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Kinetic characteristics of purified tyrosinase from different species of *Dioscorea* (yam) in aqueous and non-aqueous systems

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ABSTRACT

Yam tubers could serve as a cost-effective source of tyrosinase for various applications. Here a novel but cheap method of purification, and some properties of the enzyme from four species of yam tubers having high tyrosinase activity are described. The native and subunit molecular weights of each of the purified tyrosinase were between 41–58 kDa and 24–27 kDa respectively. Optimum pH and temperature were 6.5 and 50 °C respectively. Tyrosinase from tubers of *Colocasia esculenta* retained more than 50% activity in ethanol (≤60%). All the purified enzymes were activated in 40% ether by between 120 and 170%, and maintained 100% residual activity at up to 65% ether for 17 h. Both the K_m and V_{max} increased in 40% ether, leading to a corresponding increase in k_{cat}/K_m. All the enzymes had both monophenolase and diphenolase activities. β-Mercaptoethanol and to a lesser extent, glutathione were good inhibitors. In aqueous systems, the purified tyrosinase catalyzed formation of coloured products in the presence of some substrates such as catechol, pyruvic acid and ammonia. The catalytic properties of these enzymes especially in organic solvents suggest that they may find uses in some biotechnological applications.

1. Introduction

Tyrosinases (EC 1.14.18.1) are copper-containing enzymes which are ubiquitously distributed in all domains of life [1]. They are bi-functional enzymes that catalyze both the hydroxylation of monophenols to *o*-diphenols and the subsequent oxidation of the diphenols to *o*-quinones [2]. Molecular oxygen is used by tyrosinases to catalyze the two different enzymatic reactions: (1) the ortho-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and (2) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The active quinones polymerize spontaneously to the macromolecular melanin [3].

In plants, it is required for the biosynthesis of phenolic polymers such as lignin, flavonoids, and tannins [4]. Tyrosinases also play an important role in the regulation of the oxidation–reduction potential of cell respiration and in wound healing in plants [5]. Browning reactions which occur when mechanical injuries are inflicted on some plant tissues like tubers, fruits and vegetables have been associated with tyrosinase [3]. This has been studied in a number of plants including potato [6], banana [7], avocado [8] and Walnut [9]. The enzymatic browning reactions are initiated by endogenous tyrosinases, which oxidize the phenolic compounds present in the plant tissues. Inhibitors of tyrosinases have been studied extensively [3], since browning reactions in fruits and vegetables cause changes in food products' organoleptic properties and appearance, leading to a short shelf life and a lower market value [10].

Due to the ability of tyrosinases to react with phenolic compounds, these enzymes have been proposed for use in a variety of biotechnological, biosensor and biocatalysis applications [11]. Non-aqueous enzymology or catalysis in organic solvent is such a rapidly developing research area that has attracted interest from chemists, biochemists and chemical engineers. This is reflected by the explosive growth of literature in this area [12]. It is generally accepted that when enzymes are placed in organic media, they exhibit altered properties such as enhanced thermostability [13], increased substrates specificity [14], molecular memory [15], and the ability to catalyze reactions that are kinetically or thermodynamically impossible in aqueous solution [16].

As a result, enzymatic catalysis in organic solvents has a variety of applications, which include chiral resolution of pharmaceuticals, synthesis of fine chemicals, enantioselectivity and regioselective polymerization [17]. Tyrosinase activities in organic solvents have been studied using mostly tyrosinase from lower organisms such as mushroom and bacteria. Bacterial tyrosinase has been studied in pure organic solvents [18] and aqueous media containing water

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miscible-solvents [19]. The results from these studies suggest that catalytic activities of tyrosinase vary from one species to the other.

Some limited information has been provided on tyrosinase from yam tubers [20] in aqueous systems. In addition to the fact that the purification scheme adopted in that work gave a low yield, only one cultivar-*Dioscorea bulbifera* was well characterized. There are many species of yam grown in different regions of the world. We have decided to provide basic biochemical information in aqueous and non-aqueous systems on tyrosinase, from those species that are grown in Southwestern Nigeria, where little or no information is currently available, essentially for possible technical application of the enzyme and also for control of browning reactions in the tubers.

2. Materials and methods

2.1. Materials

Tubers were obtained from seven (7) yam cultivars grown around Ile-Ife, South Western Nigeria. The yam cultivars were authenticated at the herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

2.1.1. Chemicals

3,4-Dihydroxyphenyl-L-alanine (L-DOPA), catechol, resorcinol, caffeic acid, DL-tyrosine, phenol, 1-naphthol, pyrogallol, lysozyme, α-chymotrypsinogen A, horse radish peroxidase, bovine serum albumin (BSA), ovalbumin, N,N,N′,N′-tetramethylethylenediamine (TEMED), Coomassie brilliant blue R-250 and glutathione were obtained from Sigma Chemical Company, St. Louis, USA. CM-Sepharose CL-6B, QAE-Sephadex A-50 and Sephadex G-100 were purchased from GE Healthcare Bio-sciences, Uppsala, Sweden. Molecular weight standard for SDS-PAGE was obtained from Carl Roth GmbH, Karlsruhe, Germany. Other chemicals were of analytical grade and were obtained from reputable chemical suppliers.

2.2. Methods

2.2.1. Yam homogenate preparation

The yam tubers were peeled and 50 g each were homogenized in 50 mM sodium phosphate buffer, pH 6.5 at 4 °C. The 30% homogenates were centrifuged at 10,000 × *g* for 30 min at 4 °C in order to obtain clear crude supernatants. The supernatants were assayed for tyrosinase activity and stored at −20 °C when not used immediately. Four cultivars having the highest tyrosinase activity were selected for purification.

2.2.2. Standard procedure for tyrosinase activity assay

Tyrosinase activities with L-3,4-dihydroxyphenylalanine (L-DOPA) were determined in crude soluble yam supernatants and routinely during purification according to the method of Lerch and Etlinger [21]. This involves monitoring the change in absorbance at 475 nm of the assay mixture in a spectrophotometer. An assay mixture of 1 ml contained in final concentration 50 mM sodium phosphate buffer pH 6.5, 1 mM L-DOPA and appropriate concentration of the enzyme that would cause absorbance change of between 0.02 and 0.07/min. A complete assay mixture in which the enzyme was replaced with distilled water served as blank. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product (*o*-dopaquinone) per minute at 475 nm under the specific assay condition (ε of product = 3600 M^{-1} cm^{-1}).

2.2.3. Protein concentration determination

The protein concentrations in the crude homogenates, partially purified tyrosinase and purified tyrosinase were determined using Bradford method [22]. Bovine serum albumin (BSA) was used as the standard protein.

2.2.4. Enzyme purification

2.2.4.1. Purification by ion-exchange chromatography on CM-Sepharose. Eighteen millilitres (18 ml) equivalent to 60, 80, 200 and 340 mg of protein in the supernatants of *Dioscorea praehensilis*, *Dioscorea alata*, *Dioscorea rotundata* and *Colocasia esculenta* respectively were layered separately on CM-Sepharose ion-exchange column (1.0 cm × 10 cm) equilibrated with 50 mM phosphate buffer pH 6.5. Elution was done with the equilibration buffer, and fractions of 1 ml each were collected at a flow rate of 18 ml/h. A linear gradient of 0–1 M NaCl in the elution buffer was applied to elute bound proteins. Fractions with tyrosinase activity were pooled.

2.2.4.2. Purification by ion-exchange chromatography on QAE-Sephadex A-50. Active CM-Sepharose pools equivalent to 60, 53, 124 and 170 mg protein of *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* respectively, were layered on a freshly prepared QAE-Sephadex A-50 ion-exchange column (2.5 cm × 10 cm) which had been equilibrated with 10 mM Tris–HCl buffer pH 7.0. Fractions of 2 ml each were collected at a flow rate of 18 ml/h. Elution was done with the equilibration buffer. A gradient of 0–1 M NaCl in the elution buffer was applied to the column to elute bound proteins. Fractions with tyrosinase activity were pooled and lyophilized.

2.2.4.3. Purification by gel-filtration chromatography on Sephadex G-100. The lyophilized re-dissolved samples from QAE-Sephadex A-50 equivalent to 18, 13, 16 and 32 mg protein of *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* respectively were layered separately on Sephadex G-100 gel-filtration column (1 cm × 50 cm), which had previously been equilibrated with 50 mM phosphate buffer pH 6.5. Elution was done with the equilibration buffer at a flow rate of 12 ml/h. Fractions with tyrosinase activity were pooled and lyophilized. The lyophilized samples were re-dissolved in minimal volume of 50 mM phosphate buffer pH 6.5 and used for characterization.

2.2.5. Determination of native and subunit molecular weight

Native molecular weight was determined on Sephadex G-100. The column was calibrated with the following molecular weight standard marker proteins: bovine serum albumin (67 kDa), ovalbumin (45 kDa), peroxidase (40 kDa), α-chymotrypsinogen A (25 kDa), and lysozyme (14.4 kDa). The native molecular weights of tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* were obtained by interpolation of K_{av} values on the standard curve. For the subunit molecular weight, SDS-polyacrylamide gel electrophoresis was run on 12% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel, according to the method of Laemmli [23]. The subunit molecular weights of the purified tyrosinase were obtained by interpolation of the R_m values on the standard curve.

2.2.6. Effect of organic solvents on the tyrosinase activity

The organic solvents were introduced into the reaction mixture to achieve 0–90% final concentration for DMSO, ethanol, methanol, acetone, chloroform, and ether. The residual activities were measured under standard assay condition earlier mentioned. The activity of tyrosinase on L-DOPA in 50 mM phosphate buffer, pH 6.5 without the organic solvents was taken to be 100%. To observe the possibility of spontaneous oxidation of L-DOPA, control experiments without enzymes were performed in all the organic solvents.

2.2.7. Stability of tyrosinase in ether

The stability of tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* in ether where there was improved activity, were investigated by incubating the enzyme solution at 30 °C for