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HYDROPHOBIC CHROMATOGRAPHIC PURIFICATION OF ETHYLENE-ENHANCED CHLOROPHYLLASE FROM *CITRUS UNSHIU* FRUITS

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Key Word Index—*Citrus unshiu*; Rutaceae; chlorophyllase; ethylene; hydrophobic chromatography.

Abstract—Ethylene-enhanced chlorophyllase from *Citrus unshiu* fruits was purified to a homogeneous state after solubilization with sodium cholate, using acetone precipitation and hydrophobic chromatography. The enzyme adhered to phenyl Sepharose CL-4B in 3M KCl and was eluted with a linear gradient of Triton X-100 (0–0.5%). Its MW (SDS-PAGE) was 27 000. The enzyme behaved as a protein of MW 110 000 on Sephacryl S-200 gel filtration. The enzyme showed a specific activity of 0.069 μmol chlorophyllide *a* produced/min/mg protein. This purification procedure is a rapid method for obtaining pure chlorophyllase.

INTRODUCTION

The previous paper [1] reported that C_2H_4-enhanced chlorophyllase was purified to a homogeneous state after solubilization with Triton X-100, by using ion exchange chromatography, gel filtration and hydroxylapatite chromatography. However, this procedure involves six steps for purification to homogeneity. Therefore, I have devised a better method for purifying the enzyme.

Hydrophobic chromatography is a fairly new technique in protein purification. In particular, *O*-alkyl linked Sepharoses, which contain no charged groups, are likely to provide new and powerful tools in the fractionation and isolation of membrane-bound proteins after solubilization with detergents [2]. It is reasonable to consider that chlorophyllase which is expected to be a hydrophobic enzyme as are such membrane proteins as cytochrome *c* oxidase [3] should be particularly suited for separation by hydrophobic chromatography.

The present paper reports on the purification of enhanced chlorophyllase by hydrophobic chromatography.

RESULTS AND DISCUSSION

Nearly all purification methods for the solubilization of bound chlorophyllase have a step that involves treatment with an organic solvent (*n*-butanol) [6] or detergent (Triton X-100) [1, 4, 8]. The advantage of using sodium cholate is its higher selectivity in the solubilization membrane proteins compared to Triton X-100 [3]. However, the presence of sodium cholate limits further purification of chlorophyllase. The suitability of hydrophobic chromatography for the purification of chlorophyllase was investigated. Preliminary results suggested that KCl and Triton X-100 are useful for adsorption and desorption on phenyl Sepharose CL-4B. However, all attempts of adsorption and desorption on phenyl Sepharose CL-4B in the presence of sodium cholate were unsuccessful. Therefore, the proteins obtained by acetone precipitation of the sodium cholate-containing extract were made up in Triton X-100 to give an enzyme-Triton X-100 complex, which could then be purified by hydrophobic chromatography on phenyl Sepharose CL-4B. Optimum conditions for adsorption and desorption of the enzyme were determined by using combinations of various ionic strength of salts [KCl and $(NH_4)_2SO_4$], and of detergents (Triton X-100 and sodium cholate). However, none of them except the following would adsorb or desorb the enzyme. The enzyme adhered to a phenyl Sepharose CL-4B column in 3 M KCl and was eluted with a linear gradient of Triton X-100 concentration (0–0.5%) (Fig. 1). After sample application, the column was washed with 50 ml (about 6 column vol.) of 100 mM phosphate buffer containing 3 M KCl. There was no enzyme activity in the washings. On addition of Triton X-100 two symmetrical peaks of protein were eluted. The chlorophyllase activity was associated with, and gave the same elution profile, as the second peak of protein.

Table 1 summarizes the purification data. The purified enzyme was homogeneous according to the criteria of polyacrylamide disc gel electrophoresis and the enzyme activity on cut gels was associated with a protein band. The enzyme was eluted at a position corresponding to a MW of 110 000 on Sephacryl S-200 gel filtration. SDS-PAGE of the enzyme gave one band corresponding to a protein of MW 27 000. On the other hand, the gel-filtration profile of the acetone powder extracts showed two active fractions of MWs 110 000 and 27 000 on a Sephacryl S-200 column [1]. This showed that the low MW

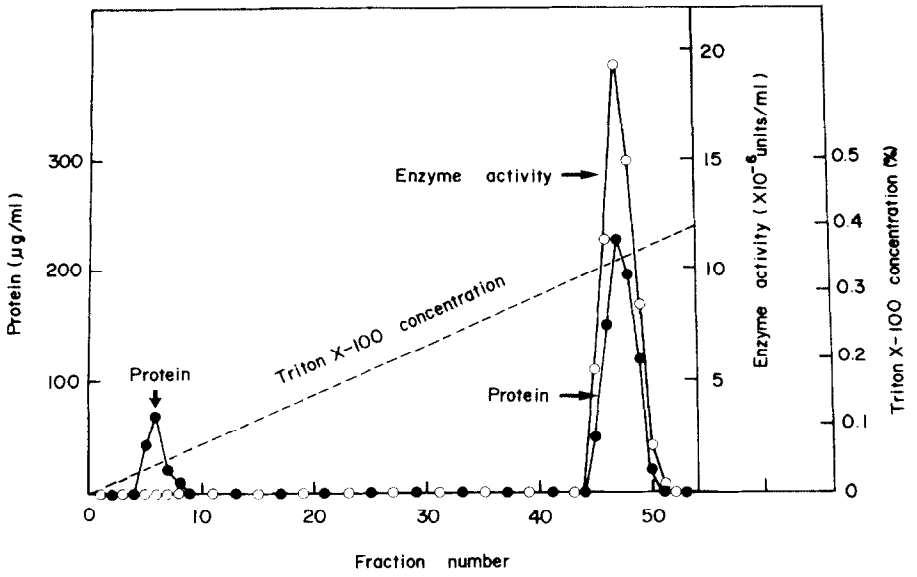

Fig. 1. Hydrophobic chromatography of C_2H_4-enhanced chlorophyllase on phenyl-Sepharose CL-4B (see Experimental for details). Total vol. 82 ml.

protein (27 000) was a basic unit of this enzyme. The high-MW (110 000) fraction was assumed to be a tetramer of the basic unit. The minimum MW (27 000) of SDS-treated *Citrus* chlorophyllase is almost the same as those of the enzyme from *Phaseolus* (30 000) [4] and *Beta vulgaris* (30 000–38 000) [5], and is smaller than those of the enzyme from *Chlorella* (38 000) [6] and *Phaeoductylum tricornutum* (38 000) [7]. The purified enzyme showed a specific activity of 0.069 μmol chlorophyllide *a* produced/min per mg protein.

The optimum pH for the purified chlorophyllase was 7.0 with both Pi and borate buffers. The pH values for half-maximal activity were 6.2 and 8.1, respectively. The enzyme showed almost the same activity with both buffers at the optimum pH. Purified chlorophyllases are roughly classified by their optimum pH into two groups; enzymes with an acidic optimum such as those from *Ailanthus* (pH 4.52) [8] and tea leaves (pH 5.8) [9], and enzymes with a neutral optimum such as those from *Chlorella vulgaris* (pH 7.2–7.3) [10], sugar beet (pH 7.1) [5] and tobacco [12]. Therefore, the C_2H_4-enhanced enzyme belongs to the latter group.

The K_m was 2.65 μM for chlorophyll *a*. This value is similar to those reported by other workers [10, 11] and is lower than those of the enzymes for *Ailanthus* [8], *Phaseolus* [4] and *Ailanthus* [13]. The optimum temperature was 20°. This is lower than that for the chlorophyllase from *Chlorella protothecoides* [11], *Beta vulgaris* [5] and *Thea sinensis* [9]. The activation energy was 8.9 kcal/mol. Thermal stability was measured in 0.01 M Pi buffer (pH 7.0) by heating at various temperatures for 10 min. The temperature for 50% denaturation was 60°. Residual activity was almost retained by heating at 55° for 10 min and completely lost by heating at 70° for 10 min. The activation energy for denaturation of the enzyme was 62 kcal mol.

This new purification procedure takes only 2 days and is a rapid method for obtaining pure chlorophyllase.

EXPERIMENTAL

Plant materials. The fruits of *C. unshiu* were obtained from a local farm in Nov. about 170 days after anthesis. The fruits were treated with 120 ppm C_2H_4 for 12 hr and kept in normal room air at 20° as described previously [14]. Twenty

Table 1. The purification of C_2H_4-enhanced chlorophyllase from *Citrus unshiu* fruits

Purification step	Total activity (under)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (–fold)
Acetone powder extract	15.13	3656	0.0041	100	1
40% Acetone precipitation	2.41	57.6	0.0419	15.9	10.1
Phenyl-Sepharose CL-4B	2.07	30.0	0.0690	13.7	16.6

four hr after the end of the C_2H_4 treatment chlorophyllase activity of the C_2H_4-treated fruits was increased to the maximum; which was about 20-fold that of the non-treated fruits.

Preparation of Me_2CO *powder.* The peel from the fully C_2H_4-induced fruits homogenized in a blender with cold (−20°) Me_2CO for 3 min. The homogenate was allowed to stand for 6 hr at 4° in the dark, then filtered through a Buchner funnel. The residue was dried *in vacuo* and stored in a desiccator under red. pres. at room temp. Under these conditions, the enzymatic activity of the Me_2CO powder was kept without loss for at least 3 years.

Enzyme activity. The chlorophyllase activity was determined according to the method of ref. [8] with slight modification. The reaction mixture contained 30 mM Pi buffer (pH 7.0) containing 0.2% Triton X-100, 23 μM chlorophyll *a*, and enzyme in a total vol. of 3.0 ml. After incubation at 20° for 5 min, the reaction was terminated by the addition of a mixture of 3 ml hexane, 2 ml Me_2CO, 1 ml 2-butanone, and 0.2 ml 0.25 M NaOH. The mixture was vigorously shaken and allowed to stand for 10 min. Chlorophyllide *a* in the Me_2CO layer was determined spectrophotometrically by using 74.9 mM cm at 667 nm [8]. One unit of cholrophyllase is defined as the amount of enzyme which catalyses the production of 1 μmol chlorophyllide *a*/min.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed according to the method of ref. [15]. After being run with a constant current of 2 mA gel, the gels were stained with 0.0025% Coomassie brilliant blue, destained and stored in 7% HOAc. SDS-electrophoresis was carried out according to the method of ref. [16]. To dissociate the sample prior to electrophoresis, SDS and 2-mercaptoethanol were each added to a final concn of 5%, and the mixture incubated for 30 min at 100°. The electrophoresis was performed at 8 mA gel for *ca* 5 hr at 20°.

Gel filtration for MW determination. A Sephacryl S-200 column (1 × 45 cm) was equilibrated with 5 mM Pi buffer containing 0.2% Triton X-100 and 0.1 M KCl. The column was calibrated with cytochrome *c* (MW 13 000), trypsin (MW 24 000), peroxidase (MW 44 000), and L-amino acid oxidase (MW 140 000). V_0 and V_i were determined using Blue Dextran 2000 and DNP-alanine, respectively.

Preparation of chlorophyll a. Chlorophyll *a* was extracted from spinach leaves with 80% Me_2CO and was then pptd with dioxane. The chlorophyll *a* was further purified by powder sugar column chromatography [17].

Protein content. This was determined by the method of ref. [18] using bovine plasma γ-globulin as the standard.

Activation energy. This was determined by incubating the enzyme in Pi buffer (0.01 M, pH 7.0) at temps. ranging from 20 to 80° for 10 min.

Activation energy for denaturation. Activation energy for denaturation was determined by incubating the enzyme in Pi buffer (0.01 M, pH 7.0) at 55°, 58°, 60°, 63°, and 66° for 30 sec, 1 min, 3 min, and 5 min. Residual activity was determined by the procedure described above.

Purification of chlorophyllase. The Me_2CO powder (100 g) was ground to a fine powder using a mill and extracted with 2 l. 0.01 M Pi buffer (pH 7.0) containing 1.0% sodium cholate, 0.03 M KCl and 10% (v/v) glycerol at 25° for 30 min. The extracts were filtered through cloth. The suspension was centrifuged at 12 000 *g* for 20 min. Cold Me_2CO (−20°) was added to the supernatant to 40%. The ppt was collected by centrifugation and dissolved in 5 mM Pi buffer (pH 7.0) containing 0.2% Triton X-100 and 0.1 M KCl was added to the enzyme soln to 3 M. The soln was applied to a small column of phenyl Sepharose CL-4B (1 × 10 cm, bed vol. about 10 ml) pre-equilibrated with 100 mM Pi (100 ml) containing 3 M KCl. The column was washed with 50 ml of the same Pi soln, and was then eluted with a linear gradient of Triton X-100 (0–0.5%, total vol. 200 ml).

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