[Chem. Pharm. Bull. 25(12)3283—3288(1977)]

UDC 577.152.01.02 : 581.192

Further Studies on the Properties of Violet-colored Acid Phosphatase from Soybean[1]

Sadaki Fujimoto, Tsutomu Nakagawa, and Akira Ohara

Kyoto College of Pharmacy[2]

(Received May 2, 1977)

The properties of the acid phosphatase isolated in a homogeneous state from soybean (*Glycine max*) have been investigated in detail. The enzyme catalyzed the hydrolysis of a wide variety of phospholylated compounds including phosphomonoesters, nucleotide mono-, di- and triphosphate, and inorganic pyrophosphate. No activity was detected for nicotinamide adenine dinucleotide and diphenyl phosphate. The enzyme activity was inhibited by orthophosphate, arsenate, fluoride, molybdate, and heavy metal ions including Cu^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+}. Treatment of the enzyme with chelating agents, reducing agents and oxidizing agents resulted in inactivation of the enzyme. The reduction of the absorbance at 540 nm of the enzyme was observed in parallel with the loss of the enzyme activity by the treatment with ethylenediaminetetraacetic acid and rongalit, but not with H_2O_2. The enzyme had a molecular weight of approximately 240000. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate suggested that the enzyme dissociated into subunits with molecular weight of approximately 60000. The amino acid and carbohydrate composition of the enzyme were also determined.

Keywords——acid phosphatase; metalloenzyme; soybean; manganese; violet-colored protein

Acid phosphatase has been identified in a large number of bacteria, animal and plant tissues.[3] But little is known as regards the characteristics of plant acid phosphatase. Recently violet-colored acid phosphatases containing manganese were found in sweet potato,[4] rice plant cultured cells[5] and spinach leaves.[6] The properties of colored enzymes, however, is not yet made clear.

In the course of our studies on violet-colored acid phosphatase, we also isolated violet-colored enzyme containing manganese from soybean (*Glycine max*) in a homogeneous state.[7] The enzyme was characterized by violet color, having an absorption maximum at around 540 nm. The purification procedure of the enzyme has been reported in a previous paper.[7] The present paper describes a more detailed study on the properties of the enzyme.

Materials and Methods

Materials——The following substances were purchased: *p*-nitrophenyl phosphate, diphenyl phosphate, α-glycerophosphate, pyridoxal phosphate, pyrophosphate, ethylenediaminetetraacetic acid (EDTA), *o*-phenanthroline and α,α-dipyridyl from Nakarai Chemical Co.; 5′-adenosinetriphosphate (5′-ATP), 5′-adenosinediphosphate (5′-ADP) and nicotinamide adenine dinucleotide (NAD^+) from Kojin Co., 5′-adenosinemonophosphate (5′-AMP), 3′-AMP, 2′-AMP, fructose 1,6-diphosphate, fructose 6-phosphate, β-glycerophosphate

1) A part of this study was presented at the 49th Annual Meeting of the Biochemical Society of Japan, Sapporo, September 1976.
2) Location: 5 *Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto, Japan.*
3) V.P. Hollander, "The Enzymes," Vol. IV, ed. by P.D. Boyer, Academic Press Inc., New York, 1971, p. 449.
4) K. Uehara, S. Fujimoto, and T. Taniguchi, *J. Biochem.* (Tokyo), **70**, 183 (1971); *idem, ibid.*, **75**, 627 (1974).
5) I. Igaue, H. Watabe, K. Takahashi, and A. Morota, *Agric. Biol. Chem.* (Tokyo), **40**, 823 (1976).
6) S. Fujimoto, T. Nakagawa, S. Ishimitsu, and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **25**, 1459 (1977).
7) S. Fujimoto, T. Nakagawa, and A. Ohara, *Agric. Biol. Chem.* (Tokyo), **41**, 599 (1977).

and chymotrypsinogen A from Sigma Chemical Co.; $NADP^+$ from Oriental Yeast Co.; γ-globulin (human) and serum albumin (bovine) from Mann Research Lab.; ovalbumin from Nutritional Biochem. All other reagents used were of analytical grade.

Preparation and Assay of the Acid Phosphatase——The acid phosphatase from soybean was prepared as described in a previous paper.[7] Protein concentration was determined spectrophotometrically at 280 nm in 0.01 M phosphate buffer, pH 6.0, using the factor, $E_{1\%}^{1\,cm}=21.4$.[7] The acid phosphatase activity was routinely determined by measuring the rate of liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate, at 35° and pH 5.5, as described in a previous paper.[7] The enzyme activity for a number of other phospholylated compounds was determined under the above condition by measuring the rate of liberation of orthophosphate as described in a previous paper.[4]

Analysis of Amino Acid and Carbohydrate——The purified enzyme preparation was hydrolyzed under vacuum in 6 N HCl for 24, 48, and 72 hr at 110°. Amino acids were analyzed on a Hitachi 034 Liquid Chromatography by the method of Spackman *et al.*[8] Cystine was measured as cysteic acid after performic acid oxidation.[9] Tryptophan was determined with *p*-dimethylaminobenzaldehyde by the method of Spies and Chambers.[10] The total content of neutral carbohydrates of the protein was measured by the method of Dubois *et al.*[11]

Results and Discussion

Substrate Specificity

The ability of the enzyme to catalyze the release of orthophosphate from a number of phospholylated compounds is summarized in Table I. The enzyme catalyzed the hydrolysis of phosphomonoesters, nucleotide mono-, di- and triphosphate and inorganic pyrophosphate. No activity was detected for NAD^+ and diphenyl phosphate. As can be seen from Table I, the substrate specificity of the enzyme resembles most other acid phosphatases of plant origin.[3]

Table I. Substrate Specificity of the Acid Phosphatase

Substrate	Relative activity (%)	Substrate	Relative activity (%)
p-Nitrophenyl phosphate	100	Glucose 6-phosphate	63
5′-ATP	84	α-Glycerophosphate	82
5′-ADP	88	β-Glycerophosphate	67
5′-AMP	58	Pyridoxal phosphate	63
3′-AMP	47	$NADP^+$	52
2′-AMP	37	NAD^+	0
Fructose 1,6-diphosphate	82	Diphenyl phosphate	0
Fructose 6-phosphate	65	Pyrophosphate	75

The enzyme activity was expressed in per cent against that for *p*-nitrophenyl phosphate.

Effect of Various Substances

Table II summarizes the effect of various substances on the activity of the acid phosphatase. As has been shown for a number of other acid phosphatases,[3] the soybean acid phosphatase was also strongly inhibited by Cu^{2+}, Zn^{2+}, Ag^+ and Hg^{2+} at 1 mM. And also molybdate, orthophosphate and arsenate were found to be potent inhibitors of the enzyme.

No divalent cation had a stimulating effect on the enzyme activity. The presence of metal-complex agents such as EDTA in the assay had also no influence on the activity. Thus the enzyme seems to have no requirement for the external addition of metal ions for its optimal acitivity.

8) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
9) C.H.W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
10) J.R. Spies and D.C. Chambers, *Anal. Chem.*, **20**, 30 (1948).
11) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Robers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

Table II. Effect of Various Substances on the Activity of the Acid Phosphatase

Substance added	Relative activity (%) Concentration (M)					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
None	100	100	100	100	100	100
$Cu(CH_3COO)_2$		29	95			
$Zn(CH_3COO)_2$		15	66			
$Mg(CH_3COO)_2$		95				
$Cd(CH_3COO)_2$		98				
$Mn(CH_3COO)_2$		103				
$Pb(CH_3COO)_2$		97				
$CaCl_2$		101				
$FeCl_3$		68	98			
$Ni(NO_3)_2$		102				
$FeSO_4$		99				
$CoCl_2$		100				
$HgCl_2$		0	4	51	87	96
$AgNO_3$		53				
KH_2PO_4		63				
$Na_2B_4O_7$		55				
Na_2HAsO_4		41				
KF		32	68			
$(NH_4)_6Mo_7O_{24}$		0	7	18	35	53
EDTA	98					
o-Phenanthroline	95					
α,α'-Dipyridyl	96					
KCN	99					
Cysteine	97					

The activity was determined by incubation of the acid phosphatase (0.04 μg) for 10 min at 35° in the presence of 3 μmol of *p*-nitrophenyl phosphate and the test substance in a total volume of 3.0 ml of sodium acetate buffer, pH 5.5. The activity was expressed in percent against no addition run.

Inactivation of the Acid Phosphatase by the Treatment with Chelating Agents.

In a previous paper,[7] we showed that soybean acid phosphatase was metalloenzyme containing manganese similar to the enzymes from sweet potato,[4] rice cultured cells[5] and spinach leaves.[6] To determine whether or not the metal is an essential component of the enzyme, the enzyme was preincubated with chelating agents such as EDTA, *o*-phenanthroline and α,α'-dipyridyl. Figure 1 shows the results. Preincubation of the enzyme with EDTA or *o*-phenanthroline resulted in significant inactivation of the enzyme with time. After 60 min preincubation EDTA- and *o*-phenanthroline-treated enzymes lost 48 and 37% of the original activity, respectively. This may be due to removal of metal(s) from the enzyme. It was of interest, therefore, to study the effect of metal ions on the enzyme which was inactivated by the treatment with chelating agents. To EDTA-treated enzyme, in which the activity was reduced to 50% of the original activity, were added various metal ions such as Mn^{2+}, Ca^{2+}, Zn^{2+} and Co^{2+} to a final concentration of 1 mM. The mixture was then preincubated at 35° for various times and assayed for the enzyme activity. No significant reactivation of the EDTA-treated enzyme was observed, however. This result may be ascribed to the denaturation of the enzyme due to the removal of the metal ion(s) from the enzyme molecule.

12) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
13) A.L. Shapiro, E. Vinuela, and J.V. Maizel, Jr., *Biochem. Biophys. Res. Commun.*, **28**, 815 (1967).

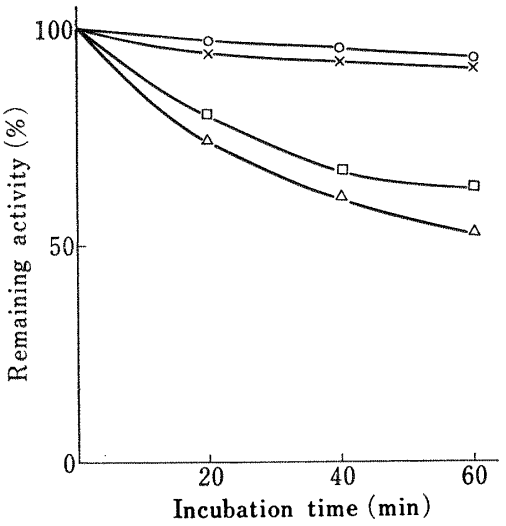

Fig. 1. Inactivation of the Acid Phosphatase by Incubation with Chelating Agents

The acid phosphatase (2 μg) was preincubated with 1 mM chelating agents at 20° in 0.75 ml of 0.05 M Tris–HCl buffer, pH 8.5. After various time, aliquots were withdrawn and then assayed for the enzyme activity. ○; control sample, ×; α,α'-dipyridyl, □; *o*-phenanthroline, △; EDTA.

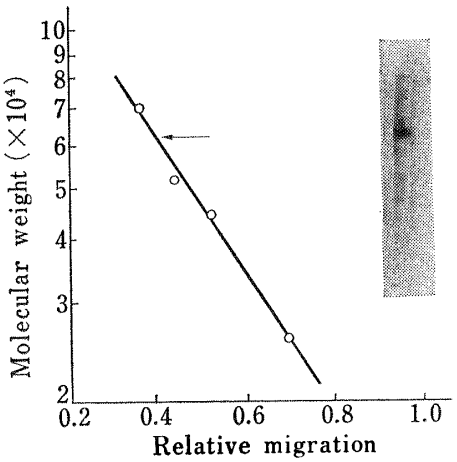

Fig. 2. Polyacrylamide Disc Gel Electrophoresis of the Acid Phosphatase in the Presence of Sodium Dodecyl Sulfate

Standard proteins and the acid phosphatase were heated at 50° for 3 hr in the presence of 1% sodium dodecyl sulfate and 1% β-mercapthoethanol and subjected to electrophoresis at 8 mA per tube as described by Shapiro *et al.*[13] The gel contained 7.5% acrylamide. After electrophoresis, proteins were stained with Coomassie brilliant blue. The molecular weight of standard proteins were plotted on a semilogarithmic scale against the distance of migration from the top of the gel relative to that of bromophenol blue as tracking dye (bovine serum albumin, human γ-globulin (H chain), ovalbumin, and chymotrypsinogen A in this order from top to bottom of the line). Mobility of the acid phosphatase subunit is noted by the arrow. The inset shows the electrophoretic pattern of the acid phosphatase.

Molecular Weight

The molecular weight of the acid phosphatase was estimated to be approximately 240000 by Sephadex G-200 gel filtration according to the method of Andrews,[12] using a series of proteins of known molecular weight as standard.

When the enzyme was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Shapiro *et al.*[13], a single protein band was obtained as shown in the inset of Fig. 2. Molecular weight of the enzyme subunit was determined to be approximately 60000 from the calibration curve as shown in Fig. 2. These results indicate that the enzyme is composed of four subunits having an identical molecular weight.

Amino Acid and Carbohydrate Composition

Amino acid analysis was performed after acid hydrolysis as described under "Materials and Method." The result is summarized in Table III together with the data cited from ref. 14. Comparing with the amino acid composition of the sweet potato enzyme,[14] the soybean enzyme has the low content of tyrosine and tryptophan.

The presence of carbohydrate in the purified acid phosphatase from soybean was indicated by a positive periodic acid–Schiff reaction in the disc electrophoretic study.[7] Analysis by the phenol sulfuric acid method indicated a neutral sugar content of 13.4% by weight (measured in equivalents of glucose). A significant amount of glucosamine was also detected in acid hydrolyzates of the enzyme.

14) K. Uehara, S. Fujimoto, T. Taniguchi, and K. Nakai, *J. Biochem.* (Tokyo), **75**, 639 (1974).

Table III. Amino Acid Composition of the Acid Phosphatase

Amino acid	Mole %	
	Soybean	Sweet potato[14]
Lysine	4.86	4.22
Histidine	5.19	3.89
Arginine	4.86	4.76
Aspartic acid	11.59	10.59
Threonine	6.06	6.70
Serine	6.97	7.68
Glutamic acid	8.42	7.89
Proline	6.30	6.59
Glycine	6.83	7.46
Alanine	6.30	4.32
Valine	6.78	7.03
Methionine	1.20	2.05
Isoleucine	4.42	4.32
Leucine	7.70	6.38
Tyrosine	5.10	8.22
Phenylalanine	4.95	4.43
Tryptophan	1.25	3.24
Cysteic acid	1.20	—

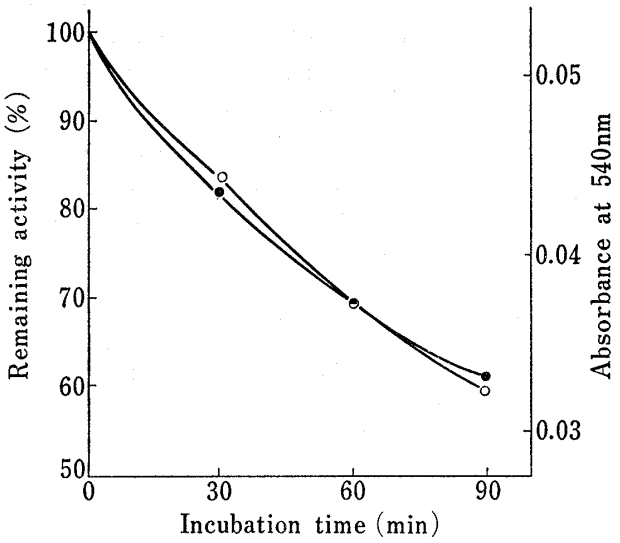

Fig. 3. The Correlation between the Enzymatic Activity and the Absorbance at 540 nm of the Acid Phosphatase by the Treatment with EDTA

The acid phosphatase (1 mg) was preincubated with 10 mM EDTA, at 20° in 2.0 ml of 0.05 M Tris–HCl buffer, pH 8.5. After various time, aliquots were withdrawn and then assayed for the enzyme activity and determined the absorbance at 540 nm of the enzyme. ○; acid phosphatase activity, ●; absorbance at 540 nm.

Table IV. The Correlation between the Enzymatic Activity and the Absorbance at 540 nm of the Acid Phosphatase by the Treatment with Reducing and Oxidizing Agents

Reagents	Concentration (mM)	Activity (%)	Absorbance at 540 nm (%)
None	—	100	100
Rongalit	30	41	45
$NaHSO_3$	30	88	82
H_2O_2	40	58	99
$K_3Fe(CN)_6$	40	100	99

The acid phosphatase (1 mg) was preincubated with the indicated reagents, at 20° in 2.0 ml of Tri–HCl buffer, pH 8.5. After 90 min, aliquots were withdrawn and then assayed for the enzyme activity and determined the absorbance at 540 nm of the enzyme.

Nature of Violet Color of the Acid Phosphatase

The acid phosphatase of soybean is violet-colored manganese-protein with an absorption maximum at around 540 nm[7] and as described in former section the enzyme was inactivated by the prolonged treatment with chelating agents. It was interesting to know if the absorbance of the enzyme might be altered by the treatment with chelating agents. The correlation between the activity and the absorbance at 540 nm was followed during the treatment of the enzyme with EDTA. The results are shown in Fig. 3. The reduction of the absorbance at 540 nm was observed in parallel with the loss of the enzyme activity. This result suggests that manganese in the enzyme molecule attributes not only to the enzyme activity but also to violet color of the enzyme.

Furthermore, the change of the activity and the absorbance at 540 nm of the enzyme were determined when the enzyme was treated with reducing or oxidizing agents. Table

IV shows the results. The rongalit-treated enzyme was inactivated to 41% of the original activity and in parallel to this inactivation the reduction of the absorbance at 540 nm was observed. On the other hand, the reduction of the absorbance at 540 nm did not occur when the enzyme was inactivated by the treatment with H_2O_2. On the basis of the above results and of the observation that the red-purple color is characteristic of model Mn(III) complexes rather than of Mn(II) complexes,[15] it may be concluded that violet color of the acid phosphatase from soybean is due to the ligand field of manganese prosthetic groups and the manganese in the enzyme molecule is Mn(III).

Acknowledgement The authors express their thanks to professor K. Uehara, Osaka University, for his encouragement. They are also indebted to Mr. S. Ishimitsu, Kyoto College of Pharmacy, for amino acid analysis. They wish to thank to Mr. R. Setsuie and Mr. T. Yamaguchi for the technical assistance in preparing the enzyme. This work was supported in part by grant from the Ministry of Education of Japan.

15) G.S. Sastry, R.E. Hamm, and K.H. Pool, *Anal. Chem.*, **41**, 857 (1969).