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Extraction of Soybean Meal Proteins with Salt Solutions at pH 4.5

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Extractable Kjeldahl nitrogen increased with increases in concentration of sodium or calcium chloride until a maximum of 65% of the nitrogen in the flakes was extracted. This maximum occurred with 0.3 *N* calcium chloride or 0.7 *N* sodium chloride. Without added salts, the pH 4.5 extract contained only 2S and 7S ultracentrifuge components. Up to 0.3 *N* sodium chloride 2S protein increased, whereas the 7S component did not reach a maximum until 0.7–0.8 *N* salt. The 11S component began to dissolve at 0.3 *N* salt and was completely solubilized at 0.8 *N*. The 15S component did not dissolve until concentrations of salt were greater than 0.4 *N* and increased in extractability up to 0.8 *N* sodium chloride. Calcium chloride extracts contained increasing amounts of 2S and 7S fractions up to 0.2 and 0.3 *N*, respectively. The 11S component began to dissolve at 0.1 *N* and increased in solubility up to 0.4 *N* calcium chloride. The 15S material did not dissolve significantly below 0.2 *N* and increased in extractability up to 0.4 *N* calcium chloride.

Distilled water extracts of defatted soybean meal have a pH of 6.5–6.7 and contain about 80–90% of the meal proteins (Smith *et al.*, 1938). When extractions are carried out with dilute salt solutions near this pH, extractability of the proteins depends upon salt concentration. As the concentration increases less protein is dissolved, until a minimum of about 46% of the total Kjeldahl nitrogen is extracted with 0.1 *N* sodium chloride and only about 22% is extracted with 0.018 *N* calcium chloride. After the minimum in solubility is reached, more protein dissolves as the salt concentration of the extraction solvent is further increased. In this respect, soybean proteins are unique as compared to the proteins of flax, rye, wheat, and barley (Smith *et al.*, 1938). If the pH is lowered to the isoelectric region of 4.6 during water extraction of soybean proteins, only 9% of the total Kjeldahl nitrogen dissolves (Smith and Circle, 1938). Adding salts during extraction at this pH increases dispersion of the protein. This increase contrasts with the minimum in extractability found near neutral pH.

Ultracentrifugal analysis of water-extractable soybean proteins reveals four components with sedimentation coefficients of approximately 2, 7, 11, and 15S (Svedberg units). Changes occur in the distribution of these components as a function of sodium and calcium chloride concentrations used for extraction near neutral pH (Wolf and Briggs, 1956). Preferential extraction of some proteins occurs as salt concentrations are varied. We examined extractability of soybean meal proteins at pH 4.5 as a function of sodium and calcium chloride concentrations and determined the ultracentrifugal composition of the various extracts.

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EXPERIMENTAL SECTION

Protein Extraction Procedure. Harosoy soybeans (1964 crop) were cracked, dehulled, and flaked. The flakes were defatted by four batchwise extractions with pentane–hexane (boiling range 33–57°) and then air dried. Nitrogen content of the defatted flakes was 9.1%, corrected for 10.6% moisture. Protein extracts were prepared with a ratio of 20 ml of extractant/g of defatted flakes, except in preliminary experiments in which a 40:1 extraction ratio was used. After addition of the extractant to the flakes, the pH was quickly adjusted with 1 *N* HCl to 4.5 and maintained while stirring for 45 min. The slurry was then centrifuged for 15 min at 19,000 × *g*. The resultant supernatant was filtered through Whatman No. 1 filter paper to remove a small amount of floating material. Nitrogen analyses were performed on the filtered extracts.

Ultracentrifugation. A 4-ml portion of extract was equilibrated by dialysis against pH 7.6, 0.5 ionic strength, phosphate buffer containing 0.01 *M* 2-mercaptoethanol (Wolf and Briggs, 1959). Preliminary studies indicated no changes were brought about in the samples by dialysis against the phosphate buffer. Calcium chloride extracts were first dialyzed against 0.4 *N* sodium chloride to remove the calcium ions before dialysis against phosphate buffer. After dialysis and centrifugation for 15 min at 19,000 × *g*, the extracts were diluted to 5 ml with buffer.

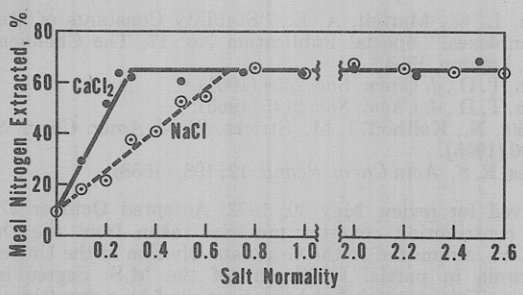

Figure 1. Nitrogen extracted from defatted soybean flakes as a function of salt concentration at pH 4.5. Straight lines are fitted to the data for convenience.

The dialyzed samples were analyzed at 23–26° in a Model E Spinco ultracentrifuge operated at 47,660 rpm. A double sector 30-mm cell was used, and photographs were taken 48 min after the start of the run (except as noted) with a bar angle of 70°. From these ultracentrifuge patterns, corrected for radial dilution, were calculated compositions of the extracts (Wolf and Briggs, 1956). The maximum amount of protein extracted with either sodium or calcium chloride at pH 4.5 was less than the quantity of protein extracted with water at pH 6.7. To show which fractions were insolubilized at pH 4.5, the area for each fraction of the salt extracts was expressed as a percentage of the area for the corresponding fraction in a water extract. In some experiments the samples were equilibrated with 0.1 ionic strength, pH 7.6, phosphate buffer before a second ultracentrifuge run (Roberts and Briggs, 1965).

Nitrogen Analysis. The nitrogen content of the samples was determined by the micro-Kjeldahl procedure of Johnson (1941).

RESULTS AND DISCUSSION

Protein Solubility and Salt Concentration. The percentage of Kjeldahl nitrogen extracted from soybean meal as a function of sodium and calcium chloride concentrations at pH 4.5 is shown in Figure 1. The solubility curves are uncorrected for nonprotein nitrogen, which accounts for 3–8% of the total nitrogen content of defatted soybean meal (Becker *et al.*, 1940). The amount of nitrogen extracted increased from 10% in the absence of salts to about 65% with 0.3 *N* calcium chloride or with 0.7 *N* sodium chloride. Further increases in salt concentration failed to solubilize additional protein.

Even if the salt concentration is expressed as ionic strength, the data points for calcium chloride and sodium chloride in Figure 1 are best fitted by separate lines. Moreover, the data in Figure 1 were obtained with a 20:1 solvent-to-meal ratio; results were similar with a 40:1 extraction ratio. This implies that the solutions are not saturated with protein. For a given amount of nitrogen extracted, the amount of chloride ion is the same in the two salt solutions. The observed solubilizing effects of the salts must therefore be primarily cationic rather than anionic.

Sodium Chloride Concentration and Extract Composition. Figure 2 contains typical ultracentrifuge patterns obtained with sodium chloride extracts. For comparison, Figure 2 includes an ultracentrifuge pattern for proteins extracted with water at pH 6.7. Water extracted 80.6% of the total nitrogen of defatted soybean meal. Since extraction ratios and dilutions were identical, the ultracentrifuge patterns are directly comparable, and changes in areas in the patterns reflect changes in protein solubility. It is apparent from Figure 2 that the four ultracentrifugal components are solubilized to different degrees by the addition of salts to the extraction medium.

Figure 3 depicts changes in extractability of the ultracentrifugal fractions occurring with variation of sodium chloride concentration. Only concentration from 0 to 0.8 *N* NaCl is considered because this range is where the change in salt concentration has its maximum solubilizing effect. Preliminary studies showed there were no significant changes in compositions of extracts at concentrations between 0.8 and 2.6 *N*.

At pH 4.5 in the absence of added salt, only 2S and 7S fractions are extracted. Such an extract comprises the soybean whey proteins, and others have shown that the whey is made up of only 2S and 7S components (Rackis *et al.*, 1959; Wolf and Briggs, 1959). The amount of 2S and 7S fractions extracted increases with salt concentration. Extractability of the 2S fraction, however, levels off at 0.3 *N* salt, while that of the 7S fraction continues to increase up to 0.8 *N*.

Sensitivity of the 7S protein toward salt concentration changes appears to differ at pH 4.5 from its behavior at neutral pH. Whereas at pH 4.5 the quantity of 7S protein ranges from 13 to 85% of the total 7S in a water extract, Wolf and Briggs (1956) found only small changes in the amount of 7S component extracted as a function of changes in sodium chloride concentration near pH 6.

Solubilization of the 11S fraction does not begin until the salt concentration is greater than 0.2 *N* but rises more rapidly with increasing salt concentration than does that of the 2S and 7S fractions. Maximum solubility of 11S protein was not reached until 0.8 *N* sodium chloride was used. Wolf and Briggs (1956) observed that solubility of 11S protein was also sensitive to changes in sodium chloride concentration at neutral pH. The 15S fraction clearly required a higher concentration of salt to solubilize it than did the other fractions.

The maximum amount of protein solubilized with sodium chloride was less than the protein extracted with

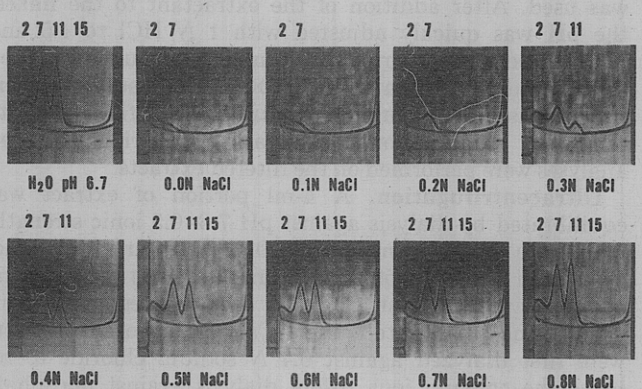

Figure 2. Ultracentrifuge patterns of pH 4.5 NaCl meal extracts. Numbers above the peaks are approximate sedimentation coefficients in Svedberg units (S).

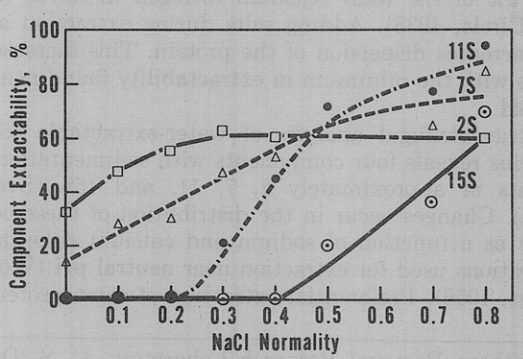

Figure 3. Component extractability as a function of NaCl concentration at pH 4.5. Each ultracentrifuge component in Figure 2 is expressed as a percentage of the area for the corresponding component in a water extract at pH 6.7.

water. It is apparent from Figure 3 that only the 11S component approached complete (95% of the amount of 11S component in a water extract) extraction in the 0.8 *N* sodium chloride extract. Decreased extractability of the proteins at pH 4.5 is accounted for primarily by the 2S and 7S fractions, which had respective solubilities of 59 and 85%, as compared to the water extract at pH 6.7. Only 75% of the 15S fraction dissolved with 0.8 *N* salt, but this fraction represented only 5% of the total water-extractable proteins. Hence it has an insignificant effect on total solubility.

Calcium Chloride Concentration and Extract Composition. Ultracentrifugal analyses of the calcium chloride extracts (Figures 4 and 5) are comparable to those with sodium chloride, except that the calcium chloride concentration required to obtain an extract with a given protein composition is considerably less than was necessary with sodium chloride.

Fractionation with Sodium Chloride at pH 4.5. Using the solubility data in Figure 3, we devised a stepwise extraction (Figure 6) to fractionate soybean proteins at pH 4.5.

The residue remaining after each extraction was reextracted once with a solution of the same sodium chloride concentration before extraction with the next higher increment of salt concentration. When the salt concentration was increased, the quantity of sodium chloride to be added was calculated by taking into consideration the volume of the previous extraction solution retained by the residue after centrifugation. The extracts were dialyzed against 0.5 ionic strength, pH 7.6, phosphate buffer containing 0.01 *M* 2-mercaptoethanol. After ultracentrifugal analysis, the solutions in 0.5 ionic strength buffer were equilibrated by dialysis with 0.1 ionic strength, pH 7.6, phosphate buffer. The lower ionic strength permits dimerization of some of the 7S globulin and thereby provides an additional means of distinguishing between the extracted proteins (Koshiyama, 1969; Roberts and Briggs, 1965). Ultracentrifugal patterns are reproduced in Figure 7. These patterns are not directly comparable regarding the relative amounts of a particular component present in different extracts, since concentrations were adjusted before ultracentrifugation. Figure 7 does demonstrate that it is possible to achieve some fractionation by adjustment of the sodium chloride concentration at pH 4.5.

On repetitive extraction, unexpectedly, no greater fractionation was achieved than that noted in Figure 7. For example, Figure 3 suggests that repeated extraction with 0.2 *N* salt will remove all the 2S fraction. Nonetheless, appreciable amounts occur in the 0.4 *N* salt extracts, and

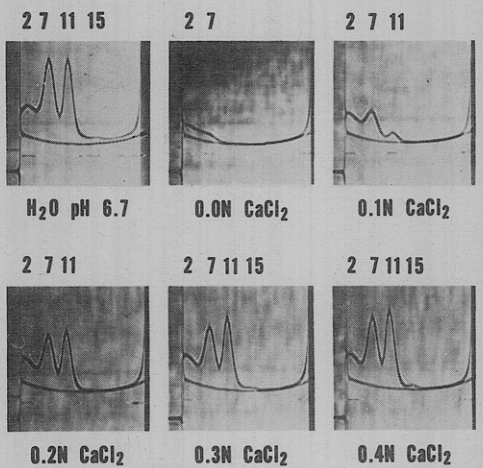

Figure 4. Ultracentrifuge patterns of pH 4.5 $CaCl_2$ extracts. Numbers above the peaks are approximate sedimentation coefficients in Svedberg units (S).

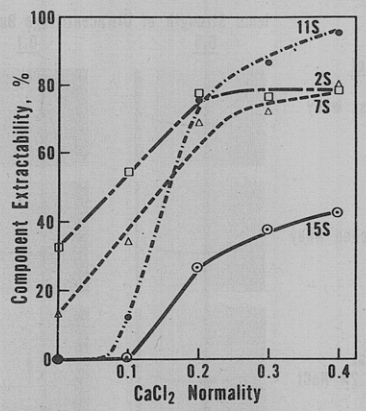

Figure 5. Component extractability as a function of $CaCl_2$ concentration at pH 4.5. Each ultracentrifuge component in Figure 4 is expressed as a percentage of the area for the corresponding component in a water extract at pH 6.7.

it is still detectable in the 1.0 *N* salt extract. Likewise, the 7S globulin that forms the 9S dimer at 0.1 ionic strength is extracted over the range of 0.2 to 1.0 *N* salt. Complex interactions between the various proteins appear responsible for this behavior. Even repetitive extraction at the same salt concentration fails to extract new globular components having higher sedimentation coefficients than components in the initial extract. Without exception, all extracts behaved this way. Also, nitrogen analysis on some of the repetitive extracts indicated that less nitrogen was extracted with each additional wash. The patterns show that the 7S $\rightleftharpoons$ 9S dimerization reaction does not occur in the whey but that it does occur in all extracts beyond the whey. The original pattern was always regenerated by dialysis back to the 0.5 ionic strength buffer.

CONCLUSIONS

Our results on the extractability of soybean proteins at pH 4.5 agree with those of Smith and Circle (1938) and extend them with the determination of protein compositions for extracts with various salt concentrations. The bulk of soybean proteins consists of globulins that are insoluble at their isoelectric points (pH 4.2–5.0) but that can be solubilized at this pH by adding salts. The protein

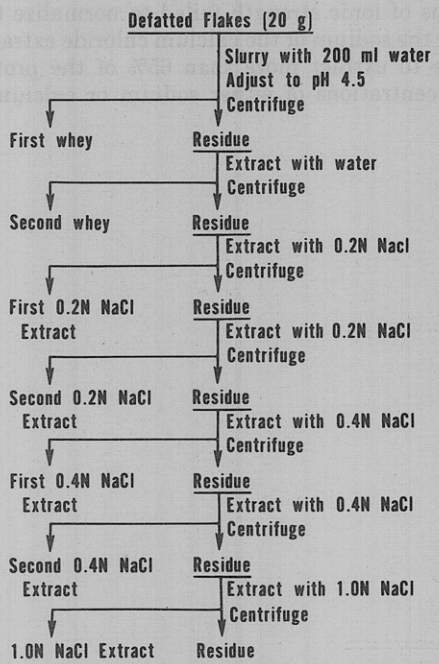

Figure 6. Repetitive fractional extraction at pH 4.5.

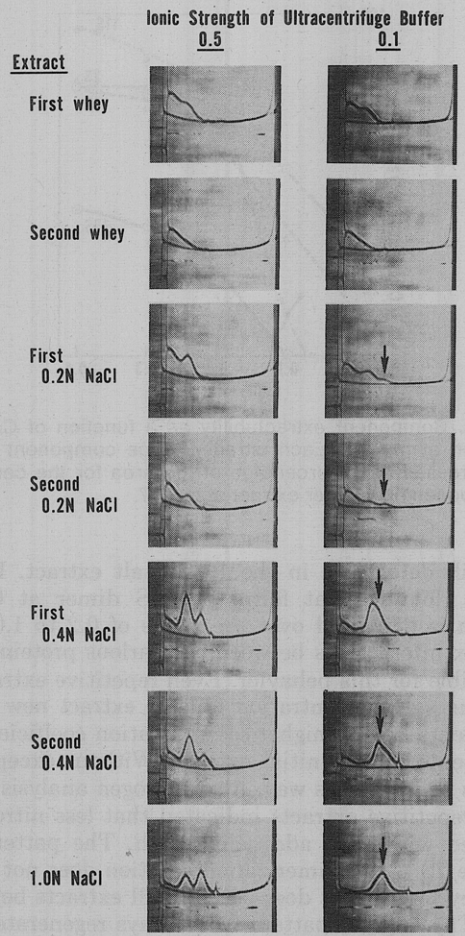

Figure 7. Ultracentrifuge patterns for repetitive fractional extracts. Patterns were photographed 40 min after the start of sedimentation. Arrows indicate the position of the 9S dimer when it is present in the 0.1 ionic strength phosphate buffer.

solubilities depend in part upon molecular size in this salting-in phenomenon. The smallest molecules (2S fraction) dissolve at the lowest salt concentration, while the largest molecules (15S fraction) are solubilized at the highest salt concentration. Specific cationic effects appear to be involved since plotting the extraction data of Figure 1 in terms of ionic strength failed to normalize the curves for either the sodium or the calcium chloride extracts.

Failure to extract more than 65% of the proteins with high concentrations of either sodium or calcium chloride (Figure 1) indicates that about 16% of the proteins were modified by the low pH treatment, since water at pH 6.7 extracted 80.6% of the total Kjeldahl nitrogen of the meal. The modification appears to be primarily a pH effect, since extraction with sodium or calcium chloride yielded the same maximum in protein solubility. Furthermore, ultracentrifugal compositions of the proteins extracted with 0.8 *N* sodium chloride (Figure 3) were similar to compositions of the proteins in a 0.4 *N* calcium chloride extract (Figure 5). Sensitivity of a portion of soybean proteins to treatment at pH 4.5 was noted previously and appeared to involve primarily the 2S and 7S fractions (Nash *et al.*, 1971; Wolf *et al.*, 1964). Figures 3 and 5 demonstrate that the 2S and 7S fractions are the major proteins contributing to the decreased extractability of the total protein with salts at pH 4.5.

Apparently the same phenomenon occurs during acid extraction and acid precipitation of soybean proteins from water extracts. Extraction and fractionation of soybean globulins at pH 4.5 and comparison with globulins at neutral pH should yield insight into the process of acid denaturation of soy protein.

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