CHROMATOGRAPHIC METHOD FOR FRACTIONATING GLOBULINS OF CORN[1]

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ABSTRACT

The globulins in water extracts of corn were fractionated by ion-exchange chromatography on carboxymethylcellulose (CMC). To increase solubility of the proteins at low pH, phytate ions were removed before chromatography. Proteins were adsorbed on unbuffered columns of CMC at low pH and ionic strength (1% acetic acid) and were desorbed by stepwise increases in the ionic strength of the eluant at constant pH (2.8). Rechromatography of components established that a true fractionation was obtained. Under conditions used, the unadsorbed material contained a true protein fraction, along with most of the yellow pigments and the nucleic acids of the extract. Evidence for an active nuclease was found.

Fundamental studies on the proteins of corn in progress in this laboratory required methods for extraction, detection, and measurement of individual protein fractions. In the work of Craine and Fahrenholtz (3) the proteins in water extracts of corn were detected by electrophoresis but resolution often was not good enough to permit quantitative measurement. In preliminary investigations chromatography of the globulins on carboxymethylcellulose (CMC) gave better resolution. In addition the protein recovery was high. Chromatography therefore had promise for determining the globulins as well as for purifying and fractionating them.

The proteins studied were extracted from corn with water and designated as globulins for easy reference (3). When the extract mixture is purified, these proteins appear to have isoelectric points in the pH range from 3 to 5 and are completely soluble below pH 3. This solubility characteristic is masked in the crude extracts by formation of insoluble protein-phytate complexes in the acidic pH range. Because of this effect on protein solubility, removal of phytate ions (3) proved necessary as a preliminary to chromatographic fractionation of the proteins on CMC.

Materials and Methods

The corn meal used for preparations of protein was that described by Craine and Fahrenholtz (3). It was made from a commercial sample

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of Schwenk 13 corn[3] and contained 8.4% moisture, 1.72% nitrogen, and 0.30% phosphorus.

Nitrogen was determined by a conventional micro-Kjeldahl procedure using mercuric oxide as a catalyst in the digestion. Total phosphorus was determined by a modification of the method of Truog and Meyer (12).

Dowex-1 (10X) was cycled twice through the hydroxide and chloride forms. The resin was then converted to the acetate form by washing with a solution of sodium acetate until the eluate was free of chloride ions.

Hercules CMC-30 was purified by treatment with 2*N* hydrochloric acid for 1 hour, followed by washing with water (settling and decantation) to remove excess acid and fine particles of CMC. This cycle was repeated until further treatment with 2*N* HCl produced no coloration in the supernatant solution. Finally the CMC was washed with 95% ethanol on a fritted-glass filter, air-dried at room temperature overnight, and dried at 100°C. for 8 hours. The dry product was ground in a mortar to a fine powder. For preparation of columns, the CMC was slurried in water, poured into the column, and allowed to settle. The column was given a final rinse with water just before introduction of the sample.

The jacketed chromatographic columns were cooled by tapwater (15° C.). Column flow rates of approximately 1 ml. per 3 minutes were maintained during all operations, except when the sample was introduced. Two-milliliter fractions were collected with an automatic, volume-control collector. The progress of a run was followed by measuring the absorbance of the solutions in the range of 240 to 340 mμ in a 1-cm. cell of a Cary recording spectrophotometer Model 11. Recoveries were based upon the absorbance at 277 mμ, which was the wave length of maximum absorbance for purified samples of the protein. A linear relationship between concentration of absorbing materials and absorbance was assumed.

Experiments and Results

Purification of Extract for Chromatography. A water extract was obtained by stirring 240 g. of meal and 1,200 ml. of water for 1 hour at 1°–2°C. Residual meal was removed by centrifugation. The supernatant solution (about 900 ml.) contained 0.4 mg. of nitrogen and 0.4 mg. of phosphorus per ml. The phosphorus content of the extract was reduced to 10% or less of its original value by two treatments

[3]Mention of trade names or products does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

with 20-g. quantities of moist Dowex-1 (10X) in the acetate form as described earlier (3). Adequate removal of phytate ions was indicated by complete solubility of the protein when the pH of the extract was adjusted to 2.8.

After lyophilization, the resin-treated extract was dissolved in 100 ml. of water, and the pH of the solution was adjusted to 2.6 with 0.5*N* hydrochloric acid. The solution was dialyzed against two 2-liter quantities of 1% acetic acid at 2°C.; the two dialysis periods were held to a minimum by stopping the dialysis when the conductance of the dialysate became constant (e.g., 2.5 and 1.5 hours, respectively). The conductance of the final dialysate was approximately that of 0.0005*M* sodium chloride in 1% acetic acid. After centrifugation to remove slight turbidity, the supernatant solution, containing 1.37 mg. of nitrogen per ml., was frozen and stored at –18°C.

Chromatographic Fractionation. The general procedure for chromatography involved: (a) adsorption of the sample from 1% acetic acid, which provided low ionic strength (about 0.002) combined with low pH (2.8) and high buffering capacity; (b) rinsing the column with 1% acetic acid to remove unadsorbed material; and (c) stepwise elution of adsorbed material with increasing ionic strength at constant pH. Ionic strength was increased by adding sodium chloride to the 1% acetic acid; in all subsequent references to the chromatography the use of 1% acetic acid solutions may be assumed. The column used for the chromatography had an approximate volume of 38 ml. (9 by

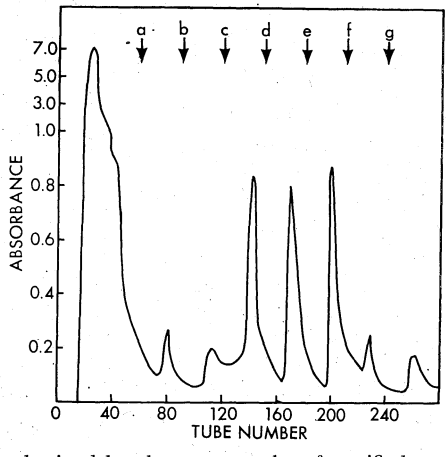

Fig. 1. Fractions obtained by chromatography of purified corn extract applied in 1% acetic acid. After a rinse with 1% acetic acid, elutriants, which were 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.5*M* sodium chloride in 1% acetic acid, were placed on the column at points a, b, c, d, e, f, and g, respectively, and the absorbance at 277 mμ was measured on the effluent.

610 mm.) and held about 6.5 g. of CMC. Twenty milliliters of the preparation containing 27.4 mg. of nitrogen were placed on the column, which then was rinsed with 60 ml. of 1% acetic acid. Stepwise elutions were made with successive 60-ml. quantities of 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.5*M* sodium chloride in 1% acetic acid. In Fig. 1 the absorbance at 277 mμ is plotted against the tube number of the fractions. A large portion (58%) of the absorbance came through in the unadsorbed fraction. Although some material was removed with each elutriant, the largest fractions were those removed with elutriants containing 0.05, 0.1, and 0.2*M* sodium chloride. Calculations based on the results in Fig. 1 showed a recovery of approximately 86%. Several replicate chromatographs gave patterns similar to Fig. 1.

Absorption Spectra and Protein Recoveries. In part of these studies the chromatographic separation was modified so that all of the desorbed protein occurred in one fraction. For this purpose the column was eluted with 0.5*M* sodium chloride in 1% acetic acid after removal of the unadsorbed fraction by rinsing with 1% acetic acid. The re-

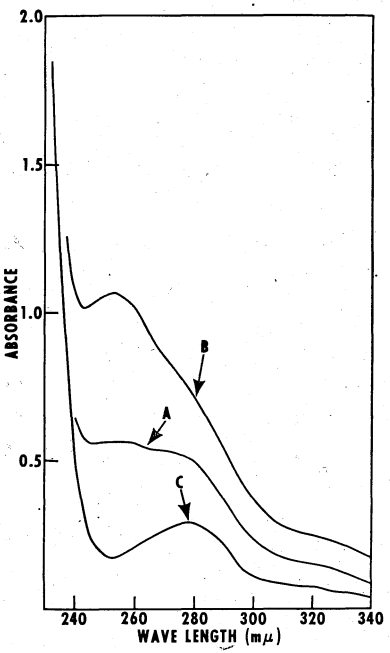

Fig. 2. Absorption spectra: Curve *A* for the purified initial corn extract; curve *B* for the entire unadsorbed fraction from the chromatographic separation; and curve *C* for the adsorbed material eluted in one step by 0.5*M* sodium chloride, corresponding to a combination of the eluted fractions of Fig. 1. All represent the same nitrogen concentration (approximately 0.05 mg. per ml.) — curve *A* as replotted and curves *B* and *C* as observed.

sulting unadsorbed fraction and total desorbed fraction had sufficient concentration of protein to permit determining both nitrogen content and ultraviolet absorption spectrum.

The absorption spectrum curves for the purified initial extract, the unadsorbed fraction, and the total desorbed fraction are shown in Fig. 2. To facilitate comparisons, the absorbance data for the initial extract was replotted so that all the curves in Fig. 2 correspond to the same nitrogen concentration (approximately 0.05 mg. per ml.). Curve *A*, for the preparation of initial extract, has a maximum at 255 mμ, a shoulder at approximately 280 mμ, and appreciable absorption in the range of 300 to 340 mμ. Although there is absorption around 280 mμ as expected of protein solutions, the curve definitely is not typical of purified proteins. The strong absorption in the region of 260 mμ is typical of nucleic acids.

The absorption in the 250–260 mμ range is accentuated in the spectrum of the unadsorbed portion, curve *B*. The maximum is at 252 mμ with only a slight shoulder around 280 mμ. Nitrogen and phosphorus determinations on a comparable unadsorbed fraction before dilution showed 0.58 mg. nitrogen and 0.016 mg. phosphorus per ml. Spectral analysis of individual tubes of the unadsorbed fraction (the initial fraction in Fig. 1) showed some concentrating of nucleic acids in the early portion (tubes 19–33) and of protein in the later portions (tubes 51–61), but no distinct separation. Most of the absorption from 300 to 340 mμ, which is evident in curves *A* and *B* of Fig. 2, occurred in tubes 17–25 of the unadsorbed fraction and corresponded to the yellow pigments of the corn extract.

The total desorbed fraction gave the spectral curve *C* of Fig. 2. It resembles a typical protein spectrum with a minimum at about 250 mμ and a maximum about 277 mμ. The individual tubes and combined fractions eluted in the run shown in Fig. 1 also gave patterns similar to curve *C* in Fig. 2.

The presence of nucleic acids, which have high specific absorbance even at 277 mμ, places some limitations on the use of ultraviolet absorption for measuring the distribution of proteins in the chromatographic fractions. The apparent recovery of protein as measured by absorbance will be higher than the actual recovery when the ratio of nucleic acids to protein is higher in the fraction than in the original mixture, and lower when the ratio is lower. This effect was rather marked, for example, in the gross separation of the corn extract into unadsorbed and total desorbed fractions. On the basis of absorbance at 277 mμ and of nitrogen analysis, respectively, the unadsorbed fraction (higher in nucleic acids) contained 64 and 43% of the initial

material; the total desorbed fraction (lower in nucleic acids) contained 22 and 40%; and the over-all recovery was 86 and 83%.

The relative distribution of protein among individual tubes of the desorbed fractions was indicated with reasonable accuracy by the absorbance at 277 mμ, as demonstrated by a limited number of analyses of column effluents by the colorimetric protein method of Lowry *et al.* (6). In these fractions the interfering effect of nucleic acids is small, since the ratio of nucleic acids to protein is low and probably fairly constant. The absorbance method therefore was adopted for evaluating the chromatographic fractionations. Advantages of the method included retention of the entire sample for further study, and applicability to the small quantities of material in the effluent fractions.

Rechromatography of Fractions. Each of the four major fractions shown in Fig. 1 was rechromatographed. One-half of the unadsorbed fraction was used without further treatment, while each of the three eluted fractions was freed of sodium chloride by a short dialysis against 1% acetic acid, as described for chromatographic fractionation of the extract. The column for rechromatography of the unadsorbed fraction was the same size as for the original fractionation; thus, ample adsorption capacity was ensured. For the three eluted fractions a smaller column was used, having a volume of about 15 ml. (7 by 380 mm.) and holding about 2.5 g. of CMC.

After the fraction was placed on the column, unadsorbed material was displaced by rinsing with 1% acetic acid (50 ml. for the large column and 40 ml. for the small one). For elution, 30 ml. of each elutriant was used. Typical results of rechromatography of the four major fractions (Fig. 1) are given in Fig. 3, where absorbance at 277 mμ is plotted against the tube number.

The originally unadsorbed fraction was not adsorbed on rechromatography (Fig. 3, A). The recovery was approximately 98%, and no peaks were observed in the elution steps.

Rechromatography of the three fractions originally eluted with 0.05*M*, 0.1*M*, and 0.2*M* sodium chloride resulted in Fig. 3, B, C, and D. The main elution peak in each case was obtained at the same sodium chloride concentration as used in eluting the original fraction. Recoveries in the main peak from each of the three fractions were 50, 47, and 35% as measured by absorbance at 277 mμ; in all cases about 17% was eluted in the peak before and about 9% in the peak after the main one. Unadsorbed material was recovered in each of the three rechromatographs (Fig. 3, B, C, and D), accounting for about 20%

of the total original absorbance. Total recoveries from the column were 92, 94, and 83% of the original absorbance.

The unadsorbed fraction had an absorption maximum at about 255 mμ, indicating that the absorbance resulted primarily from nucleic acids. The effectiveness of rechromatography for eliminating nucleic acid contaminants from the main protein components also was demonstrated by calculating the ratio of absorbance at 280 mμ to that at 260 mμ (A280:A260). The fractions before rechromatography had A280:A260 ratios from 1.2 to 1.4. For individual tubes of the main elution peak of the rechromatographs the ratios had in-

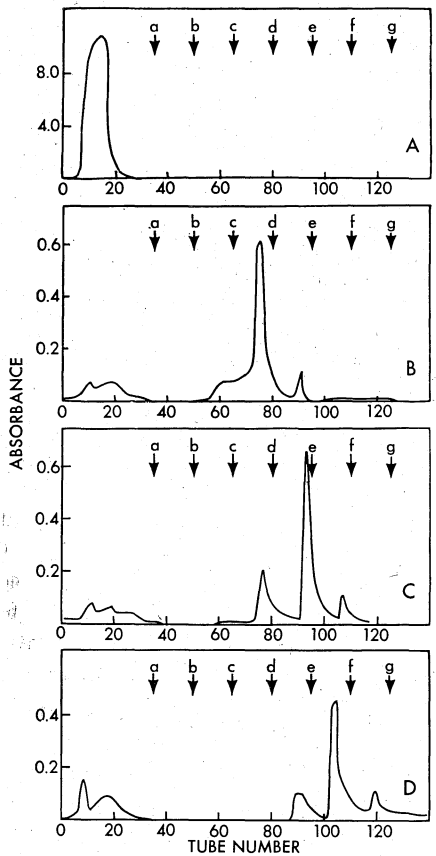

Fig. 3. Rechromatography of fractions obtained by chromatography of purified corn extract applied in 1% acetic acid. After a rinse with 1% acetic acid, elutriants, which were 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.5*M* sodium chloride in 1% acetic acid, were placed on the column at points a, b, c, d, e, f, and g, respectively, and the absorbance at 277 mμ was measured on the effluent. A, the unadsorbed fraction; B, the fraction eluted with 0.05 *M* sodium chloride; C, the fraction eluted with 0.1*M* sodium chloride; D, the fraction eluted with 0.2*M* sodium chloride.

creased to 1.4 to 1.6. A ratio of 1.75 has been given for highly purified proteins by Taylor (11), whereas for nucleic acids the ratio is about 0.5.

Effect of Prolonged Dialysis on Initial Extract. A lyophilized resin-treated extract prepared as described was dissolved in 50 ml. of 1% acetic acid and dialyzed against several changes of 1% acetic acid at 2°C. for a total of 48 hours instead of the minimum time (about 4 hours). After removal of a slight amount of precipitate by centrifugation, the supernatant solution contained 0.86 mg. of nitrogen per ml. The total nitrogen content of this preparation was only 63% of that obtained with the short dialysis time. The ultraviolet absorption spectrum of the preparation was similar to the protein-type curve, Fig. 2, C, instead of curve *A*.

Discussion

The presence of a proteolytic enzyme in water extracts of corn has been reported previously (3). This protease was found to have a maximum activity at 25°–45°C. at about pH 4.5, with no observed activity at 0°C. at any pH. In the present studies prolonged dialysis (48 hours) of the initial extract resulted in a nitrogen recovery nearly 40% below that obtained on short dialysis (4 hours). This loss could result either from proteolysis or the presence of slowly dialyzing nitrogen compounds. If proteolysis is responsible, the activity of the enzyme at 0°C. might be ascribed to the treatment with the anion-exchange resin, resulting in either an increased solubility of the enzyme or removal of some inhibitors. The results of prolonged dialysis also provided evidence of nuclease activity. The lower absorbance and the change in absorption curve from nucleic acid-type (Fig. 2, A) to nearly protein-type (Fig. 2, C) indicate a high loss of nucleic acids, as a result, presumably, of enzymatic hydrolysis to units small enough to be removed by dialysis. The amount of nucleic acids lost, however, would not account for the decrease in nitrogen recovery on prolonged dialysis. Although a decrease in nucleic acid content of extracts would be advantageous in studies of corn globulins, the accompanying possibility of enzymatic degradation of proteins is objectionable. Since the short dialysis time was effective in preventing extensive nitrogen loss, this procedure was used for preparing samples to determine the success of protein fractionation on CMC.

The two conditions for adsorption of protein on CMC emphasized in this work are low-ionic strength and low pH, attained by using 1% acetic acid as solvent. At low-ionic strength adsorption is enhanced, as previously reported (1,5,9,10) and as confirmed in these studies on corn extracts. This effect presumably results from competition be-

tween protein and inorganic ions for exchange sites; the amount of protein adsorbed increases with decreasing ionic strength. Use of low pH at which the protein exists as a cation further promotes retention of protein on CMC by ion exchange. The extensive elution of protein at high-ionic strength gave a fractionation scheme based upon the variable affinity of the CMC for the different proteins in the mixture. Reproducible chromatographic patterns were obtained by this method. As others have noted (5,9), the affinity between proteins and CMC was low enough to allow recovery of most of the protein. The recovery of material absorbing at 277 mμ and of nitrogen was in the range of 82–86%.

Evidence that the initial chromatographic fractionation separated protein components or specific protein fractions was provided by rechromatography of the eluted fractions as reported for other naturally occurring mixtures of nitrogenous compounds (7). On rechromatography most of the protein of each fraction appeared at the original elution position of that fraction, with some material in adjacent peaks. In each case the peak preceding the main peak contained a larger portion than the one following it. Tailing of each eluted fraction in the original chromatographic patterns caused portions of the "tail" to occur in the rechromatograph of the succeeding fraction.

Failure of the unadsorbed fraction to be retained on CMC when rechromatographed from 1% acetic acid indicated that it also represented a true fraction and did not result from exceeding the column capacity in the adsorption step. The protein character of the nitrogenous constituents of this fraction was indicated by a high atom ratio of nitrogen to phosphorus (about 80 when compared with 2 to 5 for nucleic acids), although nucleic acids accounted for a large part of the absorbance at 277 mμ as shown by ultraviolet spectra. Most of the nucleic acids and the yellow pigments in the corn extract remained in the unadsorbed fraction.

The proteins fractionated in this study are only a portion of the globulins of corn. A more complete extraction of the globulins was obtained recently in this laboratory (2) with the use of a carbonate buffer. After methods of completely eliminating possible protease activity have been found, fractionation and quantitative assay of the more complete protein group will be possible with the procedure described here. As the method is simple, gives high recovery, and provides good resolution, its application to other protein mixtures is readily visualized. The ultracentrifuge work of Quensel (8) and Danielson (4) indicates that the same globulins exist in the different

species of the *Gramineae*. This hypothesis could be more adequately established by a study of characteristics other than molecular weight, for example by a combination of chromatography on CMC with electrophoresis.

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