STUDY OF PHYTASE AND FLUORIDE EFFECTS IN GERMINATING CORN SEEDS[1]

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ABSTRACT

Properties of phytase were studied, with endosperm-scutellar tissue of corn seedlings 4 days old. The corn phytase lacked a strict substrate specificity. Optimum incubation temperature of the enzyme was 50°C., optimum pH was 5.6, and the Michaelis constant (K_m) was 0.9×10^{-4} moles per liter. The highest total enzyme activity was located in the fraction at $1,700 \times g$; the supernatant at $20,000 \times g$ was the location of the highest specific enzyme activity. The enzyme survived high temperature, was activated by calcium chloride, and was inhibited by sodium fluoride. Fluoride prevented phytin breakdown and inhibited growth during corn germination.

During seed germination, corn plants depend on the endosperm and scutellar tissues for the raw materials required for germination and growth. Three major types of hydrolytic enzymes—amylases, proteinases, and esterases (lipase and phosphatase)—are responsible for hydrolysis of carbohydrates, proteins, and fats and phosphates, respectively (1). Plant seeds contain 50 to 88% of the total organic phosphate as the phytin form (2).

DeTurk *et al.* (3) and Weiss (4), in their studies of chemical transformation of phosphates in germinating corn seeds, demonstrated that the phytin content decreased as germination proceeded with increasing time. No observation, however, was made of the possible association of phytin breakdown with phytase activity and the characterization of the enzyme responsible for the phytin decomposition during corn germination.

Aims of the present work were (a) to determine the properties of the enzyme hydrolyzing phytin of germinating corn endosperm-scutellar tissues compared to those of enzymes reported as phytases from other sources (5,6,7,8), and (b) to study the influence of fluoride on inhibition of the enzyme, on the phytin content of the tissue, and on growth of root tissues during corn germination.

Materials and Methods

Preparation of Experimental Materials. Uniform-sized seeds of corn (*Zea mays* L. var. Marcross) were lightly dusted with fungicide (Spergon, U. S.

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Rubber Co.). The surface-sterilized corn seeds were placed embryo down on paper toweling. The toweling was rolled and stood for germination in a moist chamber containing the various concentrations of sodium fluoride or double-distilled water for controls. Germination was conducted in darkness at 25°–27°C. The germinated plants were harvested at intervals of 24, 48, 72, and 96 hr. Then the endosperm and scutellar tissues (called endosperm-scutellum) were separated from the root (hypocotyl and radicle) and the epicotyl. Root lengths were measured to determine growth rates. For assays of phytase activity, the endosperm-scutellar tissues were kept at –20°C. until use. For analyses of inorganic and phytin phosphorus, materials were killed and homogenized in a solution containing methanol-chloroform-water (12:5:3, v./v.) and were kept in a freezer at –20°C. overnight (9). The materials then were dried at 60°C. in an oven and ground to a fine powder in a mortar.

Determination of Inorganic Phosphorus by Isobutyl Alcohol Colorimetric Method. A suitable aliquot of the sample (about 1 g.) was analyzed by the method of Pons *et al.* (10), except that the reagent quantities were reduced proportionally to the amount of sample.

Determination of Phytin Phosphorus. The present method was essentially that described by Pons *et al.* (10) with minor modifications.

(a) Sodium phytate. One-half gram of the finely ground endosperm-scutellar tissue was weighed in a small thimble and extracted with a solvent containing absolute ethyl alcohol and benzene (32.4 and 67.6 wt.%, respectively) for 4 hr. in a Soxhlet Extractor. The sample was dried at room temperature overnight and extracted for 2 hr. with 20 ml. of 2% (v./v.) hydrochloric acid containing 10% (w./v.) sodium sulfate. The mixture was centrifuged at 10,000 $\times$ *g* for 30 min., and 15 ml. of the supernatant was transferred to a 50-ml. graduated conical tube. Two drops of phenolphthalein was added, and the pH was adjusted to about 8 with sodium hydroxide and hydrochloric acid. The material was brought to 25 ml. with water, mixed with 5 ml. of ferric chloride (10), heated in boiling water for 15 min., cooled in ice-cold water for 20 min., and then centrifuged. The supernatant was decanted carefully and the residue washed three times with 3 ml. of 0.6% (v./v.) hydrochloric acid by centrifugation. The washed residue was suspended in 5 ml. of hot water, combined with 2 ml. of 1*N* sodium hydroxide, and heated in boiling water for 15 min. After cooling, the mixture was centrifuged and the supernatant was decanted into a 50-ml. volumetric flask. The residue was washed with three 5-ml. portions of hot water by centrifugation. All washings were combined with the first supernatant and made up to 50 ml. with water.

The sodium phytate so prepared contained 0.03 to 0.08% inorganic phosphorus, depending upon the content of the free inorganic phosphorus environment. However, corrections were made for the phytin phosphorus contents by determining the inorganic phosphorus contamination.

(b) Determination of inorganic phosphorus contamination. One milliliter of the sodium phytate preparation was analyzed for the free inorganic phos-

phorus by the isobutyl alcohol colorimetric method already described.

(c) Digestion of sodium phytate preparation. One milliliter (or 2) of the sample containing not more than 1.5 mg. of phosphorus was digested according to the method of Pons *et al.* (10).

(d) Determination of total phosphorus by reduced molybdate colorimetric method. A suitable aliquot (about 1 ml.) of digested sodium phytate sample was analyzed for total phosphorus by the procedure of Pons *et al.* (10). The amount of phosphorus in the sample was determined by reference to the standard curve.

Assay for Phytase Activity. Endosperm-scutellar tissues of corn seeds or those of the 4-day-old seedlings were ground with 0.4*M* sucrose solution (25 ml./g. initial dry weight of seeds) at 2°C. The mixture so obtained was allowed to stand for 2 hr. in the cold room. It was then filtered through two layers of cheesecloth. The strained homogenate was centrifuged at 1,700 × *g* for 5 min. The residue was suspended in 0.4*M* sucrose solution (25 ml./g. initial dry weight of seeds) and used for a relatively purified enzyme source. The supernatant was further centrifuged at 20,000 × *g* for 30 min. for study of distribution of phytase activity. The supernatant was not used for the assay of phytase activity, because it required a time-consuming process of dialysis to remove free inorganic phosphorus from the present fraction. A Beckman Model L preparative ultracentrifuge was used for fractionation of the components. Nitrogen was determined by the micro-Kjeldahl method of Ma and Zuazaga (11).

The substrate, sodium phytate, was prepared as described by Peers (8). A minor modification was introduced by washing the ferric phytate precipitate with excess 0.6*N* hydrochloric acid (about 300 ml. for 5 g. of the starting material, commercial sodium phytate). No inorganic phosphorus contamination was detected with sodium phytate so prepared, although the yield was about 60%. The phytic acid phosphorus was determined by the method outlined earlier. The concentration of phytate was adjusted with water to 1.6 mmoles.

The phytase activity was assayed for its ability to release inorganic phosphorus from phytate substrate by the procedure of Peers (8) with minor modifications. Incubation was conducted at 50°C. with the reaction mixture containing 0.8 ml. of enzyme preparation, 0.3 mmoles of acetate buffer at pH 5.6 and 3.2×10^{-3} mmoles of phytate in a total of 2 ml. The assay mixture, after a 3-hr. incubation, was mixed with 1 ml. of 10% (w./v.) trichloroacetic acid and centrifuged. Portions of the clear supernatant were used for the determination of orthophosphate phosphorus by the isobutyl alcohol colorimetric method described above. One unit of phytase was defined as the amount of enzyme releasing 1 γ of orthophosphate in 1 hr. under the standard assay condition described. All values were corrected by those obtained from the zero-time enzyme assays. The entire experiment was repeated twice, and all determinations were carried out in duplicate.

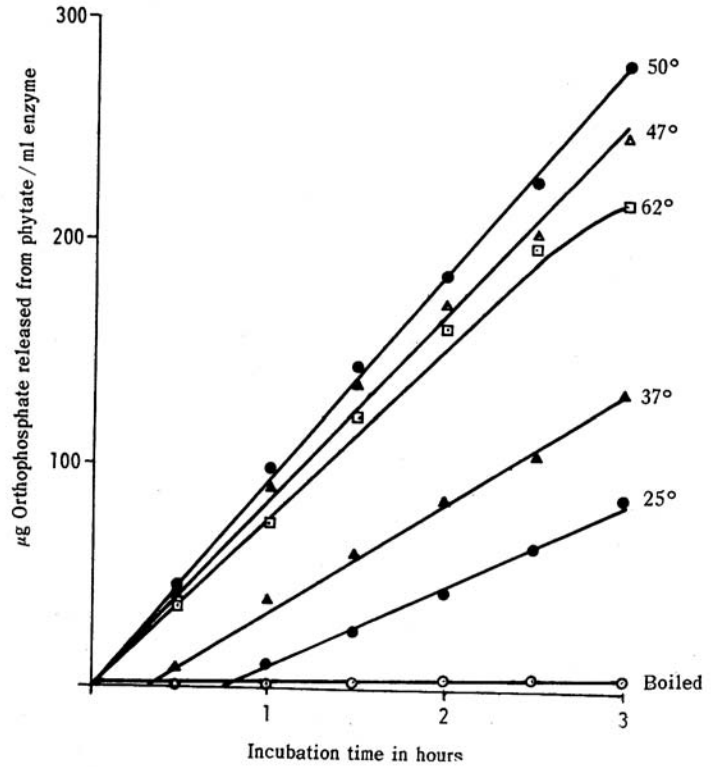

Fig. 1. Effect of temperature on the time course of phytase activity. Enzyme assay was conducted according to the standard procedures described in the text, except that incubation was at various temperatures ranging from 25° to 62°C.

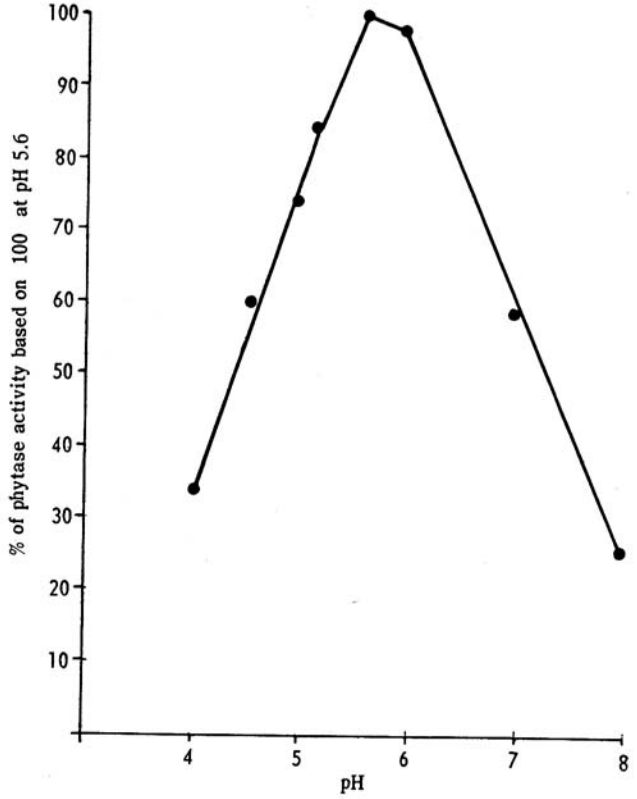

Fig. 2. Influence of pH on phytase activity. Enzyme assay was conducted by the standard procedures described in the text, except that incubation was in the various pH buffers such as acetate buffer at pH 4, 5.2, and 5.6; tris-maleate buffer at pH 6, 7, and 8.

Results and Discussion

Properties of Phytase in Endosperm-Scutellum of Corn Seedlings 4 Days Old. (a) Effect of incubation temperature. Figure 1 indicates the time-activity curve of phytase at various incubation temperatures. The optimum temperature of activity was about 50°C. for the 3-hr. period. Sobolev (2) indicated that the high optimum activity temperature was one of the characteristics of the phytase enzyme. For the optimum temperature of wheat phytase, Kolobkova (12) and Peers (8) found 55°C., and Lüers and Silbereisen (13) reported 48°C. in malt. Later experiments (Fig. 3) on the thermal inactivation of phytase indicated that the enzyme was inactivated above 50°C. The high temperature for the optimum activity may be partly a reflection of this thermostability. Temperatures of 25° and 37°C. did not activate the enzyme until the mixture was incubated for about 40 and 20 min., respectively. The enzyme preparation boiled for 2 min. had practically no activity.

(b) Time course activity. As shown in Fig. 1, the time course activity curve at 50°C. indicated that the decomposition of phytate proceeded at a

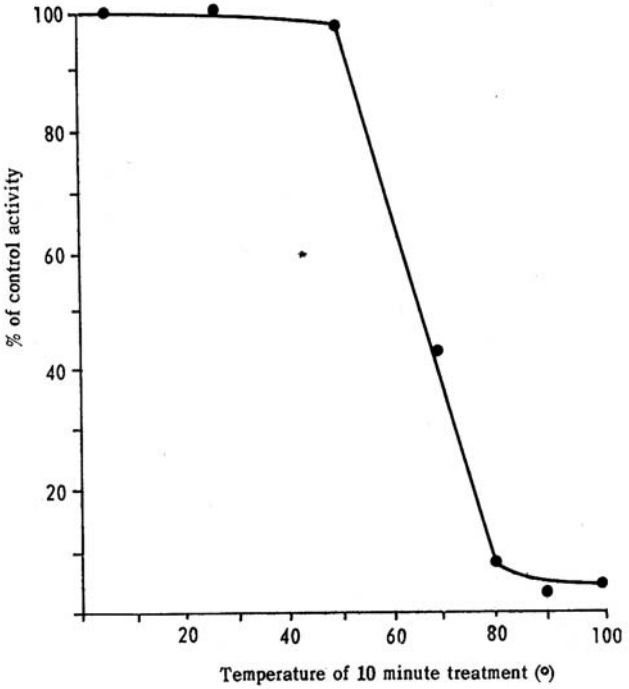

Fig. 3. Thermal inactivation of phytase enzyme. Assay method was the same as that described in the text, except that aliquots of the crude enzyme preparation were combined with acetate buffer at pH 5.6 and the mixture was heated at various temperatures for 10 min.

constant rate during the 3-hr. incubation period. This suggested that the enzyme was not significantly inhibited by hydrolysis products. The linearity of the relation between time and the amount of phytate decomposition permitted the expression of enzyme activity as γ phytate phosphorus released by 1 ml. of enzyme preparation in 1 hr.

(c) Effect of pH. The optimum pH for the enzyme activity was about 5.6 (Fig. 2). The enzyme activity diminished very rapidly on either side of this optimum. The single sharp peak of the present enzyme response to the various levels of pH suggested a single enzyme operation in this system. Various optimum pH values of phytases reported are 5.2 for malt phytase (13), 5.2 for wheat phytase (8), and two peaks (pH 5 and 7) observed in germinating lettuce seeds (7).

(d) Effect of heating. As shown in Fig. 3, assays with the preheated enzyme preparation at the various temperatures indicated that heating up to 50°C. for 10 min. did not depress the enzyme activity. However, at 60°C., the activity was depressed to about 40% of the control value and at 80°, 90°, and 100°C. was practically completely inhibited. Peers (8), in his study of the thermal inactivation of wheat phytase, reported that the preheated enzyme preparation at 80°C. for 10 min. did not lose any activity and that at 90°C. there was about 40% of the enzyme activity of the control. The resistance of phytase to heat-treatment seemed to vary, depending on the different sources of enzyme.

(e) Effect of substrate concentration. As shown in the table below, the

Phytate *mmoles*	*Phytate Phosphorus* *µg./hr./ml. enzyme*
6.4	42.0
3.2	34.3
1.6	30.8
0.8	21.2
0.27	15.4
0.09	11.5

(Michaelis constant = 0.91×10^{-4} moles/liter; see ref. 14.)

initial reaction velocities were measured according to the standard assay method described in the text except that 0.5 times dilution of the original enzyme homogenate was used. The Michaelis constant (K_m) was calculated from the graph of 1/v against 1/s (v = velocity as enzyme units, s = substrate concentration as molarity). The Michaelis constant has been reported to range between 1×10^{-2} and 1×10^{-5} moles/liter (14). Since the Michaelis constant (0.91×1^{-4} moles/liter) of the present enzyme fell in a rather low range, it was assumed that the corn phytase enzyme in this study possessed a relatively high affinity for substrate. Peers reported Michaelis constants of 0.25×10^{-3} and 0.33×10^{-3} moles/liter for crude and purified wheat phytase, respectively (8).

(f) Substrate specificity. As seen in Table I, the present enzyme hydrolyzed phytate substrate to a significantly higher degree than beta-glycerophosphate. The data indicated that the corn phytase seemed to be a phosphomonoesterase of broad specificity. The lack of a strict substrate specificity of phytase was indicated by Sobolev (2) as one of the characteristics of the

TABLE I

SUBSTRATE SPECIFICITY OF PHYTASE[a]

	Enzyme Activity			
	Sodium Phytate (P Released)		Beta-Glycerophosphate (P Released)	
mM NAF	*γ/hr./ml. enzyme*	%	*γ/hr./ml. enzyme*	%
Control	104	100	34	100
0.1	88	85	27	87
1	72	69	22	71
10	53	51	17	54

[a] Enzyme assay was conducted with 3.2×10^{-3} mmoles of sodium phytate and the same concentration of beta-glycerophosphate for phosphomonoesterase according to the standard procedures as described in the text.

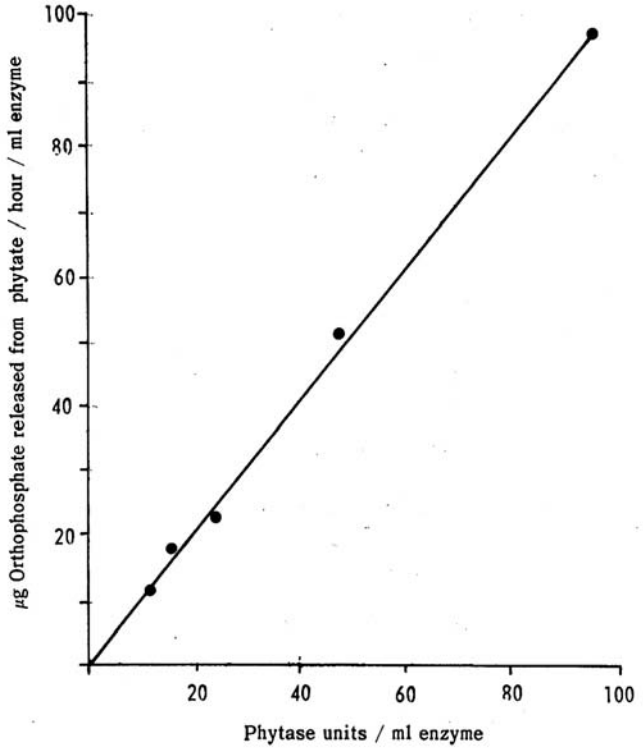

Fig. 4. Effect of enzyme concentrations on phytase activity. Enzyme assay conditions were the same as those described in the text, except that the assay mixture contained various units of enzyme preparation.

TABLE II

DISTRIBUTION OF TOTAL NITROGEN AND PHYTASE ACTIVITY IN FRACTIONS ISOLATED FROM ENDOSPERM-SCUTELLUM TISSUE HOMOGENATE FROM CORN SEEDLINGS 4 DAYS OLD

FRACTION	TOTAL NITROGEN	TOTAL ACTIVITY (PHYTATE P RELEASED)		SPECIFIC ACTIVITY (PHYTATE P RELEASED)
	γ/hr.	γ/hr.	%	γ/hr./mg./N
Homogenate	16.1	1,615	100	100.3
1,700 × *g* fraction	12.7	960	66[a]	75.6
20,000 × *g* fraction	0.6	46	3[a]	76.7
Supernatant	1.6	456	31[a]	285.0
Recovery	14.9	1,462	91	...

[a] Percent distribution based on recovery of total enzyme activity.

TABLE III

EFFECT OF VARIOUS SALTS ON PHYTASE ACTIVITY[a]

SUBSTANCE ADDED	ACTIVITY (PHYTATE P RELEASED)	PERCENT OF CONTROL	SUBSTANCE ADDED	ACTIVITY (PHYTATE P RELEASED)	PERCENT OF CONTROL
	γ/hr./ml. enzyme			γ/hr./ml. enzyme	
Control	103.0	100	Sodium nitrate	105.1	102
Sodium fluoride	48.4	47	Calcium chloride	124.6	121
Sodium sulfate	104.0	101	Potassium cyanide	98.8	96
Magnesium sulfate	101.9	99	Sodium azide	112.2	109

[a] Enzyme assays were conducted by the standard procedures as described in the text, except that each of the various salts (0.02 mmoles of each in a total volume of 2 ml.) was added to each reaction mixture.

phytase enzyme. The level of fluoride-inhibited enzyme activity with phytate was similar to that of fluoride-inhibited enzyme activity with glycerophosphate.

(g) Effect of enzyme concentrations. As shown in Fig. 4, responses of phytase to the various concentrations of enzyme were found to be linear up to 98 units of enzyme tested.

(h) Distribution of phytase. As shown in Table II, the highest total phytase activity was located at the 1,700 × *g* fraction. However, when the enzyme activity was expressed in specific activity, the highest activity was located in the supernatant at 20,000 × *g*. Mayer (7) reported that all the phytase activity in germinating lettuce seedlings resided in the supernatant after centrifugation at 18,000 × *g*. The present finding of the high total corn phytase activity associated with the heaviest particulates differed from Mayer's findings on lettuce phytase.

(i) Action of various salts on phytase activity. As shown in Table III, addition of magnesium ions did not increase the phytase activity; however, the activity was activated by calcium chloride. The increased activity caused by the addition of calcium chloride was probably due to disturbance of the enzyme reaction equilibrium through removal of calcium phosphate. Sodium

TABLE IV

EFFECTS OF VARIOUS CONCENTRATIONS OF SODIUM FLUORIDE ON *in Vitro* AND *in Vivo* PHYTASE ACTIVITY[a]

In Vitro			*In Vivo*		
Fluoride Concentrations	Phytase Activity (P Released)		Fluoride Concentrations	Phytase Activity (P Released)	
mmoles	*γ/hr./ml. enzyme*	*% of control*	*mmoles*	*γ/hr./ml. enzyme*	*% of control*
0	98	100	0	98	100
0.05	93	95	....	....	....
0.1	85	87	0.1	105	107
0.5	81	82	....	....	....
1	75	77	1	96	98
5	52	53	....	....	....
10	45	46	10	89	91

[a] For *in vitro* assays, procedures were the same as those described in the text except with addition of various amounts of sodium fluoride to the reaction mixture. For *in vivo* assays, all conditions were the same as those for *in vitro* assays except that the enzyme suspensions were prepared from materials germinated in the various concentrations of sodium fluoride.

fluoride at the level of 10 m*M* concentration significantly inhibited the enzyme activity. As can be seen in Table IV, the enzyme inhibition induced by fluoride was proportional to the various concentrations of sodium fluoride ranging from 10 to 0.005 m*M*. The inhibitory effect of such low fluoride concentration on the phytase may also explain the possible inhibition of the phytase activity by fluoride *in vivo*.

Effect of Fluoride on Phytin and Phytase in Endosperm-Scutellum of Seedlings during Corn Germination and Growth. The content of phytin in the endosperm-scutellar tissues showed a rapid decrease during germination (A in Fig. 5). The corn seeds contained initially 2.64 mg. phytin phosphorus per g. original dry weight of seeds. This represented about 87% of the total phosphorus content of seeds. After 96 hr. of germination only about 30% of the total phytin phosphorus was detected in these tissues. The continuous decrease in phytin phosphorus with time paralleled the corresponding increase in the content of inorganic phosphorus (B in Fig. 5). The amount of in-

TABLE V

EFFECT OF VARIOUS CONCENTRATIONS OF FLUORIDE ON PHYTATE CONTENTS OF ENDOSPERM-SCUTELLAR TISSUES DURING CORN SEED GERMINATION AND GROWTH

Germination Media	Phytate P Released Germination and Growth Period				
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours
	γ/g.[a]	*γ/g.*	*γ/g.*	*γ/g.*	*γ/g.*
Water	0	135	492	1,089	1,886
NaF					
0.1 m*M*	0	25	217	915	1,820
1 m*M*	0	18	30	725	1,719
10 m*M*	0	10	24	152	715

[a] Initial dry weight of seeds.

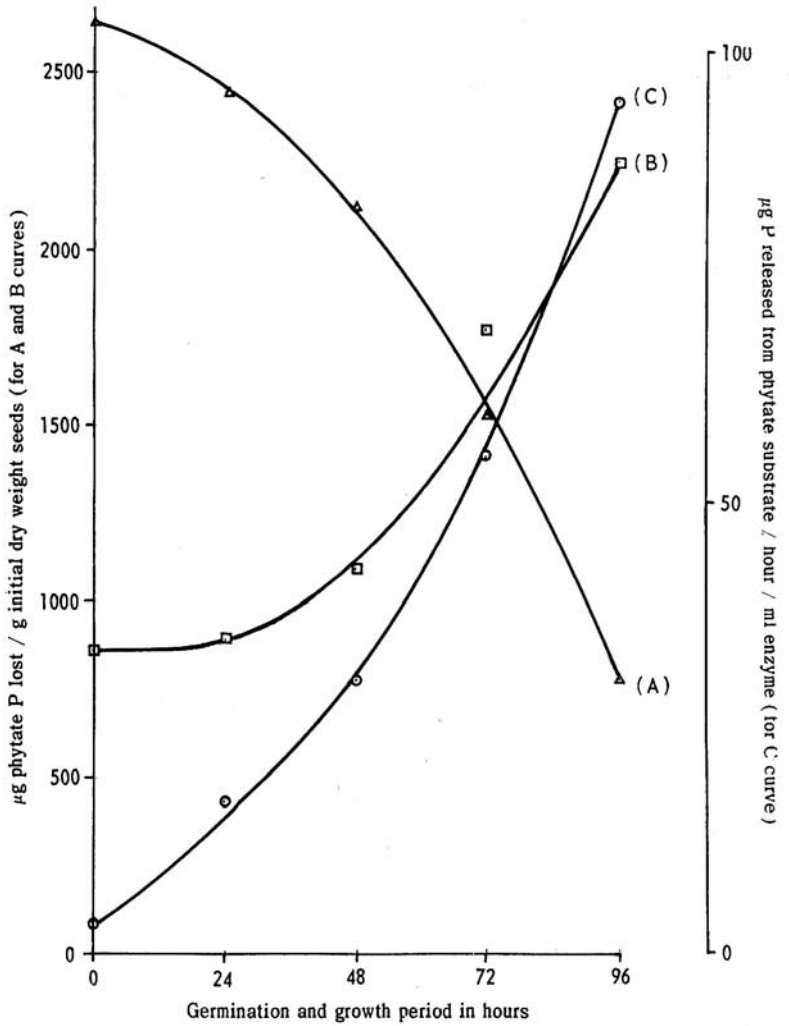

Fig. 5. Decreasing phytin content (A), increasing total inorganic phosphorus (B), and increasing phytase activity (C) of endosperm-scutellar tissues of seedlings during corn germination and growth. (See text for the enzyme assay.)

organic phosphorus increased almost three times that at zero time during the 96-hr. germination period. The diminishing phytin phosphorus and the simultaneously increasing inorganic phosphorus in germinating seeds also were reported in rice seedlings (6) and cotton seedlings (5). Assays for the ability of phytase to release orthophosphate from phytin in these tissues indicated that the enzyme activity showed a steady rise as the seed germination proceeded with time. The enzyme activity increased about 40 times that at zero time germination during the 96-hr. germination period (C in Fig. 5).

It is evident that phytin phosphorus was released by the action of corn phytase and that the resulting product, inorganic phosphate, rose in the endosperm-scutellar tissues with increasing time of germination.

As shown in Table V, the release of phytin phosphorus was prevented in the endosperm-scutellar tissues of corn seeds germinated and grown in the various concentrations of fluoride. Amounts of phytin phosphorus breakdown decreased as the fluoride concentrations in the germination medium increased. Release of orthophosphate from phytin was prevented by fluoride more significantly at the 24-hr. germination period. As corn seed germination was prolonged with time, phytin phosphorus released in the fluoride-treated tissues

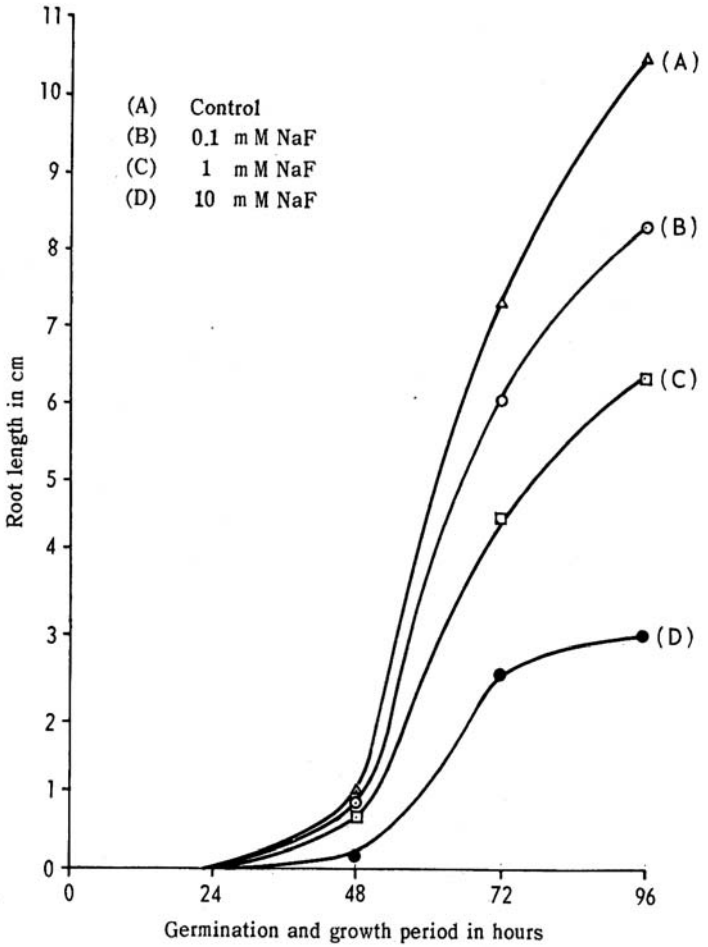

Fig. 6. Effect of various concentrations of fluoride on seedling root growth rates during corn germination and growth. Each value on the growth curves was based on the average of 70 to 100 seedling root length measurements. No measurements were possible at the first 24-hr. period of germination because initiation of root growth was negligible.

approached the control values. As shown in Table IV, the inhibition of the ability of phytase to dephosphorylate phytin in *in-vitro* assays increased as the fluoride concentrations rose; however, the effect of fluoride on the phytase activity was not pronounced in *in-vivo* assays. The concentration of 10 m*M* fluoride *in vivo* inhibited the phytase activity only at the level of about 10% of the control value, whereas 10 m*M* and 0.1 m*M* of sodium fluoride *in vitro* prevented the activity at about 50 and 87% of the control, respectively. Since levels of the present enzyme inhibition were found to be proportional to the fluoride concentrations in the *in-vitro* assays (Table IV), the low levels of the enzyme inhibition observed in the *in-vivo* assays were probably due to dilution in the enzyme assay if the influence of fluoride on the enzyme was reversible.

Germination and growth of corn seeds in the medium containing the various concentrations of fluoride resulted in a reduction in the growth rate of the seedling roots (Fig. 6). Levels of the seedling root growth inhibition were found to be related closely to fluoride concentrations.

Phytin has been reported to be the calcium, magnesium, and potassium salt of phytic acid. More than 80% of the total starchy seed organic phosphate resided in this single molecular species (2). As demonstrated in the experiments indicated in Fig. 5, another notable feature of phytin was its rapid decomposition and the liberation of its phosphorus caused by the phytase activity during germination. The dephosphorylation of phytin was also prevented by fluoride (Table V). Did any relation exist between the prevented phytin decomposition in the endosperm-scutellar tissues and the inhibited seedling root growth rates?

The dephosphorylation of phytin induced by phytase was postulated to provide germinating plants with soluble phosphate (2,7,15). Sobolev (2), in culturing germinating flax in a solution containing ^{32}P radioactive compound, reported that the growing seedlings relied on the endogenous organic phosphate-originated inorganic phosphorus more than on the exogenous orthophosphate supply in its turning over with phosphate esters such as nucleotides. Weiss (4), in his study of the phosphate transformation in 11 species of germinating seeds, reported that the inorganic phosphorus released from phytin compounds in germinating seeds was utilized mainly for nucleic acid synthesis of the growing embryonic plants. His finding was also supported in experiments conducted by Ergle and Guinn (5); these authors, in their exploration of changes of phosphate compound during germination of cotton seed, demonstrated that the increasing orthophosphate contents coupled with the diminishing phytin amounts in seeds paralleled the increasing RNA and DNA contents in the embryonic plants during a 6-day growth period. The phosphates other than the nucleic acids were not comparable to the orthophosphate content. The accumulated inorganic phosphorus was assumed to be used mainly for synthesis of nucleic acids in the young, growing embryonic organisms.

It is known that the rate of protein synthesis is a measure of the rate of growth (16). According to established concepts of the reactions involved in enzyme protein formation (17), amino acids are activated first with ATP. The activated amino acids are then linked to specific soluble RNA molecules. These are transferred to the ribosome of microsomal components where the

soluble RNA's amino acids are joined to complementary sites on an RNA template, and the amino acids are placed in the proper position for peptide synthesis. Any factors which influence either RNA metabolism or protein synthesis could be reflected in the growth rate.

The foregoing results indicate that fluoride inhibits phytase enzyme *in vitro,* prevents the dephosphorylation of phytin in endosperm-scutellar tissues, and retards root growth of corn seedlings. There occurs continuous turnover between inorganic phosphorus and nucleotides in RNA metabolism. It is likely that the limited supply of phytin-originated orthophosphate may possibly be one of the factors which inhibit the growth rate of fluoride-treated corn seedling roots.

Summary

Some properties of phytase enzyme were studied, using the $1,700 \times g$ fraction of endosperm-scutellar tissue homogenate of corn seedlings 4 days old. The corn phytase lacked a strict substrate specificity.

The optimum incubation temperature of the enzyme was 50°C., optimum pH was 5.6, and the Michaelis constant (K_m) was 0.91×10^{-4} moles/liter. The highest total enzyme activity was located in the fraction at $1,700 \times g$; the supernatant at $20,000 \times g$ was found to be the location of the highest specific enzyme activity. The enzyme survived high temperature (up to 50°C.), was activated by calcium chloride ($0.01M$), and was inhibited significantly by sodium fluoride ranging from 10 to 0.05 mM.

Rapid decomposition of phytin content was correlated with steady rises of phytase activity and inorganic phosphate in the endosperm-scutellar tissues of germinating corn seedlings. Fluoride, however, prevented the dephosphorylation of phytin compound in the tissues and retarded the rate of seedling root growth during germination.

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