Effect of pH on the Binding of Calcium Ions by Soybean Proteins

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ABSTRACT

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A calcium ion-selective electrode to measure free Ca^{2+} activity was used to study the binding of calcium ions to soy protein as a function of pH. pH strongly affects the extent of Ca^{2+} binding because hydrogen ions compete with calcium ions for the same binding sites on the protein molecule. These binding sites were identified as being associated with the side-chain carboxyl groups of the aspartic and glutamic acid residues and with the imidazole group of the histidine residues. The number of Ca^{2+} binding sites was found to be 43 per 10^5 g of low-phytate protein. The affinity of the binding sites for calcium ions was shown to increase as pH increased over the pH range of 4–9, since the binding constant increased with increasing

pH. Significant calcium ion binding did not occur much below pH 3. Between pH 3 and 7, a small change in pH results in a large change in the amount of Ca^{2+} bound. Above pH 7, no further change occurs in calcium ion binding. Thus, a critical pH range was found that corresponds to the ionization (deprotonation) of the side-chain carboxyl and imidazole groups. On the basis of the linear Scatchard plots, which were obtained for the binding data, these binding sites appear to be equivalent and independent. That is, all sites have the same intrinsic association constant and are noninteracting.

Work is being done to enhance the nutritional, functional, and flavor qualities of soy proteins, with the objective of increasing the utilization of oilseeds in human foods. For example, the effects of phytate on mineral bioavailability and protein functionality have received much attention. Consequently, some investigators have published information concerning interactions between Ca^{2+}, phytate, and soy protein (Saio et al 1968, Okubo et al 1976). However, little consideration seems to have been given to small ion-protein interactions of cereal food systems in which phytate is essentially absent. As the trend of recent work is clearly toward the development of soy proteins low in phytate, a better understanding of the ionic interactions of soy proteins is likely to lead to new or improved products and even to better methods of phytate removal. In addition, small ion-protein interactions might have important implications in cereal processing technology. Therefore, this study was undertaken to gain a better fundamental understanding of small ion-protein binding phenomena in cereal food systems. Thus, the intent of using calcium ion and soy protein as reactants was to exemplify a simplified representative system.

Several investigators have studied the interaction between calcium ions and various soybean protein preparations. Equilibrium dialysis was the method used by Appu Rao and Narasinga Rao (1975) and by Saio et al (1968) to measure calcium ion binding by soybean proteins. Another method of measuring small ion binding to macromolecules, that of gel-filtration chromatography, was used by Sakakibara and Noguchi (1977). Both of these methods require buffered reaction solutions and long equilibration or elution times. Hence, the effect of hydrogen ions on the interaction between calcium ions and soybean proteins was not studied very extensively.

The present study was designed to further clarify the interaction between calcium ions, hydrogen ions, and whole soybean proteins by studying the association-dissociation equilibria of unbuffered reaction solutions over a wide pH range. Electrometric methods employing ion-selective electrodes have been widely used in studies of small ion-protein interactions (Carr 1952, 1953a, 1953b, 1953c;

Ho and Waugh 1965; Aguanno and Ladenson 1982; Moore 1969). Carr (1972) has given a good, brief review of the basic concepts and techniques involved. In the study reported here, potentiometry was employed to simultaneously measure hydrogen-ion and calcium-ion activities during hydrogen ion titrations of soy protein solutions.

Most of the previous work was on highly purified homogeneous protein fractions such as the 11S (Appu Rao and Narasinga Rao 1975, Sakakibara and Noguchi 1977) and 7S (Appu Rao and Narasinga Rao 1976) fractions or the cold insoluble fraction (Saio et al 1968). These protein fractions were isolated from whole, raw soybeans. In the present study, unfractionated soy protein isolates were used. These were prepared from a commercially supplied defatted soy flour.

MATERIALS AND METHODS

Preparation of Protein Isolates

Untreated soy protein isolate. Honeymead defatted soy flour having a protein content of 51% and a nitrogen solubility index of 74% was extracted with dilute NaOH at pH 12 for 1 hr at room temperature, using a flour-to-solvent ratio of 1:10. At this pH, protein solubility is at a maximum, whereas phytate solubility is at a minimum (Hartman 1979). Thus, a phase separation of the two occurs and phytate is removed during the subsequent centrifugation. The insoluble residue was removed by centrifugation at 2,000 rpm ($600 \times g$) for 20 min. The crude protein solution was dialyzed against 4.5 volumes of distilled water for 108 hr at 4°C with a change of water after 60 hr. Proteins were precipitated at pH 4.5 with dilute HCl. The protein suspension was centrifuged at 2,000 rpm for 20 min. The protein residue was freeze-dried, ground to a fine powder, and stored at 4°C.

Deionized soy protein isolate. The presence of phytate ion in the protein isolate was of concern, because phytate is a calcium-binding anion that is present in significant amounts in soybeans (Saio et al 1968, Wolf and Cowan 1971, Appu Rao and Narasinga Rao 1975, Cheryan 1980). Although the above protein isolation procedure results in a product having a low phytate level (Wolf 1972, Cheryan 1980), a further reduction in phytate was desirable

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to ensure against any interference. Therefore, phytate, Ca^{2+}, and other ions were removed through the use of a deionizing procedure similar to that reported by Ho and Waugh (1965). A portion of the untreated soy protein isolate was dissolved in 0.01 M HCl, using an isolate-to-solvent ratio of 1:40. The protein solution was combined with mixed-bed ion-exchange resin (Bio-Rad Laboratories, AG501-X8(D), 20–50 mesh), using a protein solution-to-resin ratio of 20:1, and was stirred gently for 15 min. Deionization precipitated the protein, which was then separated from the resin beads by washing through a 48-mesh sieve with 0.01 M HCl. The protein suspension was combined with fresh resin in the same proportion as before. After gentle stirring for 15 min, the washing was repeated with deionized water. The suspension was centrifuged at 2,500 rpm (940 $\times$ g) for 30 min. The deionized protein was freeze-dried, ground to a fine powder, and stored at 4°C.

Preparation of Protein Suspensions

Protein suspensions (1%, w/v) were prepared by dispersing soy protein isolate in KCl solution (10–200 mM, depending on the desired ionic strength of the suspension), adjusting the pH to 7.0 with measured volumes of 0.808M and 0.1M KOH, and quantitatively transferring to a volumetric flask with KCl solution. This procedure allows the exact ionic strength of the suspension to be computed (neglecting the ionic strength contribution of the protein). If a suspension were to contain added calcium ions, then a measured volume of $CaCl_2$ solution was added before the protein was dispersed. Corresponding blank solutions (without protein) were prepared for each protein suspension.

Reagents and Solutions

All chemicals were analytical reagent grade. Solutions were prepared using deionized distilled water. KCl and $CaCl_2$ were dried in a vacuum oven at 90°C for 2 hr and stored in a desiccator. KCl (10, 50, 100, and 200 mM) was used to adjust ionic strength. KOH (0.808M and 0.100M) and HCl (1.00M) were used as titrants. A 250 mM Ca^{2+} solution was prepared from anhydrous $CaCl_2$. This solution was diluted to give 25 mM Ca^{2+} and 2.5 mM Ca^{2+} solutions. Appropriate volumes of these were added to protein suspensions and to blank solutions to give the desired Ca^{2+} concentrations upon final dilution.

The following pH buffers were used to standardize the pH meter: 1.93, 4.00, 7.00, 9.20, and 11.00. All were commercially prepared except the pH 9.20 buffer, which was 0.05M sodium borate.

The calcium ion-selective electrode was calibrated with the following Ca^{2+} standards: 0.025, 0.25, 2.5, and 25 mM. These were prepared by appropriate dilutions of the 250 mM Ca^{2+} solution. The 0.025 and 0.25 mM standards were 10 mM with respect to KCl, and the 2.5 mM standard was 1 mM with respect to KCl.

Apparatus

An improvised microburette was made from a B-D Yale Turberculin glass syringe (1.00 cc in 10-μl divisions). A small Teflon stopcock was fitted to the syringe tip, and a 2-in. piece of Teflon capillary tubing was attached to the stopcock. The capillary tubing served as a titrant delivery tube. A small-diameter rubber O-ring was slipped over the syringe plunger. Since the syringe was mounted in a vertical position over the reaction vessel, the tight-fitting O-ring served as a plunger stop to prevent unwanted delivery of titrant.

Reaction vessels were 100-ml, low-form glass beakers. Stirring was done on a magnetic stirrer insulated on top by a foam pad. pH was measured with a Beckman Expandomatic pH meter and a Sargent-Welch combination pH electrode (S-30072-75). Ca^{2+} was measured with an Orion model 93-20 calcium electrode and an Orion model 90-02 double-junction reference electrode connected to an Orion model 701 digital pH/mV meter.

Hydrogen Ion Titration Procedure

A measured aliquot of well-mixed protein suspension was transferred to a reaction vessel. An equal volume of corresponding blank solution was transferred to another reaction vessel, and a stream of nitrogen was passed over both samples for 30 min before

titrating to minimize pH changes induced by CO_2 (Perlmann 1972). Just before titration, the pH meter was standardized with the appropriate set of buffers for the pH range to be titrated.

Also, just before a titration, the calcium electrode response was measured by taking mV readings of the four Ca^{2+} calibration standards. These data are used to construct a calibration curve that relates subsequent mV readings of the calcium electrode to Ca^{2+} activity. Thus, the method is a direct measurement method for Ca^{2+}.

Initial pH and calcium electrode mV readings were taken before titrant was added. All titrations were done at room temperature. Titrants were added in 10-μl increments while the reaction solution was being stirred at a constant rate. pH and calcium electrode mV readings usually stabilized within 2 min of titrant addition, except in the region of low buffering capacity, where up to 5 min was required for the pH to stabilize. Protein suspensions were titrated with acid to pH 2.00, while the blank solutions were titrated to pH 1.60. On the alkaline side, protein suspensions were titrated to pH 12.00 and the blank solutions to pH 12.40. By using small, accurately measured volumes of titrant, the protein concentration and the ionic strength are kept essentially constant. In fact, the volume increase due to added titrant was never more than 0.98%, which is negligible compared to the other sources of error, such as the inaccuracy of the pH measurements at the extreme acid end of the curve (Steinhardt and Beychok 1964).

Calculations

The number of H^+ dissociated per 10^5 g of protein was calculated as explained by Malik and Jindal (1968).

RESULTS AND DISCUSSION

The mineral compositions of the two soy protein isolates used in this study are shown in Table I. In addition, the following analytical results were obtained for untreated isolate: protein (N $\times$ 6.25), 105.8% on dry-weight basis; ash, 1.2% on dry-weight basis; and moisture, 1.7%.

For the purposes of this study, phosphorus was assumed to be totally phytate phosphorus. Hence, only total phosphorus is reported for these isolates. The ion-exchange treatment effectively and efficiently deionized the isolate (89% of the starting protein was recovered). In fact, the levels of phosphorus and calcium were lower than those found in the ion-exchange-treated soy protein isolate of Brooks and Morr (1982), who reported phosphorus and calcium levels of 0.092 and 0.039%, respectively. The untreated isolate of the present study had lower levels of phosphorus and calcium than the control isolate of Brooks and Morr (1982), who reported 0.876 and 0.190% P and Ca, respectively. Appu Rao and Narasinga Rao (1975), in a calcium-binding experiment using unfractionated soybean proteins before and after removal of phytates, reported phosphorus levels of 0.88 and 0.10%, respectively. Saio et al (1968), in their study of phytate and calcium interactions with soybean protein, used a cold insoluble-fraction protein having 0.422% phosphorus.

Hydrogen ion titration curves were made at different ionic strengths in the absence of added Ca^{2+} so that we could see whether

TABLE I
Mineral Composition of Soy Protein Isolates[a]

Mineral	Moisture-free Protein (%)	
	Untreated	Deionized
Na	0.024	0.007
K	0.080	0.003
Ca	0.076	0.028
P	0.374	0.069

[a] Duplicate 1.0-g samples were dry ashed at 550°C overnight in Pt dishes and analyzed for Na, K, and Ca by flame atomic absorption, using a Varian Techtron model 1250 atomic-absorption spectrophotometer. For Ca analysis, the samples were diluted to 0.5% lanthanum. P was determined by the microcolorimetric method (AOAC 1980), using a B + L Spectronic 20.

the protein isolates used in this study had the same number of potential calcium ion-binding sites (ie, ionizable amino acid side-chain groups) as the soybean protein preparations of other investigators. Basically, a hydrogen ion titration of a protein measures the number of protons (H^+) dissociated from the molecule as a function of pH. Since the various ionizable amino acid side-chain groups present on protein molecules will dissociate their protons at characteristic pHs, the number of each kind of group can be counted from the titration curve (Tanford 1962). Table II shows the results of this group-counting procedure for the two protein isolates used in this study and compares these results to those of other investigators.

The group-counting procedure is somewhat subjective and arbitrary as regards the determination of the pH ranges in which the various groups are titrated (Tanford 1962). Thus, the pH range assigned to each kind of group by each author is included for comparison. Ranges, rather than absolute values, are given for the number of groups counted in each class. This reflects differences in the titration curves due to variations in a number of experimental conditions, including method of protein preparation, protein purity and homogeneity, ionic strength, acid or alkali denaturation, and time factors. In spite of these limitations, the number of groups in each class is essentially the same for all the proteins. Also, the total number of groups is the same.

Hydrogen ion titrations in the presence of calcium ions were made, and the resulting curves were compared to those obtained in
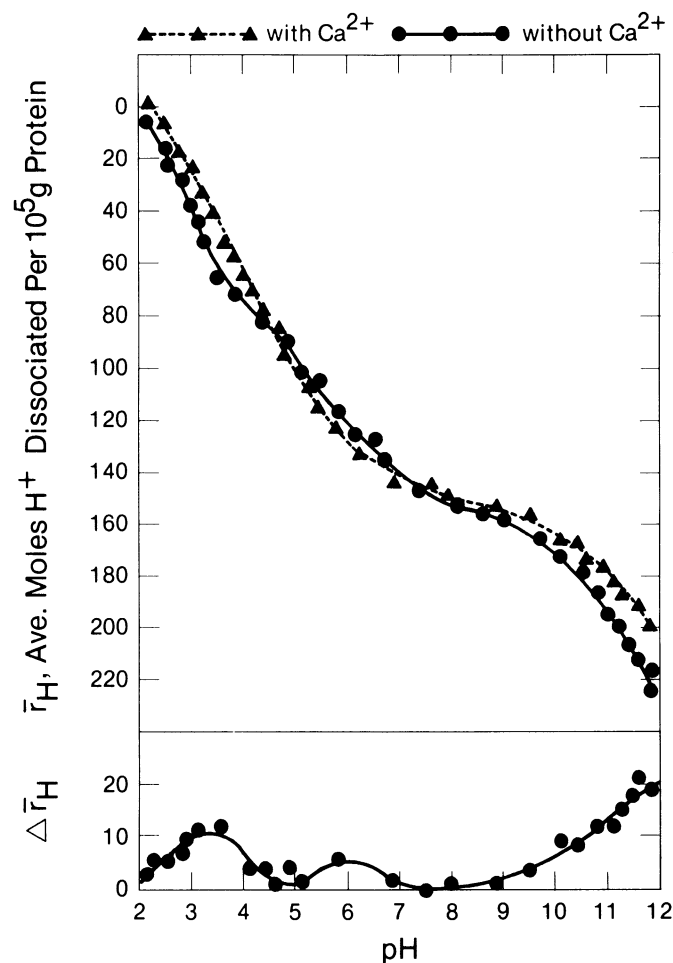

Fig. 1. Hydrogen ion titration curves and difference curve of deionized protein. Upper curves: ●—● = no added Ca^{2+}, ionic strength 0.01; ▲---▲ = 25 moles of Ca^{2+} added per 10^5 g of protein, ionic strength 0.02. Lowermost curve is the difference between the upper curves.
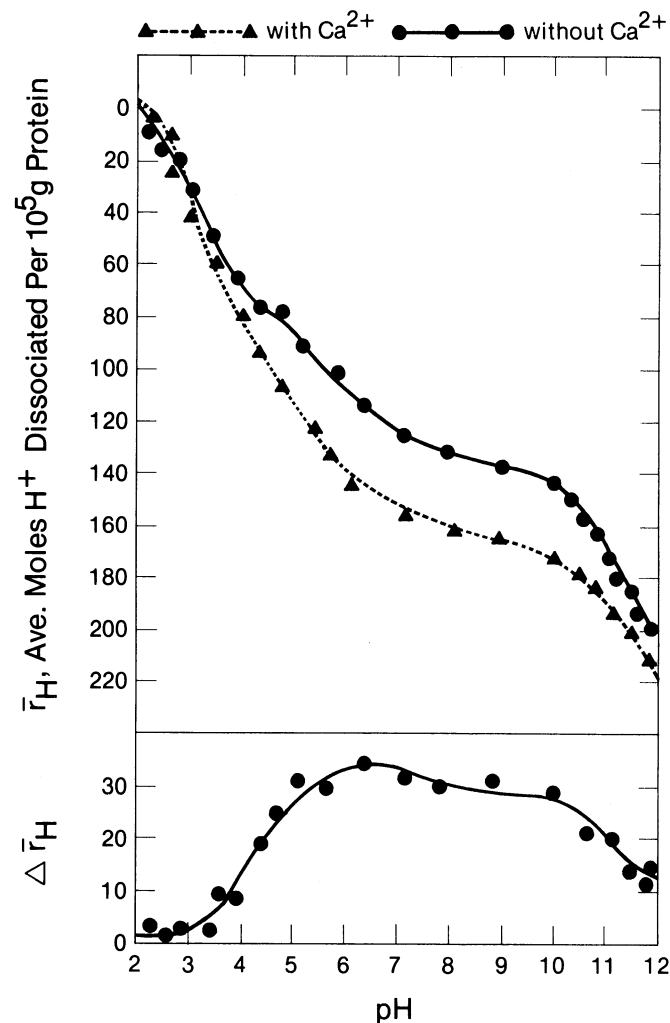
Fig. 2. Hydrogen ion titration curves and difference curve of untreated protein. Upper curves: ●—● = no added Ca^{2+}, ionic strength 0.01; ▲---▲ = 25 moles of Ca^{2+} added per 10^5 g of protein; ionic strength 0.02. Lowermost curve is the difference between the upper curves.

TABLE II
Ionizable Groups in Soybean Protein as Reported by Various Investigators

Ionizable Groups	Malik and Jindal (1968)		Catsimpoolas et al (1971)		Puri and Bala (1975)		This Study[a]	
	No. of Groups per 10^5 g of Protein	pH Range Titrated	No. of Groups per 10^5 g of Protein	pH Range Titrated	No. of Groups per 10^5 g of Protein	pH Range Titrated	No. of Groups per 10^5 g of Protein	pH Range Titrated
Acidic[b]	94	3.0–5.5	136–140	2.0–6.5	110–128	2.0–6.5	115–143	2.0–6.5
Neutral[c]	18	6.0–8.0	14–20	6.5–8.5	12–16	6.5–7.5	25–30	6.5–9.5
Basic[d]	46	8.0–11	33–44	8.5–12.0	68–72	7.5–11.7	56–65	9.5–12.0
Total	158	⋯	183–204	⋯	190–216	⋯	205–230	⋯

[a] Data from titrations of: untreated protein at ionic strength 0.01, 0.05, 0.10, and 0.20; and deionized protein at ionic strength 0.01.
[b] α-Carboxyl and side-chain carboxyl of Asp and Glu.
[c] Imidazole of His.
[d] α-Amino, side-chain amino of Lys, and phenolic of Tyr. Does not include guanidyl of Arg.

the absence of calcium ions. When hydrogen ion titration curves made in the absence and in the presence of calcium ions are compared, a difference will be seen in the positions of the curves if calcium ions are bound to the protein molecule at the same binding sites where hydrogen ions are bound. The difference is such that, at a given pH, more hydrogen ions will be dissociated in the presence of calcium ions than in the absence of calcium ions. Thus, a difference curve can be plotted to show the degree of change caused by calcium ion binding. Figure 1 shows the hydrogen ion titration curves and a difference curve of deionized protein in the presence and in the absence of calcium ions. The curves indicate that Ca^{2+} binding begins at about pH 4, because the curves begin to diverge appreciably and a significant difference is noticed. This pH region corresponds to the ionization (deprotonation) of carboxyl groups. At approximately pH 6, where imidazole groups would dissociate their protons, the maximum difference between the curves occurs, and 35 more hydrogen ions are dissociated in the presence of Ca^{2+} than in the absence of Ca^{2+}.

Figure 2 shows the hydrogen ion titration curves and the difference curve of untreated protein in the presence and absence of calcium ions. The difference between the H^+ titration curves is small in the regions of carboxyl and imidazole ionization, and there is no consistent pattern of more dissociated H^+ in the presence of Ca^{2+}. Apparently, the small amount of phytate present is enough to mask the competitive binding effects between H^+ and Ca^{2+}.

Calcium-binding curves were made from calcium ion-selective electrode measurements of free Ca^{2+} activity as a function of pH. Since the total amount of Ca^{2+} in the system is known, the amount bound at a given pH is derived by the difference between total Ca^{2+} and free Ca^{2+} (Gurd 1970). Figure 3 shows the calcium-binding curves obtained for untreated and deionized protein.

With untreated protein, some Ca^{2+} binding occurred down to pH 2.0. The amount bound did not change up to pH 3.0. From pH 3.0
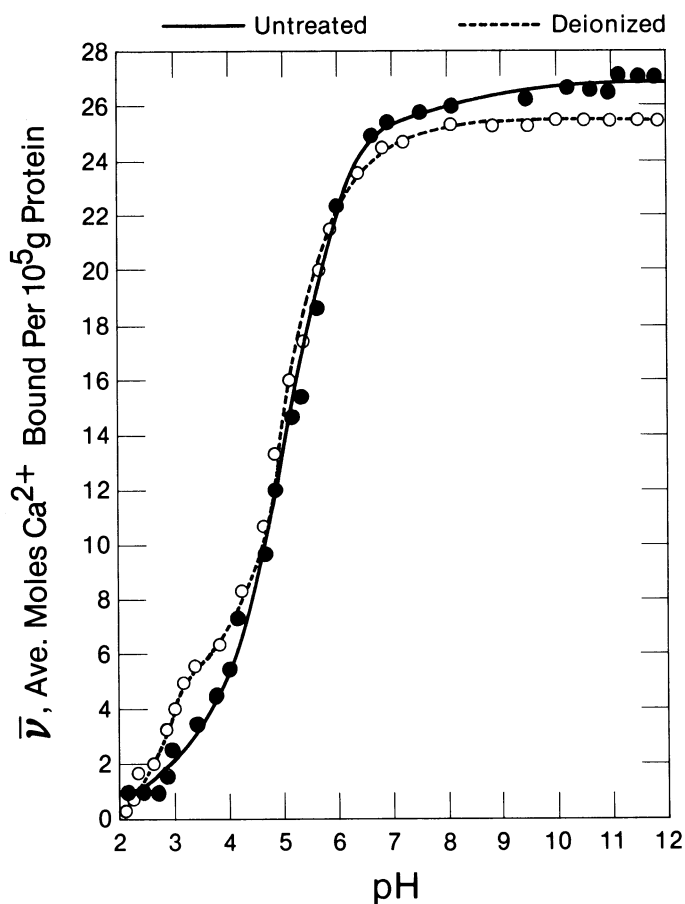
to 6.5, the amount of bound Ca^{2+} increases rapidly, showing that there is a critical pH region in which a small change in pH results in a large change in the number of Ca^{2+} bound. In other words, calcium ion binding is highly sensitive in this pH range. At pH 7 and above, little or no significant change in bound Ca^{2+} is seen. With deionized protein, there is no Ca^{2+} binding at pH 2.0. The curve ascends immediately as pH increases beyond 2.0, in contrast to untreated protein. From pH 4.0 to 7.0, the curve for deionized protein is essentially the same as that for untreated protein. The difference in $\bar{\nu}$ between pH 7 and 12 occurs because the untreated protein contains slightly more total Ca^{2+} than the deionized
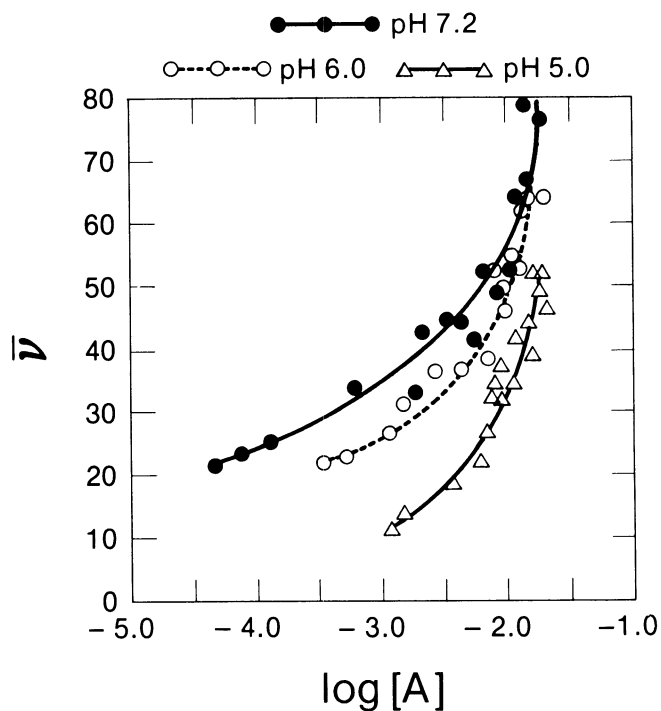

Fig. 4. Binding of Ca^{2+} by untreated protein as a function of [A], the free Ca^{2+} activity in moles per liter. ●——● = pH 7.2, o---o = pH 6.0, △---△ = pH 5.0, $\bar{\nu}$ = average moles of Ca^{2+} bound per 10^5 g of protein.

Fig. 3. Binding of Ca^{2+} as a function of pH. ●—● = untreated protein (27 moles of Ca^{2+} per 10^5 g of protein), O---O = deionized protein (25.8 moles of Ca^{2+} per 10^5 g of protein); ionic strength 0.02.
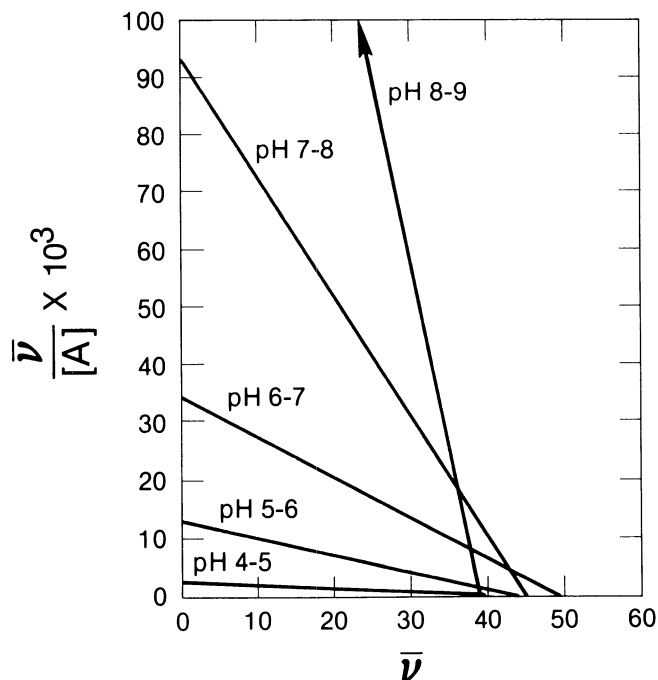

Fig. 5. Scatchard plots of Ca^{2+} binding data at various pH ranges.

TABLE III
TABLE III
Values of N and K Obtained from Scatchard Plots

pH Range	N (binding sites/10^5 g protein)	K (binding constant)
4–5	39.0	0.07×10^3
5–6	44.0	0.31×10^3
6–7	49.0	0.71×10^3
7–8	45.0	2.07×10^3
8–9	38.5	6.58×10^3
Average	43.1	
Standard deviation	4.4	

protein. Thus, the only significant difference between the two curves occurs below pH 4.0. The small amount of phytate present in the untreated protein might preferentially bind Ca^{2+} at low pH, resulting in a horizontal curve in this pH region. According to Graf (1983), phytate does, indeed, bind Ca^{2+} at pH 2.0. As pH increases above 3.0, the ionizable groups of the protein assume the dominant Ca^{2+} binding role, and Ca^{2+} binding increases with increasing pH as the ionizable groups become deprotonated. The deionized protein is essentially phytate-free, yet some Ca^{2+} is bound even at low pH. Since only the carboxyl groups of the protein would ionize in the low pH range, these must be acting as the Ca^{2+} binding sites up to about pH 6. In the intermediate pH range, from pH 6 to 7, the imidazole groups would become deprotonated, thus acting as Ca^{2+} binding sites.

In contrast, other investigators did not observe Ca^{2+} binding at low pH. Appu Rao and Narasinga Rao (1975) reported that binding did not occur at pH 5.5. Sakakibara and Noguchi (1977) did not observe binding even at pH 7.0. As a result, these authors attributed Ca^{2+} binding to the imidazole groups but not to the carboxyl groups.

Binding was also studied as a function of free Ca^{2+} activity using untreated protein. Figure 4 shows protein-bound Ca^{2+} versus the log of free Ca^{2+} activity at pH 5.0, 6.0, and 7.2. Ca^{2+} binding increased as free Ca^{2+} activity (ie, total calcium concentration) increased. As expected, Ca^{2+} binding increased as pH increased.

When the amount of bound Ca^{2+} is plotted against the ratio of bound Ca^{2+} to free Ca^{2+}, the maximum number of binding sites per protein molecule can be found as well as the value of the binding constant. This is in accordance with the Scatchard equation (Edsall and Wyman 1958):

$$\frac{\bar{\nu}}{[A]} = K (N - \bar{\nu})$$

where $\bar{\nu}$ = average moles of Ca^{2+} bound per 10^5 g of protein, $[A]$ = free Ca^{2+} activity in moles per liter, K = equilibrium binding constant, and N = number of binding sites per 10^5 g of protein.

Scatchard plots of Ca^{2+} binding data collected at several pH ranges are shown in Fig. 5. Individual data points are not shown because they are scattered and would only obscure the essential purpose of the figure. The solid lines represent best-fit curves obtained by linear regression analysis of those data points giving the highest correlation coefficients. The equilibrium binding constant (K) is given by the slope of the Scatchard plot. The maximum number of binding sites (N) is given as the intercept of the plot on the abscissa. Table III gives a summary of the results obtained from the Scatchard plots.

The equilibrium binding constant is a measure of binding strength (Edsall and Wyman 1958). It gives an indication of the affinity of calcium ions for their binding sites on the protein molecule. Thus, as the value of the binding constant increases, the ions are more tightly bound. Both Fig. 5 and Table III show that the binding constant increases as pH increases. At low pH—from pH 4 to 5, for example—calcium ions are quite loosely bound because they are in direct competition with H^+ for the binding sites on the protein molecule. On the other hand, at high pH (from pH 8 to 9, for example), calcium ions are tightly bound; that is, the binding sites have a high affinity for calcium ions. The carboxyl and

imidazole groups would be completely deprotonated in this pH range. Therefore, calcium ions would not be competing with hydrogen ions for these particular sites. Appu Rao and Narasinga Rao (1975) obtained K values at pH 7.8 of 5.3×10^3 for unfractionated soybean proteins before phytate removal (total phosphorus 0.88%) and 1.6×10^3 after phytate removal (total phosphorus 0.10%). In comparison, I obtained a K value of 2.07 $\times 10^3$ for untreated protein (total phosphorus 0.37%) at a comparable pH (pH 7—8 in Table III). Allowing for the differences in total phosphorus, these values for the equilibrium binding constant are seen to be in close agreement. Also in agreement with Appu Rao and Narasinga Rao (1975) is my finding that the binding data are best fitted to linear Scatchard plots. This indicates that the binding sites are equivalent and independent (Tanford 1961, Edsall and Wyman 1958).

The maximum number of Ca^{2+} binding sites was found to be 43 per 10^5 g of protein. This is much larger than the values obtained by other investigators. Appu Rao and Narasinga Rao (1975), for example, reported an N value of 32 for unfractionated protein before phytate removal, and Saio et al (1968) reported a maximum of 24 moles Ca^{2+} bound per 10^5 g of cold insoluble fraction protein. The larger N value of the present study can hardly be attributed to phytate interference since, as noted earlier, my untreated protein had a lower level of total phosphorus than did similar preparations of the investigators cited above. The number of Ca^{2+} binding sites may depend on the degree of dissociation and, especially, on any conformational changes accompanying the dissociations. Soybean proteins are known to undergo irreversible dissociation at high pH (Wolf 1972), and it is conceivable that the proteins used in this study were denatured because of the highly alkaline conditions during extraction. Therefore, this important difference in the method of protein preparation may account for the difference in the number of binding sites. On the other hand, the protein of this study had the same number and kind of ionizable side-chain groups as those typically found in proteins prepared under milder pH conditions. Explaining differences in the number of binding sites is difficult because the exact nature of the Ca^{2+} binding sites is so poorly understood. Finally, differences in the methods of measuring Ca^{2+} binding may account for the large variation in N values.

This study showed that potentiometry can be sucessfully used to measure the binding of calcium ions to soy protein. More importantly, evidence was presented that supports the idea that there are two types of binding sites on the protein molecule. First, it was shown that calcium ion binding takes place in the pH region where side-chain carboxyl and imidazole groups are dissociated. Both groups bind divalent cations (Gurd and Wilcox 1956, Tanford 1962) and therefore represent likely Ca^{2+} binding sites. Second, the total number of binding sites was found to be much larger than could be accounted for by imidazole group binding alone; there are only about 18 imidazole groups per 10^5 g of protein (Malik and Jindal 1968), but 43 binding sites per 10^5 g of protein were found. Therefore, the remainder of the binding sites must be associated with the side-chain carboxyl groups of the aspartic and glutamic acid residues.

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