this observation and shows that the two major vicilins are degraded at a different rate. The different behavior of vicilins 4 and 6 likely relates to the association strength and to the oligomeric structure of these two proteins. Indeed, the vicilins are very similar in peptide and sugar composition, but they differ in molecular weight and surface hydrophobicity (Cerletti, 1982; Bonomi et al., 1983). This suggests that the peptides are differently assembled. Protomers appear to be more tightly associated in globulin 6 (Bonomi et al., 1983), and this protein undergoes proteolysis by trypsin less readily than globulin 4 (Semino et al., 1983). Our present data show that in early germination stages, although globulin 6 is quantitatively more degraded by endogenous proteases, it displays for a longer time its original protomer composition. This speaks for higher structural requirements in the molecule: once attacked, it breaks down and the products are separated to another fraction.

The later onset of breakdown in legumins is likely related to a more compact structure due to presence of interpeptide disulfide bonds that are not present in the vicilins and of less extensive interruption of covalent continuity in the peptides in the dormant seed (Restani et al., 1981; Cerletti, 1982).

Globulin 1, i.e., conglutin γ, is not degraded during the germination period investigated: this is probably related to its resistance to proteolysis by trypsin (Duranti et al., 1983) and by endogenous endopeptidases (Casero et al., 1983). This behavior may depend on the higher content of bound sugar and higher surface hydrophobicity of this protein (Duranti et al., 1981; Bonomi et al., 1983; Semino et al., 1983).

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Interaction of Phytate with Mustard 12S Protein

N. V. Kishore Kumar Murthy[1] and M. S. Narasinga Rao*

Binding of phytic acid with the mustard 12S protein was studied in the pH range 5.0–1.0 by the techniques of precipitation, turbidimetry, electrophoresis, and electrometric titration. The binding increased with decreasing pH. At pH 3.0, maximum binding of 170 mol of P/mol of protein was observed, which agreed with the basic amino acid content of the protein. Only one class of binding sites was indicated. Electrophoretic studies indicated that even at pH 3.0 some amount of protein was still left in solution. Electrometric titration studies indicated the possibility of the presence of soluble protein–phytate complexes in the system. At pH 3.0, calcium inhibited the formation of protein–phytate complex.

Phytic acid, the hexaphosphoric ester of myoinositol, is a constituent of many legumes and oilseeds including mustard seeds. It reduces the bioavailability of essential minerals such as calcium, zinc, magnesium, etc. by interacting with them (Cheryan, 1980; Cosgrove, 1980). The presence of phytic acid hampers peptic digestion of the protein in the alimentary canal (Barre, 1956). A number of proteins of animal and vegetable origin are known to form insoluble complexes with phytic acid below their isoelectric point (Posternak, 1965). The protein–phytate complex could also make zinc and other minerals biologically less available (Cheryan, 1980). Earlier studies on the interaction of phytic acid with proteins have shown that the interaction is dependent on the pH of the medium. At pH values below the isoelectric point of the protein, phytate binds directly to the protein cation, and at pH values above the isoelectric point, it binds to the protein through an alkaline-earth metal (Cheryan, 1980).

Mustard meal contains 5–7.5% phytic acid, depending upon the variety (Jones, 1976). Mustard seeds contain two

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India.
[1]Present address: Hypertension Research Laboratory, Henry Ford Hospital, Detroit, MI 48202.

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classes of proteins, a high molecular weight protein (12S) constituting 25% of the total and a low molecular weight species (1.3S) that constitutes the rest. The 12S protein is the major single protein present in the seed that has been isolated to homogeneity whereas the 1.3S species is reported to consist of a number of proteins (Lonnerdal and Janson, 1972).

An understanding of the interaction of phytate with mustard proteins may help in developing methods for elimination of phytic acid from mustard flour. Although the interaction of phytic acid with a number of proteins such as soybean, black gram, and corn (Reddy et al., 1982) has been studied, to our knowledge no reports are available on the interaction of phytic acid with mustard proteins.

MATERIALS AND METHODS

Mustard seeds (*Brassica juncea* variety Varuna, RT-59) grown in the year 1978–1979 were obtained from Haryana Agricultural University, Hissar, India. The dehulled seeds were defatted by repeated extraction with hexane, ground to pass through an 85-mesh (BSS) sieve, and used.

All the chemicals used were of analytical reagent grade.

Methods. *Protein Isolation.* Mustard 12S protein was isolated by the method of Gururaj Rao et al. (1978). The precipitated protein was dissolved in 0.1 M NaCl, dialyzed against water, and used.

Protein and Phytate Concentration. Protein concentration was determined spectrophotometrically by using an $E^{1\%}_{1\ cm}$ of 9.9 (Gururaj Rao and Narasinga Rao, 1981). Phytate was hydrolyzed by using 10 N H_2SO_4 and the inorganic phosphorus was estimated by the method of Taussky and Shorr (1953). Phytate concentration is expressed as phytin phosphorus.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE of the mustard 12S protein was carried out in 0.02 M glycine–HCl buffers of the desired pH, ranging from 4.0 to 2.0, using 7.5% gels at 5 mA/tube. The gels were stained for 30 min in 0.5% amido black in 7% acetic acid and destained in 7% acetic acid.

Interaction of Phytate with Mustard 12S Protein. (1) By Precipitation. The measurements were maded at room temperature (~28 °C) in water adjusted to the desired pH with 1 N HCL. An aliquot (5 mL) of protein solution (4.4 μM) was taken in the reaction vessel of a Radiometer pH meter and the pH adjusted to the desired value by the addition of 1 N HCl. Known amounts of aqueous sodium phytate solution of neutral pH were added. The volume was made up to 10 mL with water and readjusted to the desired pH value. After 30-min incubation at room temperature, the solutions were centrifuged at 5000 rpm for 20 min and the phosphorus (phytin phosphorus) content of the supernatant was estimated. The protein content of the supernatant was determined by measuring the absorbance at 280 nm against the water blank. Controls containing only protein adjusted to the same pH values were also prepared and used for applying suitable corrections for protein concentration. From these values, the number of moles of P bound per mole of protein was calculated by using a molecular weight of 230 000 for mustard 12S protein (Gururaj Rao and Narasinga Rao, 1981).

When the protein concentration (2.2 μM) and phytate concentration (2.2×10^{-4} M) were held constant, the pH was varied from 2.0 to 5.0. Similarly, at pH 3.0 and a protein concentration of 2.2 μM, the concentration of phytate was varied from 2.2×10^{-5} to 2.2×10^{-4} M.

(2) By Turbidimetry. In this method, the turbidity developed upon addition of phytate to the protein solution was determined by measuring the absorbance at 600 nm.

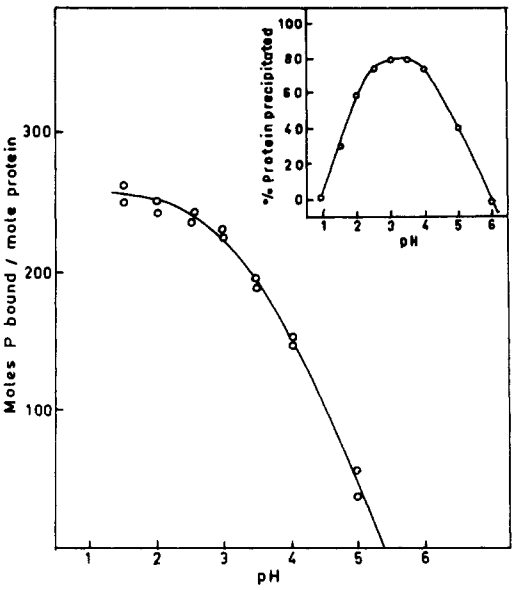

Figure 1. Effect of pH on the binding of phytate by mustard 12S protein. (Inset) Percent protein precipitated by phytate as a function of pH.

In all the cases the reaction was allowed to proceed for 1 min. The effect of pH (range 2.5–5.0), phytate concentration, protein concentration, and calcium concentration on the interaction was studied.

Electrometric Titration. An aliquot (5 mL) of a 1% protein solution (4.4×10^{-5} M) at pH 5.0 was taken in the glass reaction vessel of Radiometer pH meter. To this different volumes of stock phytate solution, also adjusted to pH 5.0, were added to give protein:phytate ratios ranging from 1:25 to 1:100. It was observed that upon the addition of phytate solution, the pH increased by 0.6–0.7 pH unit. The solutions were titrated back to pH 5.0 by using standard HCl (0.118 N) in 10-μL increments with an "Agla" micrometer syringe. In the blank, water adjusted to pH 5.0 was used in place of the protein solution. The number of hydrogen ions bound per mole of protein was calculated from these data according to the method of Tanford (1955).

All the pH measurements were made using a Radiometer pH meter (Titrator TTT-2) at a constant temperature of 30 ± 0.1 °C.

RESULTS AND DISCUSSION

The amount of phytate phosphorus bound per mole of protein increased with a decrease in pH and reached a value of 220 mol of P/mol of protein at pH 3.0 (Figure 1). Below this pH there was only a marginal increase in the value.

The amount of protein precipitated in the presence of phytate increased as the pH was reduced (Figure 1, inset) up to pH 3.0. Below this pH, solubilization of the precipitate occurred as evidenced by a decrease in the percent protein precipitated. At pH 1.0, complete solubilization of the precipitate occurred.

Low pH (up to pH 3.0) has been shown to cause dissociation and denaturation of the protein molecule (Kishore Kumar Murthy and Narasinga Rao, 1983). As all the binding sites on the protein are fully exposed, binding of phytate at pH 3.0 could be expected to be complete and the number of moles of phytate P bound would represent the total number of basic amino acid residues present in it. The increase in the amount of phytate phosphorus bound per mole of protein at these low pH values (Figure 1) could be due to the fact that more than one phytate molecule may be bound per basic amino acid residue.

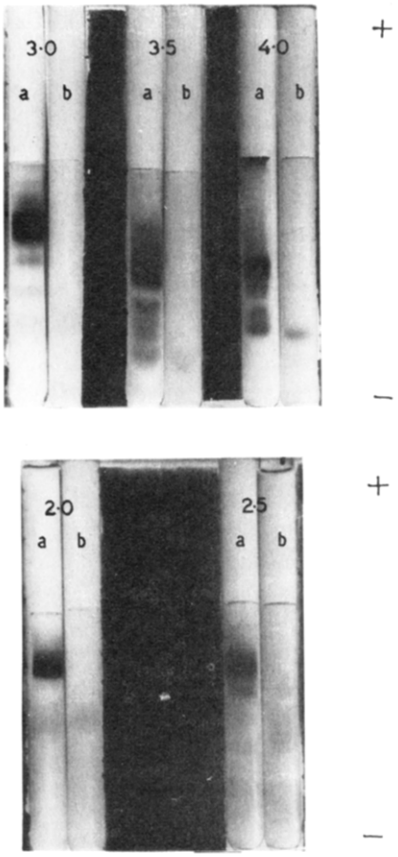

Figure 2. Effect of reaction pH on the mustard 12S protein–phytate interaction followed by PAGE (0.02 M glycine–HCl buffer of the desired pH). The numbers in the figure refer to the reaction pH. (a) Mustard 12S protein at the respective pH. (b) Mustard 12S protein plus phytate at the same pH.

Courtois (1957) has attributed the dissolution of the protein–phytate complex at low pH values to the displacement of the phytic acid (from the protein) by HCl and the simultaneous solubilization of both the components. However, Okubo et al. (1976) and Omasaiye and Cheryan (1979) have reported the occurrence of soluble protein–phytate complexes at pH 2.0 in the case of soybean protein.

As can be observed from Figure 1 (inset) not all the protein had precipitated even at pH 3.0 and at a phytate:protein ratio of 100. This indicated that some amount of protein still remained in solution after the precipitation of the complex. To study the nature of this protein fraction remaining in solution, electrophoresis of the supernatant was performed.

The experiments were carried out in the pH range 4.0–2.0 using a 0.5% protein solution adjusted to the desired value with HCl. Stock phytate solution was added to give a final phytate to protein ratio of 100. The solutions were left for 30 min at room temperature and then centrifuged at 10 000 rpm for 20 min. The supernatant was subjected to electrophoresis. Protein solution to which no phytate was added was used as a control. A fast-moving component was present in the gels, showing that one of the "subunits" remained in solution at all the pH values studied (Figure 2). From the intensity of this band it could be seen that the concentration of this component decreased up to pH 3.0, below which it again increased. These results are compatible with those presented in Figure 1 (inset). The protein component remaining in solution as observed in the electrophoretic patterns could represent an acidic subunit of the mustard 12S protein, which did not interact with phytate. An alternative explanation can be that the two low molecular weight [11 000 and 20 000 (Gururaj Rao, 1980)] subunits of mustard 12S protein may form soluble complexes instead of insoluble complexes with phytate since the formation of insoluble complexes with phytic acid is known to be dependent on the molecular weight of the protein (Courtois, 1957).

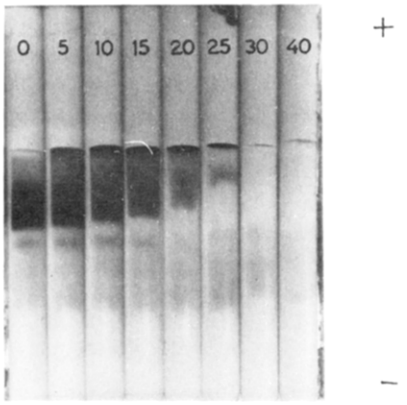

Figure 3. Effect of phytate concentration on the mustard 12S protein–phytate interaction followed by PAGE (0.02 M glycine–HCl buffer, pH 3.0). Numbers in the figure refer to the ratio of phytate to protein.

Effect of Phytate Concentration. The effect of phytate concentration on the binding was studied at pH 3.0 by using a protein concentration of 2.2 μM.

Even though the amount of protein precipitated increased gradually from a ratio of 1:20 to 1:40, the amount of P bound per mole of protein did not change, thus indicating that a critical ratio of P bound per mole of protein was needed for the precipitation to occur.

At low concentrations of phytate (ratio 1 to 10) where no precipitation occurred, soluble protein–phytate complexes may be formed. Gel electrophoresis showed that at ratios of 1:5 to 1:15 slow-moving components were present (Figure 3). These diffuse bands could represent polydisperse soluble aggregates formed upon interaction with phytate. At a ratio of 1:20, the amount of protein in the supernatant was very much reduced. Even in this case an aggregate that was unable to penetrate the gel was observed. At a ratio of 1:25 also this aggregate was seen along with a faint band that had penetrated. No slow-moving bands were observed at ratios of 1:30 and 1:40. However, in all the cases, the fast-moving component that was seen at a ratio of 1:100 (Figure 2) was also observed.

Electrometric titration of the phytate–protein mixture was also conducted to follow the interaction at pH 5.0. The results are expressed as number of hydrogen ions bound per mole of protein. These values correspond to the number of moles of P bound per mole of protein since the hydrogen ions added to the system in the course of the titration would replace the phytate molecules bound to the protein.

The number of hydrogen ions bound per mole of protein increased with the addition of phytate to the system (Figure 4). At a ratio of 1:50 (protein:phytate) the number of hydrogen ions bound per mole of protein reached a value of 50 (Figure 4, inset). Above this protein:phytate ratio, the number of hydrogen ions bound per mole of protein remained constant. This value of 50 mol of P bound/mol of protein corroborates well with the value obtained by precipitation studies at pH 5.0 (Figure 1). At a ratio of 1:25 no precipitation was observed but hydrogen ion titration studies indicated that 33 mol of P was bound/mol of protein, suggesting the formation of soluble complexes.

Barre and van Huot (1965a) in their studies with human serum albumin have shown that phytic acid binds to the

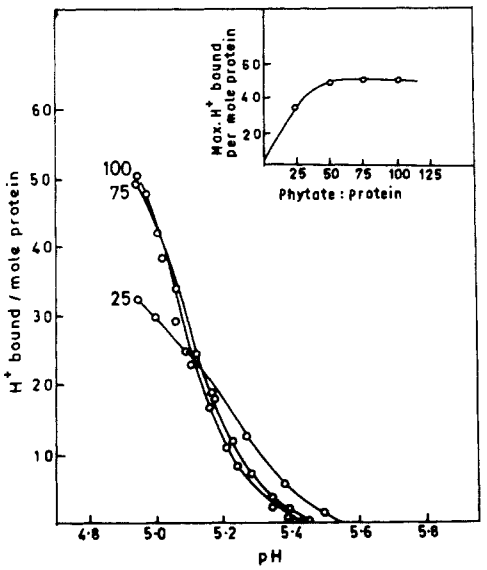

Figure 4. Effect of phytate addition on the electrometric titration curve of mustard 12S protein. Numbers in the figure refer to the phytate to protein ratio. (Inset) Maximum number of H^+ bound per mole of protein as a function of the phytate to protein ratio.

basic amino acid residues in the order lysine, histidine, and arginine whereas in ovalbumin the order was arginine, lysine, and histidine (Barre and van Huot, 1965b). They have attributed this to the easy accessibility of certain amino acids that are located on the surface whereas the others were believed to be in the interior of the protein molecule and hence available only at highly acid pH values.

In their studies with glycinin, Okubo et al. (1976) have reported the presence of two classes of binding sites: primary binding sites, which are freely accessible, and secondary binding sites, which are masked. In our present study, no steps were observed in the precipitation curve, suggesting that all the binding groups were of the same class.

Turbidimetric Studies. Turbidity measurements were also made to determine the number of binding sites. The increase in absorbance of the protein solution at 600 nm was measured as a function of phytate concentration at different pH values in the range 5.0–2.5.

Typical sigmoidal curves were obtained when the absorbance at 600 nm was plotted as a function of phytate concentration expressed as phosphorus (Figure 5). The development of turbidity and attainment of plateau region occurred at lower concentrations of phytate at higher pH values. As the pH was decreased, higher concentrations of phytate were needed to attain these conditions. It was also observed that the maximum turbidity developed upon phytate binding decreased with a decrease in pH up to 3.0, below which the value increased again. This reduction in turbidity up to pH 3.0 may be due to the dissociation of the mustard 12S protein at these pH values (Kishore Kumar Murthy and Narasinga Rao, 1983).

The midpoints of the sigmoidal curves can be taken to represent the phytate concentration at which the complex precipitates. The P bound per mole of protein corresponding to this concentration of phytate is taken to represent the number of sites participating in the primary binding process (Okubo et al., 1976).

A plot of the amount of P bound per mole of protein as a function of pH gave a straight line that intercepts the x axis at pH 5.95 (Figure 5, inset). This represents the pH at which there would be no phytate binding.

The amount of phytate bound to the protein at pH 3.0 was 170 mol of P/mol of protein. This should represent

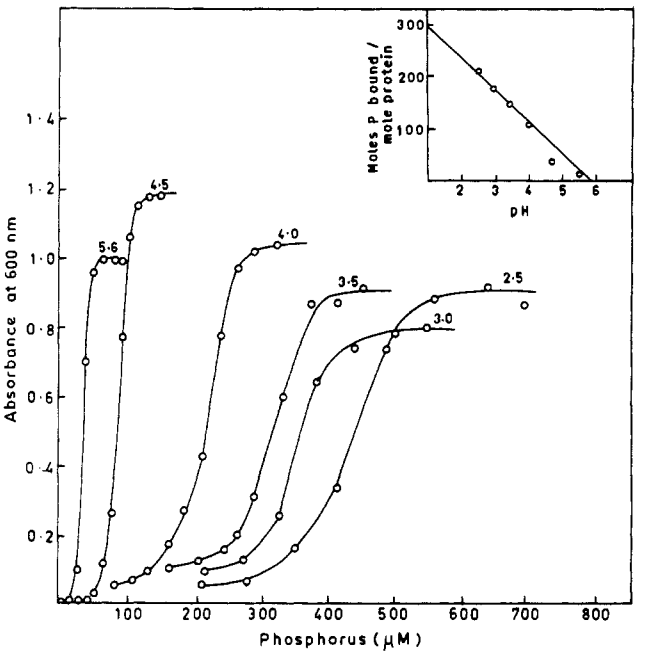

Figure 5. Effect of pH and phytate concentration on the absorbance of mustard 12S protein at 600 nm. Numbers in the figure refer to the pH of interaction. (Inset) Plot of moles of P bound per mole of protein as a function of pH. Protein concentration = 2.2 μM.

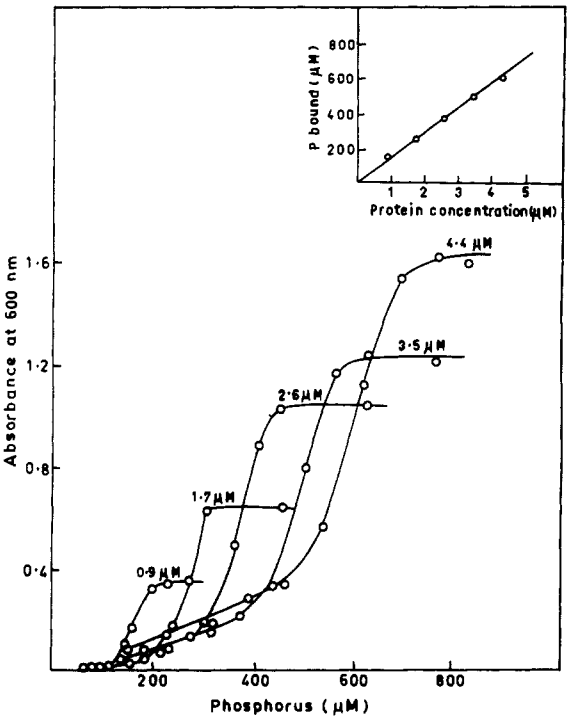

Figure 6. Effect of protein and phytate concentration on the absorbance of the mustard 12S protein at 600 nm. Numbers in the figure refer to the protein concentration. (Inset) Plot of moles of P bound as a function of protein concentration. Reaction pH = 3.0.

the total number of basic amino acid residues of the protein molecule.

Effect of Protein Concentration. The effect of protein concentration on phytate binding at pH 3.0 was studied by turbidimetry. Protein concentrations in the range 0.87–4.35 μM were used.

At each protein concentration a sigmoidal curve was obtained when the absorbance at 600 nm was plotted as

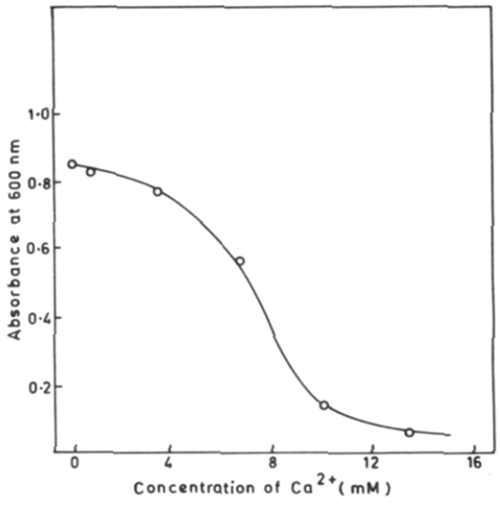

Figure 7. Effect of Ca^{2+} concentration on the absorbance of the mustard 12S protein–phytate complex at 600 nm. Protein concentration = 2.2 μM. Reaction pH = 3.0.

a function of phytate concentration (Figure 6). The turbidity values increased with protein concentration. The phytate concentration corresponding to the onset of precipitation was determined from the midpoints of the curves. A plot of the amount of P bound as a function of protein concentration was linear and the straight line passed through the origin (Figure 6, inset). From this graph, the amount of phytate bound to the protein under these conditions was found to be 160 mol of P/mol of protein. This is in good agreement, within limits of experimental error, with a value of 170 mol of P/mol of protein obtained by measuring phytate binding as a function of pH (Figure 5, inset).

The value obtained for the amount of phytate P bound to the protein by turbidimetric studies (160–170 mol of P/mol of protein) agrees well with the basic amino acid content of the mustard 12S protein reported by Mackenzie (1973). The basic amino acid content of the 12S protein from a number of cultivars of *B. juncea* varied from 150 to 170 residues/mol of protein (Mackenzie, 1973).

The amount of phytate bound to the protein at pH 3.0 estimated by precipitation experiments gave a value of 220 mol of P/mol of protein. Thus there was a discrepancy between the values obtained from the precipitation and turbidity measurements. In the precipitation experiments, the value is calculated from the phosphorus and protein content of the supernatant. It is probable that some amount of phytate would be adsorbed physically by the precipitated protein–phytate complex. This would reduce the phytate concentration in the supernatant and thus lead to a higher value of phytate binding. It would thus appear that the values from turbidimetric measurements are more reliable.

Effect of Calcium. Calcium has been shown to dissociate the protein–phytate complex formed at low pH values (Barre et al., 1955; Okubo et al., 1976) as well as to inhibit the formation of the complex (Okubo et al., 1976).

The inhibition of the formation of the protein–phytate complex by calcium in the case of mustard 12S protein was studied at pH 3.0 by turbidimetry. A protein concentration of 2.2 μM and a protein to phytate ratio of 1:80 were used. The calcium concentration ranged from 2 to 14 mM.

The turbidity of the protein–phytate mixture decreased upon addition of Ca^{2+}, and the extent of the decrease increased with an increase in Ca^{2+} concentration (Figure 7). Thus Ca^{2+} inhibited the formation of the protein–phytate complex and at 14 mM Ca^{2+} total inhibition was observed. However, up to 4 mM Ca^{2+}, inhibition was insignificant.

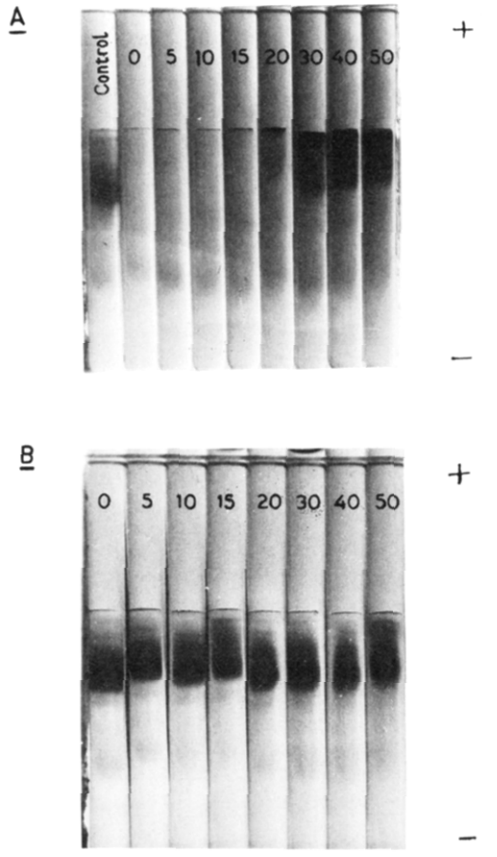

Figure 8. (A) Effect of Ca^{2+} concentration on the PAGE pattern of the mustard 12S protein–phytate complex. (B) Effect of Ca^{2+} concentration on the PAGE pattern of mustard 12S protein. Buffer: 0.02 M glycine–HCl buffer, pH 3.0. Numbers in the figures refer to the calcium concentration (mM).

Similar results have been reported by Okubo et al. (1976) in their studies on the inhibition of binding of phytate to glycinin by calcium. Dissolution of the protein–phytate complex at higher Ca^{2+} concentrations, at pH 3.0 (Figure 8), has also been reported by these workers.

Registry No. Phytic acid, 83-86-3; calcium, 7440-70-2.

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Level of Application and Period of Exposure Affecting Accumulation and Distribution of Chromium-51 and Zinc-65 in Hydroponically Grown Kale, Bush Beans, and Soybeans

Heidi A. Schmitt and Connie M. Weaver*

These experiments were conducted to assess the efficiency of various conditions for intrinsic labeling of kale, soybeans, and bush beans with ^{51}Cr and ^{65}Zn. Various harvest times, exposure levels, and exposure periods were employed and nuclide concentration, percent of applied dose accumulated by various plant parts, and nuclide distribution were calculated. ^{51}Cr and ^{65}Zn concentration increased proportionally with increasing level of application. The efficiency of incorporation of ^{51}Cr or ^{65}Zn varied little within each plant species among various treatment levels. Exposure period and level of ^{51}Cr and ^{65}Zn affected the nuclide concentration significantly ($P < 0.05$) but not the distribution of ^{51}Cr or ^{65}Zn. Radionuclide concentration was generally greater when plants were exposed throughout the growth cycle than for shorter periods of time, but the greater efficiency of incorporation into soybean seeds occurred when plants were exposed during the reproductive period of growth.

Chromium and zinc are essential elements for human nutrition. Plant foods are a dietary source of both elements. Biologically labeled plant tissues can be used to conveniently study the form and localization of micronutrients, the bioavailability of micronutrients from specific foods, and the effect of processing on trace element content and chemical associations. Knowledge of accumulation and distribution of chromium and zinc in plants throughout the plant growth cycle is necessary for the efficient production of intrinsically labeled plant material.

Whereas zinc has an intermediate mobility in plants (Epstein, 1972), chromium accumulates in the roots of plants and is poorly translocated to the shoots (Lahouti and Peterson, 1979; Turner and Rust, 1971). Although Skeffington et al. (1976) reported a greater transfer of ^{51}Cr from roots to shoots of barley plants when applied as CrO_4^{2-} rather than Cr^{3+}, others have concluded that accumulation of chromium in plants is independent of the form of administration (Huffman and Allaway, 1973; Blincoe, 1974; Starich and Blincoe, 1982). Cary et al. (1977b) presented evidence to suggest that Cr^{3+} is rapidly converted to Cr^{6+} when added to soils, but the CrO_4^{2-} anion is the form taken up by the plant from the soils. In an attempt to further understand accumulation behavior of chromium and zinc in plants, three experiments were conducted in which a selection of plants was exposed to ^{51}Cr and/or ^{65}Zn via a circulating nutrient solution. Radionuclide concentration and efficiency of accumulation of an applied dose for various plant parts were determined as a function of exposure period, level of radionuclide application, and stage of maturity when plants were harvested.

Department of Foods and Nutrition Purdue University, West Lafayette, Indiana 47907.

MATERIALS AND METHODS

Plants were germinated under conditions described previously (Levine et al., 1982) in a hydroponic system consisting of six compartments holding 85 L each of the circulating nutrient solution culture. The composition of the nutrient solution (pH 5.5) was 0.22 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM EDTA, 0.26 mM $K_2HPO_4 \cdot 3H_2O$, 0.37 mM NH_4NO_3, 0.30 mM $MgSO_4 \cdot 7H_2O$, 0.67 mM KOH, 16 μM $FeSO_4 \cdot 7H_2O$, 34 μM $ZnSO_4 \cdot 7H_2O$, 35 μM H_3BO_3, 15 μM $NaMoO_4 \cdot 2H_2O$, 2.3 μM $CuSO_4 \cdot 5H_2O$, 0.10 μM $Co(NO_3)_2 \cdot 6H_2O$, and 13.5 μM $MnCl_2 \cdot 4H_2O$. Unlabeled nutrient solution contained no added stable chromium (<0.045 ppb of Cr as determined by atomic absorption spectroscopy). Seeds were supported by using BR-8 grow blocks (Famco, Inc., Medina, OH), and when further support was necessary, as for soybeans, the plants were suspended from a support wire with string. Radionuclides, $^{51}CrCl_3$ (351 mCi/mg Cr) and $^{65}ZnCl_2$ (23 mCi/mg Zn), were administered to the plants via the circulating nutrient solution. The treatments and harvest schedule for all three experiments are given in Tables I and II, respectively.

Experiment I: Trilevel ^{51}Cr and ^{65}Zn Experiment. Soybeans (*Glycine max* L. Merr. var. "Verde"), bush beans (*Phaseolus vulgaris* L. var. "Blue Lake"), and kale (*Brassica oleracea* var. *acephala* D.C. "Dwarf Blue Curled Vates") were germinated, and the plants were continually exposed to one of three levels of ^{51}Cr or ^{65}Zn commencing 1 week from germination throughout maturation. Nutrient solution and nuclides were replaced weekly.

Harvests for both ^{51}Cr and ^{65}Zn were conducted 3 times during the plant growth cycle: preflowering (vegetative), flowering, and maturity for beans and premidmaturity, midmaturity, and maturity for kale (Table II). The harvest was conducted by removing the plants from the hydroponic system and cutting the roots from the stem. Roots were not analyzed. Each plant was put into separate plastic

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