(1H, singlet, Ar–H), 6.60 (1H, singlet, Ar–H), 6.68 (2H, singlet, 2×Ar–H). Styphnate, mp 128—130° (MeOH–ether). *Anal.* Calcd. for $C_{27}H_{28}O_{12}N_4S$: C, 51.26; H, 4.46; N, 8.85. Found: C, 51.26; H, 4.52; N, 8.65.

5,6,12,13-Tetrahydro-2,3,9,10-tetramethoxy-13-(methylthio)methyl-7*H*-dibenz[*b*,*f*]azonine (14)——A mixture of 1.1 g of **12**, 0.8 g of $Hg(OAc)_2$ and 100 ml of 30% AcOH was heated in a water bath for 2 hr. The mixture was worked up as above to give 0.2 g of **14**, mp 148—150° (MeOH–ether). Mass Spectrum *m/e*: 403 (M^+). NMR ($CDCl_3$) δ: 2.00 (3H, singlet, SCH_3), 6.55, 6.60, 6.70, 6.78 (4H, each singlet, 4×Ar–H). *Anal.* Calcd. for $C_{22}H_{29}O_4NS$: C, 65.48; H, 7.24; N, 3.47. Found: C, 65.61; H, 7.38; N, 3.47.

Acknowledgement We thank Mr. Y. Shida, Mr. S. Suzuki, and Mrs. K. Isobe for microanalyses and spectroscopic measurements. We also thank Mr. T. Ogawa for his technical assistance.

[Chem. Pharm. Bull. 25(6)1459—1462(1977)] UDC 577.152.01.07 : 581.192

Purification and Some Properties of Violet-colored Acid Phosphatase from Spinach Leaves

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(Received September 29, 1976)

The violet-colored acid phosphatase was purified from leaves of spinach (*Spinacia oleracea*). The purified preparation was found to be homogeneous by electrophoresis and ultracentrifugation. Concentrated solution of the enzyme had an intense violet color with a broad absorption band between 410 nm and 700 nm. The peak was at around 530 nm. The molecular weight of the enzyme was approximately 92000. The enzyme was composed of two subunits of equal size and contained manganese. The result of staining of the enzyme in disc electrophoresis gel by periodic acid–Schiff reagent indicated that the enzyme was glycoprotein.

Keywords——acid phosphatase; violet-colored acid phosphatase; spinach; phosphatase purification; manganese; *Spinacia oleracea*

Acid phosphatases have been isolated in various states of purity from many different living organisms, and there have been numerous reports dealing with the properties of these enzymes.[2] Violet-colored acid phosphatase containing manganese have been found recently in sweet potato[3] and rice plant cultured cells.[4] However, the substantial nature of the color, manganese requirement and subcellular distribution of the enzyme are still unknown.

During the course of our investigations of the colored acid phosphatase, it was found that leaves of spinach (*Spinacia oleracea*) also contained a violet-colored acid phosphatase containing manganese. In this communication we report the results of purification and some properties of this enzyme.

Material and Method

Materials——Spinach leaves were obtained from a local market.

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1) Location: 5 *Nakauchicho, Misasagi, Yamashina-ku, Kyoto, 607, Japan.*
2) V.P. Hollander, "The Enzymes," Vol. IV, ed. by P.D. Boyer, Academic Press Inc., New York, 1971, p. 449.
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Methods——The acid phosphatase activity was determined by measuring the rate of liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate at 35°. An assay mixture contained, in a final volume of 3.0 ml, 600 μmoles of sodium acetate buffer (pH 5.5), 3 μmoles of *p*-nitrophenyl phosphate and enzyme fraction. The reaction was stopped by addition of 1.0 ml of 0.5N NaOH and absorbance was measured at 410 nm. The unit of the enzyme activity was expressed as micromoles of substrate hydrolyzed per min under the condition described above, taking $1.7 \times 10^4\,\text{M}^{-1}\,\text{cm}^{-1}$ as the molar extinction coefficient for *p*-nitrophenol.

The concentration of protein was determined by measuring the absorbance at 280 nm, assuming that the extinction coefficient, $E^{1\%}_{1cm}$, at 280 nm is 10.0, unless otherwise stated.

Polyacrylamide disc gel electrophoresis by the method of Williams and Reisfeld[5] was carried out with β-alanine–acetic acid buffer (pH 4.5) at room temperature for 90 min at 180 V with a current of 3 mA per tube. After electrophoresis, glycoprotein in the gel was stained by periodic acid–Schiff (PAS) reagent.[6] The protein was also stained with Comassie Brilliant Blue.

Results and Discussion

An outline of the purification procedure for spinach leaf acid phosphatase is presented in Table I. Spinach leaves (18 kg) were homogenized with 36 liter of 0.01M phosphate buffer

Table I. Purification of Acid Phosphatase from Spinach Leaves

Procedure	Total protein[a] (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	340701	16540	0.05
Ammonium sulfate fractionation (0 to 80%)	152133	16242	0.11
Ammonium sulfate fractionation (35 to 80%)	223780	11065	0.47
Acetone fractionation (0 to 50%)	14052	8856	0.63
CM–Cellulose	449.0	4680	10.4
Hydroxylapatite	43.0	2303	53.6
CM–Sephadex C–25	13.8	1677	121.5
Sephadex G–100	6.8	1324	194.6
Sephadex G–100	5.1	1203	235.8

a) In the crude extract through CM-Cellulose steps, the concentration of pro tein was determined from absorbance at 280 and 260 nm.[7] In the hydroxylatite through Sephadex G–100 steps, the concentration of protein was determined as described in the text.

(pH 6.5) (Buffer A) in Waring blender. The homogenate was filtered through a layer of Hyflo super-cel. To the filtrate (46.1 liter) was added ammonium sulfate to 80% saturation. After standing overnight, the precipitate was collected by centrifugation and suspended in, and dialyzed against Buffer A. The dialyzed solution (2.6 liter) was made clear by centrifugation and brought to 35% saturation with ammonium sulfate. The supernatant fluid was recovered after centrifugation and the enzyme was precipitated by the further addition of ammonium sulfate to give a concentration of 80% saturation. The precipitate was collected by centrifugation and dissolved in, and dialyzed against Buffer A. The dialyzed solution (1.0 liter) was brought to 50% with acetone previously chilled to −10°. The resulting precipitate was collected by centrifugation and dissolved in, and dialyzed against Buffer A. The dialyzed solution (1.64 liter) was applied to a column of carboxymethyl (CM)-Cellulose (2.5×30 cm) preequilibrated with Buffer A. After the column was washed with Buffer A, the enzyme was eluted with Buffer A containing 0.1M NaCl. The active fractions were combined (460 ml) and then charged to a column of hydroxylapatite (2.5×15 cm) preequilibrated with Buffer A. After the column was washed with 0.15M phosphate buffer (pH 6.5), the enzyme was eluted

5) D.E. Williams and R.A. Reisfeld, *Ann. N. Y. Acad. Sci.*, **121**, art. 2, 373 (1964).
6) R.M. Zacharius, T.E. Zell, J.H. Morrison, and J.J. Woodlock, *Anal. Biochem.*, **30**, 148 (1969).
7) E. Layne, "Methods of Enzymology," Vol. III, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1957, p. 451.

with 0.2M phosphate buffer (pH 6.5). The active fractions were combined (178 ml) and dialyzed against Buffer A. The dialyzed solution was charged to a column of CM-Sephadex C-25 (1.3×50 cm) preequilibrated with Buffer A. Elution of the enzyme was accomplished with a linear gradient prepared by mixing 150 ml of Buffer A with the same volume of Buffer A containing 0.3M NaCl. The active fractions were combined (76 ml) and concentrated in a collodion bag under reduced pressure. The concentrated solution was passed through a column of Sephadex G-100 (2×110 cm) preequilibrated with Buffer A containing 0.1M NaCl. Elution of the enzyme was carried out with the same buffer and acid phosphatase rich fractions were combined to give 30 ml. The Sephadex G-100 active fraction was concentrated in a collodion bag under reduced pressure and again passed through a column of Sephadex G-100 as described above. The fractions that contained the acid phosphatase activity were pooled (29 ml) and concentrated as described above and stored frozen. The concentrated solution had an intense violet color.

Upon electrophoresis on polyacrylamide gel the purified acid phosphatase migrated as a single protein band which coincided with a single zone of the acid phosphatase activity as shown in Fig. 1. This protein band was stained as a glycoprotein by the periodic acid-Schiff (PAS) reagent. The purified enzyme preparation was also found to be homogeneous, as judged by ultracentrifugal analysis (not shown in figure).

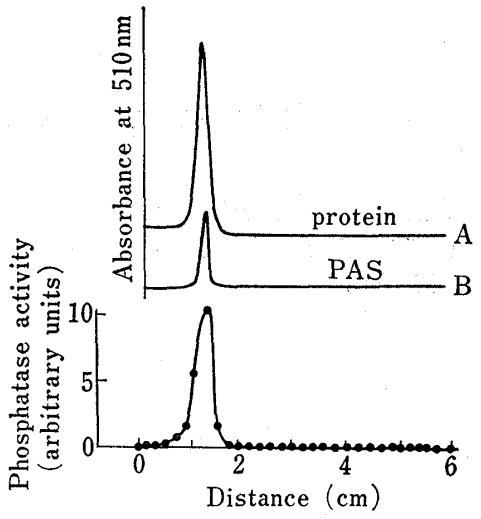

Fig. 1. Polyacrylamide Disc Gel Electrophoresis of Acid Phosphatase

The purified enzyme (80 μg) was subjected to electrophoresis under the condition described in "Material and Method". Gel A was stained with Comassie Brilliant Blue, Gel B by periodic acid-Schiff (PAS) reagent. The enzyme activity along the gel is indicated in the lower plot. The gel was cut into 2 mm sections. Each section was eluted with 2.5 ml of 0.2 M sodium acetate buffer (pH 5.5) and assayed for acid phosphatase activity.

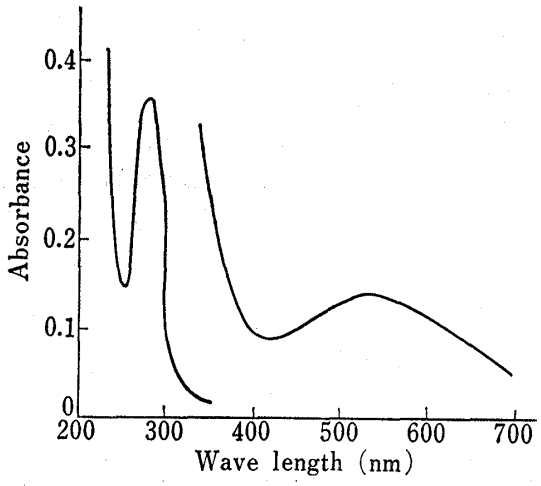

Fig. 2. Absorption Spectra of Acid Phosphatase

The spectra in the ultraviolet and visible regions were obtained with solutions containing 0.36 mg per ml and 5.4 mg per ml, respectively, of the purified enzyme in 0.01 M phosphate buffer(pH 6.5) containing 0.1 M NaCl.

Figure 2 shows absorption spectra of the purified acid phosphatase preparation. The visible region shows an absorption curve with a broad peak at around 530 nm. The ratio of absorption intensity at 280 nm to that at 530 nm was 39.1. The spectrum is similar to that shown by sweet potato[3] and rice plant cultured cell[4] acid phosphatases.

The metal analysis of the purified acid phosphatase by atomic absorption spectroscopy showed the presence of a significant amount of manganese (0.2 μg manganese/1 mg protein).

A molecular weight of approximately 92000 was obtained by gel filtration on a Sephadex G-100 column according to the method of Andrews.[8] By the disc electrophoretic determina-

8) P. Andrews, *Biochem. J.*, **96**, 595 (1965).

tion of the molecular weight in the presence of sodium dodecyl sulfate,[9] it appears that this enzyme is composed of two identical subunits with a molecular weight of approximately 50000.

The purified acid phosphatase was active toward *p*-nitrophenyl phosphate (relative activity (R.A): 100), α-glycerophosphate (R.A: 55), β-glycerophosphate (R.A: 67), pyridoxal phosphate (R.A: 41), fructose 6-phosphate (R.A: 28), fructose 1,6-diphosphate (R.A: 34), glucose 6-phosphate (R.A: 36), 2′-AMP (R.A: 25), 3′-AMP (R.A: 70), 5′-AMP (R.A: 14), ADP (R.A: 55), ATP (R.A: 55), $NADP^+$ (R.A: 51), and inorganic pyrophosphate (R.A: 98). No phosphodiesterase activity was detected toward NAD^+ and diphenyl phosphate. The enzyme had pH optimum around pH 5.5 with the K_m value of 3.9×10^{-4}M for *p*-nitrophenyl phosphate.

Acknowledgements Thanks are due to Prof. K. Uehara, Osaka University, for his encouragement. The authors express their thanks to Mr. Y. Sakamoto of the Faculty of Medicine, Osaka University for carrying out the ultracentrifugal analysis. They are also indebted to Nissei Sangyo Co., Ltd. for metal analysis. They wish to thank Mr. H. Nakamura and Mr. M. Nishihashi for the technical assistance in preparing the enzyme. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

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[Chem. Pharm. Bull. 25(6)1462—1468(1977)]

UDC 547.567.3.04 : 581.192

Studies on Quinones and Related Compounds in Higher Plants. V.[1] Model Experiments for the Preparation of Tritium-labeled Precursors for the Biosynthetic Studies of Prenylnaphthoquinone Congeners[2]

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(Received September 29, 1976)

Methods for the preparation of [3′-^{2}H]-4-(2′-carboxyphenyl)-4-oxobutanoic acid and [4,8-$^2H_2$]-2-carboxy-4-hydroxy-α-tetralone (CHT) starting from 3-methoxyphthalic anhydride have been demonstrated as model experiments for the preparation of several ^{3}H-labeled compounds necessary for the elucidation of the biosynthetic pathways of prenylnaphthoquinone congeners occurring in *Catalpa ovata*.

Keywords——prenylnaphthoquinones; biosynthesis; precursors; 4-(2′-carboxyphenyl)-4-oxobutanoic acid; 2-carboxy-4-hydroxy-α-tetralone; labeling methods

Recently we reported studies on the biosynthesis of prenylnaphthoquinone congeners of *Catalpa ovata* G. Don such as catalpalactone (**1**), catalponol (**2**) and 4,9-dihydroxy-α-lapachone (**3**) which are biosynthesized *via* 4-(2′-carboxyphenyl)-4-oxobutanoic acid (**4**).[1,4,5] As part of continuing efforts to clarify the mechanism of prenylation on their biosynthetic

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5) S. Ueda, K. Inoue, T. Hayashi, and H. Inouye, *Tetrahedron Letters*, **1975**, 2399.