JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5939–5947 5939
DOI:10.1021/jf900424b

# Study of Calcium−Soy Protein Interactions by Isothermal Titration Calorimetry and pH Cycle

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The aim of this work was to understand Ca-induced soy protein (nonhydrolyzed, NH; or hydrolyzed, H) aggregation and to characterize the involved interactions using ITC and pH cycle. The endothermic signals obtained upon titration of soy proteins with Ca were fitted with a one set of sites model. NH soy proteins bound more Ca than H soy proteins (∼52 and ∼2 mg of Ca/g of proteins, respectively). The binding constant *K* indicated the easier Ca binding onto H soy proteins than for NH soy proteins. The exothermic part involved by electrostatic interactions was completely hidden by the strong endothermic signal from the water molecule release. Ca binding onto soy proteins should be described as a $H^+/Ca^{2+}$ exchange. Whatever the soy proteins, the positive value of heat capacity changes indicated a reduction in the number of surface-exposed polar residues. Ca-induced soy protein aggregation was irreversible for pH cycle to 3.5.

**KEYWORDS: Interaction; ITC; enthalpy change; binding affinity; electrophoretic mobility**

## INTRODUCTION

Calcium (Ca) is a mineral essential for humans, especially during growth, pregnancy, lactation, and old age to act against osteoporosis. Yet, most of the world population does not satisfy the recommended daily intake of Ca (*1*) of about 1000 mg/day. More than 70% of dietary Ca comes from dairy products because casein micelles of milk are a natural vector of Ca. Nevertheless, dairy products cannot be consumed by specific populations due to nutritional reasons such as lactose intolerance (*2*) or cow's milk protein allergy (*3*). Soy milk, a vegetable beverage with a milky appearance, is traditionally prepared from soaked soybeans by grinding in water (water to bean ratio between 8:1 and 10:1), filtering, and heating. With a similar protein concentration but without lactose and cholesterol, soy milk constitutes an interesting alternative to cow's milk. However, its Ca concentration (15 mg/100 g) is 8 times lower than that in cow's milk (*4*).

Most of the time, efforts to fortify soy milk with Ca have been largely unsuccessful. Ca supplementation of soy milk at concentration levels required for fortification induced extensive soy protein aggregation (*5*, *6*). Ca tends to coagulate the proteins, forming precipitates and causing gelation of the soy milk during storage. However, this soy protein aggregation can be overcome under carefully controlled conditions, and several authors reported their ability to fortify soy milk with Ca at the same or higher level than in cow's milk, either with stabilizer or complexant addition or with Ca encapsulation. Hirotsuka et al. (*7*) fortified soy milk with Ca by means of a Ca−lecithin liposome system. Calcium ions were enveloped with a soy lecithin membrane before addition to soy protein dispersion to prevent coagulation and precipitation. In this way, instability phenomena were not observed in Ca-fortified soy milk (60 mM $Ca^{2+}$). Zemel and Shelef (*8*) fortified soy milk with Ca using an alkali metal polyphosphate salt as chelating agent to suppress aggregation. Their patented (0195167) product had a refrigerated shelf life of 3 weeks. The stabilization of soy milk fortified with calcium gluconate by sodium hexametaphosphate (SHMP) as a sequestering agent and Ca−D-saccharic acid as a stabilizing agent was studied by Rasyid and Hansen (*9*). SHMP and Ca−D-saccharic acid were effective for reduction of the calcium ion activity of soy milk and sequestering and stabilizing agent may act in synergy. Yazici et al. (*10*) reported Ca fortification of soy milk (200 mg/100 g) with calcium lactogluconate and various amounts (0−1.50% w/w) of SHMP or potassium citrate. Nevertheless, besides all of these chemical methods of stabilization, the effect of pH cycle has never been investigated on the restructuring and stability of Ca-induced soy proteins aggregates, which could be then used as possible vectors for further Ca delivery.

In this study, Ca−soy protein interactions at the origin of the formation of Ca-induced soy protein aggregates were thermodynamically characterized using isothermal titration calorimetry (ITC), a technique of choice in the study of mineral−protein interactions. Several studies reported the use of ITC for the thermodynamic characterization of Ca binding onto purified

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 Published on Web 06/16/2009 

proteins such as α-lactalbumin, calmoduline, and lysozyme (*11*−*14*). However, to our knowledge, ITC has never been applied to the study of Ca interactions in complex systems such as soy protein dispersions. Then, the ability of a pH cycle to induce restructuring and stabilization of Ca−soy protein aggregates was studied. Indeed, as a physicochemical parameter, pH has a major influence on the ionization state of proteins and on the expected electrostatic interactions involved between charged molecular species such as Ca and soy proteins. pH cycle, carried out using a reactor experimental approach, was applied to Ca-supplemented soy protein dispersions, and the ionized calcium variations were related to structural changes in the protein colloidal phase. Thus, this study gives a better understanding of mechanisms occurring upon Ca-induced soy protein aggregation through the characterization of the involved interactions by two complementary methods: ITC and pH cycle.

## MATERIALS AND METHODS

**Preparation of Soy Protein Dispersions.** *Thermodynamic Characterization of Ca*−*Protein Interactions.*

*(a) Whole Soy Protein Fraction.* Nonhydrolyzed (NH) and hydrolyzed (H) soy protein dispersions were prepared from their respective soy protein isolate (NH-SPI, non-GM soy protein isolate 760; H-SPI, non-GM soy protein isolate 219; The Solae Co., Barcelona, Spain) in Milli-Q water at 1% (w/w) protein concentration. The study of H soy proteins, in comparison with NH soy proteins (similar SPI amino acid composition, Supporting Information), was carried out to determine the effect of hydrolysis on the thermodynamic characteristics of Ca−soy protein interactions. NH and H soy proteins were compared for their ability to bind Ca, especially on the stoichiometry value (*N*) and the binding constant (*K*). The presence of insoluble protein aggregates in SPI, up to 56 and 50% for NH-SPI and H-SPI, respectively, led to large protein sedimentation. To minimize this physical instability, an appropriate stabilization treatment was applied. Soy protein dispersions were first sheared at 13500 rpm during 5 min (ultraturrax, Ultralightnin, Advantech, Strasbourg, France), and then homogenization was carried out at 500 bar during 5 cycles using a high-pressure homogenizer (EmusiFlex-C3, Avestin, Canada). Finally, desalting was performed on a PD10 desalting column (Amersham Biosciences, Buckinghamshire, U.K.) to remove ions initially present, especially Ca and phytate. Indeed, as a Ca complexant, phytate could interfere with the interactions occurring between Ca and soy proteins. Samples were stored at −20 °C until ITC analysis.

*(b) Soluble Soy Protein Fraction.* Soy protein dispersions were reconstituted in Milli-Q water at 4.2% (w/w) protein concentration. Soy protein aggregates were discarded after acidification (HCl, 1 M, Sigma Aldrich, Seelze, Germany) of protein dispersions in a thermostated vat ($T_{exptl}$ = 20 °C, 150 rpm) to reach the isoelectrical pH of soy proteins ($pH_i$ 4.5) and centrifugation (Centrifuge Sigma Bioblock, Osterode, Germany) at 3034*g*, 35 min, 20 °C (*15*). The supernatant containing soluble soy proteins was collected and back neutralized (NaOH, 1 M, Sigma Aldrich) to reach the initial pH of soy protein dispersions (~7.1−7.2). Finally, desalting was performed on a PD10 desalting column, and samples were stored at −20 °C until ITC analysis.

*(c) Protein Concentration Determination.* Protein concentration, contained in whole or soluble protein fraction collected after the desalting step, was determined according to the Bradford method. A calibration curve was performed with bovine serum albumin solution at various concentrations ranging from 0 to 1.35 mg/mL.

*Reactor Experimental Study.* NH and H soy protein dispersions were prepared from their respective SPI in distilled water at 4.2% protein concentration (w/w). Comparison of NH and H soy protein dispersions enabled us to determine the effect of hydrolysis on $Ca^{2+}$ variations between the soluble and the colloidal phase and the Ca-induced soy protein aggregates. Soy protein dispersions were stabilized as described previously.

**Thermodynamic Characterization of Ca−Soy Protein Interactions.** By ITC, thermodynamic characterization of the interactions is obtained in addition to the molar ratio of mineral binding onto proteins and to the binding constants. ITC measures the binding equilibrium directly by determining the heat involved on association of a ligand with its binding partner. In a single experiment, the value of the binding constant (*K*), the stoichiometry (*N*), and the binding enthalpy ($\Delta H$) are determined. The free Gibbs energy ($\Delta G$) and the entropy ($\Delta S$) are calculated from the binding constants. Finally, the temperature dependence of $\Delta H$, measured by performing the titration at various temperatures, describes the heat capacity changes ($\Delta C_p$).

Thermodynamic characterization of Ca−soy protein interactions was done using a VP-ITC microcalorimeter (MicroCal, North Hampton, MA). Before each experiment, Ca and soy protein dispersions were degassed during 7 min. The sample and the reference cells were respectively filled with soy protein dispersions and with Milli-Q water. The titrant, a calcium chloride solution (CC, $CaCl_2 \cdot 2H_2O$, Carl Roth GmbH, Karlsruhe, Germany) prepared in Milli-Q water, was injected stepwise into the sample cell. The measurements were performed under constant stirring (300 rpm) at constant temperature (4, 25, or 50 °C, ±0.2 °C). Small aliquots of titrant were successively injected into the sample cell, and the total number of injections varied according to the experiment in order to reach soy protein saturation. The time between two successive injections was 300 s. Because of possible sample dilution during the equilibration time preceding the measurement, the first injection was ignored in the analysis of data. Each injection produced a characteristic peak in the heat flow due to the released or absorbed heat. Due to the lack of information about the expected heats of the studied systems, the reference power of the ITC instrument was set at 15 $\mu$cal $s^{-1}$. The titration parameters are summarized in **Table 1** for the whole and the soluble protein fraction study. Each experiment was duplicated.

The titration of CC solution into Milli-Q water was studied as the reference and determined the heat of dilution of ligand. This reference experiment, carried out in the same way as the titration with protein sample, was subtracted from the sample data. The corrected binding isotherms were fitted by ORIGIN software using the "one set of sites" built-in curve fitting model. Fitting runs were repeated until the $\chi^2$ value was not reduced anymore. Although this model did not fit perfectly the experimental points, this was the most appropriate for the determination of the thermodynamic parameters ($N$, $K$, $\Delta H$, $\Delta S$) in our experiments. The usual binding curves plotted in kilocalories per mole of injectant versus the molar ratio (mol of Ca/mol of protein) are presented in this study as kilocalories per mole of injectant versus the mass ratio (g of Ca/g of protein). This presentation is more appropriate due to the complex mixture of soy proteins.

Similar experiments of ITC were scaled up for whole protein fraction (NH or H soy protein) and followed with Ca ion selective electrode (Ca ISE, Sentek, Braintree, U.K.) and Nanosizer ZS (HPPS 5001, Malvern Instrument Ltd., Worcestershire, U.K.) to determine the ionized calcium and the electrophoretic mobility variations upon CC titration.

**Ionized Ca Variations upon pH Cycle.** *Ca Supplementation.* After 5 min of system stabilization, NH or H soy protein dispersions were supplemented with 25 mmol of CC/kg of dispersion ($CaCl_2 \cdot 2H_2O$, analytical grade powder, Carl Roth GmbH, Karlsruhe, Germany) to reach a Ca concentration level similar to that found in cow's milk (30 mmol/kg). CC was chosen for its high solubility in water and its common use. Ca-unsupplemented soy protein dispersions constituted the references.

*pH Cycle Procedure.* After a Ca equilibrium period (2 h), soy protein dispersions were subjected to pH cycle, using a titrator (Multiburet

**Table 1.** Titration Parameters for the Study of Calcium−Soy Proteins Interactions in the Whole and in the Soluble Protein Fraction (NH, Nonhydrolyzed; H, Hydrolyzed)

| sample | $T_{exptl}$ (°C) | [CC] (mM) | no. of injections | titration |
|---|---|---|---|---|
| whole fraction | | | | |
| NH soy protein | 25 | 25 | 32 | 2 $\mu$L × 5 and 5 $\mu$L × 27 |
| H soy protein | 25 | 25 | 32 | 2 $\mu$L × 5 and 5 $\mu$L × 27 |
| soluble fraction | 4 | 5 | 50 | 2 $\mu$L × 5 and 5 $\mu$L × 45 |
| NH soy protein | 25 | 5 | 50 | 2 $\mu$L × 5 and 5 $\mu$L × 45 |
| | 50 | 5 | 50 | 2 $\mu$L × 5 and 5 $\mu$L × 45 |
| | 4 | 20 | 50 | 2 $\mu$L × 5 and 5 $\mu$L × 45 |
| H soy protein | 25 | 20 | 40 | 2 $\mu$L × 5 and 5 $\mu$L × 35 |
| | 50 | 25 | 50 | 2 $\mu$L × 5 and 5 $\mu$L × 45 |

2S, Crison, La Souterraine, France). The thermostated reactor ($T$ = 25 °C) was equipped with a four-bladed 90° impeller (R 100 impeller: 6 cm diameter) and rotated at 150 rpm (Lightnin LabMaster Mixer). Acidification was performed by addition of 1 M HCl in soy protein dispersions until a minimal pH value ($pH_{min}$) of 3.5 was reached, which was chosen below the isoelectrical pH of soy proteins ($pH_i$ 4.5). Directly after acidification, neutralization was performed by addition of 1 M NaOH. Acid and base were both added to dispersions at a rate of 0.25 mL/min. pH cycle was stopped when the initial pH of soy protein dispersions (7.1−7.2) was reached. At the end of each step of the kinetic ($T_1$ = system stabilization, $T_2$ = Ca equilibrium period, $T_3$ = acidification, and $T_4$ = alkalinization), soy protein dispersions were sampled for further analysis. Each kinetic was duplicated.

*Experimental Setup*. The whole kinetic was followed by pH-meter (Radiometer analytical, Remiremont, France) and Ca ISE (Sentek). The continuous monitoring of pH and $Ca^{2+}$ was performed using a data logger (Almemo 8990-8 V5, Ahlborn, Holzkirchen, Germany) and its adapted software (AMR WinControl for Almemo, Akrobit, Gera, Germany). $Ca^{2+}$ concentration was determined throughout the pH cycle, taking into account the dilution effect due to acid and base addition and the volume of sample collected for further analysis. Ca ISE was calibrated daily at 25 °C. This experimental setup and Ca ISE calibration were described previously (*16*).

**Physicochemical and Structural Characterization of Soy Protein Dispersions.** *Apparent Viscosity ($\eta_{app}$) Measurement*. The experiments in reactor were scaled down for $\eta_{app}$ determination using a stress-controlled StressTech rheometer (Rheologica, Scheelevägen, Sweden). $\eta_{app}$ was measured with a custom-built four pales right angle paddle geometry adapted to particulate system. The 100 $s^{-1}$ shear rate remained constant throughout the experiments. $\eta_{app}$ of soy protein dispersions was followed throughout the pH cycle. During the first 5 min, $\eta_{app}$ was measured on unsupplemented soy protein dispersions. Then, dispersions were supplemented with CC, and after 5 min of Ca equilibrium period, pH cycle began. Data acquisition frequency was set to 60 s. Each kinetic was duplicated.

*Particle Size ($D_{4,3}$) Measurement*. The size distribution of particles present in soy protein dispersions was determined by static light scattering (SLS), using a Mastersizer S granulometer (Malvern Instrument Ltd., Worcestershire, U.K.). A He/Ne laser (5 mW power; $\lambda$ = 632.8 nm) passed through a 0.5 mm width measurement cell, into which the mixed dispersion was injected. The light scattered by the sample was converged by a reverse Fourier 300 mm focusing lens (0.45 mm focal distance) and directed onto 42 photodiodes localized at various angles. Volume diameter ($D_{4,3}$) measurements were carried out for CC-supplemented and pH-cycled soy protein dispersions at the end of each step of the kinetic ($T_1$, $T_2$, $T_3$, and $T_4$). Samples of soy protein dispersion diluted in Milli-Q water at ambient temperature were introduced in the small volume dispersion unit to reach an obscuration value comprised within 10−15%. The refractive index was set at 1.5295 for proteins and at 1.33 for the solvent (*17*). The data acquisition was made by Malvern software (Sizer Sv2.17). Each experiment was duplicated.

*Electrophoretic Mobility ($\mu_E$) Measurement*. $\mu_E$ measurements were performed using a Nanosizer ZS (HPPS 5001, Malvern instrument Ltd.) by means of laser Doppler electrophoresis. The sample was put into a standard capillary electrophoresis cell equipped with gold electrodes. The measurement was performed at 25 °C. $\mu_E$ measurement is related to the global electrical charges of the particles in motion in an aqueous medium and is commonly used as an indicator of dispersion stability.

$\mu_E$ was measured in pH-cycled soy protein dispersions, for initial pH and each pH unit throughout the pH cycle (pH 6.5, 5.5, 4.5, 3.5), during acidification and back neutralization. $\mu_E$ was also determined in soy protein dispersions after CC supplementation. For such measurements, samples were diluted in filtered Milli-Q water (pore size = 0.22 $\mu$m, Millex GP, Millipore, Carrigtwohill, Cork, Ireland). Electrophoretic mobility units ($\mu m \cdot cm \cdot s^{-1} \cdot V^{-1}$) will be referred to as emu in the following.

## RESULTS AND DISCUSSION

As a mineral essential for life, Ca supplementation of soy milk constitutes a nutritional alternative to cow's milk. Yet, soy protein aggregation is induced unless carefully controlled conditions are maintained. The aim of this work was to understand mechanisms occurring upon Ca-induced soy protein aggregation and to characterize the Ca−soy protein interactions involved by means of two complementary scientific approaches: ITC and pH cycle studies. A better understanding is needed for further use of soy protein aggregates as a possible vector of Ca.

**Thermodynamic Characterization of Ca−Soy Protein Interactions.** *Whole Soy Protein Fraction*. The binding titration curves of whole soy protein dispersions by CC are shown in **Figure 1a**. Binding of $Ca^{2+}$ to soy proteins was an endothermic process ($\Delta H > 0$). On the contrary, the dilution enthalpy of CC (25 mM) in Milli-Q water was negative and consequently exothermic ($\Delta H < 0$; result not shown). This reference signal increased with CC concentration as shown previously (*18*) but was much smaller in magnitude than the CC−soy protein complexation enthalpy. Moreover, the increase of ionic strength due to calcium supplementation was taken into consideration in the enthalpy measured through the reference experiment subtraction. In this range of concentration, the heat of dilution of soy protein dispersion was almost constant and could be neglected compared to the heat of titration of CC into soy protein dispersion.

Upon CC titration, the binding enthalpy decreased for NH and H soy protein dispersions to reach saturation (**Figure 1a**). Whatever the soy protein dispersion, the binding titration curve was fitted by a one set of sites model, which means that any number of sites $N$ have the same binding constant value ($K$, $M^{-1}$) and the same enthalpy ($\Delta H$, kcal/mol of injectant). Several studies previously characterized Ca−soy protein interactions and also concluded that Ca binding sites onto soy protein were equivalent and independent (*19*−*23*).

The binding titration curves varied with the nature of soy proteins studied (NH or H). Once the saturation was reached (plateau value), the mass ratio of Ca bound to soy proteins (g of Ca/g of proteins) was determined at half-inflection point. NH soy proteins saturated at much higher Ca loads than H soy proteins. The mean value of calcium ions bound to soy proteins is presented in **Table 2** and compared with that in other investigations on Ca−soy protein interactions. The value of calcium ions bound to NH soy proteins (52 mg of Ca/g of protein) was higher than the values reported in the literature except the results of Kumagai et al. (*22*), which were higher. On the other hand, results of calcium ions bound to H soy proteins (2 mg of Ca/g of protein) were within the range of values commonly mentioned for NH soy proteins. These differences in the amount of Ca bound onto soy proteins could have several explanations such as the kind of soy proteins studied (11S or 7S globulins or whole soy protein fraction), the content in native or aggregated forms, and the method of sample preparation (i.e., pH of extraction, ionic strength, Ca and phytate deionization procedure, use of ultraturrax and high pressure in this study). Also, the number of Ca binding sites may depend on the degree of dissociation of soy globulins and on any conformational changes accompanying the dissociation. Therefore, different methods of protein preparation may account for the difference in the number of calcium ions bound to soy proteins. As well, differences in the methods of measuring $Ca^{2+}$ binding (i.e., ITC, Ca ISE, gel filtration, dialysis, etc.) may account for large variation in the quantification of Ca−soy protein interactions. The huge difference observed with the study of Kumagai and co-workers (*22*) comes from the fact that they evaluated the Ca bound to one amino acid of 140 Da on average. In this case, each carboxylic acid function in $C_\alpha$ can react under ionized form $COO^-$with $Ca^{2+}$. In the case of proteins, this carboxylic acid function is involved in an amide bound and consequently less available for interactions with calcium ions.

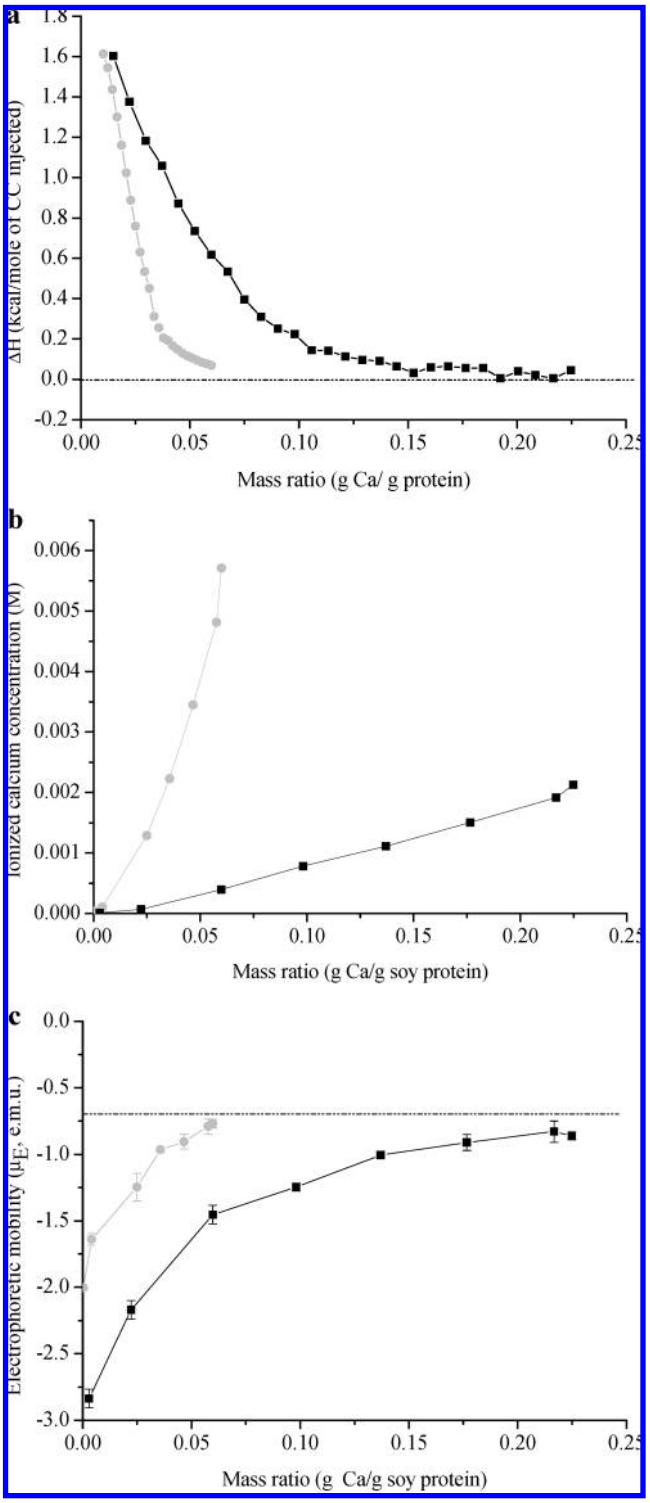


**Figure 1.** Binding titration curve of soy protein dispersion by CC (25 mM, **a**), ionized calcium concentration (M, **b**), and electrophoretic mobility variations (emu, **c**) as a function of mass ratio (g of Ca/ g of soy protein): black squares, nonhydrolyzed; gray circles, hydrolyzed. *T*, 25 °C; initial pH, 6.17 and 6.21; final pH, 5.32 and 5.57, for NH and H soy protein dispersions, respectively.

Comparison of the binding constant value ($K$, $M^{-1}$) for H and NH soy proteins showed that H soy proteins had a higher binding constant affinity for Ca (stronger slope $K$, **Figure 1a**). Hence, H soy proteins bind more easily calcium ions than the NH soy proteins, which may be related to fewer conformational modifications and structural rearrangements upon CC titration than in NH soy proteins. Hence, Ca accessibility is favored into the Ca binding sites.

The ionized calcium concentration ($M$), determined on similar titration experiments than ITC, was set out as a function of the mass ratio (g of Ca/g of soy proteins, **Figure 1b**). Upon CC titration, the ionized calcium concentration increased in soy protein dispersions and was higher in the H soy protein dispersion as compared to the NH one (**Figure 1b**). These variations in ionized calcium concentration were indirectly related to Ca binding onto soy proteins. More Ca−soy protein interactions occurred in NH soy proteins as compared to H soy proteins, despite the same concentration in each acid amino acid able to bind calcium ions in both SPIs. In accordance with ITC results, H soy proteins are saturated at lower mass ratio (g of Ca bound/g of protein) than NH soy proteins (**Figure 1a**,**b**). Hence, soy protein hydrolysis may involve modification of the environment of the Ca-binding sites, involving distinct aspects of protein struture, which would be then unfavorable for Ca fixation onto the target amino acid (lower $N$ value for H soy proteins). On the other hand, such modification would facilitate Ca binding onto the remaining Ca binding sites and may explain the higher $K$ value obtained for H soy proteins. According to Ca ISE results, the enthalpy drop observed in ITC experiment (**Figure 1a**) was related to the decrease of the amount of $Ca^{2+}$ bound to soy proteins (similar to ref (*24*)).

To evaluate the contribution of electrostatic interactions on Ca−soy protein interactions, Ca binding titration was performed in the same conditions as during the ITC experiment while followed by electrophoretic measurements. The variations of electrophoretic mobility (emu) are plotted as a function of mass ratio (g of Ca/g of soy protein, **Figure 1c**). The electrophoretic mobility measured in soy protein dispersions increased upon CC titration from −2.8 and −2.0 emu for NH and H soy proteins, respectively, and reached the same plateau value at about −0.8 emu for both dispersions saturated in Ca. These electrophoretic mobility variations, indicative of electrostatic interactions upon Ca binding onto soy proteins, could partially explain the fact that NH soy proteins bound more calcium ions than the H ones. This result was unexpected because electrostatic attractive interactions are reported to be highly exothermic (*18*). Hence, the exothermic part of the thermodynamic signal involved by electrostatic interactions was completely hidden by the strong endothermic signal (**Figure 1a**).

Yet, whatever the soy proteins, the entropy ($T\Delta S$) overcame the binding enthalpy ($\Delta H > 0$; **Table 3**) and kept the $\Delta G$ negative. Hence, Ca binding onto soy protein was entropically driven and occurred spontaneously ($\Delta G < 0$). A positive volume variation observed by dilatometry (*25*) accompanied the aggregation of soy proteins by Ca and was the consequence of the release of ordered water molecules on the soy protein surface at the aggregating sites. Hence, not Coulomb interactions but rather liberation of water molecules, from either the hydration shell of the calcium ions or dehydration of the hydrophobic core of soy proteins, may be the driving energy for the binding of multivalent ions onto soy proteins [similar to Sinn et al. (*18*, *26*)]. In accordance with Kroll (*21*), Ca binding onto soy proteins should be rather described as a counterion $H^+/Ca^{2+}$ exchange, more energetically neutral (less exothermic phenomenon) than the electrostatic forces involved.

*Soluble Soy Protein Fraction*. The effect of temperature on Ca-soluble soy proteins interactions was studied to determine the heat capacity changes ($\Delta C_p$). $\Delta C_p$ is closely related to changes in the exposure of both hydrophobic surfaces and polar residues to water, occurring upon protein (un)folding (*27*−*29*). Yet, contribution of the latter to the $\Delta C_p$ is smaller and additionally can be canceled by the burial of the polar groups in the protein binding center (*30*). $\Delta C_p$ values offer direct information on the extent of

**Table 2.** Stoichiometry of the Calcium−Soy Protein Interaction Determined by ITC: Comparison with Other Investigations

| authors, year | pH | mol of $Ca^{2+}/10^5$ g of protein | mg of Ca/g of protein | sample |
|---|---|---|---|---|
| this study | 6.17 | 129 | 51.7 | NH-SPI[a] |
| | 6.21 | 5.5 | 2.2 | H-SPI[b] |
| Kumagai et al., 1998 | 7.4 | 986 | 394.4 | globulin |
| | | 707 | 282.8 | phytate free globulin |
| Kroll, 1984 | 6−7 | 22.5−25 | 9−10 | whole soy protein |
| Sakakibara and Nogushi, 1977 | 6 | no interaction | no interaction | 11S |
| | 7 | no interaction | no interaction | |
| | 8 | 3.7−11.5 | 1.4−4.6 vs [CC][c] | |
| Appurao and Narasinga Rao, 1976 | 5.5 | negligible | negligible | 7S |
| | 7.8 | 9−10 | 3.6−4 | |
| Appurao and Narasinga Rao, 1975 | 5.5 | negligible | negligible | 11S |
| | 7.8 | 14−17 | 5.6−6.8 | |
| Saio et al., 1967 | | 24 | 9.6 | CIF[d] |

[a] NH-SPI, nonhydrolyzed soy protein isolate. [b] H-SPI, hydrolyzed soy protein isolate. [c] [CC], CC concentration added. [d] CIF, cold insoluble fraction.

**Table 3.** Binding Parameters Obtained after Fitting the Binding Titration Curves of the Soluble Soy Proteins with the One Set of Sites Model (Mean Values of Two Duplicated Experiments)

| sample | $T$ (°C) | $K$ ($M^{-1}$) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal $mol^{-1}$ $K^{-1}$) | $N$ (g of $Ca/10^5$ g of protein) | $N$ (mg of Ca/g of protein) |
|---|---|---|---|---|---|---|
| NH-SPI[a] | 4 | nd[c] | nd | nd | nd | nd |
| | 25 | $7.3 \times 10^{03}$ | 1.341 | 22.2 | 240 | 2.4 |
| | 50 | $7.0 \times 10^{03}$ | 2.772 | 26.2 | 309 | 3.1 |
| H-SPI[b] | 4 | $2.6 \times 10^{03}$ | 0.430 | 17.2 | 1170 | 11.7 |
| | 25 | $1.3 \times 10^{03}$ | 1.356 | 18.8 | 1268 | 12.7 |
| | 50 | $7.6 \times 10^{02}$ | 2.610 | 21.3 | 1607 | 16.1 |

[a] NH-SPI, nonhydrolyzed soy protein isolate. [b] H-SPI, hydrolyzed soy protein isolate. [c] nd, not determined.

conformational changes of proteins (*31*, *32*). A decrease in $\Delta C_p$, observed in protein folding and other self-assembly processes involving proteins (*33*), indicates a decrease in exposure of hydrophobic surfaces (*34*). On the contrary, a positive $\Delta C_p$ is relevant to the protein unfolding. The extensive hydration and a reduction in the number of surface-exposed polar residues can make the $\Delta C_p$ sign positive (*30*, *35*).

The binding titration curves of soluble soy proteins (NH or H) displayed similar trends at various temperatures (4, 25, and 50 °C; results not shown). The curves were fitted with a one set of sites model to obtain the related thermodynamic parameters (**Table 3**). As the experimental data were not perfectly fitted with this former model, the values of $N$, $K$, $\Delta H$, and $\Delta S$ should be rather considered as qualitative values. At higher temperature, enthalpic changes ($\Delta H$) induced by the calcium binding reactions were more endothermic and the stoichiometry values ($N$) increased. The heat capacity changes ($\Delta C_p$) were determined as the slope of the curve in the plot of the binding enthalpy ($\Delta H$, kcal $mol^{-1}$ °$C^{-1}$) versus temperature. $\Delta C_p$ values were positive and were set at 57 and 48 cal $mol^{-1}$ °$C^{-1}$ for NH and H soy proteins, respectively. $\Delta C_p$ determined for NH soy proteins must be handled with care because this value was obtained from two distinct temperatures only. The difference between $\Delta C_p$ values determined for NH and H soy protein was not significant. The positive values of $\Delta C_p$ indicate a reduction in the number of surface-exposed polar residues, which can be related to the burial of charged ligand and protein polar groups inside the binding sites.

*Comparison of Calcium−Proteins Interactions between Soluble and Whole Soy Protein Fraction*. Whatever the soy proteins (NH or H), the soluble fraction showed a lower endothermic signal upon CC titration than the whole soy proteins fraction. However, a different behavior was observed between NH and H soy proteins on the $N$ value measured. For NH soy proteins, fewer interactions were measured between Ca and soluble soy proteins and indicated that this latter fraction was less sensitive to Ca than the NH whole soy protein fraction. On the contrary, the H soluble soy protein fraction bound more Ca than the H whole soy protein fraction. Hence, the soluble peptide released upon hydrolysis might be enriched in Ca binding sites. H soluble soy protein would be an efficient vector for further Ca supplementation.

**Ca Equilibrium between Soluble and Colloidal Phase in Soy Proteins Dispersions.** *$Ca^{2+}$ Variations during pH Cycle*. Ionized calcium concentration was plotted as a function of pH and compared for NH and H soy protein dispersions, CC-supplemented and subjected to pH cycle down to 3.5 (**Figure 2**). Whatever the soy protein dispersion, the initial $Ca^{2+}$ concentration measured ($T_1$) was almost negligible before CC supplementation. After CC supplementation, the $Ca^{2+}$ concentration increased in the soluble phase due to CC solubilization into $Ca^{2+}$ and $Cl^-$. Yet, only 15 mM $Ca^{2+}$ for NH soy protein and slightly more for H soy proteins (∼16 mM) were measured in comparison to the 25 mM CC added ($T_2$). This difference could not be explained by a partial solubilization of CC, due to the high solubility of this Ca salt (100 g $L^{-1}$ at 20 °C for $CaCl_2 \cdot 2H_2O$). Instead, $Ca^{2+}$ interactions either with anions of the soluble phase (i.e., phosphate anions) or with soy proteins should rather be expected. Upon acidification from initial pH to about pH 4.0, near the $pH_i$ value of soy proteins commonly reported in the literature (*36*, *37*), soy proteins initially negatively charged underwent a diminution of the number of charges, which involved the release of $Ca^{2+}$ previously captured upon CC supplementation. Consequently, $Ca^{2+}$ concentration increased to reach a maximum value at about pH ∼4.0, set at 22 and 25 mM $Ca^{2+}$ for NH and H soy proteins dispersions, respectively. Hence, one part of supplemented Ca seems irreversibly bound to NH soy proteins. From $pH_i$ to pH 3.5 ($T_3$), soy proteins are globally positively charged and the ionized calcium concentration decrease could possibly be explained by the calcium ion interaction with anions contained in the soluble phase (i.e., citrate, phosphate, etc.). Upon neutralization ($T_3-T_4$), inverse phenomena occurred and ionized calcium concentration curves were

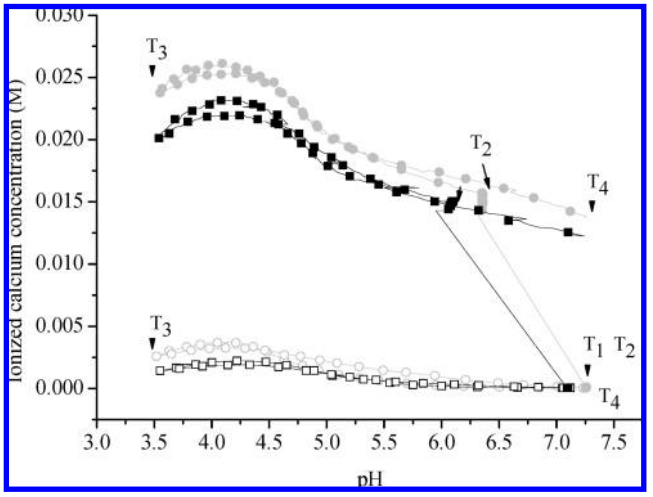


**Figure 2.** Ionized calcium concentration (M) as a function of pH: comparison of NH and H soy protein dispersions, CC-supplemented (25 mmol/kg), and subjected to pH cycle ($pH_{min}$ 3.5, $T_{exptl}$ = 25 °C). $T_1$ = probe stabilization, $T_2$ = calcium equilibrium period, $T_3$ = acidification, $T_4$ = alkalinization. Squares: NH soy protein dispersion; circles, H soy protein dispersions; open symbols, no CC supplementation; solid symbols, CC supplementation.

almost superimposable. Above $pH_i$, proteins became more and more negatively charged and were then able to bind $Ca^{2+}$, which decreased in the soluble phase.

$Ca^{2+}$ variations were almost similar for both references and for both CC-supplemented soy protein dispersions (NH or H). However, significantly higher values were measured for H soy protein dispersions, in reference and in CC supplemented dispersions. These $Ca^{2+}$ profiles could be related to the buffering capacity variations (results not shown). The higher the $Ca^{2+}$ concentration, the higher was the buffering capacity during the pH cycle. This implies that $Ca^{2+}$ solubilized from soy proteins was replaced by protons (acting then on the buffering capacity) and confirms the ITC hypothesis. Ca binding onto soy proteins can be described as a counterion $Ca^{2+}/H^+$ exchange, rather than solely electrostatic interactions. Moreover, the higher buffering capacity for H soy protein dispersions, in reference and in CC-supplemented dispersion, as compared to NH soy proteins, is in accordance with the higher $K$ value determined by ITC.

From these results, soy proteins can be considered as a Ca "pump", which can easily release or absorb Ca as a function of pH variations.

A scheme of Ca equilibrium in soy protein dispersion during pH cycle is proposed in **Figure 3**. Similarly to the salt equilibrium proposed by Brulé (*38*) in cow's milk, Ca equilibrium exists in soy protein dispersion between $Ca^{2+}$, the anions contained in the soluble phase, and the soy proteins considered as charged entities. Indeed, phosphate mineral is present in the soluble phase of reconstituted soy protein dispersions. Nevertheless, in soy protein dispersions, phosphate exists in the majority under phytate form. Moreover, acid amino acids (contained in high proportion in SPI) have a p$K$ value of around 4.5−4.7. Hence, for pH higher than p$K$, aspartic acid and glutamic acid are negatively charged and can interact with $Ca^{2+}$.

To summarize, above $pH_i$, Ca is in equilibrium both with the proteins as a charged entity and with the free anions as well. Below $pH_i$, Ca is in equilibrium only with anions contained in the soluble phase or indirectly with soy proteins through anion bridges. The maximum $Ca^{2+}$ concentration is measured at about the $pH_i$ region.

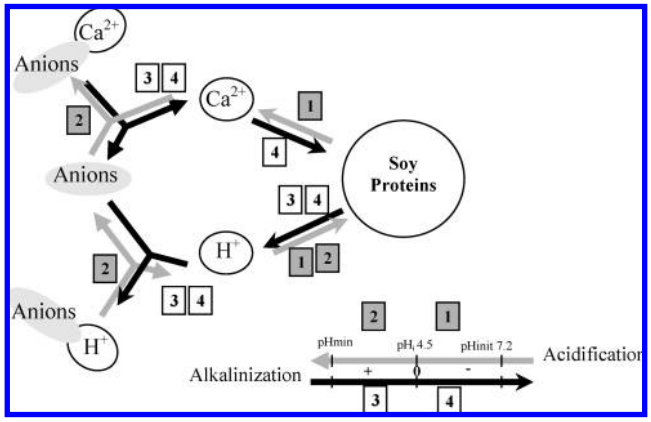


**Figure 3.** Schematic presentation of calcium equilibrium in pH cycled soy protein dispersion.

*Physicochemical and Structural Characterization of the Protein Colloidal Phase during pH Cycle.*

*Apparent Viscosity Variations ($\eta_{app}$).* For reference NH soy protein dispersion ($T_1$ identical to $T_2$), acidification from initial pH ($T_1$) down to pH 3.5 ($T_3$) showed two opposite trends (**Figure 4a**). First, $\eta_{app}$ increased to a maximal value corresponding to $pH_i$ then decreased to pH 3.5 ($T_3$). Upon acidification ($T_1-T_3$), the $\eta_{app}$ increase was related to an increase in the particle size (result not shown). Around the protein $pH_i$ region, where the global charge of protein is null, attractive interactions predominated between soy proteins, inducing their aggregation with a maximum $\eta_{app}$ measured. Below $pH_i$, aggregates were solubilized with a decrease of $\eta_{app}$; yet, $\eta_{app}$ measured at $T_3$ was higher than that at $T_2$. During alkalinization ($T_3-T_4$) an inverse phenomenon was observed in accordance with electrophoretic mobility variations reported hereafter (**Table 4**), yet with lower variations in $\eta_{app}$ than during acidification ($T_2-T_3$). A slight difference was measured between the initial (4.5 mPa·s, $T_1$) and the final $\eta_{app}$ (8 mPa·s, $T_4$), reflecting the persistence of aggregated proteins in the system. $\eta_{app}$ variations were in accordance with particle size measurements, which was partially reversible upon pH cycle ($T_1-T_4$; results not shown).

In CC-supplemented NH soy protein dispersion, CC addition induced an $\eta_{app}$ increase from 4.5 mPa·s ($T_1$) to 23 mPa·s ($T_2$), related to the Ca-induced soy protein aggregation (*6*, *37*, *39*−*42*). Indeed, upon CC supplementation, the $D_{4,3}$ irreversibly increased from about 50 $\mu$m ($T_1$) to about 240 $\mu$m ($T_2$) for NH soy protein dispersion (results not shown). Upon pH cycle ($T_2-T_4$), the same overall trends were observed in CC-supplemented sample as in the corresponding reference. However, CC-supplemented soy protein dispersion appeared more viscous over the range $pH_i$−pH 3.5 ($T_3$) than the reference, suggesting the involvement of more interactions between molecular species once CC supplementation was made. At the end of the pH cycle ($T_4$), $\eta_{app}$ was higher than in reference (17 and 8 mPa·s, respectively).

For reference H soy protein dispersion (**Figure 4b**), the initial $\eta_{app}$ was 3.7 mPa·s ($T_1 = T_2$). Upon pH cycle, the same $\eta_{app}$ variations as in NH soy protein dispersion were observed but the fluctuations were smaller. $D_{4,3}$ increased with acidification ($T_2-T_3$, acid-induced aggregation) and was partially reversible upon alkalinization ($T_3-T_4$, results not shown). The final $\eta_{app}$ measured after the pH cycle ($T_4$) was 7.1 mPa·s. These $\eta_{app}$ variations indicated that the protein−protein interactions formed during acidification ($T_2-T_3$) were not fully dissociated during back alkalinization ($T_3-T_4$).

As previously observed for NH soy protein dispersion, CC supplementation induced a rapid increase of $\eta_{app}$ of H soy protein

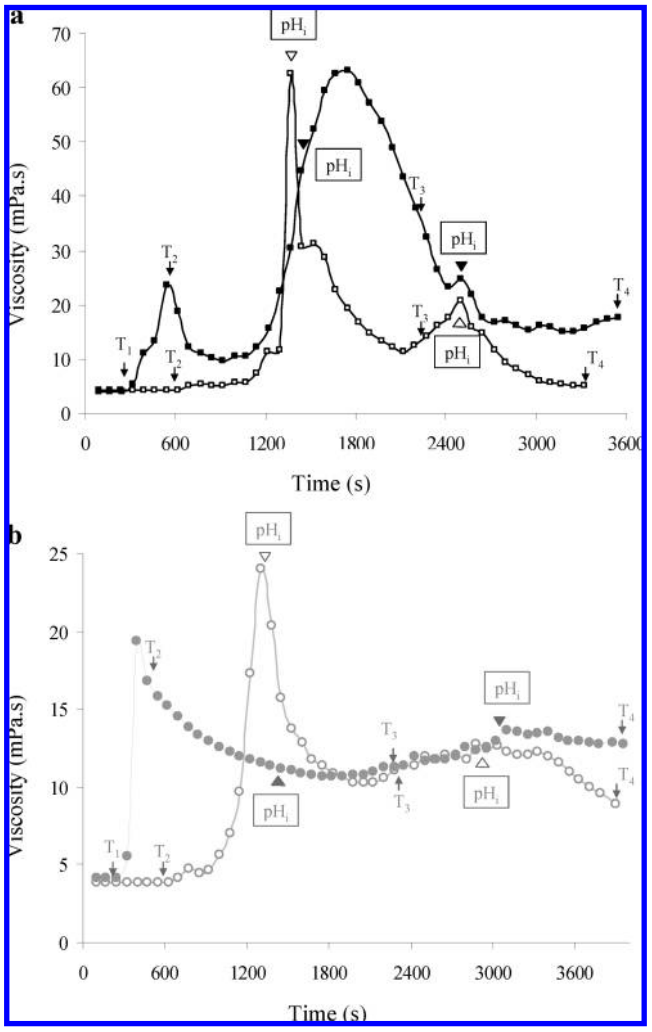


**Figure 4.** Apparent viscosity measured at a shear rate of 100 $s^{-1}$ in reference and CC-supplemented NH and H-soy protein dispersion (25 mmol/kg), subjected to pH cycle ($pH_{min}$ 3.5; $T_{exptl}$ = 25 °C) as a function of time. $T_1$ = end of probe stabilization, $T_2$ = end of calcium equilibrium period, $T_3$ = end of acidification ($pH_{min}$ 3.5), $T_4$ = end of alkalinization (pH 7.1−7.2). $pH_i$ 4.8 and 4.2 for NH and H soy protein dispersion, respectively. Squares, NH soy protein dispersion (**a**); circles, H soy protein dispersions (**b**); open symbols, no CC supplementation; solid symbols, CC supplementation.

**Table 4.** Electrophoretic Mobility Values ($\mu_E$) for Reference NH and H Soy Protein Dispersions Subjected to pH Cycle (0 mmol of CC/kg, $pH_{min}$ 3.5, $T_{exptl}$ = 25 °C)

| | NH soy protein dispersion | | | H soy protein dispersion | |
|---|---|---|---|---|---|
| pH | $\mu_E$ (emu) | $\sigma$ (emu) | pH | $\mu_E$ (emu) | $\sigma$ (emu) |
| 7.1 | −3.2 | 0.015 | 7.24 | −3.2 | 0.059 |
| 6.35 | −2.7 | 0.046 | 6.53 | −2.8 | 0.032 |
| 5.5 | −1.3 | 0.194 | 5.54 | −1.8 | 0.036 |
| 4.5 | 0.3 | 0.054 | 4.5 | −0.3 | 0.048 |
| 3.5 | 2.7 | 0.049 | 3.5 | 0.9 | 0.039 |
| 4.5 | −0.1 | 0.035 | 4.5 | −0.2 | 0.011 |
| 5.5 | −2.3 | 0.136 | 5.5 | −1.7 | 0.100 |
| 6.5 | −2.4 | 0.155 | 6.5 | −2.2 | 0.055 |
| 7.1 | −3.6 | 0.170 | 7.24 | −2.7 | 0.139 |

dispersion from 3.7 mPa·s ($T_1$) to 18 mPa·s ($T_2$) (**Figure 4b**). This $\eta_{app}$ change was related to the thickening of dispersion due to Ca-induced soy protein aggregation. Upon CC supplementation, $D_{4,3}$ irreversibly increased from about from 20 $\mu$m ($T_1$) to about 40 $\mu$m ($T_2$) for H soy protein dispersion (results not shown). The effect of pH cycle ($T_2-T_4$) had less influence on $\eta_{app}$ variations as compared to the CC supplementation effect ($T_1-T_2$). Because the initial $\eta_{app}$ was not restored ($T_1/T_4$), irreversible $Ca^{2+}$−soy protein interactions were expected.

*Electrophoretic Mobility Measurements*. $\mu_E$ values are shown in **Table 4** for NH and H soy protein dispersions subjected to pH cycle to 3.5. $\mu_E$ was negative at initial pH of soy protein dispersions (7.1−7.2, $T_1$): −3.2 and −3.2 emu for NH and H soy protein dispersions, respectively. $\mu_E$ increased with acidification (i.e., became less negative), to reach a zero value around the $pH_i$ of soy proteins (∼4.5), which was experimentally determined at pH 4.8 and 4.2 for NH and H soy protein dispersions, respectively. For pH values lower than $pH_i$, $\mu_E$ became positive. At the end of acidification ($pH_{min}$ 3.5, $T_3$), NH and H soy protein dispersions had $\mu_E$ values of 2.7 and 0.9 emu, respectively. After a whole pH cycle ($T_4$), $\mu_E$ was shifted from −3.2 to −3.6 emu for NH soy protein dispersion and from −3.2 to −2.7 emu for H soy protein dispersion. These shifts may be partly due to the changes in ionic strength due to acid and base addition. $\mu_E$ values were also measured for NH and H soy protein dispersions, after CC supplementation (results not shown). Whatever the soy protein dispersion, the $\mu_E$ value increase (∼ +2 emu) upon CC supplementation ($T_1-T_2$) indicated $Ca^{2+}$−soy protein interactions of electrostatic nature, with the neutralization of negative protein charges by $Ca^{2+}$. This $\mu_E$ variation could explain the Ca-induced aggregation of soy protein dispersions as explained hereafter.

*Relationship between $Ca^{2+}$ Variations and Soy Protein Phase Behavior as a Function of pH Cycle*. All of the studied parameters ($[Ca^{2+}]$, $\eta_{app}$, $\mu_E$) showed two distinct behaviors during acidification and the reverse events upon alkalinization, when the $pH_i$ region was traversed. For reference soy protein dispersions, with acidification from initial pH ($T_1$) to $pH_i$, $Ca^{2+}$ concentration increased (endogenous Ca release from soy proteins) due to a diminution of $\mu_E$ of soy protein ($\mu_E$ became less negative) and $\eta_{app}$ increased. The maximum value of each parameter was reached in the $pH_i$ region, where the $\mu_E$ value was null and the maximum protein aggregation occurred. With acidification from $pH_i$ to pH 3.5 ($T_3$), $Ca^{2+}$ decreased in the soluble phase. Because soy proteins were positively charged below $pH_i$, $Ca^{2+}$ would rather be chelated with anions present in the soluble phase and/or linked to soy proteins through anion bridges. Reverse phenomena occurred upon alkalinization as described further. From pH 3.5 ($T_3$) to $pH_i$, $Ca^{2+}$ increased due to its solubilization from anions. In the meantime, to counteract alkalinization and in relation to their buffering properties, soy proteins released protons and became less and less positively charged. Due to the diminution of their ionization state, soy protein interactions increased and led to an elevation of $\eta_{app}$ to reach a maximum at $pH_i$. From $pH_i$ to initial pH of soy protein dispersion ($T_4$), $Ca^{2+}$ reintegrated soy protein aggregates. Because $\eta_{app}$ decreased, we could expect a restructuring and a redispersion of these soy protein aggregates. pH cycle to 3.5 was not reversible.

With CC supplementation ($T_1-T_2$), $D_{4,3}$ irreversibly increased whatever the soy protein dispersion, with a Ca-induced aggregation higher in NH than in H soy protein dispersion. This result would indicate a higher sensitivity of NH soy proteins for $Ca^{2+}$ than H ones (in accordance with ITC results). Once CC supplementation had been made, the formed Ca−soy proteins aggregates showed only few variations in size, whereas other parameters such as $Ca^{2+}$ concentration and $\eta_{app}$ varied throughout the pH cycle ($T_2-T_4$).

To conclude, in the absence of CC supplementation and whatever soy protein dispersion, the phenomena observed were

partially irreversible for pH cycle to 3.5 ($pH < pH_i$). Whatever the soy protein dispersion, NH or H, Ca-induced aggregation was irreversible upon pH cycle ($pH_{min}$ 3.5).

To gain a deeper understanding of calcium−soy protein interactions, other physicochemical parameters could be studied with ITC, such as ionic strength and pH, while the experiments were performed in buffer media.

## ABBREVIATIONS USED

NH, nonhydrolyzed; H, hydrolyzed; Ca, calcium; SHMP, sodium hexametaphosphate; ITC, isothermal titration calorimetry; SPI, soy protein isolate; CC, calcium chloride; Ca ISE, calcium ion selective electrode; $pH_i$, isoelectrical pH; $\eta_{app}$, apparent viscosity; $D_{4,3}$, particle size; $\mu_E$, electrophoretic mobility.

## ACKNOWLEDGMENT

We are indebted to the laboratory of RNA Maturation and Molecular Enzymology (MAEM, Nancy University, Faculty of Sciences) for ITC experiments. We thank Maria-Virginia Munoz for the achievement of rheometry experiments.

**Supporting Information Available:** Amino acid content of nonhydrolyzed and hydrolyzed soy protein isolates. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Received February 6, 2009. Revised manuscript received April 28, 2009. We are indebted to Unilever Research and Development center of Vlaardingen (The Netherlands) for their financial support.**