Binding of Ca(II) by the 11S Fraction of Soybean Proteins

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ABSTRACT

Binding of Ca(II) by the 11S fraction of soybean proteins was determined by equilibrium dialysis at pH 7.8 and 5.5. The binding was negligible at pH 5.5. At pH 7.8 binding was decreased by the addition of 0.5M NaCl. Prior treatment with EDTA also reduced the binding. Analysis of the binding data with the Scatchard equation suggested that the probable binding site on the protein molecules was the imidazole group. Addition of Ca(II) did not cause association or dissociation of the protein. Heat coagulation was increased by the addition of Ca(II). The protein was almost quantitatively precipitated at 1.0×10^{-2}M Ca(II). This precipitation was decreased by the addition of NaCl. Binding studies with the unfractionated soybean proteins indicated that Ca(II) was bound by the proteins as well as the phytate impurities. When these were removed Ca(II) appeared to be bound by the proteins at the imidazole group.

Soybean proteins consist mainly of four fractions which are usually designated as 2S, 7S, 11S, and 15S, based on their sedimentation coefficient (1). The 11S fraction can be preferentially precipitated with Ca(II) and the supernatant can be used for the preparation of the 7S fraction (2). It was therefore of interest to us to determine the binding of Ca(II) by the various fractions and to determine the

effect of such binding on their physico-chemical properties. In this communication we report the results of such a study on the 11S fraction. For comparison, binding studies with the unfractionated soybean proteins have also been made. In this case the impurities in the protein preparation such as phytate also appear to bind Ca(II). Saio et al. have studied protein-calcium-phytic acid relationships in soybean proteins (3,4).

MATERIALS AND METHODS

Improved Pelican variety soybeans were used in this investigation. The dehulled seeds were defatted by extraction six times with hexane. The defatted meal was dried in air, powdered, and passed through a 60-mesh sieve. Calcium-45 in the form of calcium chloride was purchased from Bhaba Atomic Research Center, Bombay.

Methods

Unfractionated soybean proteins were prepared by stirring defatted soybean flour for 30 min. with water or 1M NaCl using a meal to solvent ratio of 1:10. The insoluble residue was removed by centrifugation at 6,000 r.p.m. for 20 min. This solution was then dialyzed against the appropriate buffer for 24 hr.

Phytate impurities were removed from the unfractionated proteins by the method of McKinney et al. (5) using ammonium sulfate for precipitating the proteins.

Preparation of 11S Fraction

This was prepared by the method of Wolf et al. (6). The homogeneity of the preparation was tested by ultracentrifugation and polyacrylamide gel electrophoresis. The ultracentrifuge pattern revealed the presence of 15S and 7S components whose proportion did not amount to more than 10% of the total.

Protein Concentration

This was determined by measuring the absorbance of the protein solution at 280 nm. For converting absorbance to concentration the following values were used for $E^{1\%}_{280\ nm.}$; unfractionated soybean proteins, 11.8; unfractionated soybean proteins after removal of phytate impurities, 8.0; 11S fraction, 9.2. These values were determined by using a series of solutions of known protein concentration which was estimated by micro-Kjeldahl nitrogen method and by the use of a factor of 6.25 to convert nitrogen to protein.

Equilibrium Dialysis

Two or 5.0 ml. of protein solution in the appropriate buffer was dialyzed for a period of 40 hr. at 30° C. against 5.0 or 10.0 ml. of buffer solution containing various amounts of Ca(II). Corresponding "blanks" containing only the buffer solution were also run. At the end of the equilibration period, the concentration of Ca(II) in the outside solutions was estimated; the inside protein solution could not be used since the protein was found to quench the scintillation. From the difference in the Ca(II) concentration of the "blank" and experimental, the number of Ca(II) ions bound by 100,000 g. of protein was calculated.

Estimation of Calcium

Cold calcium chloride solution was mixed with Ca-45. The concentration of this was determined by titration with standard EDTA solution. Solutions of known concentration were prepared from this stock solution. The radioactivity of these solutions was determined by counting with a Beckman liquid scintillation counter, LS 100. The composition of the scintillation liquid was: 200 mg. POPOP in 100 ml. absolute methanol, 20 ml. ethylene-glycol, 4 g. PPO, and 60 g. naphthalene made to 1 liter with dioxane. The efficiency of the counting was 90%. From the calibration curve of c.p.m. vs. concentration, the concentration of the unknown was read. No decay corrections were applied since a calibration curve was prepared for each set of experiments.

Carbohydrate Estimation

The method of Montgomery (7) was used. Aliquots of 2.0 ml. of 0.3% protein solution were used.

Phosphorus Estimation

Phosphorus was estimated by the method of Taussky and Shorr (8), on 0.2 ml. of 1.0% protein solution.

Ultracentrifugation

The experiments were made at room temperature (25°), using a Spinco Model E analytical ultracentrifuge fitted with phase plate Schlieren optics and RTIC unit. The measurements were made using 1% protein solution. The $S_{20,w}$ values were calculated by the standard procedure (9).

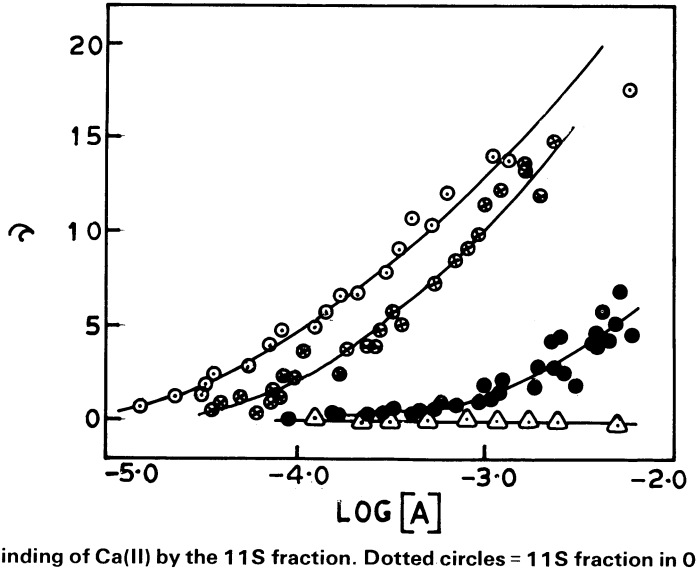

Fig. 1. Binding of Ca(II) by the 11S fraction. Dotted circles = 11S fraction in 0.1M borate buffer of pH.7.8; x'd circles = 11S fraction after EDTA treatment in 0.1M borate buffer of pH 7.8; solid circles = 11S fraction in 0.1M borate buffer of pH 7.8 containing 0.5M NaCl; dotted triangles = 11S fraction in 0.1M acetate buffer of pH 5.5 containing 0.5M NaCl.

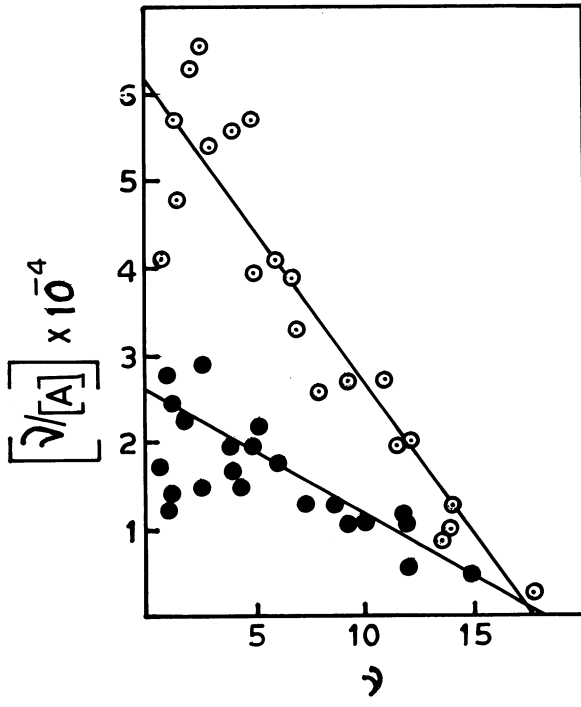

Fig. 2. Scatchard plot of the binding data. Dotted circles = 11S fraction in 0.1M borate buffer of pH 7.8; solid circles = 11S fraction after EDTA treatment in 0.1M borate buffer of pH 7.8.

Heat Coagulation

Five milliliters 0.5% protein solution in borate buffer was heated for 15 min. at various temperatures from 30° to 97° C. For each temperature a separate aliquot was used. At the end of 15 min. the solution was cooled to room temperature. Since the precipitate could not be sedimented by centrifugation even at 18,000 r.p.m., the turbidity was measured to determine the extent of coagulation. This was determined by measuring transmittance at 540 nm. in a Bausch and Lomb colorimeter; as turbidity increased transmittance decreased. From the measurement a quantity [(To–T/To) × 100] was calculated, where To is the transmittance of unheated solution, and T is that of heated solution. This quantity was taken as a measure of coagulation. No differences in the readings were obtained when the hot or cooled solution was used for measurement.

Precipitation of Proteins with Ca(II)

To 2.0 ml. 1.0% protein solution, in buffer, varying amounts of $CaCl_2$ in buffer solution were added and the final volume was made to 5.0 ml. The solution was maintained at 30° C. for 6 hr. in a constant-temperature water bath. At the end of the period the resulting precipitate was removed by centrifugation at 6,000 r.p.m. for 15 min. The absorbance of the supernatants, after dilution whenever necessary, was read at 280 nm. From the absorbance of the original solution and

that of the supernatant, the percentage of protein precipitated was calculated. The experiments were done with the 11S fraction, and unfractionated soybean proteins before and after removal of phytate impurities.

Other Measurements

The absorbance measurements were made at room temperature with a Carl-Zeiss spectrophotometer. The pH measurements were made with a Radiometer pH meter TTT2.

Reagents

All the reagents used were of analytical reagent grade. The solutions were prepared in deionized water.

RESULTS AND DISCUSSION

Figure 1 gives data on the binding of Ca(II) by the 11S fraction v, the number of Ca(II) ions bound per 100,000 g. protein is plotted against the logarithm of free Ca(II) concentration, (A). The binding was determined at two different pH values, 7.8 (0.1M borate buffer) and 5.5 (0.1M acetate buffer containing 0.5M NaCl). At pH 5.5, the binding was negligible. At pH 7.8 where considerable binding was observed, the addition of NaCl at a concentration of 0.5M decreased the binding. Soybean proteins are known to be associated with metal ions (10). To remove any possible metal ion impurities, especially Ca(II), the 11S fraction was mixed with 5mM EDTA solution and then dialyzed extensively against the borate buffer. Binding studies were made with EDTA-treated 11S fraction also. EDTA treatment reduced the affinity of 11S fraction for Ca(II).

TABLE I. THE VALUES OF n, k, HISTIDINE, PHOSPHORUS, AND CARBOHYDRATE CONTENT

Protein	Buffer	k	n	No. of histidine residues per 100,000 g.	Phosphorus %	Carbohydrate (glucose units) %
11S Fraction	Borate buffer	3.4×10^3	17	17[1]	...	...
11S Fraction	Borate buffer + 0.5M NaCl	0.2×10^3	16	17	...	...
11S Fraction after EDTA treatment	Borate buffer	1.4×10^3	18	17	...	...
Unfractionated soybean proteins	Borate buffer	5.3×10^3	32	14[2]	0.88	2.3
Unfractionated soybean proteins after removal of phytates	Borate buffer	1.6×10^3	14	14	0.10	2.0

[1]Ref. (21).
[2]Ref. (22).

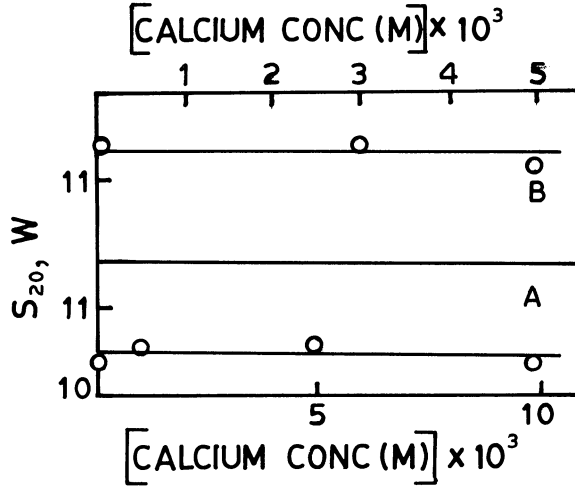

Fig. 3. Variation of $S_{20,w}$ of the 11S fraction with Ca(II) concentration. A = 0.1M borate buffer of pH 7.8 containing 0.5M NaCl; B = 0.1M borate buffer of pH 7.8.

The binding data were analyzed with the Scatchard equation to determine the association constant and the maximum number of ions bound (11). The equation is $\upsilon/A = k(n-\upsilon)$ where k is the association constant and n is the maximum number of ions bound. For a set of equivalent binding sites the plot would be a straight line with the intercept on the abscissa giving n, and the slope giving k. If the binding involves more than one type of binding sites, the plot would be nonlinear.

The Scatchard plots are given in Fig. 2. In spite of the scatter in the experimental points, the binding data could be fitted to a linear plot. By least squaring of the data, values of k and n were obtained and are given in Table I. A linear plot was obtained with the 11S fraction after EDTA treatment also. Whereas the value of n did not appear to change the value of k decreased. The binding values obtained in the presence of 0.5M NaCl (in borate buffer) were low and showed considerable scatter; hence analysis with the Scatchard equation was a little uncertain. However, here also it was observed that n value was nearly the same but k decreased considerably. EDTA treatment reduced the binding affinity by a factor of ~2 and the addition of NaCl reduced it by ~20. Thus it appeared that the binding occurred at the same set of binding sites with the 11S fraction before and after EDTA treatment and also in the presence of NaCl. The 11S fraction did not contain any phosphorus or carbohydrate impurity (Table I) and the binding could be attributed solely to the protein.

The effect of EDTA treatment or the addition of NaCl to the buffer was to reduce the affinity of the protein for Ca(II). Wolf and Briggs (12) have reported that the addition of EDTA to the 11S fraction of soybean protein caused a conformational change probably involving dissociation into subunits. This conformational change could be reversed by the addition of metal ions such as Ca(II), Mg(II), and Ba(II). It was probable that the decreased affinity of the 11S fraction for Ca(II) ions after EDTA treatment was due to this conformational

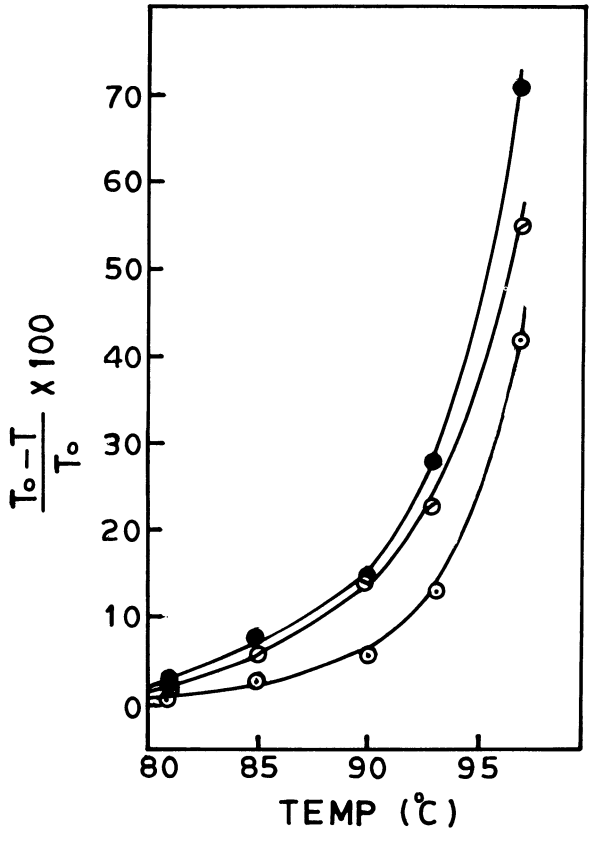

Fig. 4. Heat coagulation of the 11S fraction. Dotted circles = no Ca(II); x'd circles = 4 × 10^{-4}M Ca(II); solid circles = 6 × 10^{-4}M Ca(II).

change. Wolf and Briggs (13) have reported that when the NaCl concentration is increased from 0.1 to 1.0M, the $S_{20,w}$ value of the 11S fraction decreases from 11.1 to 10.5 indicating either a conformational change or dissociation of the 11S fraction. It was therefore probable that the decreased binding of Ca(II) by the 11S fraction on addition of 0.5M NaCl was due to this effect.

The maximum number of Ca(II) ions bound agreed well with the histidine content of the 11S fraction (Table I). Thus the probable binding sites were the imidazole groups of the histidine residues. Ca(II) ions can combine with only the unprotonated imidazole groups. Since the pK value of imidazole groups of the 11S fraction is 7.0 (14), these would be fully protonated at pH 5.5 and thus would be unable to combine with Ca(II). At pH 5.5 there was practically no binding of Ca(II) (Fig. 1). Binding took place only in the pH region where the imidazole groups were able to dissociate.

The effect of Ca(II) on the electrometric titration curve of the 11S fraction was also determined. In the pH region below pH 4.5 (where the carboxylic groups would combine with H^+ ions), the titration curves with and without added Ca(II)

were superposable. However, above pH 5.0, where the dissociation of imidazole group would begin, more hydrogen ions were released in the presence of Ca(II) than in its absence. The extent of this difference increased with the concentration of added Ca(II). Theoretically, the difference in the number of H^+ ions dissociated, in the presence and absence of added Ca(II), should pass through a maximum and again become zero at some pH ~2 pH units above the pK of imidazole groups. However, a straight forward interpretation of the difference curve is difficult because of the fact that around and above neutral pH, Ca(II) ions combine with the hydroxyl groups of the alkali used for the titration. However, the following observations suggested that the probable binding sites were the imidazole groups: a) n agreed with the histidine content of the 11S fraction; b) the titration curve showed differences in the region of imidazole dissociation and c) at pH 5.5 there was practically no binding of Ca(II).

The effect of Ca(II) binding on the physicochemical properties of the 11S fraction was also determined. The 11S fraction undergoes a dissociation reaction which is influenced both by pH and ionic strength (15,16). The $S_{20,w}$ of the 11S fraction was determined in the presence of various concentrations of Ca(II) at pH 7.8 with and without added NaCl. In both the cases the added Ca(II) did not affect the $S_{20,w}$ values (Fig. 3). The $S_{20,w}$ value of the 11S fraction was observed to

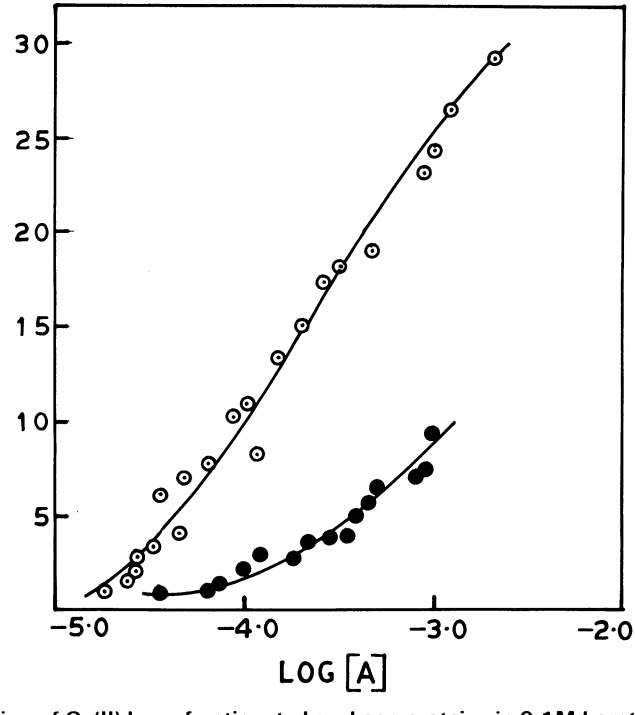

Fig. 5. Binding of Ca(II) by unfractionated soybean proteins in 0.1M borate buffer of pH 7.8. Dotted circles = unfractionated soybean proteins; solid circles = phytate-free unfractionated soybean proteins.

be lower in the buffer containing 0.5M NaCl; this agrees with the results of Wolf and Briggs (13).

The effect of Ca(II) on the heat coagulation of the 11S fraction was studied. The turbidity that developed on heating the 11S fraction could not be removed by centrifugation even at 18,000 r.p.m. Therefore no quantitative estimate of the amount of protein precipitated due to heat coagulation could be made. However, it could be seen qualitatively that the turbidity at any temperature increased with added Ca(II) (Fig. 4). We were interested in determining the temperature corresponding to the midpoint of the curve, if plateau regions could be obtained. However, no such plateau regions could be obtained. Qualitatively it may be concluded that the addition of Ca(II) shifted the equilibrium at any particular temperature in favor of the insoluble form of the 11S fraction. Catsimpoolas et al. (17) have studied the effect of low concentrations of Ca(II), Mg(II), and Al(III) on the thermal coagulation of glycinin (11S fraction) at 90° in phosphate buffer of pH 7.6 containing 0.4M NaCl. The turbidity due to thermal aggregation in the presence of Ca(II) was lower than in the presence of other cations. It is not clear if the effect on thermal coagulation is related to the extent of binding of metal ions by the protein.

The rate of hydrolysis of the 11S fraction by α-chymotrypsin was observed to

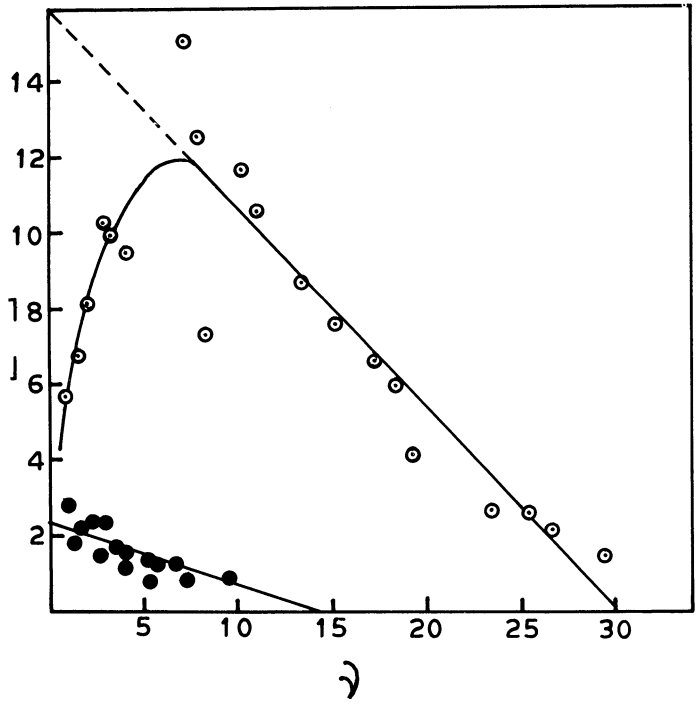

Fig. 6. Scatchard plot of the binding data in 0.1M borate buffer of pH 7.8. Dotted circles = unfractionated soybean proteins; solid circles = phytate-free unfractionated soybean proteins.

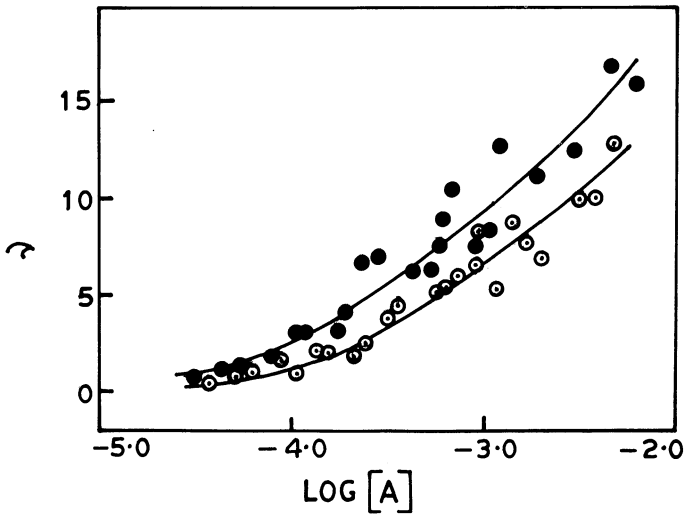

Fig. 7. Binding of Ca(II) in 0.05M *tris*-buffer. Dotted circles =phytate-free unfractionated soybean proteins after EDTA treatment; solid circles = 11S fraction after EDTA treatment.

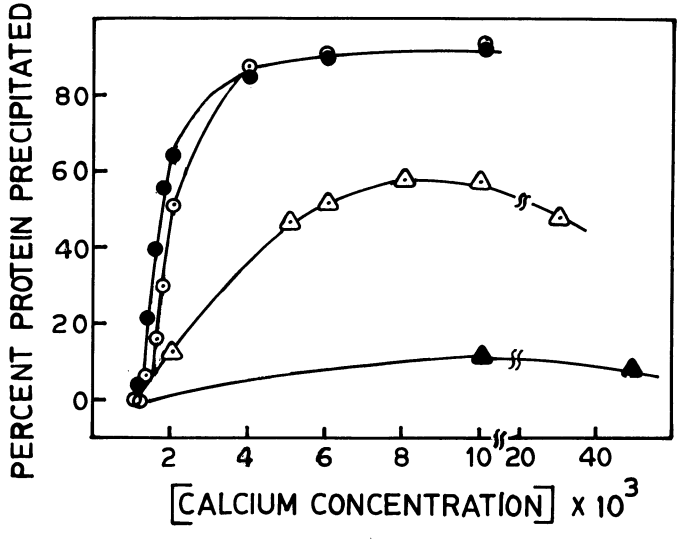

Fig. 8. Precipitation of the 11S fraction by Ca(II) (0.1M borate buffer of pH 7.8). Dotted circles = 11S fraction; solid circles = 11S fraction after EDTA treatment; dotted triangles = 11S fraction before EDTA treatment in buffer containing 0.1M NaCl; solid triangles = 11S fraction before EDTA treatment in buffer containing 0.2M NaCl.

be increased by the addition of Ca(II). Markus (18) has shown that the conformation of the substrate protein may affect the rate of hydrolysis. It would, therefore, be tempting to conclude that Ca(II) changes the conformation of the

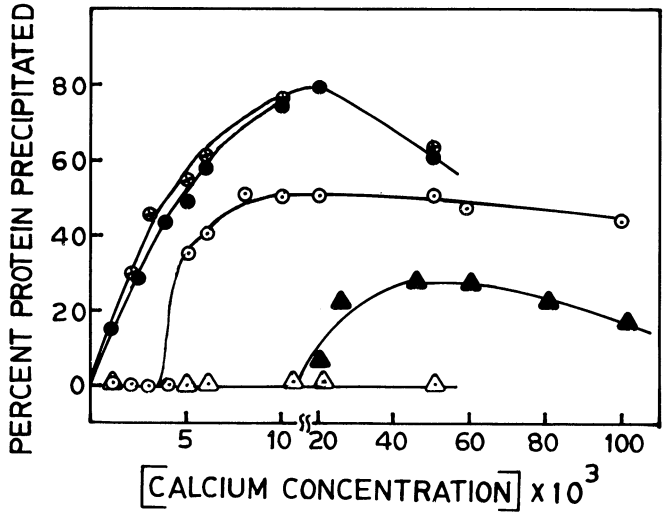

Fig. 9. Precipitation of unfractionated soybean proteins by Ca(II) (0.1M borate buffer of pH 7.8). Dotted circles = unfractionated soybean proteins; solid circles = phytate-free unfractionated soybean proteins; x'd circles = phytate-free unfractionated soybean proteins after EDTA treatment; dotted triangles = phytate-free unfractionated soybean proteins in buffer containing 0.2M NaCl; solid triangles = unfractionated soybean proteins in buffer containing 0.2M NaCl.

11S protein. However, this should be tempered with the known effect of Ca(II) on the activity of α-chymotrypsin (19).

Binding Studies with the Unfractionated Soybean Proteins

The binding of Ca(II) by the unfractionated soybean proteins was also determined in 0.1M borate buffer of pH 7.8 to compare the binding with that of the 11S fraction. However, a difficulty arose in making such a comparison. In Fig. 5, the binding data obtained with the unfractionated protein both before and after removal of phytate impurities are given. The phytate-free proteins bound much less Ca(II). This clearly showed that the Ca(II) ions were bound by the impurities in the unfractionated protein. The Scatchard plots obtained from the binding data also showed striking differences. The Scatchard plot of the phytate-free proteins was linear; however, the phytate-containing proteins gave an abnormal Scatchard plot (Fig. 6). The value of n obtained from the phytate-free proteins corresponded to the histidine content of unfractionated soybean proteins (Table I). On the other hand, the value of n obtained with the phytate-containing proteins was much higher than the histidine content, suggesting that either group other than the imidazole groups of the protein or nonprotein impurities also bound Ca(II).

In addition to the phytate and nucleic acid impurities in the unfractionated soybean proteins, the carbohydrates could also bind Ca(II) under the experimental conditions used. The unfractionated soybean protein contained carbohydrates (Table I). It is known that borate complexes with the carbohydrate (20) to give anionic complexes which may also combine with

Ca(II), because of their negative charge.

To eliminate any complications that may arise from carbohydrate-borate complexes, and phytate and metal ion impurities, the binding data were obtained in 0.05M *tris*-buffer of pH 7.8 with the phytate-free material after EDTA treatment. These data along with the binding data of the 11S fraction, after EDTA treatment in *tris*-buffer are given in Fig. 7. The unfractionated protein bound less Ca(II) than the 11S fraction. The other fractions of unfractionated soybean proteins perhaps bound less Ca(II). It was also possible that some fractions did not bind Ca(II) at all. Preliminary results with the 7S fraction indicate that this fraction binds less Ca(II) than the 11S fraction. The above discussion clearly shows that measurements made on the unfractionated proteins must be interpreted with circumspection.

In the procedure for the preparation of the 7S fraction from the unfractionated proteins, the 11S fraction is preferentially precipitated by the addition of 0.05M Ca(II). This would suggest that the precipitability of the 11S fraction is greater than that of the other fractions. Therefore the precipitation of both the unfractionated proteins and the 11S fraction was measured in 0.1M borate buffer of pH 7.8 at various concentrations of Ca(II). In Fig. 8 the data with the 11S fraction are given. At a concentration of 5.0×10^{-3}M Ca(II), more than 90% of the protein was precipitated. The EDTA-treated protein precipitated to a slightly greater extent than the untreated up to a concentration of 4.0×10^{-3}M Ca(II), and above this the precipitation was practically the same with both the preparations, although the EDTA-treated protein bound less Ca(II) than the untreated. If any quantitative significance could be given to the observed differences, it would suggest that the precipitability is perhaps more related to the conformation than the amount of Ca(II) bound. It was also observed that in the case of the untreated 11S fraction the addition of NaCl to the buffer reduced the precipitation of protein by Ca(II). At 0.2M NaCl concentration there was hardly any precipitation of the protein even at 5.0×10^{-2}M of Ca(II) concentration.

In Fig. 9 the precipitation data with the unfractionated soy bean proteins are given. In the case of the unfractionated proteins which contained phytate impurities even at very high concentrations of Ca(II) (1.0×10^{-1}M) only about 50% of protein was precipitated. However, the removal of phytate impurities increased the extent of precipitation. Thus at 1.0×10^{-2}M Ca(II) about 80% of the protein was precipitated. Here again there was no relation between the precipitability and the affinity for Ca(II) ions. The phytate-free proteins bound less Ca(II) but were precipitated to a greater extent. In the case of phytate-free material EDTA treatment increased the precipitability slightly, as in the case of the 11S fraction. In this case also the addition of NaCl reduced the extent of precipitation by Ca(II). The effect was more marked in the case of phytate-free proteins, where at 0.2M NaCl concentration there was practically no precipitation. Thus the ability to be precipitated by Ca(II) appeared to be more dependent upon the conformation of the protein than on its ability to bind Ca(II).

In some cases it was observed, that at higher concentrations of Ca(II) the extent of precipitation decreased and the protein was being solubilized again. The significance of this observation is not apparent and further work is needed to explain this observation.

Acknowledgment

The authors are grateful to Mr. Pramod, Regional Research Laboratory, Hyderabad, for the ultracentrifuge measurements.

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[Received November 12, 1973. Accepted June 4, 1974]