTI and L for LSW is shown in Figure 1b.

T_p values of L were comparable between 0 and 20% (w/w) water content, with a decreasing tendency when the water content exceeds 30%. At 40% water, T_p values of both fractions were similar to those reported by Sorgentini and Wagner[2] for an aqueous dispersion of soybean whey proteins at 70% w/w water content. The analysis of Figure 1a and b clearly evidenced that inversion in the T_p of KTI and L by the effect of water content is attributed to the strong influence of this condition on the thermal behavior of KTI. While this protein is more heat stable than L in anhydrous conditions, a rapid destabilization of KTI occurs near 15.0−16.0% /w/w water, and finally KTI is noticeably less thermostable that L at higher water contents. When the LSW thermograms were performed setting a heating rate of 10 °C/min (Figure 1b), the peak temperatures were at least 5 °C higher than those observed for assays conducted at 5 °C/min when the water content was low (0−10.0% w/w). Nevertheless, the thermal stability of L and KTI reversed at the same water content (15.0−17.0% w/w).

For LSW at 0−20.0% w/w water content, the total denaturation enthalpy of proteins was in the range 90−180 J/g protein, which is 5−10 times higher than those reported for the denaturation of whey soy proteins in diluted dispersions.[2] This could be attributed to the stabilizing effect of sugars and salts of the whey. The presence of divalent cations (such as calcium and magnesium), the high ionic strength, and the low water activity are some possible factors that increase the thermal stability of soy proteins and other food proteins.[2,20,21] The latter author reported an increase of denaturation enthalpy of lysozyme with increasing the polyol concentration, due to a decrease of water activity. Thermodynamic data support the hypothesis that the thermal stabilization of protein by polyols is attributed to a preferential solvent interaction effect, which strengthens the hydrophobic interaction of the protein. The oligosaccharides present in the soy whey could have a similar effect, inducing the formation of aggregates more thermally stable of KTI and L. An increase in the enthalpy value of the trasitions of these proteins could also be due to the formation of aggregates via glycosilation. It should be noted that during the heating of LSW in anhydrous conditions, glycosilation reactions occur, simultaneously or prior to denaturation, leading to the formation of aggregates of KTI and L.[4] In a previous work, Oste et al.[22] have already reported that the conformation of KTI is modified by Maillard-type reactions, detected by heating solid mixtures of KTI and sugars to 120 °C. The salts and nonprotein nitrogen compounds (∼7.0%) in the whey are factors that accelerate the Maillard reaction. The high values of ΔH in the absence of water or at low water content can also be attributed to the rupture of hydrogen bonds and covalent bonds in the protein structure (deamidation, desulfuration, or decarboxylation) when the heating is carried out at temperatures higher than 100 °C.[23] The rupture of a covalent bond always requires approximately 20 times the required energy for the rupture of a hydrogen bond.[24] Besides, in anhydrous conditions, the rupture of a hydrogen bond requires more energy (approximately 5.0−6.0 kcal/mol) than in the presence of water (0.5−1.5 kcal/mol).[25]

In DSC assays with hydrated LSW samples, total denaturation enthalpy (ΔH_{total}) decreased with the water content, giving the linear correlation (ΔH_{total} = 297.18 − 4.59 × WC, R = −0.9947), where WC is the water content for LSW. According to this correlation, when WC increases above 60% w/w the effect of sugars and salts of the whey is negligible, because LSW samples showed ΔH_{total} in the range 12−20 J/g protein, which is in the order of those reported for aqueous dispersions of isolated soybean whey proteins.[2] This decreasing tendency also was observed by Sessa[14] for 7S and 11S fractions in cracked bean and soy flour in the moisture content range between 4.0% and 65.0%.

It is noteworthy that the LSW sample contains near 50% w/w total carbohydrates, and these can crystallize only when the sample is stored for long periods. In the present study, a noncrystallized LSW sample was used. This fact discards the

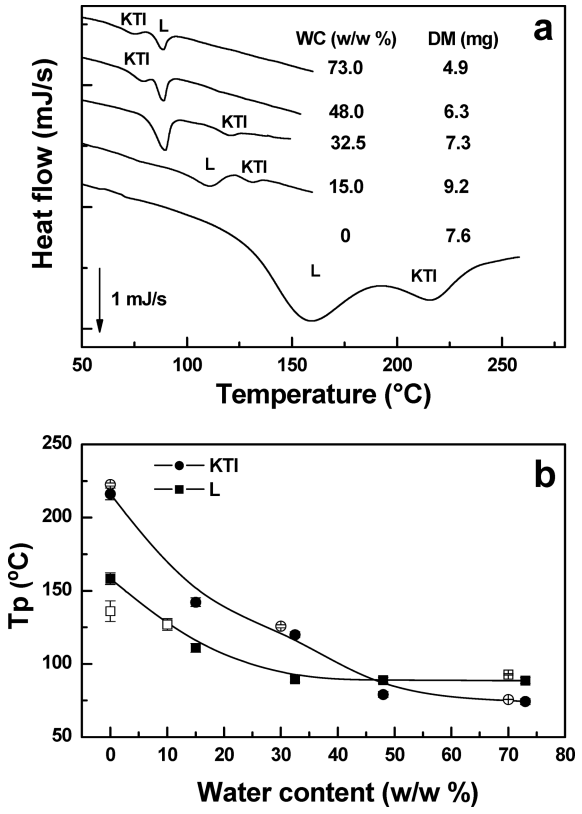

Figure 2. (a) DSC thermograms of whey soy proteins (WSP) in anhydrous conditions and at different water contents (WC, 15.0−73.0 w/w %). Heating rate: 5 °C/min. DM: dry mass. (b) Effect of water content (w/w %) on peak temperatures (T_p, °C) of Kunitz trypsin inhibitor (KTI) and lectin (L) for WSP sample. T_p values for purified KTI (○) and L (□) are also included. At 70.0% w/w water content, T_p values for purified KTI and L were reported by Sorgentini and Wagner.[2] Heating rate: 5 °C/min.

possibility that the high value of enthalpy can be attributed to sugar melting.

Thermal Behavior of Whey Soy Proteins (WSP) at Different Water Contents. The thermal behavior of WSP sample was analyzed by DSC (at 5 °C/min) in aqueous dispersions at several water contents (0−73.0% w/w, Figure 2a). The DSC thermogram of the dispersion of WSP at 73% w/w water exhibited two transitions corresponding to KTI (T_p = 74.37 ± 0.35 °C) and L (T_p = 88.52 ± 0.02 °C). This result is consistent with the values previously reported by Sorgentini and Wagner,[2] although these authors used whey soy proteins prepared by salting out with ammonium sulfate. In anhydrous conditions, the WSP thermogram presented two large endotherms at 159.0 and 216.0 °C corresponding to L and KTI, respectively. In addition, for the same sample, an additional modulated DSC assay revealed a T_g value of 128.0 ± 0.6 °C, prior to denaturation of KTI and L. This behavior is in accordance with previously reported data for other proteins in anhydrous conditions.[26]

It has been generally accepted that the compact protein structure unfolds during denaturation. The unfolding is accompanied by breaking and reforming of the intermolecular and intramolecular interactions.[27] Water molecules play an important role in the breaking and reforming of these interactions. These effects will be reflected in the increase of denaturation temperature when water content decreases. Reducing the water level would destabilize the unfolded state relative to the native state, because the unfolded species have a more extensive interface with solvent than the folded species. Destabilization of the unfolded state results in a rise in denaturation temperature.[15]

The thermograms of hydrated WSP samples, unlike what was observed with LSW, showed a decrease of T_p values, both KTI and L, at low water contents (Figure 2b). Between 0 and 30.0% w/w water, the T_p of KTI was always higher than that of L, which reached a minimum and constant value from 30.0% w/w water. However, the T_p of KTI exhibited a decreasing tendency in the whole range of moisture contents, resulting in an inversion of the thermal sensitivity when 50% w/w water is reached. In this point, a crossing of the curves corresponding to KTI and L was effectively observed (Figure 2b). At higher water contents (>50% w/w), KTI was less thermally stable than L. When DSC assays were performed on purified KTI and L, the thermal behavior was similar with respect to the same proteins in WSP sample. In anhydrous conditions, T_p values of main peak for KTI and L were 222.6 ± 0.5 and 136.0 ± 7.0 °C, respectively. These temperatures also decreased when the water content increased, also showing an inversion of thermal stability (Figure 2b).

A comparative analysis of Figures 1b and 2b shows that the inversion was observed at higher water contents for WSP with respect to LSW, which could be explained by the aforementioned release of water during heating of LSW as a consequence of the high sugar content in the whey. In a DSC study performed on purified 7S and 11S fractions, it was reported that both fractions are denatured at high temperatures at low water content (∼130 and 180 °C, respectively) and exhibited a decrease in denaturation temperature when the moisture was increased.[16] Moreover, Sessa[14] reported that at "apparent" zero moisture, electrophoretically pure fractions of 11S, 7S, and KTI are denatured at 210.2 ± 0.2, 218.5 ± 0.6, and 203.6 ± 0.5 °C, respectively. In our work, the total dehydration was achieved due to it was performed using P_2O_5 as desiccant, which reflected in a T_p approximately 20 °C higher for KTI (222.6 ± 1.0 °C). Sessa[14] also found that increasing water content decreases the denaturation temperature with the same trend in the three fractions.

With regard to denaturation enthalpy, the thermograms of WSP samples (also seen with LSW) showed that the enthalpy involved in the denaturation of KTI was always less than that of L (Table 1). It is evident that the ΔH values (total, KTI, and L) for anhydrous WSP were significantly higher than those of hydrated samples ($p < 0.05$), exhibiting the same tendency with respect to purified KTI and L (27.2 ± 2.2 and 90 ± 15 J/g, respectively). Although in hydrated conditions (15.0−73.0% water content) some ΔH values were significantly different ($p < 0.05$), these values remained in the same order. At 73% water, total enthalpy was approximately 16 times lower than the corresponding value for anhydrous WSP. According to Sessa,[14] the effect of moisture on the ΔH remained constant in the assayed moisture range for KTI (from 70.0% w/w to an "apparent" zero water content). As was mentioned above, in our work, the total dehydration was reached, and in this condition, ΔH values of KTI and L for WSP sample noticeably increased, giving a total enthalpy of 216.8 ± 3.0 J/g crude protein. In the absence of water, the thermal transition involves not only a conventional denaturation process but also thermal decomposition reactions as was previously described for LSW.

Finally, to confirm definitively the thermal stabilization of KTI at low water contents, additional assays were carried out at two different water contents (32.0 and 73.0% w/w) where KTI and L

Table 1. Denaturation Enthalpies (ΔH) Obtained from Thermograms of Whey Soy Proteins (WSP) in Anhydrous Condition and at Different Water Contents[a]

	partial ΔH (J/g protein)			
water content (w/w %)	KTI	L	KTI:L area ratio	total ΔH (J/g crude protein)
0.0	36.1 ± 1.3 a	81.5 ± 1.7 a	1:2.26	216.8 ± 3.0 a
15.0	1.4 ± 0.3 b	8.9 ± 1.0 b	1:6.39	19.1 ± 1.2 b
32.0	1.3 ± 0.2 b	6.7 ± 0.7 c	1:5.35	14.8 ± 0.8 c
48.0	2.7 ± 0.2 c	4.4 ± 0.3 d	1:1.62	12.4 ± 0.5 d
73.0	2.8 ± 0.1 c	4.2 ± 0.2 d	1:1.51	13.3 ± 0.3 d

[a] Thermograms were shown in Figure 2a. Letters "a−d" within the same column followed by different superscripts were significantly different ($p < 0.05$).

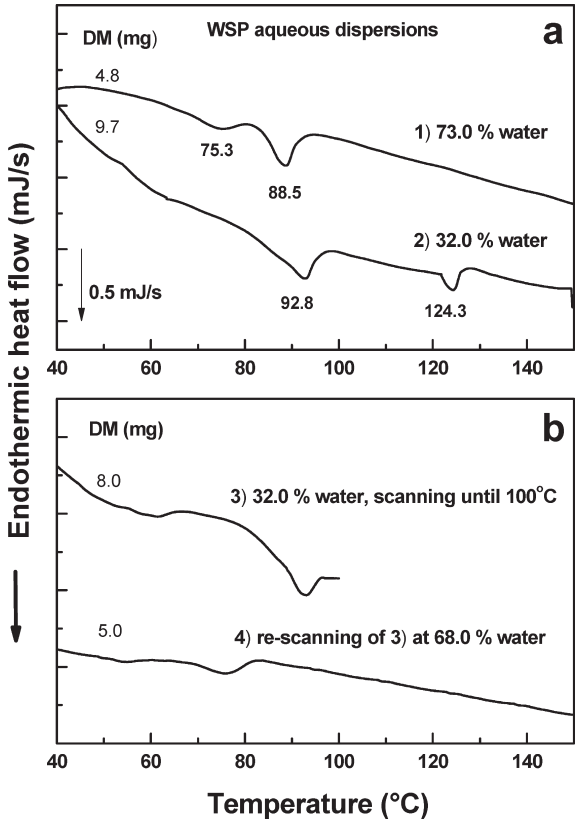

Figure 3. (a) DSC thermograms of whey soy proteins (WSP) at 73.0 and 32.0% w/w water content (runs 1 and 2, respectively). (b) DSC thermograms of WSP: Run was first carried out at 32.0% w/w water until 100 °C (run 3), then cooled and subsequently run again at 68.0% w/w water (run 4). DM: dry mass.

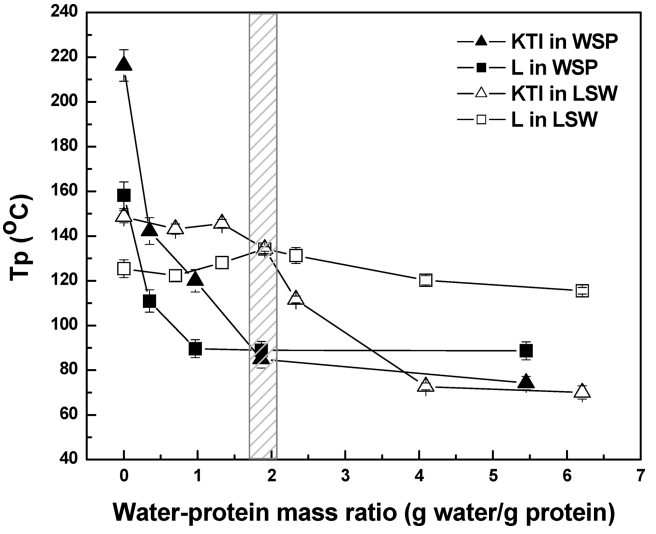

Figure 4. Effect of water−protein contents mass ratio (g water/g protein) on peak temperatures (T_p) of Kunitz trypsin inhibitor (KTI) and lectin (L) for lyophilized soy whey (LSW) and whey soy proteins (WSP). The shaded area includes the crossing point of curves corresponding to KTI and L for both samples. Protein content was determined by Lowry method.[19] Heating rate: 5 °C/min.

exhibited inverted thermal stability (Figure 3a). In a first assay, DSC run of WSP at 32.0% w/w water was performed to 100 °C, where only L was denatured (Figure 3b). Next, in a second assay, the same sample previously cooled was hydrated to reach 70.0% w/w water and run in the same conditions in a new hermetic sealed pan. A thermal transition corresponding to KTI was found at 75 °C, which confirms that at low water content this protein was more thermostable than L, the inverse behavior of what happens to water contents above 20% w/w.

Comparative Analysis of Thermal Behavior between LSW and WSP. The thermal behavior of KTI and L for WSP and LSW exhibited some important differences (Figures 1a and 2a). In anhydrous conditions, thermograms of LSW showed lesser T_p values for L and KTI (120.5 ± 3.0 and 142.3 °C ± 2.7 °C, respectively) with respect to the same proteins in the WSP sample (158.2 ± 2.6 and 216.0 ± 5.8 °C, respectively). This behavior was more evident for KTI. Besides, the baseline of thermogram corresponding to anhydrous LSW suffered a drift upward until 180 °C. This behavior, which was not observed in WSP, was attributed to an exothermic reaction of glycosylation.[4] It is assumed that this reaction is favored by the high ratio (3:1) between of carbohydrates and protein content in soy whey. The thermograms of hydrated LSW have no drift in the baseline, which would indicate a low contribution of the exothermic transition attributable to protein glycosylation (Figures 1a and 2a).

Another difference between the thermal behavior of LSW and WSP was the different water content in which the inversion in the thermal stability was observed. For LSW, the inversion occurs at 16.0−17.0% moisture, whereas for WSP sample the crossing of curves was observed at approximately 50.0% moisture (Figures 1b and 2b). However, when these T_p values were plotted as a function of water and protein content mass ratio (W/P, expressed as g water/g protein), the inversion was observed at the same W/P value (1.9 ± 0.1, Figure 4). The zone of W/P < 1.9 g water/g protein showed the greatest differences between LSW and WSP

with respect to the effect that water content exerts on thermal behavior. For WSP, T_p of KTI and L values showed a marked dependency on the W/P (especially at W/P lesser than 1.0 g water/g protein). Conversely, the effect of water content was not evident for LSW, because no significant differences between T_p values were observed ($p < 0.05$). At W/P values lower than 1.0 g water/g protein, the water molecules would be strongly interacting with proteins in WSP, and when this ratio approaches to 0 g water/g protein, they would behave as though more strongly linked. Thus, the thermal stability of proteins markedly increases. In a study on the thermal behavior of lysozyme at low water content, it was postulated that below 0.3 g water/g protein, the water molecules are bound by hydrogen bonds to the polar amino acid residues on the surface of proteins.[12] In a more recent paper, Zhong and Sun[15] evaluated the thermal behavior of 7S and 11S soy globulins, reporting that below 0.3−0.32 g water/g protein, water behaves as nonfreezing water.

For LSW, T_p values for KTI and L were lower than 150 °C and remained relatively constant in the range of W/P between 0 and 1.5 g water/g protein. Moreover, at W/P > 1 g water/g protein, a comparative analysis of T_p values for L and KTI in both samples revealed an increase of thermal stability for LSW sample (Figure 4). One possible explanation for this behavior is that the high sugar content tends to increase the range in which water molecules behave as bound water. According to MDSC assays, freezable water (FW) was not detected at W/P ≤ 1.3 g water/g protein (0−12.0% water content), while at W/P = 1.9 g water/g protein (17.0% water content, corresponding to thermal inversion), an incipient FW content (<0.3%) was observed. Finally, when W/P increased (>1.9 g water/g protein), the effect of water content on thermal stability of proteins was effectively observed, especially for KTI. The decrease of T_p values was attributed to the presence of free water and a weakening of the influence of sugar presence in the system.

It is possible to make a comparison between the thermal behavior of KTI and L for LSW and WSP and the data reported by Sessa[14] for 7S and 11S globulins in purified samples and defatted soy flour. The thermal behavior of purified 7S and 11S showed that for water content close to zero, thermal denaturation of both fractions occurs at T_p > 200 °C. However, when the thermal study was performed on defatted soy flour, T_p values for 11S and 7S were approximately 190 and 130 °C, respectively.[14] We must consider that soy flour contains oligosaccharides and other minor components that can affect the thermal stability of proteins in the flour. Similarly, for LSW in anhydrous conditions, the presence of sugars decreases the T_p values of KTI and L (Figure 4).

To confirm whether the sugars are responsible for the anomalous behavior of proteins in LSW sample, an additional DSC assay consisting of a mixture of sucrose−WSP in an amorphous state was carried out in anhydrous conditions. The sucrose:protein mass ratio in this system (3:1) was similar to the carbohydrate:protein mass ratio for LSW. This study revealed two different thermal behaviors depending on sugar crystallization, which was evidenced as an exothermic peak at 92.4 °C (Figure 5). For the sucrose−WSP mixture, the thermogram without sugar crystallization showed a behavior similar to the LSW sample in anhydrous conditions (Figure 1a), where the peaks at 134.0 and 152.0 °C are attributed to thermal denaturation of L and KTI, respectively. In addition, the small peak at 169.3 °C could correspond to the denaturation of BBTI, which also was observed in anhydrous LSW (Figure 1a). At temperatures

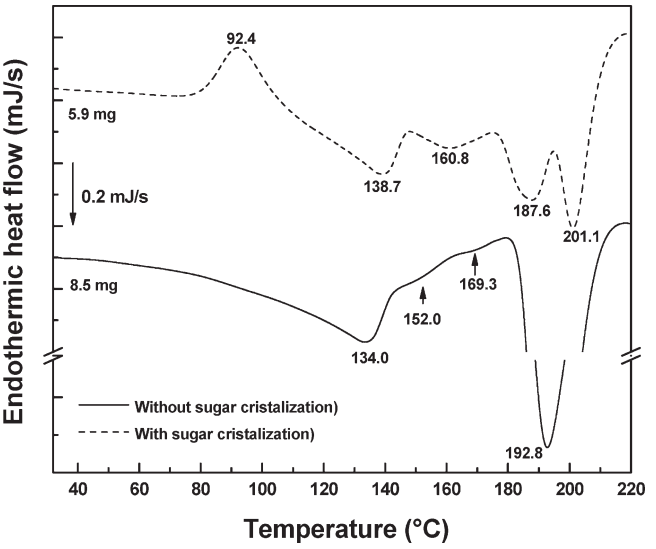

Figure 5. Modulated DSC thermograms of sucrose−WSP mixtures, without and with sugar crystallization. Heating rate: 5 °C/min.

higher than 180 °C, a large endotherm (T_p = 192.8 °C) was effectively observed as a consequence of thermal decomposition of the system. This result was also found by Sobral and Wagner[4] in a previous work. As was seen for anhydrous LSW, this thermogram also displayed an upward drift of the baseline (from 140 to 180 °C), which is attributed to the simultaneous reactions of glycosylation protein and sugar dehydration prior to decomposition.[4] When the sugar crystallization occurs, the thermogram also displayed the denaturation endotherms corresponding to L and KTI at 138.7 and 160.8 °C, respectively (Figure 5). However, the drift in the baseline was less evident due to the lower contribution of crystallized sugar to the processes of glycosylation and dehydration. Besides, an additional peak was observed at 187.6 °C, which corresponds to the melting of sugars crystals prior to thermal decomposition (T_p = 201.1 °C). According to Figure 2, the denaturation temperatures of L and KTI in anhydrous WSP were 159.0 and 216.0 °C, respectively. Thus, the presence of sucrose was responsible for the lower thermal stability of these proteins. In anhydrous conditions, sugars may suffer dehydration, releasing water to the environment, which explains why KTI and L exhibited a relatively low thermal stability for sucrose−WSP mixture and LSW sample. One should not rule out the possibility that other substances produced during dehydration of sugars (such as the furfurals) contribute to the protein destabilization. For the sucrose−WSP mixture, the high amount of sucrose leads to a decrease of about 45 °C in T_g values, this being the other possible explanation for low denaturation temperature of whey soy proteins in this system.

Conclusion. This study shows that thermal behavior of KTI and L was highly dependent on the water content and the presence of sugars, and hence was different when the heating was carried out on these proteins in the isolated state and or on soy whey. According to the overall results, two important conclusions can be obtained. First, an inversion of thermal stability of KTI and L was observed both in WSP and in LSW samples at the same water−protein content mass ratio (∼1.9 g water/g protein). KTI was more heat stable than L in anhydrous conditions and at low water content, while L was more thermostable at intermediate and high water contents. The high stability

of antitryptic factor at low moisture content indicates that strong thermal treatments on soybean and its products are always necessary, unless they are hydrated before heating. Second, sugars exert an important influence on thermal behavior of KTI and L at low water contents and especially in anhydrous conditions, where an important decrease of thermal stability of both proteins was effectively observed. This observation was corroborated both in LSW and in sucrose−WSP mixtures. T_p values of KTI and L for the LSW sample remained practically constant at water−protein contents mass ratio lower than 1.3 g water/g protein, probably due to a combined effect of decrease of T_g of the system, the absence of free water, and water liberation due to thermal decomposition reactions. During the soybean processing, substantial volumes of contaminant effluents are generated, and their conversion in products of interest for food industry requires dehydration and subsequent KTI inactivation. Indeed, due to the dramatic decrease of thermal stability of KTI as water content increases, soy whey syrups at 30% w/w moisture are microbiologically stable, noncrystallizable, and can be inactivated at relatively low temperatures (<100 °C).

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REFERENCES

(1) Pearson, A. M. Soy proteins. In *Developments in Food Proteins-2*; Hudson, B. J. F., Ed.; Applied Science Publishers: London, UK, 1983; pp 67−108.

(2) Sorgentini, D. A.; Wagner, J. R. Comparative study of structural characteristics and thermal behavior of whey and isolate soybean proteins. *J. Food Biochem.* **1999**, *23*, 489–507.

(3) Cheftel, J. C.; Cuq, J. L.; Lorient, D. Las proteínas de soja. In *Proteínas Alimentarias*; Cheftel, J. C., Ed.; Editorial Acribia S.A.: Zaragoza, Spain, 1989; pp 257−275.

(4) Sobral, P. A.; Wagner, J. R. Thermal properties of soybean whey and its proteins. In *Functional Properties of Food Components*; Lupano, C. E., Ed.; Research Signpost: Kerala, India, 2007; pp 57−76.

(5) Koshiyama, Y.; Kikuchi, M.; Fucushima, D. 2S Globulins of soybean seeds. 2. Physicochemical and biological properties of protease inhibitors in 2S globulins. *J. Agric. Food Chem.* **1981**, *29*, 340–343.

(6) Dipietro, C. M.; Liener, I. E. Heat inactivation of Kunitz and Bowman-Birk soybean protease inhibitors. *J. Agric. Food Chem.* **1989a**, *37*, 39–43.

(7) Dipietro, C. M.; Liener, I. E. Soybean protease inhibitors in foods. *J. Food Sci.* **1989b**, *54*, 606–617.

(8) Birk, Y. Proteinase inhibitors. In *Hydrolytic Enzymes*; Neuroberger, A., Brocklehurst, K., Eds.; Elsevier Science Publishers: Amsterdam, Netherlands, 1987; pp 257−300.

(9) Liener, I. E. Factors affecting the nutritional quality of soya products. *J. Am. Oil Chem. Soc.* **1981**, *58*, 406–415.

(10) Rouilly, A.; Orliac, O.; Silvestre, F.; Rigal, L. DSC study on the thermal properties of sunflower proteins according to their water content. *Polymer* **2001**, *42*, 10111–10117.

(11) Rouilly, A.; Orliac, O.; Silvestre, F.; Rigal, L. Thermal denaturation of sunflower globulins in low moisture conditions. *Thermochim. Acta* **2003**, *398*, 195–201.

(12) Fujita, Y.; Noda, Y. Effect of hydration on the thermal denaturation of lysozyme as measured by differential scanning calorimetry. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 1567–1568.

(13) Fujita, Y.; Noda, Y. The effect of hydration on the thermal stability of ovoalbumin as measured by means of differential scanning calorimetry. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 3233–3234.

(14) Sessa, D. J. Hydration effects on the thermal stability of proteins in cracked soybeans and deffated soy flour. *Lebensm.-Wiss. Technol.* **1992**, *25*, 365–370.

(15) Zhong, Z. K.; Sun, X. S. Thermal behavior and nonfreezing water of soybean protein components. *Cereal Chem.* **2000**, *77*, 495–500.

(16) Kitabatake, N.; Tahara, M.; Doi, E. Thermal denaturation of soybean protein at low water contents. *Agric. Biol. Chem.* **1990**, *54*, 2205–2212.

(17) Sousa, I. M. N.; Mitchell, J. R.; Ledward, D. A.; Hill, S. E.; Beirão da Costa, M. L. Differential scanning calorimetry of lupin and soy proteins. *Z. Lebensm.-Unters. Forsch.* **1995**, *201*, 566–569.

(18) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.

(19) Lowry, O. H.; Rosembroug, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(20) Scilingo, A. A.; Añón, M. C. Calorimetric study of soybean proteins isolates: Effect of calcium and thermal treatments. *J. Agric. Food Chem.* **1996**, *44*, 3751–3756.

(21) Matsue, S.; Tomoyuki, F.; Miyawaki, O. Effects of water activity and aqueous solvent ordering on thermal stability of lysozyme, α-chymotrypsinogen A, and alcohol dehydrogenase. *Int. J. Biol. Macromol.* **2001**, *28*, 343–349.

(22) Oste, R. E.; Brandon, D. L.; Bates, A. H.; Friedman, M. Effect of Maillard browning reactions of the Kunitz soybean trypsin inhibitor on its interaction with monoclonal antibodies. *J. Agric. Food Chem.* **1990**, *38*, 258–261.

(23) Cheftel, J.-C.; Cuq, J. L.; Lorient, D. Aminoácidos, péptidos y proteínas. In *Química de los Alimentos*; Fennema, O., Ed.; Editorial Acribia S.A.: Zaragoza, Spain, 1993; pp 374−406.

(24) Blanksby, S. J.; Ellison, G. B. Bond dissociation energies of organic molecules. *Acc. Chem. Res.* **2003**, *36*, 255–263.

(25) Sheu, S. Y.; Yang, D. Y.; Selzle, H. L.; Schlag, E. W. Energetics of hydrogen bonds in peptides. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 12683–12687.

(26) Roos, Y. H. *Phase Transitions of Foods*; Academic Press, Inc.: San Diego, CA, 1995.

(27) Careri, G.; Giansanti, A.; Gratten, E. Lysozyme film hydration event: An IR and gravimetric study. *Biopolymers* **1979**, *18*, 1187–1203.