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Phenoloxidases of perennial plants: Hydroxylase activity, isolation and physiological role

N.T. Omiadze*, N.I. Mchedlishvili, M.O. Abutidze

Agricultural University of Georgia, Sergi Durmishidze Institute of Biochemistry and Biotechnology, 240, David Agmashenebeli Alley, Tbilisi, 0159, Georgia

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ABSTRACT

The difficulties of isolation of phenoloxidases with hydroxylase activity from different plants have been discussed. Phenoloxidase with high hydroxylase activity has been isolated from tea and kivi leaves, apple (Kekhura) fruits, walnut husks and vine stems. The tea leaf phenoloxidase has been shown to be able to catalyze the hydoxylation of the natural substrate p-coumaric acid. The step in the secondary metabolism in which phenoloxidase hydroxylase activity may be included in the plant lignification process has been shown. It has been supposed that masking of hydroxylase activity of phenoloxidase might be connected with the difficulties and peculiarities of the enzyme isolation and perhaps, hydroxylase activity of perennial plant phenoloxidase is included in the process of lignin formation.

Introduction

Polyphenol oxidases (PPOs) are a group of copper-containing enzymes that catalyze o-hydroxylation of monophenols (monophenolase, also called hydroxylase or cresolase.

(EC 1.14.18.1) and the oxidation of o-diphenols into o-quinones (diphenolase, also termed catecholase or catechol oxidase EC 1.10.3.2) with oxygen as the primary oxidant or in the presence of oxygen [1–4].

These enzymes are widely distributed among animals, fungi, and plants, but many plant PPOs appear to lack monophenolase activity [5]. Monophenolase activity is always coupled to diphenolase activity. However, diphenolase activity is not always preceded by hydroxylase activity [6,7]. Activity towards o-diphenols only was found for PPOs from grape [8], field bean seed [9], strawberry [10] and sunflower seed [11]. The activity of apple PPO on tyrosine is much lower than on o-diphenols [12], which is typical for PPOs. It appears that substrate specificity of PPO is also dependent on species and cultivars [13].

The study of PPOs in plants has focused primarily on their role in the process of postharvest browning, whereby cut or damaged plant tissues turn brown due to the polymerization of PPO-generated quinones, generating phytomelanins [14]. PPO-generated quinones are highly reactive and may cross-link or alkylate proteins, leading to the commonly observed brown pigments in damaged plant tissues and plant extracts [15]. The conspicuous pigments are generally undesirable in food products, and the role of PPO in browning has prompted numerous studies on PPO in food and beverages.

In parallel, the potential roles for PPO in plant defense against pests have motivated many studies. The most frequently suggested role for PPO in plants has been in defense against herbivores and pathogens, based on the physical separation of the chloroplast-localized enzyme from the vacuole-localized substrates [16]. PPOs and their potential phenolic substrates have been considered to be physically separated from one another in intact plant cells, with most PPOs targeted to the chloroplasts, while phenolic compounds accumulate primarily in the vacuole and cell wall [5,17]. Thus, PPO activity *in vivo* has typically been associated with senescing, wounded, or damaged plant tissues in which cellular compartmentalization is lost [5,18]. The *o*-quinone–-protein complexes, formed as a consequence of cell damage, may reduce the nutritional value of the tissue and thereby reduce predation but can also participate in the formation of structural barriers against invading pathogens [17].

Aside from possible contribution of PPO as a part of a protective mechanism of plants against invading pathogens or insect pests, several other roles for PPO were also proposed. High induction of pokeweed PPO in ripening, betalain-containing fruit, was related to its involvement in the betalain pathway [19]. Tyrosine hydroxylation by PPO has been described in pokeweed, where this reaction constitutes part of the biosynthetic pathway leading to betalains [20]. Investigations by Steiner et al. [21] provided additional evidence supporting the hypothesis that PPO is involved in betalain biosynthesis of higher plants. Its likely role in auxin biosynthesis was also considered by Shaw et al. [22] For most PPOs, however, the endogenous substrates are unknown.

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* Corresponding author.
E-mail address: nino.omiadze@agruni.edu.ge (N.T. Omiadze).

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The importance of identifying PPO substrates in plants and understanding the overall phytochemical context of PPO-containing plants is emphasized by recent reports of PPO-like enzymes with biosynthetic roles as hydroxylases of secondary metabolites [23,24] reported that reaction of proteins with PPO- or laccase-generated o- and p-quinones, respectively, may be involved in formation of humus. Furthermore, thylakoid location of the enzyme led researchers to suggest that PPO may play a role in photosynthesis of functional chloroplast [17,25]. Associations of the enzyme with photosystem complexes suggested a likely role in photosynthetic electron transport [26,27]. It may function by regulating pseudocyclic photophosphorylation (ATP production with oxygen as a terminal electron acceptor) [25,28].

According to Araji et al. [18] silencing (hiding) of PPO caused major alterations in the metabolism of phenolic compounds and their derivatives (e.g.coumaric acid and catechin) and in the expression of phenylpropanoid pathway genes. Several observed metabolic changes point to a direct role for PPO in the metabolism of tyrosine and in the biosynthesis of the hydroxycoumarin esculetin *in vivo*. In addition, PPO silenced plants displayed massive (9-fold) increases in the tyrosine-derived metabolite tyramine, whose exogenous application elicits cell death in walnut and several other plant species. Overall, these results suggest that PPO plays a novel and fundamental role in secondary metabolism and acts as an indirect regulator of cell death in walnut [18].

It is also known about the participation of phenol oxidases (laccases) in the formation of lignin. Laccases can act as p-diphenol: O_2 oxidoreductases by using O_2 directly to oxidize all types of monolignols to form DHPs [29–31]. Laccases do not appear to have specifically evolved to enable plant lignin polymer formation and present a high degree of structural conservation between bacteria, fungi and plants [32].

PPO has been studied intensively for more than a century and significant advances have been made on biochemistry, molecular biology and genetics of the enzyme to date. The biochemical properties involved in the action of PPO are determined from numerous plant sources. Much attention is given to significant variations in physiochemical properties of the enzyme from different plant sources. Models explaining monophenolase and diphenolase activities of PPO have provided an additional insight to our understanding of a detailed picture of the molecular structure of PPO reactions. Genes encoding PPOs have been isolated and characterized from a variety of plants and all these studies verify the plastidic location of the nuclearly coded protein [13].

In spite of the fact that phenoloxidases have intensively been studied, it is still unknown whether any plant phenoloxidases have hydroxylase activity and what the physiological role of monophenolase is in undamaged, intact plant cells.

The purpose of the work was to isolate tea leaf phenoloxidase, study its hydroxylase activity and physiological role.

Objectives and methods

Fresh leaves of tea (*Camellia sinensis*) and kivi (*Actinidia deliciosa*) plants, fruits of apple (*Mallus domestica*), husks of walnut (*Juglans regia L.*) and stalks of grapes of grapevine (*Vitis vinifera*) were used in the experiments. Local sorts of all these plants were used in this study.

Isolation of phenoloxidase crude preparation from the investigated plants were carried out as described in [33].

Protein content in enzyme preparation was determined by the Amido Black reagent [34].

Catechol oxidase activity was determined spectrophotometrically according to Lanzarini et al. [35], using catechol as substrates. One unit of enzyme activity was defined as the amount of the enzyme sufficient to change the absorption spectrum at 420 nm by the value 0.05 in 1 min. Specific activity was expressed in units per mg protein.

Hydroxylase (monophenolase) activity was determined according to the rates of conversion of p-coumaric acid into caffeic acid and the p-cresol oxidation in the presence of proline or ascorbic acid at 530 nm [36,37]. Reaction mixture containing the enzyme solution, 1% p-cresol, and 0.2% proline was incubated for 24 h. The optical density of the reaction mixture was measured at 530 nm with a SF-26 spectrophotometer.

To determine caffeic acid two-dimensional ascending paper chromatography was carried out in *the* solvent systems: benzene - acetic acid -water (10:7:3, upper layer) and 2% acetic acid [38].

Results and analysis

Isolation of phenol oxidases from different sources is related to the certain difficulties. The enzyme easily lose hydroxylase activity during the isolation. In this point of view tea plant is very interesting. It was supposed that tea plant phenoloxidase could not catalyze hydroxylation of monophenols [39,40].

High content of phenolic compounds complicates the isolation of proteins from different parts of plants including tea leaves. The fact is that phenolic compounds bind proteins and, as a result, enzymes lose their activity [39]. Therefore, one of the most important stages during isolation of phenoloxidase is the separation of enzymes and phenolic compounds. Phenolic compounds may be separated from the protein mainly by binding them to such absorbents as polyamide, polyvinylpyrrolidone or by treating raw materials with acetone [39] Phenoloxidases from different sources were isolated from their acetone preparations [41,42].

We have developed two schemes of isolation of phenoloxidase from tea leaves.

The former scheme was based on the obtaining of the acetone preparation from fresh tea leaves and then the extraction of enzyme from it. Enzyme solution of acetone preparation from tea leaves was characterized by very low hydroxylase activity and high catecholase activity. As it can be seen from Fig. 1(1), the ratio of these two activities from the enzyme solution from acetone preparation was much higher in favour of catecholase. “Masking” of hydroxylase activity of phenoloxidase may be caused by the lost of some phenolic compounds, acting as activators of phenoloxidase, during the isolation of the enzyme. Hydroxylation function of phenoloxidase may also be lost in the acetone preparation due to the processes connected with dehydration.

As the enzyme solution obtained from acetone preparation was characterized by low hydroxylase activity, a new scheme of isolation of phenoloxidase was worked out directly from fresh tea leaves [43–45]. The results displayed in Fig. 1 (2)) show that phenoloxidase crude preparation obtained from fresh tea leaves revealed quite high hydroxylase activity.

Multiple forms of plant phenoloxidases and their substrate specificities

In the present work phenoloxidases have been isolated not only

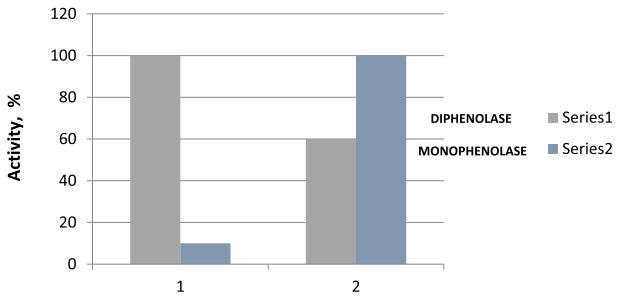

Fig. 1. The ratio of hydroxylase and catecholoxidase activities of phenoloxidse isolated by various methods.
(1) - Enzyme fraction isolated from acetone preparation of tea leaf;
(2) - Enzyme solution obtained from fresh tea leaves.

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