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FRACTIONATION OF JERUSALEM ARTICHOKE PHENOLASE BY IMMOBILIZED COPPER AFFINITY CHROMATOGRAPHY

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Key Word Index—*Helianthus tuberosus*; Compositae; Jerusalem artichoke; immobilized copper affinity chromatography; phenolase.

Abstract—Phenolase from tubers of Jerusalem artichoke was fractionated by metal chelate affinity chromatography using copper conjugated to iminodiacetic acid Sepharose 6B. Four fractions obtained after chromatography showed various specific activities, with an increase in activity of 160-fold for the first unbound enzymatic fraction, and 18-fold for a fraction eluted with glycine buffer. Electrophoresis of phenolase fractions in gradient polyacrylamide gel resulted in a pattern consisting of three major groups of bands differing in relative mobilities.

INTRODUCTION

The Jerusalem artichoke (*Helianthus tuberosus L.*) is recognised as a good source of stored inulin (fructan) but has been reported to exhibit discoloration reactions during processing [1,2] and wounding [3]. Although preliminary results [4] have indicated that this discoloration is primarily due to phenolase (polyphenol oxidase) activity (*o*-diphenol: O_2 oxidoreductase, E. C. 1.14.18.1), no attempts have been made to purify the enzyme. Purification of phenolase from other plant sources has been conducted using conventional methods including ammonium sulphate, ion exchange chromatography and gel filtration [5] or affinity chromatography with immobilized substrates [6]. In most cases, however, these methods are laborious and enzyme yields are usually low. A relatively new method, referred to as metal chelate or immobilized metal affinity chromatography, introduced in 1975 by Porath *et al.* [7] is now becoming widely accepted for the isolation of proteins and enzyme from various sources [8–11].

The present study describes the fractionation of phenolase from Jerusalem artichoke by immobilized copper affinity chromatography (ICAC). To our knowledge this is the first investigation which has employed this technique for phenolase purification.

RESULTS

Initial purification

Phenolase was precipitated from an acetone powder buffer extract between 20 and 80% ammonium sulphate. Approximately 98% of the initial enzyme activity was recovered and almost 90% of the contaminating proteins were eliminated. A resulting 8-fold increase in purification was obtained. Less than 1% of the phenolase activity containing 12% protein was precipitated in the 0–20% fraction. Desalting on Sephadex G-25 resulted in a further 11-fold increase in purification.

Immobilized copper affinity chromatography

A typical elution profile of desalted phenolase obtained with ICAC is shown in Fig. 1. When the column was eluted with equilibration buffer, two flow-through fractions of activity, P_1 and P_2 were obtained. To ensure that these fractions were not a result of column overloading, the P_1 fraction was rechromatographed. Approximately 90% of the reapplied activity was recovered as a flow-through fraction. Stepwise elution, first with glycine and then histidine, revealed two additional fractions (P_3 and P_4 respectively). The majority of the activity was present in the P_1 and P_3 fractions which contained 39 and 76 activity units respectively; fractions P_2 and P_4 contained 23 and 18 activity units respectively. Approximately 55% of the initial activity applied to the column was recovered. The overall recovery of activity was *ca* 40% when compared to the initial extract.

Polyacrylamide gel electrophoresis

Fractions P_1 and P_4, from ICAC, were analysed by polyacrylamide gel electrophoresis (PAGE) coupled with enzymatic staining for oxidase activity using catechol as a substrate. This analysis yielded an electrophoretic pattern in which three groups of bands, referred to as A, B and C predominated. A comparison of the four activity peaks indicated that the group A bands of low mobility were present only in the P_1 fraction. Group B, represented by two bands, was most apparent in the P_3 fraction. The group C bands of high mobility were found in fractions P_1–P_3. Fraction P_4 from ICAC showed faint activity bands, two of which appeared co-terminous with subspecies of group B bands, and another band intermediate to groups B and C.

DISCUSSION

In this study artichoke phenolase was separated into four fractions of activity using ICAC. This type of

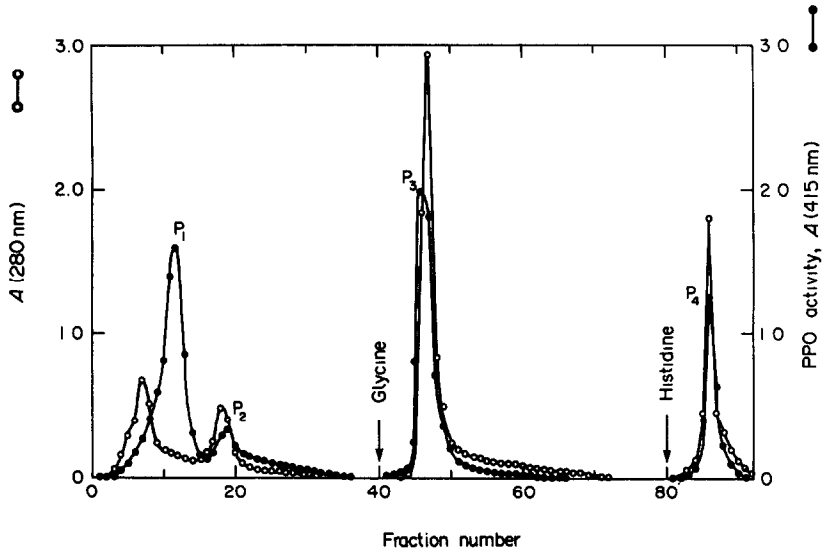

Fig. 1. Immobilized copper affinity chromatography of phenolase. Sephadex G-25 fractions containing activity were pooled and concentrated by diafiltration. A 5 ml sample containing 38 mg protein with 381 activity units was applied to the column after equilibration as described in Experimental. The column was eluted stepwise, first with 50 mM Tris–HCl, 0.15 M buffer (pH 7.5) containing 20 mM glycine and then with the same buffer containing 10 mM DL-histidine (as indicated by the arrows) at a flow rate of 15 ml/h. Fractions of 4 ml were collected. An aliquot from each sample was assayed spectrophotometrically (for phenolase activity using 10 mM catechol as the substrate.

chromatography is based on the ability of proteins to form coordination bonds with a metal due to the presence of surface chelating amino acids (metal combining sites). The differences in affinity among the phenolase fractions for the gel may reflect differences in the number of density of copper chelating amino acid groups exposed on the surface of the enzyme molecules. The fact that the P_1 and P_2 fractions were obtained as flow-through fractions may indicate that the enzyme components present do not have effective copper ion binding sites on their molecular surfaces [12]. In contrast, the P_3 and P_4 fractions were adsorbed strongly to the column. Lowering the pH from 7.5 to 4.5 or increasing the ionic strength from 0 to 0.3 M NaCl did not desorb these fractions (data not shown). A stepwise elution, however, with buffer first containing glycine and then, histidine in order of their stability constants to copper [13], resulted in their desorption. These results suggest that the enzyme components of P_3 and P_4 fractions bind to the metal chelate gel via structural sites possibly composed of either glycine or histidine residues.

Heterogeneity of plant phenolase is a well known phenomenon. Multiple forms of this enzyme from various plant sources have been distinguished on the basis of their charge, M_r and electrophoretic mobility [14–17]. The four enzymatic fractions obtained after ICAC were indicative of their heterogenity based on differences in affinity for immobilized copper. Electrophoretic analysis of phenolase fractions indicated that ICAC could be used to partially fractionate this enzyme into components differing in electrophoretic mobilities. There were, however, some similarities in electrophoretic banding patterns of phenolase among column fractions. For example, group C enzyme bands, found in enzymatic fractions with little or no affinity for immobilized copper, also were found in an enzymatic fraction strongly adsorbed to the affinity column.

EXPERIMENTAL

Jerusalem artichoke tubers (*Helianthus tuberosus* (L.) cv Columbia) grown at the Morden Research Station, Agriculture Canada, were used throughout this investigation. All procedures, unless otherwise indicated, were carried out a 4°.

Initial enzyme purification. An acetone powder was prepared from fresh artichoke tubers (1 kg) [18] and crude phenolase was extracted using 0.1 M Na-Pi (1:30 w/v) pH 6.5 containing 20 mM L-ascorbic acid. After stirring for 1 hr. the slurry was centrifuged at 4000 g for 20 min and the supernatant filtered (Whatman No. 4). The filtrate was treated with $(NH_4)_2SO_4$ and the protein fraction which pptd between 20 and 80% was dissolved in Na-Pi (0.1 M, pH 6.5) and loaded on a Sephadex G-25 column (2.6 × 70 cm) equilibrated with the same buffer. Fractions having phenolase activity and free of $(NH_4)_2SO_4$ were pooled and concentrated by diafiltration using an Amicon cell with a membrane having an exclusion limit of 10000.

Immobilized copper affinity chromatography. IDA Sepharose 6B was prepared according to ref. [17] and was used for both the working (1 × 20 cm) and guard columns (1 × 6 cm). The working column was loaded with an aqueous solution of $CuSO_4 \cdot 5H_2O$ (6 mg/ml) until metal was observed in the the eluate. The excess copper was washed off the column with water. Both columns, coupled in sequence, were equilibrated with Tris–HCl (50 mM, pH 7.5) containing NaCl (0.15 M). The guard column was used to adsorb any copper ion leakage from the working column during enzyme elution. Columns were regenerated after each run with 50 mM EDTA containing 0.5 M NaCl in 20 mM Na-Pi (pH 7.0).

Electrophoresis. Gradient polyacrylamide gel electrophoresis was performed on an LKB 2001 Vertical Electrophoresis Unit using the modified method of ref. [19]. The stacking gel consisted of 3.9% acrylamide and 0.1% N^1,N^1-methylenebisacrylamide and was prepared in 0.16 M Tris–0.25 N H_3PO_4 buffer (pH 6.9). Solutions were polymerized under UV light by the addition of 2.5 ml catalyst (0.06% ammonium persulphate and 0.002%

riboflavin) and 5 μl TEMED per 15 ml of gel solution. The separating gel consisted of 0.95 M Tris–HCl buffer (pH 8.5) and a gradient of acrylamide, bisacrylamide and glycerol ranging from 3.9 to 11.4, 0.2 to 0.6 and 0.5 to 5.0‰, respectively. This gel was polymerized under UV light by the addition of catalyst (1.9 ml) and 30 μl TEMED per 15 ml of gel solution. The electrode compartments were filled with two buffer systems. The upper tank contained 0.04 M Tris–0.04 M glycine buffer (pH 8.9) while the lower tank contained 0.06 M Tris–HCl buffer (pH 7.5). Each gel was run for 3 hr at 20° with a constant current of 30 mA. Gels were stained [20] using 10 mM catechol in 0.1 M Na-Pi (pH 6.0) containing 0.05% *p*-phenylenediamine.

Enzyme assay. Phenolase activity was determined at 30° using a biological oxygen monitor (Model 53, Yellow Springs Instrument, Co.). The total reaction mixture (3 ml) included 10 mM catechol in air saturated 0.1 M Na-Pi, pH 6, plus 10 μl of enzyme solution. One unit of activity was defined as the amount of oxygen (μM) consumed per min [21]. The air-saturated buffer contained 0.230 mM of dissolved oxygen at 30°. PPO activity of the gel chromatographic fractions was determined spectrophotometrically [22]. Protein was determined according to ref. [23] or by absorbance at 280 nm.

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