Identification of Phytate in Proteins Using Polyacrylamide Disc Gel Electrophoresis

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A technique for the identification of protein–phytate complexes on polyacrylamide disc gel electrophoresis precipitates the phytate as a white band of ferric phytate. A chromogenic reaction believed to be specific for iron was evaluated as a means to improve the visualization of the ferric phytate on the gels. The results suggest that white bands, which are formed when protein–phytate complexes on polyacrylamide gels are treated with iron solution, could be the result of nonspecific protein–iron interaction in addition to phytate–iron interaction. These findings were supported by experiments in which proteins containing no phytate gave positive staining for the reaction believed to be specific for phytate–iron complex.

INTRODUCTION

Phytate [*myo*-inositol, hexakis(dihydrogen phosphate)] is a naturally occurring organic substance found in many cereal grains, oilseeds, and legumes, e.g., wheat, maize, sesame, and soybeans (Lolas and Markakis, 1976; Maga, 1982). In most legume seeds, phytate phosphorus accounts for approximately 80% of the total phosphorus and is present primarily as a metal phytate or complexed with proteins.

Many papers (Erdman and Thompson, 1982; Maga, 1982; Serraino and Thompson, 1984) suggest that phytic acid and its derivatives decrease the bioavailability of many nutritionally essential minerals such as Ca, Mg, Fe, and Zn. In addition, this complex formation has been reported to interfere with the enzymatic degradation of proteins (O'Dell and deBoland, 1976; Singh and Krikorian, 1982). Several studies have been directed at the interactions between phytate, proteins, and various metals (Reddy and Salunkhe, 1981; Champagne et al., 1985); however, these investigations have not clearly elucidated the exact nature of these interactions.

O'Dell and deBoland (1976) indicated that formation of protein complexes could be detected on polyacrylamide disc gels and suggested that gels on which protein–phytate complexes were electrophoresed, when incubated with a ferric chloride solution, showed the presence of an opaque white band which indicated the presence of precipitated ferric phytate on the gel. The present work was directed at investigating whether the white ferric phytate band could be made more conspicuous by means of visualization of the phytate in proteins after electrophoresis on polyacrylamide gels. This involved the use of a color reaction on the gels. Beaton et al. (1961) described a technique for identifying iron in heme proteins separated by starch gel electrophoresis; a cobalt hexaammine chloride solution used to identify the heme proteins produced a green color with the iron moiety of the heme protein. This solution was investigated as a reagent that could produce a color reaction with the ferric phytate–protein on polyacrylamide gels.

MATERIALS AND METHODS

Materials. Proteins were prepared from *Phaseolus vulgaris* beans (white kidney beans, navy beans) and from *Phaseolus lutanus* beans (baby lima beans, large lima beans) according to the procedures described previously (Alli and Baker, 1980). The conditions of the extraction were selected such that they provided protein isolates having a range of phytate content between 2% and 5% (Alli and Baker, 1981). Soybean protein isolate (Archer Daniels Midland Co.), bovine β-casein (Sigma Chemical Co.), egg albumin (Anachem Co.), and bovine serum albumin (Sigma) were purchased.

Determination of Nitrogen. The nitrogen content of the proteins was determined according to the Kjeldahl method (AOAC, 1980) using an automated micro-Kjeldahl apparatus (Labconco). The protein content was calculated using the factor 6.38 for bovine β-casein and 6.25 for the other proteins.

Determination of Phytate. The phytate content of the protein fractions was determined according to the method of Haug and Lantzsch (1983), with some modifications. The protein (100 mg) was extracted with HCl solution (0.2 N, 10 mL) containing Na_2SO_4 (5%) to solubilize the phytate. The mixture was filtered, and 2.5 mL of the filtrate was diluted to 10 mL. A quantity (0.5 mL) of the filtrate was mixed with HCl solution (0.2 N, 1.5 mL) containing ferric ammonium sulfate (0.5 mM) and two drops of saturated bromine water. The mixture was heated in a boiling water bath (30 min), cooled in an ice bath (15 min), and then centrifuged (5000*g*, 30 min) to precipitate any ferric phytate formed during the reaction. The iron content of the supernatant was determined by adding 2,2′-bipyridyl/mercaptoacetic acid solution (1.5 mL) to the supernatant (1 mL) followed by colorimetric analysis at 519 nm. A standard curve was prepared using sodium phytate (Sigma).

Determination of Iron. The iron content of the protein isolates was determined by atomic absorption spectrophotometry. The protein samples were digested with acid solution (nitric acid/perchloric acid 10:2 v/v). The digested samples were then analyzed for iron using an atomic absorption spectrometer (Perkin-Elmer Model 2380). An air acetylene flame was used for ionization of the samples, and the spectrophotometer was set at a wavelength of 248.3 nm for iron detection and quantitation.

Polyacrylamide Disc Gel Electrophoresis. The electrophoretic procedures and the techniques for preparation and for staining and destaining of gels for proteins were performed according to the method described by Mauer (1971). Protein sample (1 mg) was added to spacer gel solution (0.5 mL), and 100 μL of this solution was subjected to electrophoresis (4 mA/gel) for 1.5 h. Triplicate gels were prepared for each sample. One gel was stained for protein using Coomassie Blue staining solution (1%). The second gel was subjected to the phytate staining technique using a ferric chloride solution (1.48×10^{-4} M) according to the method of O'Dell and deBoland (1976) and then stained for iron using a cobalt hexaammine chloride solution as described by Beaton et al. (1961). The third gel was stained directly with the cobalt hexaammine chloride solution of Beaton et al. (1961).

The proteins were also subjected to sodium dodecyl sulfate (SDS) electrophoresis using the procedure described by Weber

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Table I. Protein, Phytate, and Iron Contents of Proteins Used

protein source	protein,[a,c] %	phytate,[b,c] %	iron, ppm
white kidney bean[b]	67.60 (±0.148)	3.93 (±0.02)	242.4
navy bean[b]	76.00 (±0.132)	4.84 (±0.02)	264.9
baby lima bean[b]	70.08 (±0.143)	3.57 (±0.01)	59.5
large lima bean[b]	60.97 (±0.164)	2.86 (±0.02)	65.3
soybean[b]	87.46 (±0.240)	1.05 (±0.06)	158.1
egg albumin	90.20 (±1.813)	0.00	8.9
bovine serum albumin	96.66 (±0.911)	0.00	11.9
bovine β-casein	91.56 (±0.180)	0.00	260.3

[a] Protein (%) = (% Kjeldahl nitrogen × 6.25) except for β-casein (% Kjeldahl nitrogen × 6.38). [b] Protein preparations. [c] Results are means (standard deviations) of triplicate determinations.

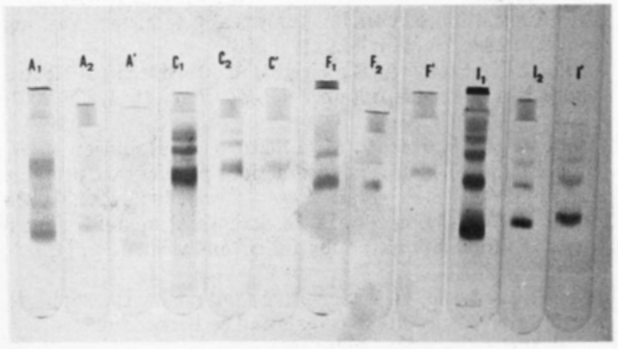

Figure 1. Electropherograms from polyacrylamide disc gel electrophoresis of egg albumin (A_1, A_2, A′), baby lima bean protein isolate (C_1, C_2, C′), navy bean protein isolate (F_1, F_2, F′), and bovine serum albumin (I_1, I_2, I′). Gels of each protein sample were stained for protein (A_1, C_1, F_1, I_1), for iron (Beaton et al., 1961), and phytate (O'Dell and deBoland, 1976) (A_2, C_2, F_2, I_2) and for iron only (Beaton et al., 1961) (A′, C′, F′, I′).

et al. (1972). Triplicate gels were prepared for each protein, and the gels were stained as described above.

RESULTS AND DISCUSSION

Table I shows the protein, phytate, and iron contents of the samples that were studied. The proteins of animal origin (casein, egg albumin, and bovine serum albumin) contained no phytate. The phytate content of the plant proteins ranged from 1.05% (soy protein isolate) to 4.84% (navy bean isolate). The phytate content of the bean proteins is similar to those reported by other workers (Alli and Baker, 1980; Jost and de Rham, 1979). The selection of proteins used facilitated the investigation of phytate detection on polyacrylamide gel electrophoresis and permitted a comparison between phytate-containing proteins and proteins with no phytate.

Figure 1 shows electropherograms of proteins that were stained for protein (A_1, C_1, F_1, I_1); well-defined protein stained (blue) bands were obtained for egg albumin (A_1), baby lima bean protein (C_1), navy bean protein (F_1), and bovine serum albumin (I_1). Gels A_2, C_2, F_2, and I_2 were subjected to the O'Dell and deBoland (1976) technique for phytate detection on the gels, followed by a colorimetric reaction of iron according to Beaton et al. (1961). The bands that showed positive staining for protein also stained "positive" for phytate, using a combination of the techniques of O'Dell and deBoland (1976) and Beaton et al. (1961). Interpretation of these results (Figure 1) based on the methods of O'Dell and deBoland (1976) and Beaton et al. (1961) would suggest that all protein fractions of all samples showed protein stain for iron; it is unlikely, however, that all protein fractions of all the samples contain iron. Egg albumin (A_2) and bovine serum albumin (I_2) contain no phytate (Table I) but gave a positive stain. This could be the result of binding of iron to protein and/

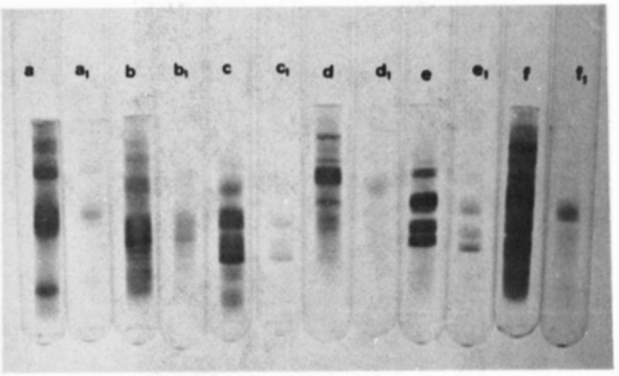

Figure 2. Electropherograms from sodium dodecyl sulfate (SDS) disc gel electrophoresis of egg albumin (a, a_1), bovine β-casein (b, b_1), baby lima bean protein isolate (c, c_1), white kidney bean protein isolate (d, d_1), large lima bean protein isolate (e, e_1), and navy bean protein isolate (f, f_1). Gels of each protein sample were stained for protein (a, b, c, d, e, f) and for iron (Beaton et al., 1961) and phytate (O'Dell and deBoland, 1976) (a_1, b_1, c_1, d_1, e_1, f_1).

or binding of the hexaammine staining reagent to protein but not the binding of iron to phytate since phytate is not present. Several papers (Champagne et al., 1985; Nelson and Potter, 1979) have reported that proteins can bind to iron when placed in iron solution. This suggests that the technique involving precipitation of ferric phytate in protein, with ferric chloride solution, on polyacrylamide gels might not represent phytate–iron interaction only but could also be the result of nonspecific protein–iron interaction. It was also considered that the colorimetric reaction of Beaton et al. (1961), when used in our experiments, might not be specific for iron. Gels A′, C′, F′, and I′ were stained for iron directly (no treatment with iron solution) using the Beaton et al. (1961) technique. The results reveal that all fractions of all proteins gave a positive stain with the hexaammine cobalt chloride solution. This suggests that the positive stain obtained with these gels could be the result of direct interaction between the protein and the iron staining reagent (cobalt hexaammine chloride) or that all of the protein fractions of all samples contain iron which produced the positive stain; however, we consider this to be unlikely. The results of iron analysis (Table I) indicate that iron was detected in all protein samples but with relatively low levels in BSA and egg albumin.

Electropherograms of SDS electrophoresis separation of proteins are shown in Figure 2. The positive stain for iron on the animal proteins which contain no phytate (egg albumin and β-casein) supports the suggestion that if in fact the cobalt hexaammine chloride solution stains specifically for iron, then the technique used by O'Dell and deBoland (1976) results in the binding of proteins with iron; consequently, the technique might not be specific for detection of phytate–iron interaction in the gels. The fact that the intensity of the stain observed with the animal proteins (egg albumin and β-casein; a_1 and b_1 in Figure 2) which contain no phytate was similar to that obtained with the plant proteins (c_1, d_1, e_1, and f_1) which contain, respectively, 3.57%, 3.93%, 2.86%, and 4.84% phytate (Table I) also suggests the lack of specificity of the technique for phytate.

Figure 3 shows the gels (SDS electrophoresis) in which the white bands should represent precipitate due to phytate–iron interaction (O'Dell and deBoland, 1976). The gels were photographed against a dark background so that the visibility of the white bands is improved. The fact that the animal proteins (egg albumin and β-casein; A⁻

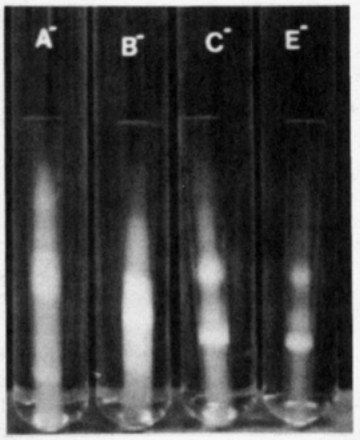

Figure 3. Electropherograms from sodium dodecyl sulfate (SDS) disc gel electrophoresis of (A^-) egg albumin, (B^-) bovine β-casein, (C^-) baby lima bean isolate, and (D^-) large lima bean isolate. Gels were stained for phytate (O'Dell and deBoland, 1976).

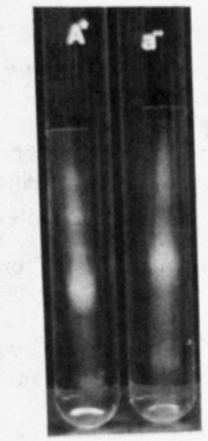

Figure 4. Electropherograms from sodium dodecyl sulfate (SDS) disc gel electrophoresis of egg albumin (a**) with phytate addition and (A*) with no phytate added. Gels were stained for phytate (O'Dell and deBoland, 1976).

and B^- in Figure 3) which contain no phytate (Table I) showed intense white bands, similar to those obtained with the phytate-containing plant proteins (C^- and E^- in Figure 3), further supports the suggestion of lack of specificity of the technique for phytate detection on the gels. The gels shown in Figure 4 represent egg albumin (A*) and egg albumin to which phytate was added (a**); both gels were treated with iron solution (O'Dell and deBoland, 1976). The results show that white bands were obtained both in the presence and in the absence of phytate and support our suggestion that the proteins may bind to iron, resulting in white bands on the gels. It is also evident from Figure 4 that behavior of the egg albumin to which phytate was added was different from that of the albumin with no added phytate. Other workers (Reddy and Salunkhe, 1981) have also reported that electrophoretic patterns of proteins which contained phytate were different from the patterns of the same proteins with the phytate removed.

CONCLUSION

The present work has indicated that the treatment with iron solution of polyacrylamide gels on which proteins containing phytate are electrophoresed might not be specific to allow identification of phytate by the phytate–iron interaction. Interaction between proteins and iron could also occur on the gels. It is also likely that, because of the reactivity of proteins, the cobalt hexaammine chloride solution which was used to indicate the presence of iron in protein molecules may actually react with the protein. In spite of this work, a method that can identify phytate in protein fractionated by gel electrophoresis needs to be developed.

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