A Protein from Wheat Flour that Binds Calcium and Stearoyl-2 Lactylate Ions[1]

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ABSTRACT

A protein component that binds Ca^{++} has been found in the water-soluble fraction of both hard red and soft white wheats. The protein was isolated by solubility fractionation in ammonium sulfate solutions and by gel-permeation chromatography. After binding calcium this protein can react further with phospholipids to form complexes of an ordered, crystalline structure. The protein with bound calcium also will form a complex with the dough improver, stearoyl-2 lactylate ion.

Metal ions derived either from the flour itself or from added "hard" water can affect the properties of doughs. For example, hard water decreases the absorption and extensibility and increases the resistance and viscosity of doughs (1). The mechanism by which these ions affect dough properties is not well understood, but might be related to an enhancement of complexing between lipids and proteins (2–8). The formation of lipid complexes of wheat protein mediated by metal ions was the subject of a previous paper (7). This paper describes isolation and partial characterization of a protein from the fraction of wheat proteins soluble in 70% saturated ammonium sulfate. This protein forms soluble complexes with calcium that can interact further with phospholipids and stearoyl-2 lactylate to form highly stable, insoluble ternary complexes.

MATERIALS AND METHODS

Materials

Water-soluble protein from Spring Wheat Long Patent and Lemhi flours was prepared by stirring unbleached flour into twice its weight of cold, distilled water and mixing for 30 min. in the cold with a propeller-type electric stirrer at slow speed. The mixture was centrifuged for 30 min. at 17,000 X g at 4°C., and the supernatant solution was dialyzed at 4°C. against several changes of deionized, distilled water. Any precipitate which formed at this point was removed by centrifuging as before. The solution was then made 2% in disodium EDTA and again dialyzed against several changes of deionized, distilled water to remove bound metal ions.

The protein component of interest was prepared from the water solubles by adding a saturated solution of ammonium sulfate until the mixture was 70% saturated with ammonium sulfate. The resulting precipitate of protein was removed by filtration and the clear solution remaining was dialyzed against several changes of deionized, distilled water in the cold, then was made 1% in disodium EDTA and again dialyzed against water. The dialyzed solution was lyophilized. The product was chromatographed in 200-mg. batches on a 55-cm. length, 3-cm. diameter column of Sephadex G-200 in water. Elution was followed with a refractive index

[1]Reference to a company or product name does not imply approval or recommendation of the product by U.S. Department of Agriculture to the exclusion of others that may be suitable.

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monitor, by absorption of light at 280 nm., and in some instances by carbohydrate analysis of the column effluent. The desired protein component was rechromatographed through the same Sephadex column. All fractions were lyophilized after chromatography and were stored at $-10°C$. until used. Sepharose 4B and DEAE-cellulose chromatography were carried out in a similar manner in a 55-cm. length, 3-cm. diameter column in water, pH 7.0, pH 7.5, or pH 8.0 buffers with a gradient of sodium chloride from 0 to 1.0M, followed in some experiments by HCl solutions to 0.1M and in some experiments by 2% EDTA solution in an attempt to remove adsorbed protein.

Phospholipids were obtained by the Folch fractionation (9,10) of bovine brain which had been frozen within 1 hr. of death. DEAE-cellulose chromatography was used for purification (11) with 0.005% 4-methyl-2,6-di-*tert*-butylphenol included in the solvent mixture as an antioxidant (12). Lipid purity was determined by thin-layer silicic acid chromatography, paper chromatography of the deacylated products, and analysis of ester:phosphorus ratio as previously described (6). For use in binding studies the phospholipids were dispersed in 0.05M *N*-ethylmorpholine buffer at pH 8.0 by ultrasonication at 20 kc. for 1 to 3 min. Phosphatidylserine dispersed in this manner has a molecular (micellar) weight

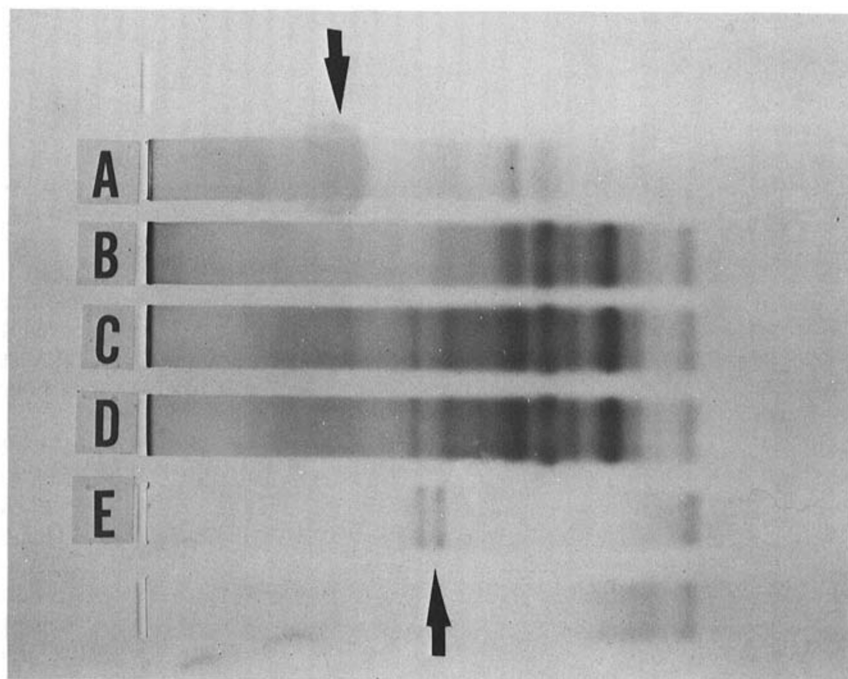

Fig. 1. Electrophoresis patterns of wheat-flour water-soluble proteins and that fraction of the water-solubles which is soluble in 70% saturated ammonium sulfate solution. The gel in this case is polyacrylamide with no urea present. A, α-gliadin marker; B, H_2O extract of flour; C, dialyzed H_2O extract; D, EDTA-treated H_2O extract; E, 70% saturated $(NH_4)^2SO_4$ solubles. Ca-binding component appears as a doublet in the absence of urea.

average of 4 × 10^6 with all hydrophilic groups exposed to the aqueous medium (13,14).

N-Ethylmorpholine from Matheson, Coleman, and Bell (Norwood, Ohio) was diluted and titrated with HCl solution to pH 8.0 to give a 0.5M stock solution which was diluted tenfold for use.

Sodium stearoyl-2 lactylate (Emplex) was from Patco Products Division of C. J. Patterson Company (Kansas City, Missouri). Clear water dispersions of 5 mg. per ml. were produced by ultrasonication at 20 kc. for 1 min. or less.

All other chemicals used were of reagent grade. Water used was distilled and deionized by passage through a Bantam mixed-bed demineralizer from the Barnstead Still and Sterilizer Co. (Boston, Mass.). The cellophane dialysis tubing used was heated to boiling in 2% sodium carbonate solution, rinsed, and heated again to boiling in deionized, distilled water before use.

Complex Formation

Complexes between the protein of interest and phospholipid or stearoyl-2 lactylate were formed in three ways. Dialysis against deionized, distilled water of a protein solution of 5 mg. per ml. which had previously been made 0.1M in calcium chloride leaves calcium bound to the protein component. Upon addition of a 5 to 10 mg. per ml. dispersion of phosphatidylserine or stearoyl-2 lactylate to the protein solution a precipitate of the complex formed. The precipitated material was centrifuged down in a small clinical centrifuge, shaken once with water to remove contaminating solubles, and again centrifuged. The proteins in the first supernatant were considered nonbound to the lipid; those in the precipitate were assumed to be complexed with the lipid. Complexes also can be formed by addition of a few μmoles of the metal ion to a clear mixture of the protein and lipid dispersion. A precipitate will then form which can be treated as above (7). Or the complex can be formed by dialysis of the protein-lipid mixture against 0.01M calcium chloride. Results are similar by each method.

Electrophoresis

Starch-gel electrophoresis in aluminum lactate pH 3.1 buffer was carried out as described by Cole and Mecham (15). Photographs were taken of the wet gels following destaining. Moving boundary electrophoresis was carried out in a Perkin-Elmer Model 38A electrophoresis apparatus using 0.5% solutions of protein fraction in 0.017M, pH 3.1, aluminum lactate buffer at 0°C. Sodium dodecyl sulfate electrophoresis was carried out in 5% polyacrylamide gel with 0.125M, pH 8.9, *tris*-borate buffer (16). A potential of 7 v. per cm. was applied across the gel, giving a current of 25 ma., for 4 hr. The gel was stained with Coomassie Blue in trichloroacetic acid solution.

Samples for amino acid determination were hydrolyzed in 6N HCl at 110° C. for 22 hr. in sealed, evacuated tubes. Carbohydrate was determined by phenol-sulfuric acid (17) with a xylose standard. Protein was determined by the Folin method (18), by ninhydrin (19), and by ultraviolet absorption. Calcium was determined by atomic absorption analysis of the protein fraction after dialysis against four changes of an 800-fold excess of 0.05M NaCl, to negate binding by charge alone, and two changes of water.

Photomicrographs were taken of the protein-calcium-phosphatidylserine complex with an electronic flash source through a polarizer.

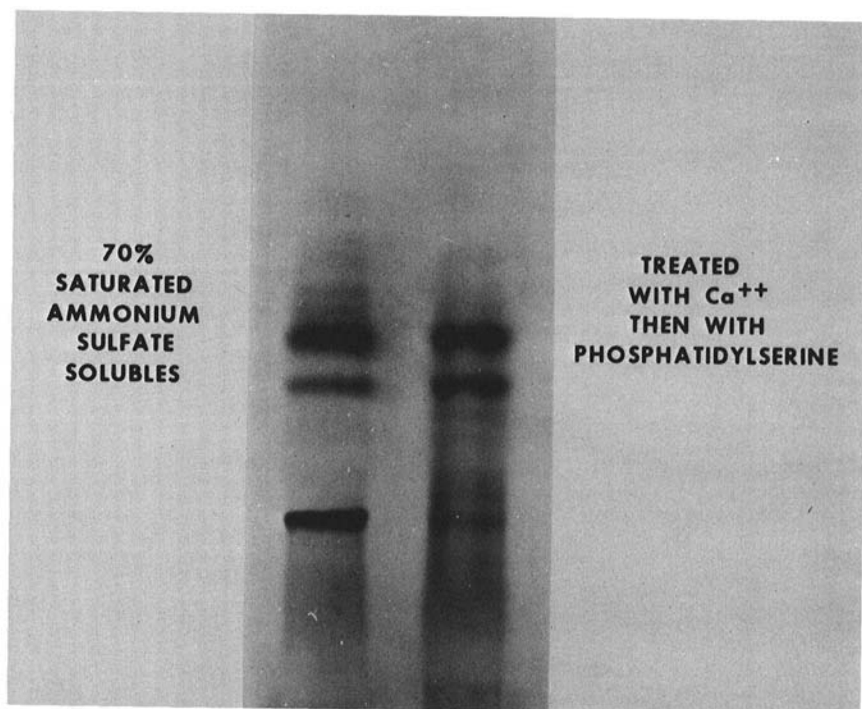

Fig. 2. Starch-gel electrophoresis patterns of the wheat protein fraction which is soluble in 70% saturated ammonium sulfate solution, and after treatment with Ca^{++} and phosphatidylserine.

Ultraviolet spectra of the protein were recorded with a Beckman Model DB recording spectrophotometer and solutions of 5 mg. per ml. concentration in a 1 cm. quartz cell in order to detect any ferulic acid absorption.

RESULTS

Binding of Phosphatidylserine to Protein

If the water-soluble fraction of wheat flour is made 0.1M in calcium chloride, then exhaustively dialyzed against water to remove nonbound calcium, two of the protein components retain sufficient bound calcium to bring about interaction with phosphatidylserine to form a lipoprotein precipitate. This is evident by removal of these two protein species from the electrophoretic pattern of the soluble proteins. As previously reported (7), treatment by calcium chloride or phosphatidylserine alone does not alter the pattern. One of these two proteins is soluble in 70% saturated ammonium sulfate solution and is the one of present interest.

Figure 1 shows the electrophoretic patterns of water-solubles extracted from Spring Wheat Long Patent flour (patterns B, C, and D) and those components of this mixture that remain soluble in 70% saturated ammonium sulfate solution (pattern E. The arrow indicates the Ca-binding component which appears as a doublet in the absence of urea). The arrow at pattern A indicates the position of α-gliadin. Treatment of the 70% saturated ammonium sulfate-solubles with calcium

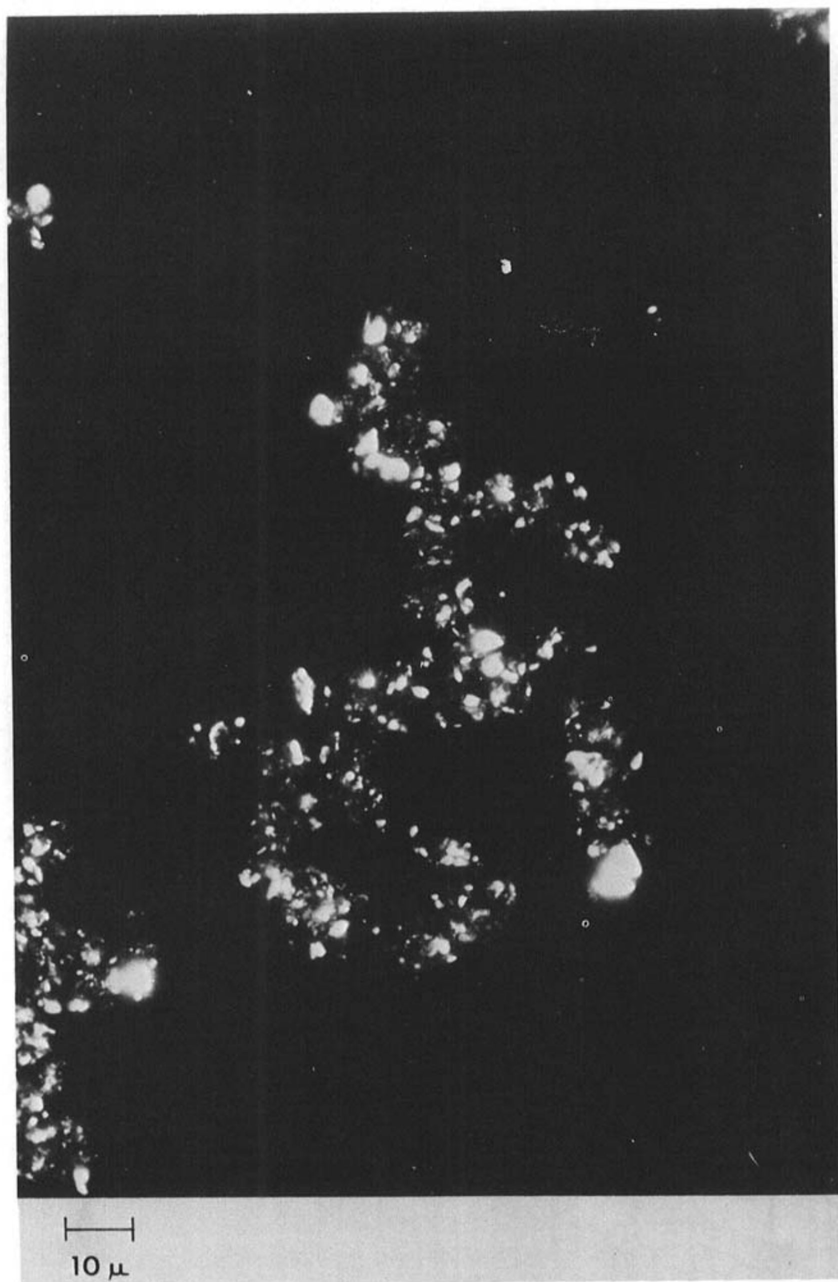

Fig. 3. Photomicrograph taken with plane-polarized light of crystalline complex formed from phosphatidylserine, calcium, and the calcium-binding protein. Particles formed in this way are typically 5 to 10 μ in diameter.

chloride and dialysis to remove nonbound calcium as before again leaves the same protein component with the capability of binding phosphatidylserine; addition of the lipid again brings about lipoprotein precipitation to remove the protein from the mixture. Results are similar when water-solubles from Lemhi flour are used in the above sequence of operations. Figure 2 shows a starch-gel electrophoresis pattern demonstrating the removal of the protein. One pattern shows the complete 70% saturated ammonium sulfate-soluble protein mixture; the other shows the mixture with the protein of interest removed by the phosphatidylserine-calcium complex. If magnesium chloride is used in the place of calcium chloride no binding of the protein to phosphatidylserine can be demonstrated. Changing the phospholipid to triphosphoinositide, however, produces similar removal of the same protein from the mixture. The phosphatidylserine-calcium-protein complex produced is of

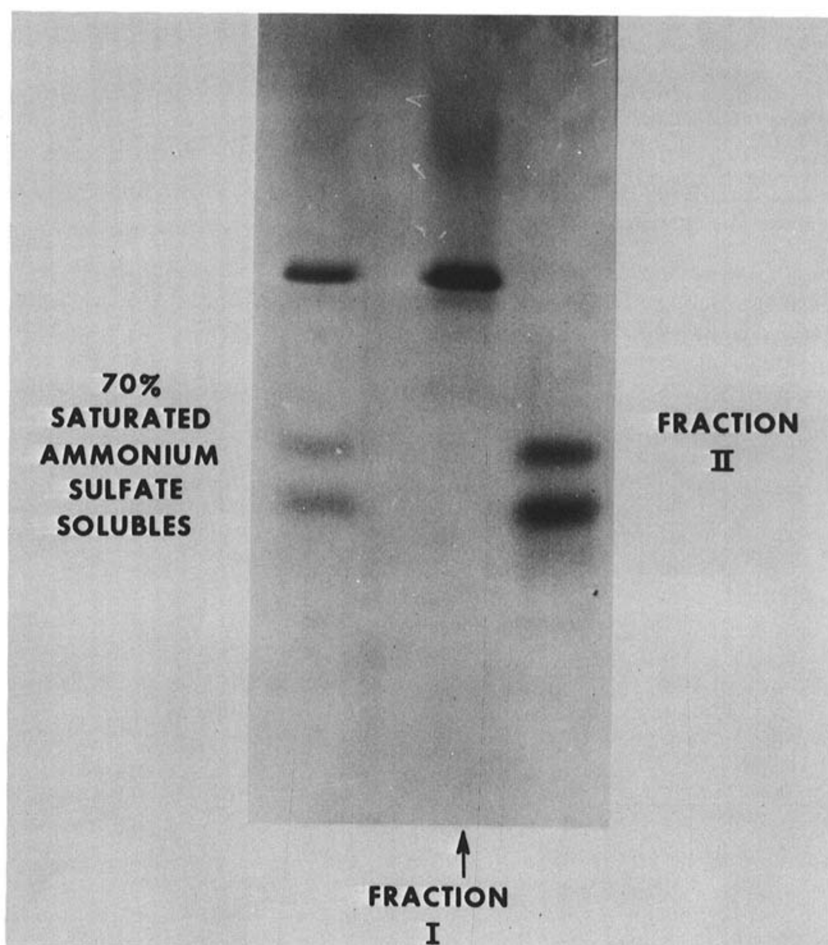

Fig. 4. Starch-gel electrophoresis patterns of the 70% saturated ammonium sulfate-soluble proteins from wheat flour and the fractions of this mixture as eluted from Sephadex G-200. The origin in this case is at the top of the figure.

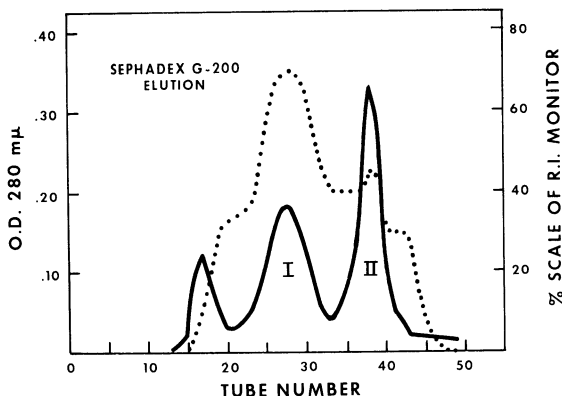

Fig. 5. The elution curve from Sephadex G-200 of the 70% saturated ammonium sulfate-solubles from wheat flour. The solid line is protein elution as followed by ultraviolet absorption at 280 nm.; the broken line is the elution curve as traced by a refractive index monitor. Fraction size was 7.5 ml.

ordered structure (Fig. 3). The crystalline complex was photographed through a microscope using plane polarized light.

Purification of the Protein Component

Chromatography of the 70% saturated ammonium sulfate-solubles through Sephadex G-200 separates the protein of interest cleanly from the other protein components of the mixture. Starch-gel electrophoresis (Fig. 4) of the mixture (pattern A), fraction I (pattern B), and fraction II (pattern C) clearly shows the separation. The elution curve (Fig. 5) indicates carbohydrate contamination of the protein fractions. This is evident from the lack of coincidence of the protein curve and the refractive index curve. Analysis of the desired protein fraction from this type of separation reveals a carbohydrate content of 61%. This figure can be reduced to 55% by treatment with 3M urea and repeated dialysis. Protein analyses gave various results depending on the method used. Ninhydrin values were highest, giving values up to 41% protein. Some Folin analyses, however, gave protein values as low as 10%. No explanation has yet been found for these differences. An intermediate value of about 25% protein was determined from absorption of light at 280 nm. in comparison to a standard of α-gliadin, which has a similar content of aromatic residues per unit weight (20).

Ferulic acid (21) and phytic acid can bind calcium and can be associated with larger molecules. The protein under study was examined for inclusion of such agents by measurement of phosphorus content and of the ultraviolet absorption spectrum. Ferulic acid exhibits a characteristic absorption at 325 nm., but no absorbance at this wavelength by the protein component was detected. Phosphorus analysis showed the fraction to contain less than 0.5 μmoles of phytic acid per g., which is negligible.

Electrophoresis of the protein was carried out in polyacrylamide gel containing sodium dodecyl sulfate along with proteins of known molecular weights. Migration distances of ribonuclease, chymotrypsinogen A, and ovalbumin fell in a straight line when plotted against molecular weight on a log scale. The migration distance of the

calcium-binding protein fell on this line at a point corresponding to a molecular weight of 16,500 ± 10%.

Removal of carbohydrate which is not part of the protein molecule was attempted by chromatography on Sepharose 4B, a permeation gel having also sulfate ion-exchange groups. Three fractions, one mostly hexose polymer, one mostly pentose polymer, and one minor contaminating protein, could be removed from the calcium-binding protein in this manner, but the protein of interest was irreversibly adsorbed on the Sepharose. It could not be removed by 0.5M NaCl or by 2% EDTA solutions. Likewise on DEAE-cellulose the desired protein component, once applied at pH 8.0, could not be removed by salt solutions, EDTA solutions, or 0.1N HCl. When applied at pH 7.5 or below to DEAE-cellulose, none of the fraction was retained on the column. The contaminating material removed from the protein, when it was adsorbed on the resins, accounted for about 70% of the applied Sephadex fraction I material after elution, lyophilization, and weighing. This lends support for the value of 25% protein in the Sephadex fraction as determined by ultraviolet absorption. Moving boundary electrophoresis showed that the carbohydrate was separable from the protein component, but once separated from carbohydrate the protein precipitates, and is thereafter totally insoluble. This might explain the irreversible adsorption on ion-exchange resins. The protein may also be removed from carbohydrate by salt precipitation which yields a similar insoluble product. This has been useful in one instance in providing material for amino acid analysis.

Table I lists the amino acid composition of the protein component under study. The values were calculated from results in terms of μmoles of amino acid in a given weight of sample with an assumed molecular weight near 16,500. With a method using the smallest whole integer value for the least abundant amino acids, results are the same. Rounded to the nearest whole residue the amino acid values correspond to a molecular weight of 16,780.

After exposure of the protein to calcium chloride and dialysis against four changes of an 800-fold excess of 0.05M sodium chloride and two changes of water, 0.60 calcium atoms per protein molecule were still present. Although binding constants were not measured because of the difficulty in preparing pure, soluble protein, it appears from these experiments that the equilibrium is quite in favor of a calcium-protein complex. When applied in the same manner as calcium, manganous ion, which is often used in place of calcium ion for electron-spin resonance studies of calcium-binding materials, does not bind to this protein, as evidenced by electron-spin resonance spectra of the treated protein.

TABLE I. AMINO ACID COMPOSITION OF THE CALCIUM-BINDING PROTEIN

Amino Acid	Residues per Mole[a]	Amino Acid	Residues per Mole[b]
Lysine	12.2	Glycine	13.6
Histidine	3.8	Alanine	18.4
NH_3	21.4	Cysteine	0.7
Arginine	7.0	Valine	12.0
Aspartic acid	15.4	Methionine	2.8
Threonine	11.1	Isoleucine	5.7
Serine	8.2	Leucine	13.2
Glutamic acid	15.7	Tyrosine	3.3
Proline	8.8	Phenylalanine	6.1

[a]Molecular weight from electrophoresis = 16,500.
[b]Molecular weight from amino acid analysis = 16,780.

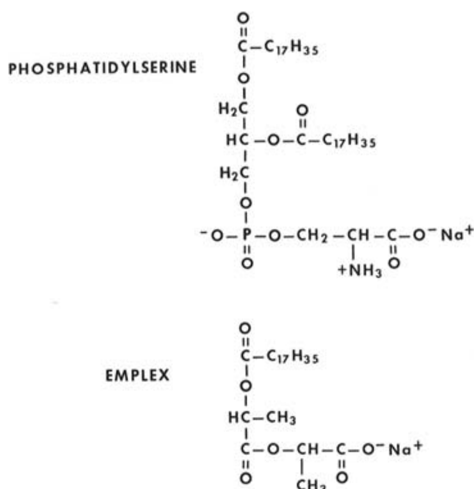

Fig. 6. Representative structures of phosphatidylserine and sodium stearoyl-2 lactylate drawn to emphasize the similarities in polarity distribution between the two molecules.

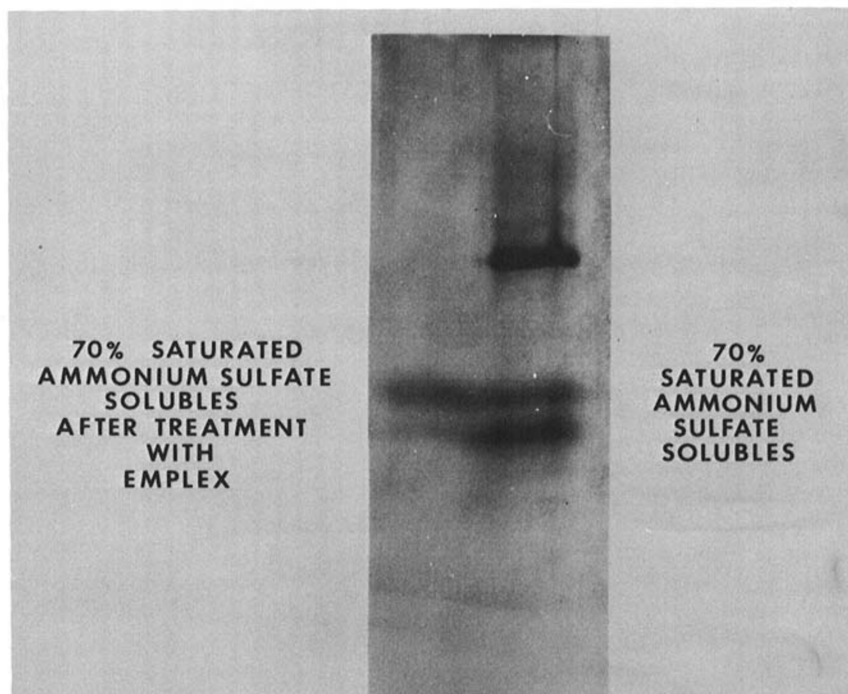

Fig. 7. Starch-gel electrophoresis patterns of the 70% saturated ammonium sulfate-solubles from wheat flour and those components of this mixture which remain in solution after treatment with Ca^{++} and sodium stearoyl-2 lactylate. The origin is at the top of the figure.

Binding of Stearoyl-2 Lactylate to Protein

Figure 6 contains representative structures of phosphatidylserine and sodium stearoyl-2 lactylate. The similarities in structure are evident, particularly with respect to polarity distribution. Stearoyl-2 lactylates exhibit interaction with the protein component in a manner similar to phosphatidylserine. Calcium, either added first to the stearoyl-2 lactylate, first to the protein, or to a mixture of both lipid and protein, again mediates a reaction to form a precipitate of the mixed protein and stearoyl lactylate. Figure 7 shows an electrophoretic gel with the patterns of the proteins soluble in 70% saturated ammonium sulfate and a portion of the same mixture after treatment with sodium stearoyl-2 lactylate and calcium. The protein of interest is clearly removed from the solution by this treatment.

DISCUSSION

The water extract from wheat flour after dialysis against water contains sufficient divalent metal ions to mediate phospholipid-protein interactions as previously described (6,7). Dialysis against EDTA solution is necessary to remove such cations that apparently are bound to various protein species in the water-soluble mixture. Previous studies of phospholipid-metal-protein binding and the present study have demonstrated that cation-protein interactions can be quite specific. The protein of present interest has provided a possible opportunity for a more in-depth study of these interactions. Even though it could not be isolated in pure, soluble form, its interactions with metal ions and lipid have been demonstrated.

Calcium probably binds to the protein at certain amino acid side groups. Associated molecules such as phytic acid or ferulic acid are not involved. Binding of calcium or lipid to the carbohydrate present also seems unlikely. The previous work concerning phospholipid binding by wheat proteins indicated that sulfur-containing amino acids and hydroxyl-containing amino acids of the proteins were involved in the binding of phospholipid-metal complexes. Amino acid analysis of the protein of present interest, however, showed that it contained fewer sulfur-containing residues than the water-soluble proteins as a whole (20) and also fewer hydroxyl-containing amino acids. The protein does contain about three times as much lysine as the water-soluble proteins as a whole, as well as more threonine, and more phenylalanine. Probably secondary structure of the protein is the determining factor in binding calcium, in that proper functional groups are brought into a spatial orientation suitable for binding the metal ion.

The protein component isolated in this study represents about 0.2% by weight of the starting flour. There is sufficient calcium present in flour (22) and in the water-solubles (23) to saturate the component. This protein could therefore be expected to undergo the complex formation reactions reported here when mixed in a dough.

Sodium and calcium stearoyl-2 lactylates have proved to be efficient dough improvers and are of increasing importance because of their value in baked wheat-flour products that are nutritionally fortified with soy flour (24) or milk solids (25). Sodium stearoyl-2 lactylate can act in the capacity of an emulsifier as well as a dough improver (26). The calcium salt, even though having limited emulsifying ability, still increases the mixing tolerance of doughs, increases loaf volume, and imparts finer grain and softer crumb to breads (27). Binding of the stearoyl-2 lactylate anion to flour protein is the suggested cause for these effects

(27) although the exact mode of action is still unknown. The present study demonstrates that such interaction can certainly occur and in a specific manner.

Possibly added sodium or calcium steroyl-2 lactylate in a dough could act as a binding agent between emulsified lipids and the flour proteins. It has been suggested (28) that stearoyl lactylates interact in the dough system with the flour proteins in much the same manner as do phospholipids. Phospholipids, being the most polar lipids, can coat lipid droplets with their polar groups exposed to interact with protein. Substituting stearoyl lactylate for phospholipid then could strengthen the link between lipid droplets, micelles, or sheets and protein in the dough matrix.

The stability of this link could be quite high as demonstrated by the stable complexes formed between the protein discussed above and stearoyl-2 lactylate. Such stabilization of lipid-protein interactions would be expected to strengthen the dough and soften the crumb of bread, effects demonstrated by sodium stearoyl-2 lactylate.

Acknowledgments

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