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Effect of pH and Ca^{2+}-Induced Associations of Soybean Proteins

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An experimental procedure was developed to characterize the solubility of the soybean protein fractions close to the isoelectric point. The results show that the 7S fraction is precipitated in a much narrower range of pH values than the 11S fraction. Surprisingly, the addition of salt to the solutions leads to increased solubility of proteins, unlike the common "salting out" effect generally expected for proteins in solution in this range of salt concentrations. The precipitation equilibria of both soybean fractions in the presence of calcium ions and electrolyte were characterized. The amount of calcium ions required to precipitate a mole of the 7S fraction is much larger than that required for the 11S fractions. The precipitation pattern can be correlated to the charge density per surface area of the proteins.

KEYWORDS: Soybean proteins; precipitation; equilibrium; pH; calcium; electric charge

INTRODUCTION

Soybean, *Glycine max*, originated in Eastern Asia, and records of human consumption date back to 30 BC in Chinese writings. It was not until after World War II that effort was put into making soybean protein widely recognized as food in western culture because of an increasing concern over a shortage of protein worldwide (*1*). Soybean proteins are valued not only for their nutritional quality but also the functional properties they contribute to food systems. Among these attributes are water holding, oil binding, and emulsifying properties.

The seed proteins of legumes, including soybeans, are albumins and globulins. Globulins, the dominant storage proteins, account for about 50−90% of seed protein. Storage globulins are grouped into two types according to their sedimentation coefficients: 7S globulins (vicilin, 7.1−8.7S) and 11S globulins (legumin, 10.1−14S) (*2*). The ratio of 11S to 7S globulins varies among cultivars.

The 7S fraction is classified into three major fractions with different physicochemical properties, designated β-conglycinin, γ-conglycinin, and basic 7S globulin. β-Conglycinin is the most prevalent of these three. γ-Conglycinin and basic 7S globulin account for less than a few percent. β-Conglycinin is a trimer with a molecular mass of 150−200 kDa. As a constituent subunit, four subunits are identified: three major subunits α (68 kDa), α' (72 kDa), and β (52 kDa), and one minor subunit γ similar in size to that of β subunit. 11S is a hexamer with a molecular mass of 300−380 kDa. As a constituent subunit, five subunits are identified: $A_{1a}B_{1b}(G_1)$, $A_2B_{1a}(G_2)$, $A_{1b}B_2(G_3)$, $A_5A_4B_3(G_4)$, and $A_3B_4(G_5)$. Each subunit is composed of an acidic polypeptide and a basic polypeptide. The acidic and basic polypeptides are linked together by a disulfide bond. Both trimer and hexamer are formed under the action of electrostatic and hydrophobic forces.

Glycinin and β-conglycinin are the two major storage proteins in the soybean. These two proteins differ greatly in molecular weight, amino acid make up, surface characteristics, and isoelectric points. It has been suggested that each of the two proteins contributes different qualities to the functional properties of the soy proteins (*3*). Their contributions to properties such as emulsification and foaming are playing an increasing role in food formulations as the food industry moves toward creating more market-driven consumer products. Both economics and consumer attitudes have driven manufacturers to use plant proteins, especially soy, to replace animal proteins, such as milk and egg proteins. It is the objective of the present work to study the potential of the soybean proteins on the basis of the interactions between the soy proteins in solution. It is these interactions, such as electrostatic, van der Waals, hydrophobic, and steric, that determine the structure and properties of gels, precipitates, and emulsions, and hence the quality of the final products.

EXPERIMENTAL PROCEDURES

Electrolyte and protein solutions were prepared by dissolving preweighed quantities of the appropriate salts and the dry protein powder in Milli-Q purified water with resistance greater than 18 MΩ. A protein concentration of 1 mg/mL was used in all experiments. All salts were analytical grade and were obtained from Fisher Scientific or Sigma-Aldrich. The pH was measured using a Cole-Parmer mV/°C/pH meter.

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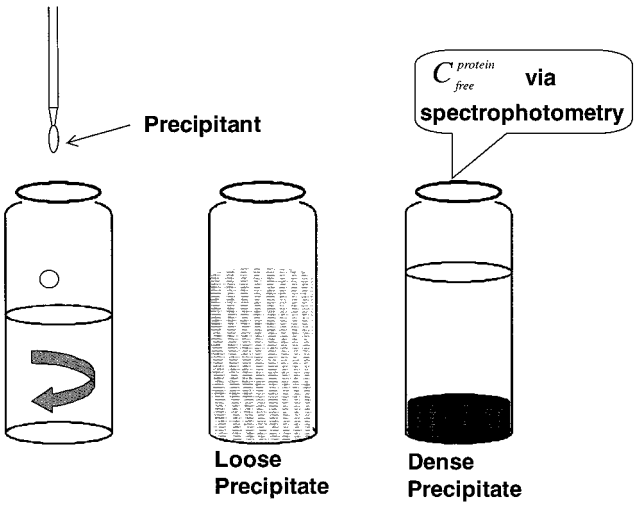

Figure 1. Schematics of the precipitation equilibrium experiment.

Table 1. Nomenclature of Chemical Compounds

entity	free	required to precipitate one mole protein	total added
calcium	C_{free}^{Ca}	$C_{Re'd}^{Ca}$	C_{total}^{Ca}
protein	$C_{free}^{protein}$	$C_{Re'd}^{protein}$	$C_{total}^{protein}$

TRIS (pH 7.5, 25 mM) was used to minimize pH variations during electrophoresis. The neutral marker was prepared as a 0.05% mesityl oxide solution in water.

The methodology developed and applied to study precipitation is simple and robust. A schematic representation is given in **Figure 1**. The soybean proteins were precipitated by the addition of Ca^{2+}, or by adjusting pH. The protein solution was separated into 10−15 volumetric vials (15 mL), and an incrementally increasing amount of precipitation agent was added to each vial. Precipitation usually occurred within a few seconds to minutes, and the milky solution was then rocked for 30 min in order to allow equilibrium conditions to be reached and the precipitate to form. After the solution was centrifuged, the top supernatant was analyzed by spectrophotometry at 280 nm to determine the amount of remaining proteins in the solution.

METHODOLOGY: PHASE EQUILIBRIUM

The precipitation behavior of the protein can be described by the concept of solubility product expression. Assuming weak ion binding, this concept suggests the formation of a stoichiometric complex of limited, but not very low, solubility. The complex starts precipitating when its solubility limit is exceeded and stops precipitating when all of the protein is converted into the complex:

$$\text{Protein} + n\text{Ca}^{2+} \leftrightarrow \text{Protein} \times (\text{Ca}^{2+})_n \qquad (1)$$

The notation used in the formulas is summarized in **Table 1**. As most of the Ca^{2+} exists in an unbound state, an additional approximation that simplifies the mathematical expressions is that the molecular fraction of the calcium bound and precipitated with the protein is much lower than that of the free ions in the solution.

Assuming that the precipitate described in eq 1 is a single solubility product, with n the number of bound Ca^{2+} ions in the complex, eq 2 expresses the relation between the added Ca^{2+} and the residual protein in the solution.

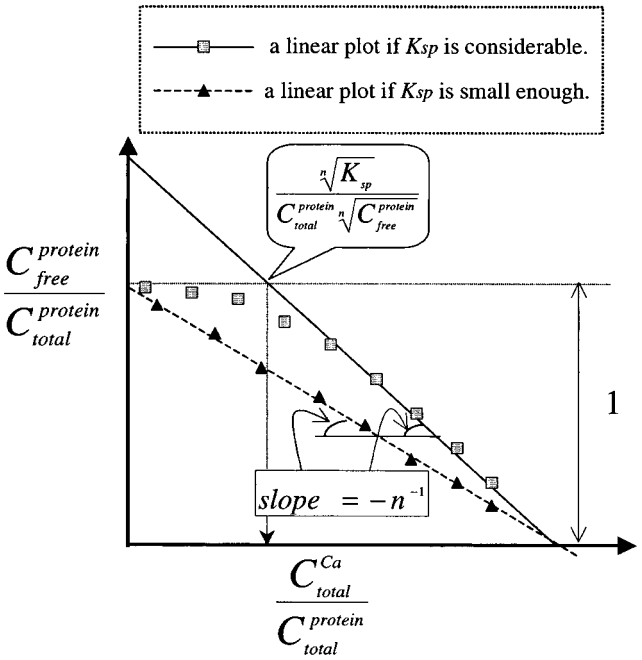

Figure 2. Explanation of the simple model used for the data fitting.

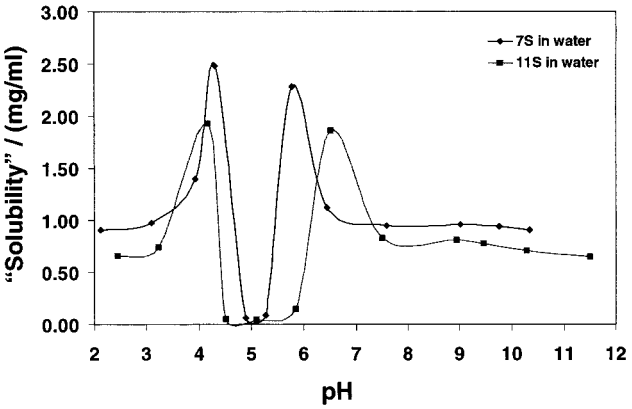

Figure 3. Solubility of soybean proteins determined by A_{280}.

$$\text{If } K_{sp} = C_{free}^{protein}(C_{free}^{Ca})^n \text{ and } n = \frac{C_{\text{Re'}d}^{Ca}}{C_{\text{Re'}d}^{protein}}:$$

There are two possible plots as shown in **Figure 2**, corresponding to eq 2. It seems reasonable to expect that the solubility product equilibrium constant (K_{sp}) for the reaction should be small compared with the total concentration of the protein ($C_{total}^{protein}$). An approximate equation is given:

$$\therefore \frac{C_{free}^{protein}}{C_{total}^{protein}} = -\frac{C_{total}^{Ca}}{nC_{total}^{protein}} + 1 + \frac{\sqrt[n]{K_{sp}}}{nC_{total}^{protein}\sqrt[n]{C_{free}^{protein}}} \qquad (2)$$

$$\text{If } \frac{\sqrt[n]{K_{sp}}}{nC_{total}^{protein}\sqrt[n]{C_{free}^{protein}}} \approx 0: \quad \frac{C_{free}^{protein}}{C_{total}^{protein}} = -\frac{C_{total}^{Ca}}{nC_{total}^{protein}} + 1 \qquad (3)$$

It shows a linear dependence and an intercept of the extrapolated line on the Y-axis. The slope of the line should be approximately equal to $-n^{-1}$, and the number of Ca^{2+} ions bound in the complex can be estimated, as shown in **Figure 2**. As most of the Ca^{2+} exists in an unbound state (i.e., K_{sp} is considerable larger), there is an induction period before proteins start to

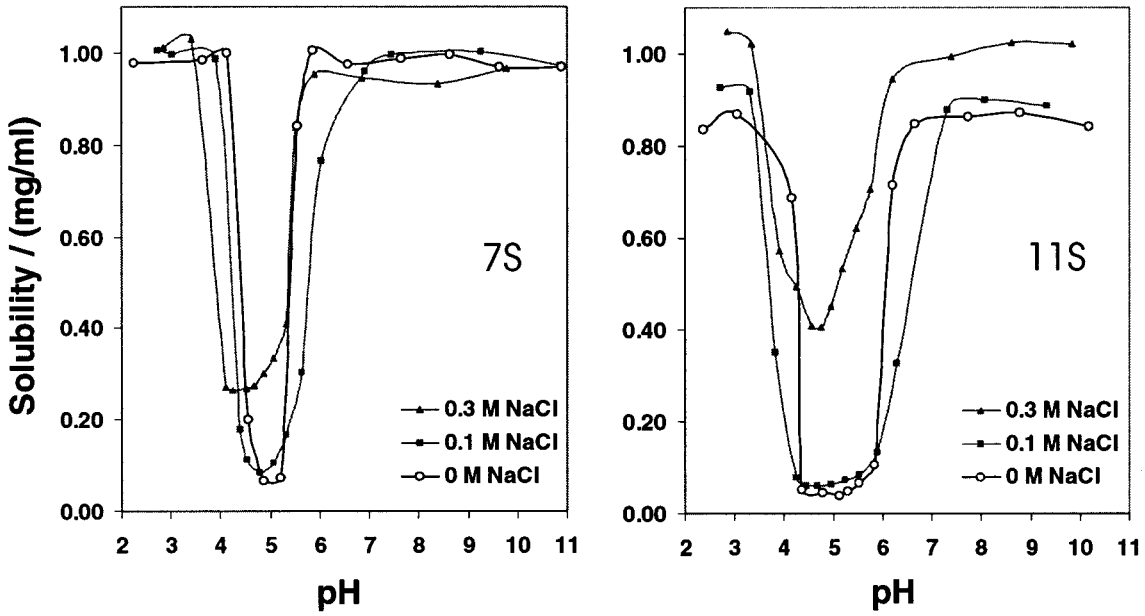

Figure 4. Effect of pH and salt concentration on the soybean protein fractions' solubility close to the isoelectric point.

precipitate. It caused the pattern of change of a new parameter obtained from the fit of the data, the intercept between the fitted line and the constant value of $C_{free}^{protein}/C_{total}^{protein} = 1$. This "precipitation threshold" value is an equivalent of the dimensionless concentration of the [protein × $(Ca^{2+})_n$] complex at which the precipitation begins.

RESULTS AND DISCUSSION

The phase equilibria in protein solutions and, in particular, the precipitation by various agents plays a significant role in the food and pharmaceutical industries. Understanding the physical basis of the processes that take place during precipitation, and theoretical modeling of the precipitation equilibria, are required in order to optimize the separation conditions and to decrease the amount of precipitant needed.

Effect of pH. Because the dispersion/aggregation state of soybean proteins depends on the environmental conditions, the pH was varied from 3 to 12, and the influence of such changes on the apparent "solubility" was analyzed by spectrophotometry. Results so obtained are shown in **Figure 3**. As the pH approached the isoelectric point, the aggregation of the soybean proteins resulted in the precipitation due to interaction between protein molecules. The electrostatic charge can be used to predict soybean protein dispersion and association. The pH is the major parameter that affects the peptide charge. As a consequence, proteins precipitate at their isoelectric points. Far from the isoelectric point, the protein molecules disperse in a solution because of electrostatic repulsion. The results show that the 7S globulin is precipitated in a much narrower interval of pH values than 11S globulin, because of the greater van der Waals and hydrophobic forces among 11S globulin molecules. The greater the attractive interaction forces are, the smaller is the solubility. Surprisingly, the addition of salt to the solutions leads to increased solubility of the proteins as indicated in **Figure 4**, unlike the common "salting out" effect generally expected for the proteins in solution. Similar shapes of the solubility profiles of curd and soy protein isolate were seen by Shen (*4*), but of different magnitude due to determination of soluble proteins by Biuret analyses. This "salting in" effect is due to thermodynamic linkage between the free energy of salt binding and solubility of the soybean proteins, however, it is usually found at NaCl concentrations that are at least an order of magnitude lower than the ones used in this study.

Effect of Ca^{2+}. The structure of soybean 7S and 11S globulins is very compact at pH 7.6 (*5*, *6*). The investigation of Ca^{2+}-induced associations of soybean proteins was carried out in this pH range to eliminate dissociations of globulins' subunits. The ions, such as Ca^{2+} or Na^{+}, will neutralize the electric charges by physical attraction if a protein molecule carries negative charges. This will result in two processes. The attractive interactions between molecules induce the protein aggregation, whereas the hydration repulsion further enhances the protein solubility due to an electrical double layer. As discussed in the Phase Equilibrium section, the number of Ca^{2+} ions needed to precipitate soybean proteins can be quantified by a simple model. **Figures 5** and **6** show the results of the experiments on Ca^{2+}-induced precipitations of soybean proteins in the absence and presence of NaCl, respectively. The precipitation begins after some initial threshold concentration of the Ca^{2+} ions is reached. As suggested by the theory described in Phase Equilibrium, this initial concentration is strongly dependent on an individual protein, becoming higher for higher solubility. The precipitation follows a decay curve that levels off at a plateau region before most of the soy protein is precipitated. The amount of residual nonprecipitated protein is also strongly dependent on salt and increases with increasing salt. 7S has a longer "induction" period due to its high solubility, compared to that of 11S. The numbers of Ca^{2+} ions needed to precipitate a mole of protein are 164 for 7S and 79 for 11S in water.

The ionic strength is an important factor in soybean protein associations, because of a "salting in" effect. Similarly to the isoelectric precipitation data, the addition of NaCl to the solutions suppresses the effect of Ca^{2+}. As shown in **Figure 7**, a much larger amount of precipitant is required and the solubility of the protein in the plateau region is higher when NaCl is added. The numbers of Ca^{2+} ions needed are changed because of the double electrical layer formed by Na^{+} addition, from 164 to 1000 for 7S and from 79 to 435 for 11S as indicated in **Figure 6**. The difference between the parameters of Ca^{2+} ion-induced precipitation of the two proteins can be explained in two alternative ways. First, it may be possible that Ca^{2+} cross-links

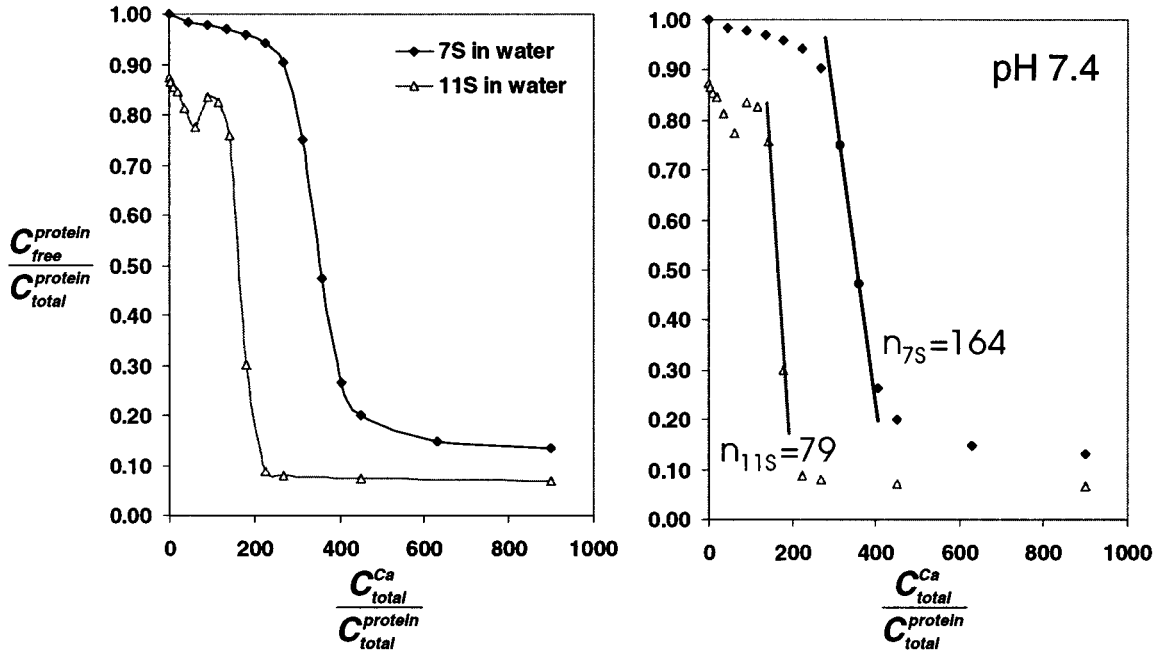

Figure 5. Data on Ca^{2+}-induced precipitation of soybean proteins in the absence of NaCl.

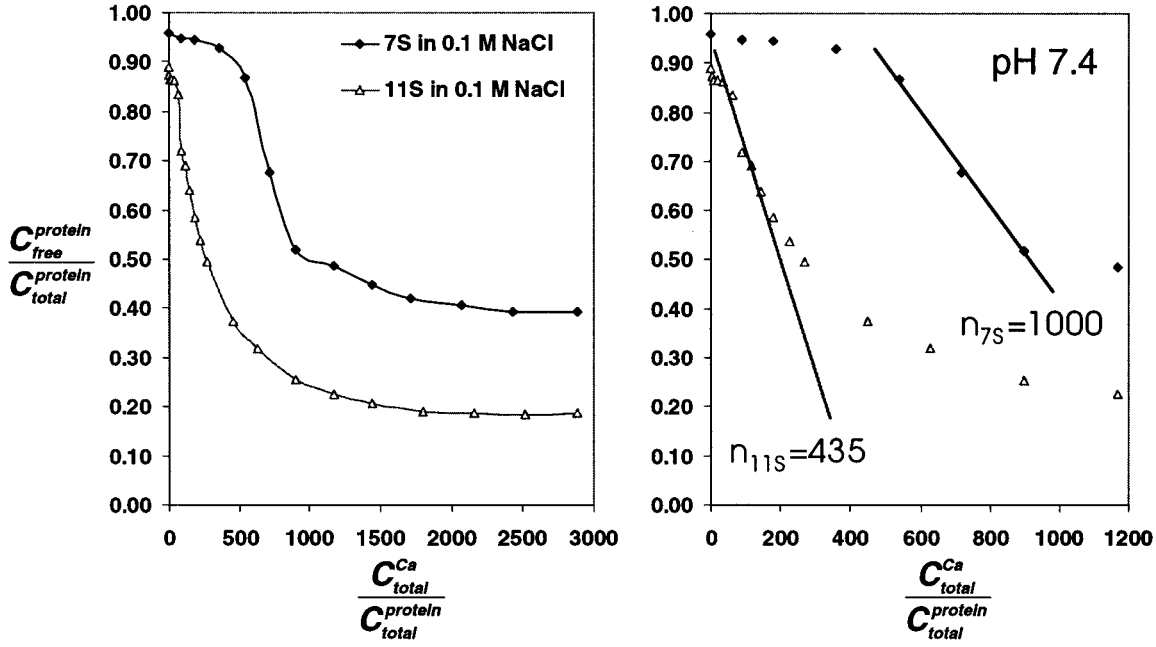

Figure 6. Effect of NaCl on Ca^{2+}-induced precipitation of soybean proteins.

the 11S protein molecules by binding at specific sites, in which case only a certain number of divalent ion bridges will be enough to precipitate the molecules in a network. The van der Waals attraction between such 11S networks will lead to easy aggregation and precipitation as observed experimentally. In contrast, 7S may form a smaller number of cross-links, smaller aggregates, and be less prone to precipitation by attractive van der Waals forces. Second, the observed sequential changes in 11S solubility in the "induction" period, which occur with increasing Ca^{2+} concentration, are also thermodynamically linked to Ca^{2+} binding. A similar finding involved an initial "salting out" and followed by a partial "salting in" was evidenced in calcium-induced associations of the caseins (*7*). The model may account for the stoichiometry only in the precipitation stage, that is, the number *n* may reflect only the number of additionally adsorbed Ca^{2+} ions to precipitate the previously formed protein$-Na^{+}$ complex.

The data obtained on the precipitation of soy proteins with weakly binding Ca^{2+} ions allows optimization of the separation process by choosing the appropriate amount of precipitant required to maximize the effect of the precipitant and to achieve the steady-state separation state. As illustrated in **Figure 7** as dotted lines, it shows the comparison of Ca^{2+}-induced precipitation of the 7S and 11S fractions procedure for quantitative. The different amounts of Ca^{2+} ions shown are those required to precipitate the same amount of the 7S and 11S fraction. That means that the method of Ca^{2+}-induced associations of soybean proteins is commercially viable to separate the two globulins or to make a mixture of two globulins by adjusting the addition of Ca^{2+}.

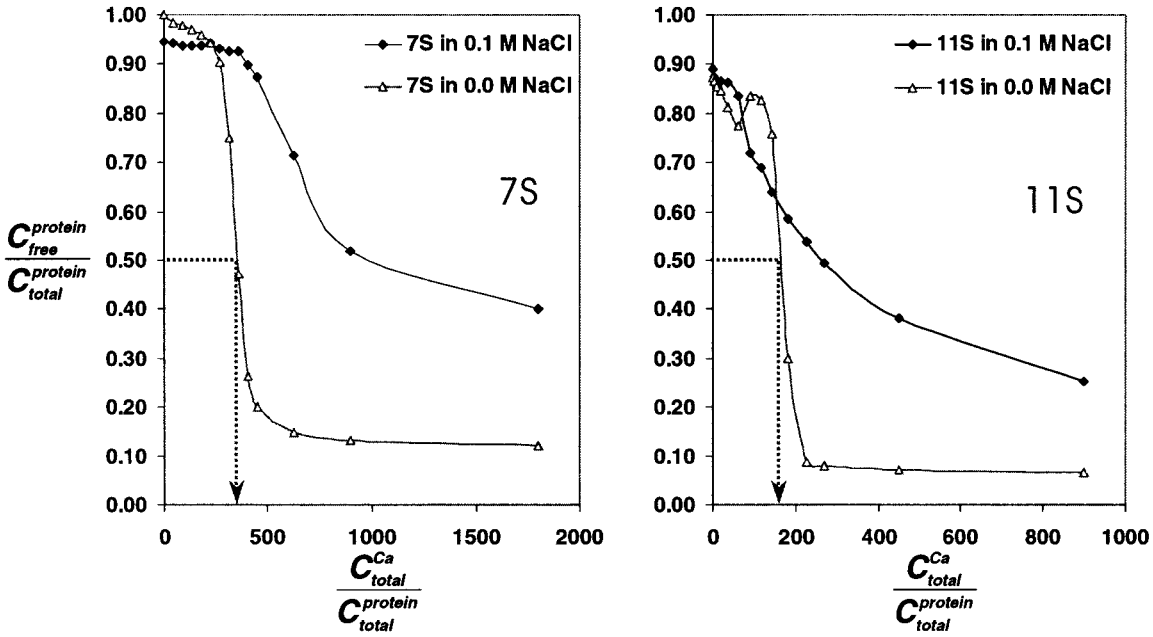

Figure 7. Comparison of NaCl effect on Ca^{2+}-induced separations.

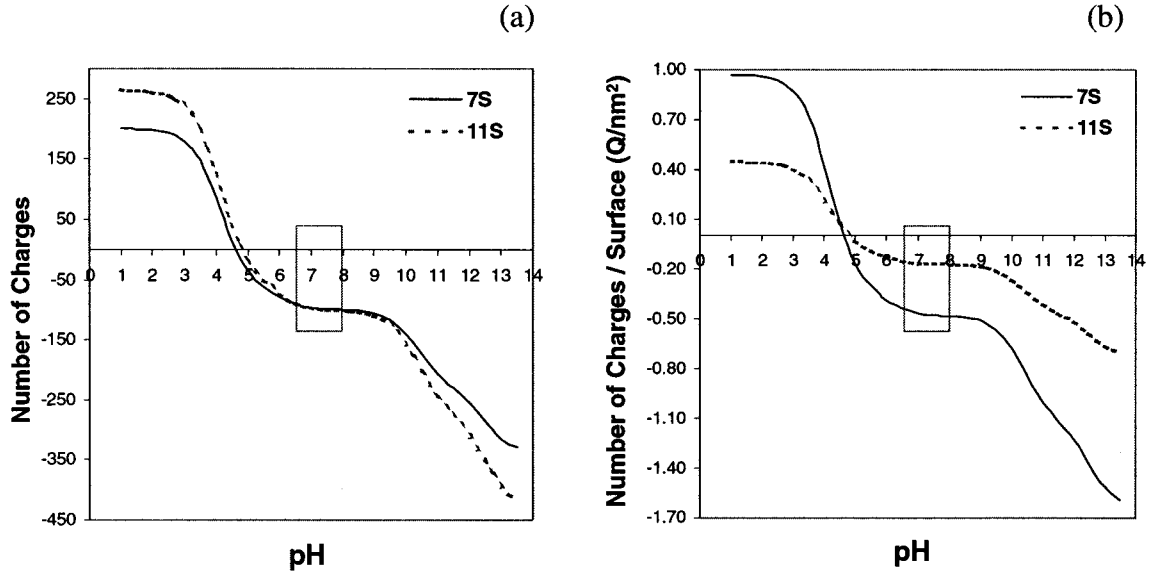

Figure 8. Comparison of the calculated charge (a) and charge density (b) of the 7S and 11S fractions.

Use of the Protein Charge Density as a Qualitative Predictor. Quantitatively, the number of Ca^{2+} ions needed can be directly correlated to the protein charge. However, the ratio of Ca^{2+} needed to precipitate the same amounts of 7S to 11S is 2.1 and 2.3 in the absence and presence of NaCl, respectively. The calculated charge on the 7S and 11S fractions is shown in **Figure 8a**. The charge is calculated from the net charge of all the polar amino acid residues in the protein at different pHs. It is seen that in the pH range of 6.5 to 8 the protein charge for both proteins changes relatively little and is on the order of 100 charges/molecule. The experimental values of *n*, however, vary from 79 to 164 for 11S and 7S in water, respectively. The inadequate amount for complete charge neutralization might result from the attractive interactions via van der Waals forces, as the 11S globulin is much bigger.

The data can be correlated and expressed via the surface charge density of the protein molecules. **Figure 8b** shows surface charge density vs pH for 7S and 11S. Obviously, the surface charge densities are significantly different for 7S and 11S, especially between pH 7 and 8. This might result in the different associations by Ca^{2+}-induced charge neutralizing. Surprisingly, the Ca^{2+} increment ratios of 7S and 11S in both absence and presence of NaCl are very close to the ratio of the charge density as illustrated in the last row of **Table 2**. The actual protein charge density will be further investigated by capillary electrophoresis to confirm that the hypothesis can be used as a predictor of Ca^{2+}-induced separation.

Table 2. Results from the Fitted and Calculated Data

	surface (nm^2)	charge (e^-)	charge density (e^-/nm^2)	*n* (Ca^{2+})	
7S	206	–98	–0.47	164(H_2O)	1000(NaCl)
11S	592	–99	–0.17	79(H_2O)	435(NaCl)
ratio	0.35	1	2.8	2.1	2.3

CONCLUSIONS

A simple theoretical relationship between protein solubility and calcium concentration is developed to explain the solubility characteristics. The precipitation of soybean proteins 7S and 11S was characterized as a function of pH and Ca^{2+}. The number of Ca^{2+} ions required to precipitate a molecule of 7S is much higher than that required for 11S. The solubility of soybean proteins increases with increasing salt concentration especially higher at pI with NaCl. And also, both proteins need more Ca^{2+} to precipitate when in the presence of NaCl, due to a "salting in" effect.

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