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ENZYMATIC HYDROLYSIS OF SOY PROTEIN ISOLATES
DSC study

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Abstract

The aim of this work was to analyze the possible use of differential scanning calorimetry (DSC) as a method to study the process of protein modifications during enzymatic hydrolysis. Results of the enzymatic hydrolysis of soy protein showed significant differences in the values of maximum deflection temperature (T_p), heat of reaction (ΔH), and width at half peak height ($\Delta T_{1/2}$), between DSC curves corresponding to the substrate, or zero time of hydrolysis, and those of the hydrolysates obtained by the action of *cucurbita* and *pomiferin* enzymes. DSC curve changes mentioned were explained by the use of gel-filtration chromatography, denaturing electrophoresis and surface hydrophobicity of the hydrolysis products obtained at 30 min of reaction.

Keywords: DSC, hydrolysis, soy proteins

Introduction

Soy proteins are often used as a component of food formulations because of the wide variety of their functional properties [1]. It is a well known fact that the alteration of protein structure by physical, chemical and/or enzymatic methods leads to changes in their functional properties. Thus, for example, severe thermal treatment results in denaturation of soy proteins leading to aggregation, decreasing solubility in aqueous solvents and increasing water imbibing capacity [2–5]. It has been also reported that deamidation and treatment at acid pH alter the surface properties of these proteins, leading to a higher ability for foam formation and stability and a slight increase of emulsifying stability [6–9].

Partial enzymatic hydrolysis enhances solubility, foaming and emulsifying properties, mainly in heat-denatured proteins [10–11].

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The study of the products of the hydrolysis reaction is generally performed by means of different types of chromatography and electrophoresis [12–14].

Differential scanning calorimetry (DSC) is a technique that makes possible to analyze physical and chemical changes involving heat absorption or release, while recording at the same time the temperatures and amounts of heat associated with the process under study. This type of calorimetry has been used, among others, to follow up first order transitions in foods, such as protein denaturation, starch gelatinization and retrogradation, lipid fusion or crystallization, etc. [15–16] as well as second order transitions (changes of heat capacity, glass transitions, etc. [17–18]. With regard to proteins, denaturation curves result from thermal changes associated with the breakdown of those bonds involved in stabilizing protein structure (e.g. hydrogen bonds, hydrophobic interactions, electrostatic interactions, etc.), and, in some cases, an additional aggregation process [19].

Partial enzymatic hydrolysis results in the breaking of peptide bonds, which may lead to changes in the secondary and tertiary structures of proteins, and then, to changes in the reaction of thermal denaturation of the modified protein molecule.

The objective of the present study was to determine whether differential scanning calorimetry is a useful tool for the follow up of changes undergone by proteins, particularly soy protein isolates, during enzymatic hydrolysis.

Materials and methods

Preparation of protein isolates

Protein isolates were prepared from defatted soy flour containing 42.02% of protein (Santista Foods, Brazil) by extraction with alkaline water (ratio water:flour 10:1 mass/v) for 2 h at room temperature; pH was kept at 8.0 by adding 2 N NaOH as needed. The suspension thus obtained was centrifuged at 1000 × g for 30 min at 4°C. The supernatant was adjusted to pH 4.5 with 2 N HCl. The precipitate separated by centrifuging at 5000 × g for 15 min at 4°C, was suspended in water (5% protein mass/v) and adjusted to pH 8.0 by adding 1 N NaOH.

Enzymes

Two proteases, *cucurbita* and *pomiferin* (both of the serine type) were used; they were obtained from the Laboratorio de Bioquímica, Facultad de Ciencias Básicas y Matemáticas, Universidad Católica de Valparaíso, Chile [20] and from the Laboratorio de Botánica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata [21], respectively.

Hydrolysis reaction

Solutions or dispersions of soy protein isolates (30 mg mL^{-1}) in pH 8.0, 0.01 M phosphate buffer were mixed with enzyme solution (0.4 mg mL^{-1} in the same buffer) in a ratio 4:1 at 40°C. Dispersions were hydrolyzed for 15, 30, 60, 180, 360 and 720 min. Freezing the

aliquots at –20°C followed by lyophilization stopped the hydrolysis reaction. A sample was obtained immediately after adding the enzyme (zero time of hydrolysis).

Hydrolysis reactions were carried out at least twice.

Degree of hydrolysis

Free amino groups were determined with the method of trinitrobenzenesulfonic acid (TNBS) [22]. To this end, 0.6 mL samples of the enzymatic reaction mixture were removed at the above-mentioned times. Then, 1 mL of sodium phosphate buffer pH 8.2 and 1.0 mL of 0.1% TNBS (v/v) solution was added. The mixture was incubated with stirring for 60 min at 50°C, followed by addition of 2.0 mL of 0.1 N HCl and keeping for 30 min at room temperature in a dark place. Triplicate absorbance readings corresponding to each tube were recorded at 340 nm; 1.5 M *L*-leucine was used as standard.

Differential scanning calorimetry

A Polymer lab (PL-DSC, Rhemotric Scientific) Differential Scanning Calorimeter was used. Runs were analyzed with Plus V5.41 software. Calibration of the equipment (temperature and heat-flow) was carried out according to ASTM Standards E967-83 and E-968-83 [23–24], respectively. Lauric acid and indium were used as temperature standards, the latter was also used as a heat-flow standard.

Analyzed samples were 300 mg mL^{-1} dispersions of protein isolate and of hydrolysates in distilled water; 15–17 mg samples were placed in hermetic aluminum capsules. An empty aluminum capsule was taken as reference.

Run conditions were as follows: rate of heating, 10°C min^{-1}, temperature range, 25–125°C. After the runs were over, capsules were punctured and the dry matter content was determined by heating in an oven at 105°C until constant mass was reached. The base line was constructed as a straight line from the beginning to the end of the DSC curve (Fig. 1, ISP). The following parameters were obtained from the DSC curves: temperature of maximal deflection, T_p and heat of reaction, ΔH. A measure of cooperativity of components contributing to the first endotherm was obtained from the width at half peak height ($\Delta T_{1/2}$). To this end total DSC curves were divided into two partial areas as shown in Fig. 1 [25].

Triplicate determinations were done throughout.

Electrophoresis

Electrophoretic runs were performed in a BIO-RAD Mini Protein I Cell. The SDS-PAGE denaturing electrophoresis was carried out in acrylamide gradients (5–15%), at 200 V and 15 mA per plate, according to the Laemmli method [26].

Lyophilized samples were dissolved in sample buffer in order to reach 2–3 μg protein per μL of dispersion; 10 μL sample per lane was placed at the start. When the runs were over, gels were fixed and stained with coomasie blue.

Molecular masses were determined using the low molecular mass standards from Pharmacia and Sigma.

Molecular exclusion chromatography

Sephacryl S300 HR resin with an exclusion range of 10-1500 kDa was employed. The column of 1.6 mm diameter and 64 cm length was equilibrated with 0.1 M pH 8.0 sodium phosphate buffer; rate of flow was 16 mL h^{-1}. Chromatographic profiles were obtained by measuring absorbance at 280 nm. An amount of sample corresponding to 2% of the total volume of the column was used in all determinations. The following protein standards were used: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chimotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

Surface hydrophobicity (Ho)

Values of Ho were determined by the hydrophobicity fluorescence probe (with ANS: 1-anilino-8-naphthalene sulfonate (Aldrich Chemical Company Inc., catalog number 21,690-9) according to the method reported by Kato and Nakai [27]. Protein concentration in the supernatants was determined by the method of Lowry *et al.* [28]. Each protein sample was serially diluted with 0.1 M phosphate buffer (pH 8.0) to obtain protein concentrations ranging from 0.005 to 0.05%. Then, 10–40 μL ANS (8.0 mM in 0.1 M buffer phosphate pH 8.0) solution was added to 2 mL of sample. Fluorescence intensity (FI) was measured at 364 nm (excitation) and 484 nm (emission) using Perkin Elmer 2000 Fluorescence Spectrometer. FI intensity was standardized by adjusting the full scale to 80% for ANS in methanol. The net FI (maximum value) at each protein concentration was determined by subtracting FI of each solution without the probe from that with the probe. The initial slope of the FI *vs.* protein concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity (Ho).

Statistical analysis

A variance analysis (ANOVA) was carried out; significant differences between different times of hydrolysis were determined by means of the Tukey test, with a level of significance alfa: 0.05. Both analyses were carried out using the SYSTAT statistical software [29].

Results and discussion

Figure 1 shows typical DSC curves corresponding to native soy protein isolates and to samples of isolates hydrolyzed with *cucurbita* for different periods of time. Non-hydrolyzed native soy protein isolate showed a typical DSC curve, characterized by two endotherms corresponding to the denaturation of β-conglycinin (7S fraction) (T_p: 79.9±0.3°C) and glycinin (11S fraction) (T_p: 95.5±0.4°C), respectively. DSC curves corresponding to the control (zero-time of hydrolysis) showed a shift of the maximal peak temperature (T_p 7S: 84.8±0.4°C, T_p 11S: 99.4±0.4°C). This fact may be ascribed to the higher salt content of these samples due to the composition of the medium in which the hydrolysis reaction was performed (Materials and methods).

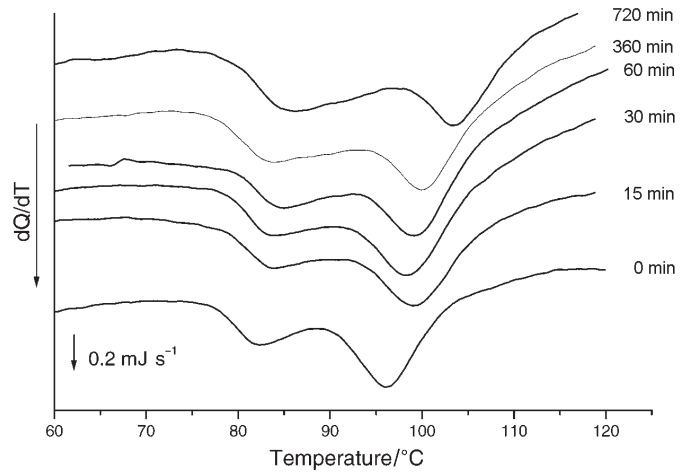

Fig. 1 Typical DSC curves corresponding to native soy protein isolates (ISP), control sample (zero time of hydrolysis) and to samples of *cucurbita*-hydrolysates obtained at different times of reaction

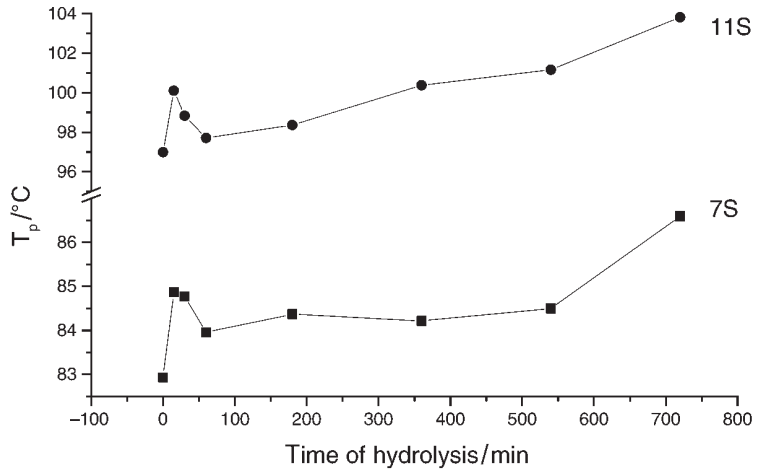

Fig. 2 Temperature of maximal deflection, T_p, corresponding to the first (7S) and second (11S) endotherms of *cucurbita*-hydrolysates obtained at different times of reaction

As the hydrolysis proceeded, a shift of the maximal peak temperatures of both endotherms, towards higher temperatures was detected. Values of T_p corresponding to the first and second endotherms in curves obtained for the samples hydrolyzed with this enzyme are shown in Fig. 2. In the case of the first peak a significant increase ($p<0.05$) was detected in the T_p of the hydrolysates with reference to the zero time of hydrolysis (control). This increase reached a maximum at 60 min of hydroly-

sis (ΔT_p with reference to the control: 6°C), then decreasing slightly (ΔT_p with reference to the control: 4–5°C) ($p<0.05$). The same tendency was observed with the second endotherm, when at 60 min of hydrolysis a shift towards higher temperatures of about 7°C ($p<0.05$) was detected. In the case of hydrolysates obtained with *pomiferin*, DSC curves were also shifted towards higher temperatures. This effect was also shown by the control (zero time of hydrolysis) (T_p 7S: 83.9±0.6°C and T_p 11S: 97.0±0.4°C) (Fig. 3). The first endotherm exhibited a shift of T_p ($p<0.05$) only with the sample corresponding to the highest degree of hydrolysis (Fig. 4). In the case of the second endotherm (Fig. 4) an increase of the thermal stability of the hydrolysate was observed at 15 min of reaction, then, a slight decrease until 60 min and again an increase for longer times of hydrolysis (180 to 720 min) ($p< 0.05$).

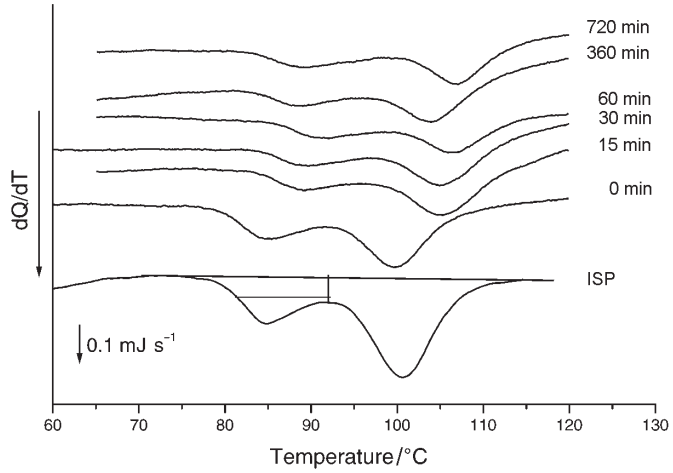

Fig. 3 Typical DSC curves corresponding to control sample (zero time of hydrolysis) and to samples *pomiferin*-hydrolysates obtained at different times of reaction

Heats of reaction required for the thermal denaturation of *cucurbita* and *pomiferin* hydrolysates and control samples are shown in Fig. 5. The analysis of the values showed, in both cases, a decrease of total ΔH with the reaction time ($p<0.05$). For *cucurbita* the decrease of total ΔH with the time of reaction was very fast, reaching at 60 min a 53% of the initial value. For *pomiferin*, after 15 min of hydrolysis, a low decrease of total ΔH values were observed (11% at 720 min for hydrolysis in respect to the initial value). ΔH values for *cucurbita* hydrolysates were considerably lower than *pomiferin* ones. ΔH value combines both exothermic and endothermic contribution of the breakup of hydrophobic and hydrogen bonds respectively and it is correlated with the content of ordered secondary structure of a protein [30]. The lowering of ΔH with the hydrolysis time suggests the presence of hydrolysis products stabilized by different amounts of hydrogen bonds and hydrophobic interactions. The modifications of enthalpy also result in changes in the shape of the endotherms in the different DSC curves, mainly the first one, ascribed for the substrate, to denaturation with β-conglycinin. According with Privalov *et al.* [31] the width of the calorimetric

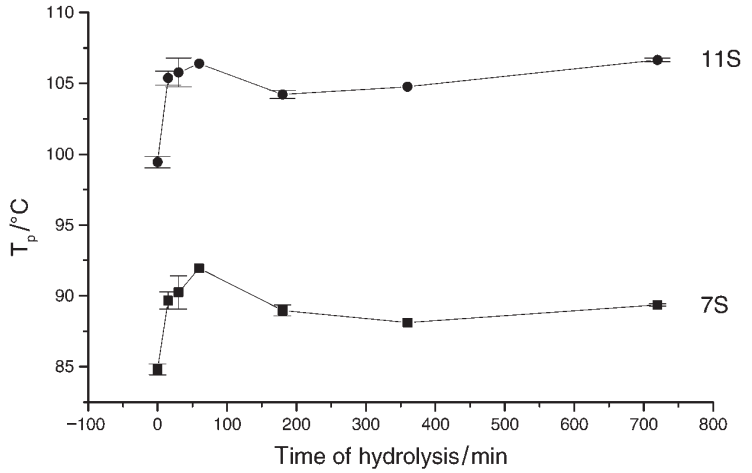

Fig. 4 Temperature of maximal deflection, T_p, corresponding to the first (7S) and second (11S) endotherms of samples *pomiferin*-hydrolysates obtained at different times of reaction

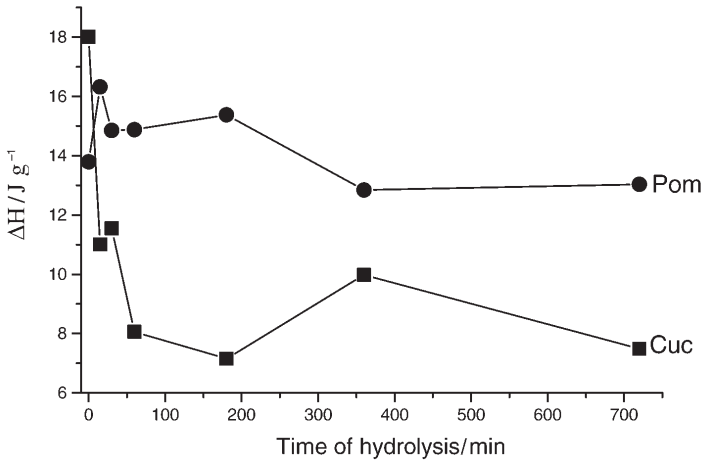

Fig. 5 Heats of reaction, Δ*H*, required for the thermal denaturation of control samples and samples hydrolyzed with *pomiferin* and *cucurbita*

transition at half peak height, $\Delta T_{1/2}$, is indicative of the cooperativity of protein unfolding. If the reaction presented a low $\Delta T_{1/2}$ the transition was considered highly cooperative. As the hydrolysis advances, either by the action of *cucurbita* or *pomiferin*, a loss of cooperativity (high $\Delta T_{1/2}$) was detected (Table 1, Figs 1 and 3).This effect is magnified at long times of reaction (180 to 720 min), especially in the case of *pomiferin* hydrolysates where a loss of 1.8-fold of the width of the first endotherm was observed ($\Delta T_{1/2}$ at 720 min/$\Delta T_{1/2}$ from control). No modification in cooperativity

was detected in the second endotherm during hydrolysis ($\Delta T_{1/2}$ at 0 time of hydrolysis: 8.2±0.1°C and 8.9±0.0°C $\Delta T_{1/2}$ at 720 min of hydrolysis: 7.05±0.1°C and 9.8±0.6°C for *cucurbita* and *pomiferin*, respectively). The broadening of the first endotherm detected indicates that hydrolysis induced changes in the protein structure of the substrate and/or is a consequence of the existence of several components, products of hydrolysis, with different thermal stability.

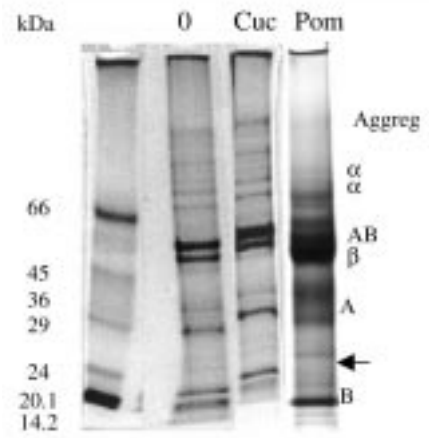

Fig. 6 Electrophoretic (SDS-PAGE) profiles corresponding to samples hydrolyzed for 30 min with *cucurbita* and *pomiferin* and to the control (zero hydrolysis time)

To determine the actual nature of the first and second endotherms of the different above-described curves, hydrolysates obtained with either enzyme were analyzed by electrophoresis in polyacrylamide gels (SDS-PAGE), in the absence of a reducing agent. Profiles corresponding to 30 min hydrolysis and to the control (zero time of hydrolysis) are shown in Fig. 6. In the case of hydrolysates obtained with *cucurbita*, a profile similar to that of the control was observed, in which α, α′, and β-7S, and the AB subunit of fraction 11S could be readily identified. Some acid and basic glycinin polypeptides (indicated as A and B) and aggregates of high molecular mass (higher than 95 kDa) were also detected. *Pomiferin* hydrolysates gave a profile different from that of the control, showing both the disappearance of aggregates of high molecular mass and of subunits α, α′ of fraction 7S and of the appearance of new polypeptides, products of hydrolysis with low and intermediate molecular mass (indicated by an arrow). At longer times of reaction it was possible to detect even higher differences; at 720 min there was a complete disappearance of aggregates of high molecular mass and the rest of β-conglycinin subunits. Besides, hydrolysis products of intermediate sizes tended to disappear, and new protein species of low molecular mass arose (results not shown).

Gel filtration chromatographic analysis of the products of the reaction supported the results obtained by electrophoresis (Fig. 7). The native soy isolate exhibited a major peak of 917 kDa comprising fractions 7S and 11S and two very minor peaks of 40.9 and 4.3 kDa, respectively. The profile obtained by hydrolysis with *cucurbita* was similar to that

of the substrate and an increase of the peaks 58.8 and 5.5 kDa was detected, corresponding to products of hydrolysis. Hydrolysates with *pomiferin* showed a decrease of the molecular mass of the first peak (479 kDa), whereas several peaks – corresponding to products of hydrolysis – appeared in the rest of the chromatogram.

Table 1 Width of the calorimetric transition at half peak height, $\Delta T_{1/2}$, corresponding to the first endotherm of thermograms of *cucurbita* and *pomiferin* hydrolysates

Hydrolysis time/min	$\Delta T_{1/2}$/°C	
	Cucurbita	Pomiferin
0	9.45±0.21	8.90±0.00
15	9.20±0.35	10.05±0.07
30	10.80±0.14	10.04±0.15
60	10.80±0.00	10.80±0.24
180	10.90±0.14	11.55±0.21
360	13.20±0.00	13.63±0.25
720	13.20±0.00	15.68±0.28

Both the electrophoretic patterns and the gel filtration chromatograms clearly indicated differences between the products of hydrolysis obtained with the two enzymes. Since in both cases there was an increase of the degree of hydrolysis (12 and 22% at 120 min of reaction and 20 and 45% at 720 min, with *cucurbita* and *pomiferin*, respectively), the above-mentioned results suggest that most of the products of hydrolysis obtained

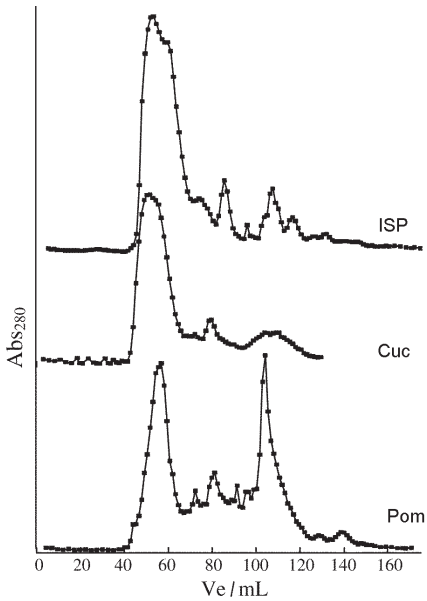

Fig. 7 Gel filtration chromatogram of native soy protein isolate (ISP) and 30 min *cucurbita* (Cuc) and *pomiferin* (Pom) hydrolysates

with *cucurbita* remain attached to the initial structure of the substrate; whereas *pomiferin* gives rise to intermediate products which are released into the medium.

Determination of surface hydrophobicity of the soluble fractions, analyzed at 30 min of reaction by means of gel filtration chromatography, showed a decrease of Ho of the components of the main peak of the hydrolysates (Ho: 2.3±0.1 and 1.6±0.1 for *cucurbita* and *pomiferin* respectively) and a significant increase of the components of lower molecular mass (Ho: 4.1±0.2 and 7.7±0.1 for *cucurbita* and *pomiferin*, respectively) in comparison with non-hydrolyzed soy isolate (Ho: 6.3±0.3 for the main peak and 2.4±0.2 for the minor protein species of low molecular mass).

Having in mind the features of the products of hydrolysis resulting from the action of both enzymes at 30 min of reaction, it becomes evident that the components contributing to the first and second endotherm do not correspond solely to the denaturation of glycinin and β-conglycinin. In the case of *pomiferin*, subunits α and α′-7S were hydrolyzed after 30 min reaction, whereas hydrolysis of β-7S subunit took 720 min. Therefore, the increase of $\Delta T_{1/2}$ of the first endotherm (at 30 min of hydrolysis), would result from the contribution in that region of the products released by hydrolysis, effect that is magnified at long reaction times. A low decrease of total heat of reaction with the hydrolysis suggests that a number and kind of bonds similar to the substrate stabilize the structures of the products.

In the case of *cucurbita* the hydrolysis-induced changes in the conformation of the substrate may lead to a residual structure stabilized by a lower amount of hydrogen bonds and/or a high proportion of hydrophobic interactions. The fact that hydrolysis products remain attached to the initial structure of the substrate could explained the low increase of $\Delta T_{1/2}$. The augment in the T_p may be attributed to a greater proportion of hydrophobic residues involved in the unfolding of the residual structure formed.

Conclusions

Results obtained in the study presented here indicate that differential scanning calorimetry is a suitable tool for studying the structural changes undergone by soy proteins during enzymatic hydrolysis. The appearance of the products of hydrolysis is evidenced by the shift of the maximum peak temperature, the broadening of the endotherms and the heat evolved during the denaturation reaction. As is also the case with other techniques, calorimetric determinations should be complemented by other techniques in order to have a complete picture of the reaction under study.

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