Purification and Characterization of Acid Phosphatases with or without Phytase Activity from Rice Bran

Toshiro HAYAKAWA, Yoshiaki TOMA* and Ikuo IGAUE

Department of Agricultural Chemistry, Faculty of Agriculture,
Niigata University, Ikarashi 2-8050,
Niigata 950-21, Japan

Received October 19, 1988

The phytases (EC 3.1.3.26) and acid phosphatases (EC 3.1.3.2) of rice bran were purified. Four acid phosphatases were purified from rice bran: F1 and F2 had phytase activity, but F3 and F4 did not. The optimum pH of F1 and F2 for phytic acid were 4.4 and 4.6, respectively, and those of F1, F2, F3, and F4 for *p*-nitrophenyl phosphate were 5.1, 5.2, 5.4, and 6.0, respectively. Their molecular weights were estimated to be about 59~70 K by SDS polyacrylamide gel electrophoresis, velocity density gradient centrifugation, and gel filtration on Ultrogel. The isoelectric points of F1, F2, F3, and F4 were 5.1, 5.1, 5.6, and 5.9, respectively. F1, F2, F3, and F4 had a violet color and F2 showed an absorption maximum peak at 560 nm. They had broad substrate specificities.

Phytic acid (IHP) or myo-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) is widespread in nature, and the principal form of phosphorus in many seeds. In cereals and meals, IHP is regarded as antinutritive factor. In rice seeds, the phosphorus of IHP represent up to 78% of the total phosphates.¹⁾ Phytase (myo-inositol hexaphosphate phosphohydrolase) hydrolyzing IHP to inositol and inorganic phosphorus (Pi) is widely distributed in plants, animals, and microorganisms.²⁾ In 1907, Suzuki *et al.*³⁾ found an enzyme in rice bran, and they tried to purify the enzyme but with little success. Later, the nature of phytases from soy bean,⁴⁾ dwarf beans,⁵⁾ lettuce seeds,⁶⁾ wheat bran,^{7~10)} mung beans,¹¹⁾ isolated aleurone particles of rice grains,¹²⁾ pumpkins,¹³⁾ pollen,^{14,15)} and barley¹⁶⁾ has been reported. However, enzyme preparations of sufficient purity suitable for characterization and structural studies have up to now been unavailable.

In this study, we describe the purification and some properties of acid phosphatases with or without phytase activity from rice bran.

Materials and Methods

Sample preparation. Rice bran of the strain *Koshihikari*, *Oryza sativa* L. *Japonica* was provided by Niigata Kennbei Co. The samples were stored at about 1~4°C.

Enzyme assay. Phytase activity was assayed in a 0.5 ml reaction mixture containing 250 μ l of 0.2 M acetate buffer pH 4.6, 50 μ l of 100 mM Na-IHP, and 200 μ l of enzyme solution. The reaction mixture was incubated at 40°C for 60 min, and the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid solution, and the liberated Pi was measured by the method of Fisk and Subbarow.¹⁷⁾ One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μ mol of Pi from substrate per hour under the assay conditions. The assay of acid phosphatase activity was as described in our previous paper.¹⁸⁾ Protein was estimated from the absorbance at 280 nm, and by the method of Lowry *et al.*¹⁹⁾

Chemicals. Na-IHP was prepared by the procedure of Nagai and Funahashi.⁷⁾ *p*-Nitrophenyl phosphate (Npp), α -naphthyl phosphate, and Fast Garnet GBC were obtained from Nakarai Chemicals Ltd., DEAE-Cellulose, and P-Cellulose from Brown Co., and DEAE-Sephadex, CM-Sephadex, Sephadex G-150, Con-A Sepharose, and Blue dextran 2000 from Pharmacia. Ultrogel AcA 34 and Ampholine were obtained from LKB. Standard proteins were purchased from Boehringer. All other reagents were

* Present address: Hoechst Japan Limited, Sapporo 060, Japan

of analytical grade.

Electrophoresis. Analytical polyacrylamide gel electrophoresis (PAGE) was done at pH 8.9 by the method of Davis.²⁰⁾ Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli except for using 2.7 M urea.²¹⁾

Velocity density gradient centrifugation. A linear 5~10% glycerol density gradient (4.6 ml) in 0.05 M sodium acetate buffer (pH 5.1) was prepared by the method of Martin and Ames.²²⁾ The activity peaks were located by monitoring the phytase and phosphatase activities. The *S* value given is the average of the values obtained with different standards.

Estimation of molecular weight. Molecular weights of the purified enzymes were estimated by SDS-PAGE,²¹⁾ velocity density gradient centrifugation,²²⁾ and Ultrogel AcA 34 chromatography by the method of Andrews.²³⁾

Isoelectric focusing. Isoelectric focusing was done in a 110 ml column (LKB-produkter) using 1% carrier ampholytes with a pH range of 4~6. The density gradient in an electrofocusing column was formed with glycerol, and electrofocusing was done at 800 V and 2°C for 48 hr.

Purification of acid phosphatase with phytase activity

Step 1. Preparation of crude juice. All operations were at 0~4°C. Unless otherwise stated. Rice bran of 6.8 kg was homogenized in 18 l distilled water with a rotary mixer for 1 hr. The homogenate was strained through four layers of gauze and centrifuged at 8000 × *g* for 20 min. The floating fraction was excluded by three layers of gauze.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant solution to 30% saturation; the mixture was kept for 30 min and centrifuged at 8000 × *g* for 20 min. The supernatant was raised to 70% saturation of ammonium sulfate. The precipitate was dissolved in a small volume of distilled water, and dialyzed for 48 hr in distilled water. The dialyzate was centrifuged to remove the inactive protein. The supernatant was purified further.

Step 3. DEAE-Cellulose column chromatography. The enzyme solution (total phytase activity: 108,700 units, acid phosphatase activity: 4,012,000 units) was put on a column (7 × 100 cm) of DEAE-Cellulose equilibrated with 0.01 M Tris-HCl buffer pH 7.5. The column was thoroughly washed with the same buffer and the adsorbed enzyme was eluted from the column with a linear gradient of 6 l of the same buffer in the mixing chamber and 6 l of the buffer containing 0.5 M NaCl in the reservoir. Acid phosphatase with phytase activity was detected in fractions 100~180 and acid phosphatase without phytase activity in fractions 210~360. Fractions 100~180 were brought to 75% saturation with solid ammonium sulfate. After for 1 hr, the precipitate formed was collected by centrifugation.

Step 4. P-Cellulose chromatography. The precipitate was dialyzed overnight against 10 liters of 0.01 M acetate buffer, pH 4.5, containing 0.01% Triton X-100. The dialyzate was put on a column of P-Cellulose (5.6 × 95 cm), which was equilibrated with the same buffer. The column was first washed with two vols. of the same buffer, then eluted with a linear gradient of 3 l of the same buffer in the mixing chamber, and 3 l of the buffer containing 0.5 M NaCl in the reservoir. Fractions of 20 ml were collected. The acid phosphatase with phytase activity (fractions 310~350) was concentrated by an Advantix Ultrafilter, N-25, and dialyzed against 0.01 M acetate buffer, pH 5.5, containing 0.01% Triton X-100.

Step 5. Con-A Sepharose chromatography. The dialyzed solution was adsorbed on a Con-A Sepharose column (2 × 30 cm) equilibrated previously with 0.01 M acetate buffer, pH 5.5, containing 0.01% Triton X-100. The column was washed thoroughly with the same buffer to remove unadsorbed protein. The adsorbed proteins were eluted with a linear gradient of 0.7 l of the same buffer in the mixing chamber and 0.7 l of the buffer containing 0.2 M methyl- α -D-glucoside in the reservoir. Fractions of 10 ml were collected. The acid phosphatase with phytase activity was resolved into two peaks (F1 and F2) eluting at 0.05 M and 0.1 M methyl α -D-glucoside contained in the buffer; those peaks were pooled and concentrated to about 2 ml in a collodion bag (Sartorius Co). The concentrated enzymes were a violet color.

Step 6. Gel filtration on Sephadex G-150. Fractions F1 and F2 were each passed through a column (2.5 × 105 cm), which had been equilibrated with 0.05 M acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.05% Triton X-100. The elution profiles of the phytase activity and of the acid phosphatase activity were identical.

Step 7. CM-Sephadex C-50 Chromatography. The concentrated fraction of F1 from Step 6 was put on a column (1.7 × 30 cm) of CM-Sephadex C-50 equilibrated with 0.01 M acetate buffer, pH 4.6, containing 0.01% Triton X-100. Elution was done with a linear gradient of 300 ml of the same buffer and 300 ml of the buffer containing 0.5 M NaCl. Fractions of 5 ml were collected. Fractions 93~102 were pooled and condensed by a collodion bag. The F2 fraction was put on the column and eluted with the buffer, changing the pH from 4.6 to 4.9 with the acetate buffer. Fractions 110~135 were combined and concentrated with a collodion bag.

Step 8. Second gel filtration through Sephadex G-150. The concentrated F1 and F2 were passed through a column (1.5 × 100 cm) equilibrated with acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.05% Triton X-100. The elution patterns of phytase activity, phosphatase activity, and protein from F1 and F2 were symmetrical. These preparations were used for all subsequent experiments on F1 and F2.

Purification of acid phosphatase without phytases

Step 4'. CM-Sephadex C-50 chromatography. The frac-

Fig. 1. CM-Sephadex Column Chromatography of the Acid Phosphatase without Phytase Activity from Rice Bran.

The dialyzate of ammonium sulfate precipitates from DEAE-Cellulose column chromatography (fractions 210~360) was put on a CM-Sephadex column (4×90 cm) and eluted prep. a linear gradient of NaCl in the loading buffer. We designated the first peak as fraction F3 (No. 400~450) and the second peak as fraction F4 (No. 470~510).

—○—, acid phosphatase activity without phytase activity; —●—, acid phosphatase with phytase activity; ----, protein; —, NaCl concentration.

tions (210~360) of DEAE-Cellulose chromatography in step 3 were brought to 75% saturation with solid ammonium sulfate. The precipitate formed was collected by centrifugation and was dialysed against 10 l of 0.01 M acetate buffer, pH 5.1, containing 0.01% Triton X-100. The dialyzate (acid phosphatase activity: 1,535,000 units) was put on a column of CM-Sephadex C-50 (4×90 cm), which was equilibrated with the same buffer. The column was washed with the same buffer. The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M using the same buffer. The acid phosphatase without phytase activity was separated into F3 and F4 fractions (Fig. 1). The fractions of F3 (400~450) and F4 (470~510) were pooled and brought to 80% ammonium sulfate saturation. After standing for 30 min, the fractions were precipitated by centrifugation for 20 min with 8000×g.

Step 5'. 2nd CM-Sephadex chromatography. The precipitated F3 fraction of 1st CM-Sephadex chromatography was dialysed against 0.01 M acetate buffer, pH 5.2, containing 0.01% Triton X-100. The dialysed enzymes were put on to a CM-Sephadex column (3×30 cm), previously equilibrated with the same buffer, and the column was washed with the same buffer. The active fraction was eluted from the column, however, much inactive proteins was adsorbed. The eluted active fraction of F3 was concentrated by an Advantic Ultrafilter, N-25. The chromatography of F4 fraction was done by the same

methods as for F3 fraction and the active fraction of F4 was not adsorbed by the column. The eluted F4 fraction was concentrated by the same methods as the F3 fraction.

Step 6'. Con-A Sepharose chromatography of F3. The concentrated F3 fraction was put on a column of Con-A Sepharose (2×30 cm) which was equilibrated with 0.01 M acetate buffer, pH 5.5, containing 0.01% Triton X-100. The column was washed thoroughly with the same buffer. The adsorbed protein was eluted with a linear gradient consisting 0~0.1 M borate buffer containing 0.01% Triton X-100. The active fractions of F3 were concentrated by an Advantic Ultrafilter, N-25.

Step 7'. Sephadex G-150 gel chromatography of F3. The concentrated F3 fraction was put on a column of Sephadex G-150 column (2×100 cm), equilibrated with 0.01 M acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.01% Triton X-100. The elution pattern of acid phosphatase activity, as well as of protein, was symmetrical. The preparation was used for all subsequent experiments on F3.

Step 7''. First DEAE-Sephadex A-50 chromatography of F4. The concentrated F4 fraction of Step 5' CM-Sephadex column was put on a DEAE-Sephadex C-50 column (2×30 cm) which was preequilibrated with 0.01 M Tris-maleate buffer, pH 6.8, containing 0.01% Triton X-100. The adsorbed protein was eluted with a linear gradient consisting of 0~0.4 M NaCl in the same buffer. The active fractions were concentrated with an Advantic Ultrafilter, N-25.

Step 8''. 2nd DEAE-Sephadex A-50 column chromatography of F4. The F4 fraction was put on a column of DEAE-Sephadex (1×30 cm) equilibrated with 0.01 M Tris-maleate buffer, pH 6.8, containing 0.01% Triton X-100. The adsorbed F4 fraction was eluted by the same method as in Step 7''. This preparation was used for all subsequent experiments on F4.

Results

Purifications

The enzyme activities were separated by DEAE-Cellulose chromatography into two major peaks: fractions 100~180 (maximum peak at 150) were acid phosphatases with phytase activities, but fractions 210~360 (maximum peak at 260) were acid phosphatases without phytase activities. In Con-A Sepharose affinity chromatography, the acid phosphatase with phytase activity was resolved into two fractions, F1 and F2. Sephadex G-150 and CM-Sephadex chromatographies could not separate the phytase activity from acid phosphatase activity. Acid phosphatase without phytase activity, F3 and F4, were adsorbed on a CM-Sephadex column at pH

Table I. PURIFICATION OF ACID PHOSPHATASE WITH PHYTASE F1 AND F2 FROM RICE BRAN

Procedure	Phytase activity						Acid phosphatase activity				
	Fractions	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification	Total activity (units)	Specific activity (units/mg)	Recovery	Purification	Phytase activity (%) ^a
Water extract		205,700	295,300	1.4	100	1	7,211,900	35	100	1	4.0
(NH ₄) ₂ SO ₄ 30 ~ 70% sat.		37,160	108,700	2.9	36.7	2	4,012,000	108	55.6	3.1	2.7
DEAE-Cellulose	F1, 2	6770	44,050	6.5	14.9	5	8,604,780	127	11.9	3.6	5.1
P-Cellulose	F1, 2	440.8	34,590	78.5	11.7	54	507,930	1152	7.0	32.8	6.8
Con A	F1	21.8	10,190	466.7	3.5	324	149,160	6832	2.1	194.6	6.8
Sepharose	F2	22.7	11,390	502.8	3.9	349	191,380	8431	2.7	240.2	5.9
1st Sephadex	F1	13.9	8920	641.5	3.0	446	169,430	12,189	2.4	347.3	5.3
G-150	F2	7.0	7440	1070.9	2.5	744	141,420	20,347	2.0	579.7	5.3
CM-Sephadex	F1	1.6	3920	2390.2	1.2	1707	74,480	45,415	1.0	1294.0	5.3
C-50	F2	2.6	6210	2388.0	2.1	1706	111,780	42,992	1.6	1224.9	6.6
2nd Sephadex	F1	1.0	3030	3030.0	1.0	2164	56,500	56,500	0.8	1609.7	5.4
G-150	F2	1.3	3505	2696.0	1.2	1926	61,300	47,154	0.9	1343.4	5.7

^a Shows % of specific phytase activity to specific acid phosphatase activity in F1 and F2.

Table II. PURIFICATION OF ACID PHOSPHATASE WITHOUT PHYTASE F3 AND F4 FROM RICE BRAN^a

Procedure	Fractions	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
DEAE-Cellulose	F3, F4	12,980	1,535,000	118.4	21.3	3.4
1st CM-Sephadex	F3	706.7	233,100	329.8	3.2	9.4
	F4	866.9	633,200	730.7	20.8	20.9
2nd CM-Sephadex	F3	155.9	144,530	927.1	2.0	26.5
	F4	151.2	491,400	3250.0	6.8	92.9
Con-A Sepharose	F3	4.0	49,900	12,470	0.7	350.4
Sephadex G-150	F3	0.8	35,000	43,750	0.5	1250.0
1st DEAE-Sephadex	F4	4.8	124,000	25,830	0.2	736.0
2nd DEAE-Sephadex	F4	0.9	38,700	43,000	0.5	1229.0

^a Purification procedure of these fractions were followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation in Table I.

Fig. 2. PAGE of Purified Acid Phosphatase with Phytase Activity (F1 and F2) and Acid Phosphatase without Phytase Activity (F3 and F4).

One or two μg of protein were used for activity visualization and 30 μg of protein were used for carbohydrate and protein staining. Symbols: A, activity; C, carbohydrate; P, protein; 1, F1; 2, F2; 3, F3; 4, F4.

5.1, however, at pH 5.2, then were eluted from the column. The eluted enzymes were much purified by CM-Sephadex chromatography.

The yields of the enzymes were phytase activity of F1, 1% and of F2, 1.2%, and the acid phosphatase activity of F1, 0.8% and F2, 0.9% (Table I). The yields of acid phosphatase activity F3 and F4 were only 0.5% (Table II). The enzyme activities are stable at 4°C for several months in the 0.01 M acetate buffer, pH 5.0~7.0, containing 0.01% Triton X-100, in a cold room.

PAGE

As shown in Fig. 2, PAGE indicated that

Fig. 3. SDS-PAGE of F1, F2, F3 and F4.

Electrophoreses were done in 10% polyacrylamide gel in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. Before electrophoreses, samples containing 0.01 M phosphate buffer, 1% SDS, 1% 2-mercaptoethanol, and about 20~30 μg protein were incubated at 50°C for 2 hr. Lane 1, F1; 2, F2; 3, F3; 4, F4. Lane S: standards proteins: I, aldolase (158 K); II, bovine serum albumin (67 K); III, ovalbumin (45 K); IV, chymotrypsinogen (25 K); V, cytochrome *c* (12.4 K).

F1, F2, F3, and F4 contained a single protein which had the phosphatases activity detected by the procedure of Ogita,²⁴⁾ using α -naphthyl-phosphate and Fast Blue B salt. All of these

Fig. 4. Effects of pHs on the Stability of F1, F2, F3, and F4.

The enzyme solutions (0.5 μ g of protein/ml) were kept at 4°C for 24 hr in 0.05 M of each buffer. After the treatment, the enzyme solutions were diluted with 0.5 M acetate buffer, pH 4.6, (phytase activity of F1 and F2) and acetate buffer, pH 5.5, (acid phosphatases activity of F1, F2, F3, and F4), then remaining activities were assayed under the standard conditions. Symbols: —●—, phytase activity of F1 and F2; —○—, acid phosphatase activity of F1 and F2; —△—, acid phosphatase activity of F3; ---○---, acid phosphatase activity of F4.

Table III. SUBSTRATES SPECIFICITY OF F1, F2, F3, AND F4

Enzyme preparation	F1		F2		F3	F4
pH of the enzyme reactions	4.4	5.2	4.6	5.2	5.4	6.0
Substrate (10 mM)	Relative activity (%)					
NPP	100	100	100	100	100	100
IHP	5.4	2.5	5.7	2.3	0	0
α -Naphthyl phosphate	24.5	35.0	41.0	48.2	31.5	34.0
β -Naphthyl phosphate	29.4	71.2	59.6	75.5	75.0	78.6
α -Glycerol phosphate	5.1	4.3	3.6	3.3	13.6	14.9
β -Glycerol phosphate	90.2	93.0	91.6	92.0	51.6	21.4
Glucose 1-phosphate	61.9	100.0	5.1	10.6	10.0	ND
Glucose 6-phosphate	7.0	7.5	5.1	5.0	6.0	15.1
Fructose 1,6-diphosphate	8.0	7.0	9.2	9.5	17.0	28.8
Adenosine 5'-triphosphate	51.1	64.0	66.7	73.5	52.0	41.6
Adenosine 5'-diphosphate	13.3	16.2	11.5	16.0	17.0	19.1
Adenosine 5'-phosphate	2.4	1.6	1.8	1.3	11.5	8.9
Inorganic pyrophosphate	50.1	60.0	53.0	58.6	52.1	58.0
Bis(<i>p</i> -nitrophenyl)phosphate	0	0	0	0	0	0
Adenosine 3',5'-phosphate	0	0	0	0	0	0

protein bands were stained by the PAS reaction.²⁵⁾

Physical properties

Molecular weight of the purified enzymes were estimated by SDS-PAGE (Fig. 3), gel filtration on Ultrogel AcA 34, and velocity density gradient centrifugation: F1, 66 K by SDS, 61 K by Ultrogel filtration and 68 K by

density gradient centrifugation; F2, 61 K, 70 K, and 68 K; F3, 61 K, 66 K, and 66 K, F4, 60 K, 59 K, and 68 K. The molecular weight of the native enzymes was similar to that for SDS-PAGE. This suggested that F1, F2, F3, and F4 may be monomeric proteins. The isoelectric points of F1, F2, F3, and F4 were F1; 5.1, F2: 5.1, F3: 5.6, and F4: 5.9. The purified F1, F2, F3, and F4 had a violet color

Fig. 5. Effects of Temperature on the stability of F1, F2, F3 and F4.

Enzyme preparations were incubated for 30 min at indicated temperatures in 0.05 M acetate buffer, pH 5.5. After the treatment, the enzyme solutions were diluted with 0.2 M acetate buffer, pH 4.6 (phytase activity of F1 and F2) and acetate buffer pH 5.5 (acid phosphatase activity of F1, F2, F3, and F4). The residual activity is expressed as a percentage of the control (not treated).

(A) symbols: —●—, phytase activity of F1; —○—, acid phosphatase activity of F1; —×—, phytase activity of F2; —x—, acid phosphatase activity of F2.

(B): —△—, acid phosphatase activity of F3; —●—, acid phosphatase activity of F4.

and F2 had a broad absorption peak at around 560 nm in 0.05 M acetate buffer, pH 5.5. We could not measure the absorption spectra of F1, F3, and F4 because of their low yields.

Effects of pH on the enzyme activity and stability

The pH-activity curve of phytase activities of F1 and F2 were a little different. The phytase activities had a narrow pH-activity curve and were active at 4.4 for F1 and at 4.6 for F2. Acid phosphatase activities were broad pH-activity curves and were most active at 5.1 for F1, at 5.2 for F2, at 5.4 for F3, and at 6.0 for F4. The pH-stability maxima were between pH 5.5~7.5, however, below pH 4.5, acid phos-

phatase rapidly lost activity, but the phytase activity was stable at pH 4.5 (Fig. 4).

Effects of temperature on activity and stability

The optimum temperatures of the four enzymes were as follows: 40°C for phytase activity of F1 and F2; 46°C, 50°C, 48°C, and 50°C for acid phosphatase activity of F1, F2, F3, and F4, respectively. Their thermal stabilities for the phytase activity of F1 and F2 retained 90% and 78% of their initial activity, and acid phosphatase activity of F1, F2, F3, and F4 retained 100% of the activity (Fig. 5).

Substrate specificity

The substrate specificity of F1, F2, F3, and F4 was investigated, and their optimum pH conditions (Table III). The substrate specificity of the enzymes were similar except for IHP and G-1-P. F1 and F2 were active against IHP, but F3 and F4 were not. F1 was inhibited by IHP above the concentration of 3 mM (data not shown). No phosphodiesterase activity was observed.

Kinetic constants for IHP and NPP

The K_{mi} values for IHP and NPP were calculated Lineweaver-Burk plots. They were 0.17 mM (F1) and 0.09 mM (F2) for IHP, 1.37 mM (F1), 1.33 mM (F2), 0.83 mM (F3), and 1.33 mM (F4) for NPP.

Effects of metal ions and chemical reagents on enzyme activity

The effects of metal ions and chemical reagents on the enzyme activities were examined using IHP and NPP as the substrates. The results are shown in Table IV. The enzyme activities were strongly inhibited by $\text{Mo}_7\text{O}_{24}^{6-}$ and Ag^+ , Zn^{2+} , Cu^{2+} , and Hg^{2+} also potently inhibited the acid phosphatase activity of F1 and F2, but phytase activities of these were only inhibited about 30~80%. Pi and F^- also caused inhibition. ICH_2COOH and PCMB inhibited phytase activities of F1 and F2 and phosphatase activity of F1 but not the acid phosphatase activity of F2, F3, and F4. Ca^{2+} enhanced the phytase activity rather than the

Table IV. EFFECTS OF METAL IONS AND SEVERAL INHIBITORS ON F1, F2, F3, AND F4

Fractions		F1		F2		F3	F4
pH of enzyme reactions		4.4	5.2	4.6	5.2	5.4	6.0
Substrates of enzyme reactions		IHP	Npp	IHP	Npp	Npp	Npp
Substances	Concentration	Relative activity					
None		100	100	100	100	100	100
ZnSO ₄	1 mM	68	9	47	11	25	23
CuSO ₄	1	71	8	62	8	50	54
HgCl ₂	1	30	3	77	26	1	1
(NH ₄) ₆ Mo ₇ O ₂₄	1	1	2	1	2	0	0
CoCl ₂	1	116	100	109	99	100	104
NaF	1	72	77	69	66	60	70
Pi	1	63	85	70	83	92	86
Pi	10	15	44	20	30	50	60
ICH ₂ COOH	10	50	79	78	95	100	107
PCMB	10	1	8	4	4	82	90
AgNO ₃	10	0	0	1	0	2	3
CaCl ₂	10	140	117	133	121	110	115
MnCl ₂	10	122	ND	116	101	95	99
MgCl ₂	10	115	100	110	100	108	111
EDTA	10	114	108	110	106	98	100
Bipyridine	10	102	103	100	100	99	97
<i>o</i> -Phenanthroline	10	100	100	100	98	100	99
8-Hydroxyquinoline	10	114	110	105	106	101	98
D(-)Tartrate	10	115	116	108	106	104	100
L(+)Tartrate	10	112	110	107	105	102	101

acid phosphatase activity of F1, F2, F3, and F4. Mn^{2+} and Mg^{2+} had little effect on all the phytase activities of F1, and F2, and on the acid phosphatase activities of F1, F2, F3, and F4. The enzyme activities of F1 and F2 were slightly activated by EDTA, hydroxyquinoline, D(-)tartrate, and L(+)tartrate.

Discussion

In this paper, we attempted to clarify the nature of IHP hydrolysis by isolating enzymes which participate in the dephosphorylation of IHP in the rice bran. We could not separate an IHP specific enzyme from acid phosphatase with phytase activity from rice bran, and the phytase activity was always about 4~6% of that of acid phosphatase activity of F1 and F2. We also isolated acid phosphatases F3 and F4 which had no phytase activity. The enzymes were monomeric proteins and their molecular

weights were slightly lower than that of the acid phosphatase from aleurone particles of rice grains.²⁶⁾ F1, F2, F3, and F4 had a violet color and the absorption peak of F2 was broad with a maximum at around 560 nm. Similar acid phosphatases have been isolated from aleurone particles of rice grains,²⁶⁾ sweet potato,^{27~30)} soybean,³¹⁾ and kidney bean.³²⁾

In our previous papers,^{33,34)} we isolated violet-colored acid phosphatase isozymes from cultured cells of rice plants. The molecular weight of the cytoplasmic enzymes were 230 K, composed of four subunits. Some properties of F1 and F2 resembled to those of the colored acid phosphatase prepared from the cultured cells in their *K_m*, *pI*, absorption spectra, substrate specificities, and inhibitors except for the molecular weights. Presumably 59 K~70 K of the colored acid phosphatases in this study were dissociated forms of the tetramer type of the cytoplasmic acid phosphatases from cul-

tured cells of rice plants. The reported pH optimum of phytase from aleurone particles of rice grains was 4.5³⁵⁾ for IHP and that reported by Yamagata *et al.* was 4.8 for acid phosphatase with NPP as a substrate. IHP-specific phytases were separated from microorganisms^{36~38,42)} and plants.^{11,14,39,40)} Our enzymes from rice bran were nonspecific acid phosphatases. They are similar to the enzymes from wheat bran,^{7~10)} navy bean⁴¹⁾ and aleurone particles of rice rains.²⁶⁾

References

- 1) F. Kurasawa, T. Hayakawa, I. Okamoto and S. Imai, *Nippon Nōgeikagaku Kaishi*, **43**, 735 (1969).
- 2) D. J. Cosgrove, "Inositol phosphates," Elsevier Scientific Publishing Co., Amsterdam, 1980, pp. 85~98.
- 3) U. Suzuki, K. Yoshimura and M. Takaishi, *Bull. Coll. Agr. Tokyo Imper. Univ.*, **7**, 503 (1907).
- 4) F. C. Mayer, R. E. Campbell, A. K. Smis and L. L. Mckinney, *Arch. Biochem. Biophys.*, **94**, 301 (1961).
- 5) L. N. Gibbins and F. W. Norris, *Biochem. J.*, **86**, 67 (1963).
- 6) A. M. Mayer, *Enzymologia*, **19**, 1 (1958).
- 7) Y. Nagai and S. Funahashi, *Agric. Biol. Chem.*, **26**, 794 (1962).
- 8) Y. Nagai and S. Funahashi, *Agric. Biol. Chem.*, **27**, 619 (1963).
- 9) P. E. Lim and M. E. Tate, *Biochim. Biophys. Acta*, **250**, 155 (1971).
- 10) P. E. Lim and M. E. Tate, *Biochim. Biophys. Acta*, **302**, 316 (1973).
- 11) N. C. Mandal, S. Burman and B. B. Biswas, *Phytochem.*, **11**, 495 (1972).
- 12) T. Yoshida, K. Tanaka and Z. Kasai, *Agric. Biol. Chem.*, **39**, 289 (1975).
- 13) M. Goel and C. B. Sharma, *Phytochem.*, **18**, 1939 (1979).
- 14) A. Hara, S. Ebina, A. Kondo and T. Funaguma, *Agric. Biol. Chem.*, **49**, 3539 (1985).
- 15) J. Lin, D. B. Dickinson and T. D. Ho, *Plant Physiol.*, **83**, 408 (1987).
- 16) K. Gabard and R. L. Jones, *Physiol. Plant*, **67**, 182 (1986).
- 17) C. H. Fisk and Y. Sabbarow, *J. Biol. Chem.*, **66**, 375 (1925).
- 18) I. Igaue, M. Nishio and F. Kurasawa, *Agric. Biol. Chem.*, **37**, 941 (1973).
- 19) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 20) J. B. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 21) U. K. Laemmli, *Nature*, **227**, 680 (1970).
- 22) R. G. Martin and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).
- 23) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
- 24) Z. Ogita, *SABCO. J.*, **2**, 91 (1966).
- 25) R. M. Zacharius, T. E. Zell, T. H. Morrison and J. J. Woodlock, *Anal. Biochem.*, **30**, 1481 (1969).
- 26) H. Yamagata, K. Tanaka and Z. Kasai, *Plant Cell Physiol.*, **21**, 1449 (1980).
- 27) K. Uehara, S. Fujimoto and T. Taniguchi, *J. Biochem.*, **75**, 627 (1974).
- 28) K. Uehara, S. Fujimoto, T. Taniguchi and K. Nakai, *J. Biochem.*, **75**, 639 (1974).
- 29) Y. Sugiura, H. Kawabe, H. Tanaka, S. Fujimoto and A. Ohara, *J. Biol. Chem.*, **256**, 10664 (1981).
- 30) S. K. Heffler and B. A. Averill, *Biochem. Biophys. Res. Commun.*, **146**, 1173 (1987).
- 31) S. Fujimoto, T. Nakagawa and A. Ohara, *Agric. Biol. Chem.*, **41**, 599 (1977).
- 32) J. L. Beck, L. A. Mcconachie, A. C. Summors, W. N. Arnild, J. D. Jersey and B. Zerner, *Biochim. Biophys. Acta*, **869**, 61 (1986).
- 33) I. Igaue, H. Watabe, K. Takahashi, M. Takekoshi and A. Molota, *Agric. Biol. Chem.*, **40**, 823 (1976).
- 34) I. Igaue, Monbusho Kagakukenyuhi Ippankenkyu B. Houkokusho, 1979, p. 27.
- 35) T. Yoshida, K. Tanaka and Z. Kasai, *Agric. Biol. Chem.*, **39**, 289 (1975).
- 36) V. K. Power and V. Jagannathan, *J. Bacteriol.*, **151**, 1102 (1982).
- 37) G. C. J. Irving and D. J. Cosgrove, *Aust. J. Biol. Sci.*, **24**, 547 (1971).
- 38) G. C. J. Irving and D. J. Cosgrove, *Aust. J. Biol. Sci.*, **27**, 361 (1974).
- 39) I. B. Maiti, A. L. Majumder and B. B. Biswas, *Phytochemistry*, **13**, 1047 (1974).
- 40) I. B. Maiti and B. B. Biswas, *Phytochemistry*, **18**, 316 (1979).
- 41) C. M. Lolas and P. Markakis, *J. Food. Sic.*, **42**, 1094 (1977).
- 42) A. H. J. Ullah and D. M. Gibson, *Preparative Biochem.*, **17**, 63 (1987).