REVIEW ARTICLES

Characterization of polyphenol oxidase from plants

LEI Dongfeng*, FENG Yi and JIANG Dazong

(The School of Life Science and Technology, Xi'an Jiao Tong University, Xi'an 710049, China)

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Abstract Polyphenol oxidase (PPO) which can mediate browning reaction is a bifunctional copper-containing enzyme encoded by plant nucleolus gene. It usually leads to excessive browning reaction which reduces the commercial profits of fruits and vegetables. In this paper, PPO genes and enzymes in plants are characterized systematically, and the latest progress is reviewed. Some clonings of PPOs genes are reported; the specific temporal and spatial expression pattern of PPOs genes is described; the model of the structure of the precursor form of catechol oxidase is introduced; the possible functions of PPOs in defending against pathogen, wounding, surrounding stress and other inducing factors are demonstrated; the induction and activation of latent PPOs in some plants is elucidated; the scheme of browning inhibition by L-cysteine is clarified; the mechanism of suicide inhibition of latent PPO and kinetic synergism are established. Furthermore, the area for future study is also discussed.

Keywords: PPO, copper-containing enzyme, latent enzyme, enzymatic browning reaction, resistance of plant, biosynthesis.

Polyphenol oxidase (PPO) encoded by plant nucleolus gene is a widespread copper-containing enzyme, and it is synthesized in initial tissue developmental stages and stored in chloroplasts of plants. One of the reasons of the deterioration of appearance and nutritional values of fruits and vegetables, which can considerably reduce the commercial profits of plant, is that PPOs enzymes cause excessive browning reaction. In recent years, more and more PPOs genes especially from the tissues or organs with high market demand have been cloned[1~11] and the properties of PPOs enzymes particularly in the developmental stages or producing process in which the quality of products easily declines have been examined. Here, the latest progress in studying PPOs with respect to molecular cloning and multiple gene family, the specific expression pattern, biochemical properties, activity inhibition[12,13], induction and activation of latent PPOs[16,17], physiological functions[18~20], catalytic mechanisms[21,22] and practical use[23~25] are reviewed thoroughly. Furthermore, the present review analyzes the data obtained, and attempts to establish, in general, some new concepts or theories and tries to predict the future research work of PPOs.

1 Molecular biological characterization of PPO

1.1 Molecular cloning and multiple gene family

Since the PPO genes from Chinese pawpaw, lichi, tea, sweet potato[26,27], tomato, bean, potato, grape, pokeberry, apple, sugarcane, tobacco, apricot, pear, peach, and loquat had been cloned, the PPO genes from hybrid poplar, banana[28], pineapple[29], coffee[30] were also cloned subsequently in recent years. The number of these PPO copy genes showed that most PPO genes belong to multifamily.

For example, the 7 copies of tomato PPO genes are located on the chromosome 8[31], and in potato, at least 6 copies of PPO genes exist[32,33]. Moreover, 3 cDNA clones of PPO show in the bean[34], and the copies of PPO gene are 2 and 4 in wheat[35] and banana[28], respectively. However, not all PPO genes of plants belong to multifamily, because only one PPO gene was found in grape veins[36]. A reported intron in the banana PPO gene[28] challenges the traditional knowledge that the plant PPO gene contains no intron. The intron may likely lead to the silence of PPO genes although no silent PPO gene has been reported so far. Therefore, the reported copy numbers of PPO genes may be less than those of the actual

* To whom correspondence should be addressed. E-mail: tewentysecond@x263.net

copies.

1.2 The specific temporal and spatial expression pattern of PPO genes

The PPO genes in different varieties of plants express themselves in different patterns and even in the same multifamily they are also expressed with specificity in different tissues and organs of the identical plant and at different developmental stages of the same tissues and organs by enabling the PPO genes to turn on or turn off via the different signal transduction way[37, 38] as the result of specific interactions between *trans*-acting factors and corresponding *cis*-elements. Therefore, temporal and spatial difference is the characteristic of PPO gene expression[39], which contributes the variable levels and/or different degree of activity to formed PPO enzymes and the specific biochemical properties and physiological functions to isozymes. Therefore, the temporal and spatial specificity and multiformity of PPO enzymes caters for the complex metabolic activity and physiological functions and ensures the plants to respond to all kinds of stresses and adapt to unpredictable unfavorable environmental conditions[39, 40].

1.3 Molecular structure of PPO

Precursor protein of 60 ~ 75 kD containing transient peptide is encoded by PPO gene in the cytoplasm, and consequently is imported onto the membrane of thylakoid and decomposed to a mature protein of 45 ~ 69 kD. At the amino-terminal domain of precursor protein, a strong hydrophilic poorly conserved extension domain (n-domain) is followed by a high homogenous and highly conserved thylakoid transient domain which is responsible for the transportation of precursor peptide from cytoplasm to chloroplast. The catalysis unit localized between the transient peptide and C-terminal extension region has two highly conserved region, CuA and CuB.

The sequence analysis of PPOs in different varieties of plants[29, 41] shows that the CuA and CuB of PPO have high homogeneity, which guarantees PPOs′ enzymatic activity. The structure of the latent precursor form of catechol oxidase from sweet potatoes (*Ipomoea batatas*) has been modeled based on the 3D structural data of mature catechol oxidase and hemocyanin from giant octopus (*Octopus dofleini*)[42, 43]. Compared with the crystal structure of mature ibCO, this model has an additional C-terminal extension peptide of about 35 amino acids (residues 342 ~ 374) which was found in the cDNA sequence but not in the purified mature form of catechol oxidase. Furthermore, at the beginning of the extension peptide, the three histidine residues (His438, His441 and His443) possibly make the precursor form have the function in the copper-uptake and the additional loop region putatively increase its flexibility. Therefore, this additional flexible region may enable the shield region of extension peptide to move away from the active site without cleavage and enable the precursor form to be activated, although its structure remains unclear.

2 Biochemical properties and physiological functions of PPO

2.1 Biochemical properties and activity control of PPOs in plants

In order to control PPO activity effectively and ultimately, and to raise the commercial profits of fruits and vegetables, biochemical properties of PPO have been investigated[44 ~ 49]. The studies show that the optimal pH of PPO is acidic (4.5 ~ 8), optimal temperature is 30 ℃ or 25 ℃[12, 13], and the molecular weight is 40 ~ 45 kD or 60 kD except that only one isoenzyme is 70 kD. The biochemical properties of PPOs in different varieties of plants are not identical, and the properties of the different PPO forms expressed by the different members of PPO multifamily in one plant are different. Furthermore, biochemical properties and contents of PPOI and PPOII separated from the same tissue in some plants by chromatography techniques show differences. By SDS-PAGE, the numbers of gel bands PPOI and PPOII of latex of *Hevea brasiliensis* are 32 and 34 kD respectively, although they possess the same pI, optimal pH and optimal temperature[50]; the optimal pH of PPOI in tobacco leaves is 7 as the substrate of catechol, but the optimal pH of PPOII is 6, and meanwhile the content of PPOI is 10-fold of PPOII[51].

The methods in inhibition of PPO enzyme can be classified into three types, chemical inhibition, physical control and molecular biological regulation methods. The chemical and hormones like thiourea, EDTA, mercapto ethanol, $NaHSO_3$, citric acid[52, 53] and ethylene[49] usually have better inhibition effect. In addition, some novel naturally extracted substance from plants like praline[54], triterpenoid glycosides[55], glutathione[5], analogue tropolone, mimo-

sine[56], cinnamic acid[57], endogenous antioxidants[14], L-ascorbate-2-triphosphate (L-AATP)[15], beta-cyclodextrin (beta-CD)[15,57], sorbic acid, benzoic acid and "enokitake" mushroom extracts[58] are also used to inhibit PPO activity more successfully without side-effect of chemical substances. Besides, other newly synthesized competitive inhibitors such as 4-hydroxybenzyl benzoate(4e) can control PPO activity of some plants[56] effectively and specifically. However, we must notice that some chemical substance can act as not only inhibitor but also activator. For example, azide has been consistently reported to act as an inhibitor of copper proteins, but it can also act as an activator of polyphenol oxidase II (PPO II) from tobacco leaves. This can be attributed to azide complexing with PPO II and forming $CuO_2^{2-}Cu$, which is the active site of the peroxide-PPO II complex in which peroxide plays the role of activator[59]. Therefore, choice of the inhibitors should be on the basis of the varieties of plants and plentiful tests.

Physical control methods usually prevent browning reaction effectively without contamination resulting from additional chemical inhibitors. These methods involve changing gas composition of storage environment[48], heat treatment, high-pressure sterilization, irradiation, microwave heating, ultrasound, osmotic rehydration. For instance, increasing temperature appropriately during curing can avoid excessive browning reaction and improve flue-cured tobacco leaves quality[60,61]. Inhibitory molecular biology methods include anti-sense RNA[62] and transgenic strategy. A variety of tomato with lower PPO activity is created by transgenic technique, and the browning reaction of potato is inhibited by the anti-sense RNA technique[63], but this variety of transgenic potato is more sensitive to attack of pathogen and herbivores[64]. Therefore, in order to inhibit the activity of PPO, we should not only create transgenic plant with lower level of expression of PPO gene and high resistance to diseases and herbivore by molecular technique[65], but also pay attention to combination of molecular, physical and chemical methods.

2.2 The possible physiological functions of PPO

In general, it is well known that PPO may play an important role in defense against pathogen and herbivore and this knowledge is supported by four facts: (1) the scab of the accumulated melanin formed by σ-quinone can prevent infection of pathogen[66]; (2) the binding of σ-quinone to protein can reduce the nutritive value of nucleophilic amino acid and seems to induce an antinutritive defense[67]; (3) σ-quinone can restrain the propagating of the bacterium[68]; (4) an increase in PPO activity has been observed in incompatible interaction[36]. Although the increase of PPO activity in many plants has been reported when the plants are wounded or attacked by wounding, pathogen and herbivore, and the roles have been proposed, neither the defined biological function nor the clear defense mechanism is understood completely. Only by determining the plant PPO defense mechanism against pathogen, wounding and herbivory, can the defined physiological functions of PPO be clarified. To do this, the transgenic plant containing resistance gene will be ideal materials available. Meanwhile, a tyrosinase has other special functions. It is involved in betacyanin biosynthesis in common portulaca and red beet; and a chalone-specific PPO is responsible for the formation of aurones in yellow snapdragon flowers[69]. In addition, as one form of oxido-reductase, PPO can also regulate the rates of the light reactions and is involved in electron transportation[70,71] and energy transform in the chloroplast.

3 Induction and activation of latent PPO

3.1 Induction of PPO

PPOs can be classified into constitutive and induced enzyme. The former is present in the cells and has activity without being induced by any form of inducing factors[29,73] while the latter is formed and has activity only after being induced. Many inducing factors of PPO have been confirmed, such as infection of pathogen, attack of herbivorous insects and pests, influence of wounding and chemical substance, damage of membrane and even substances released from neighboring wounded or herbivore attacked plants[74,75]. The manners of induction and activation of latent PPO comprise activating PPO gene, enhancing expression or synthesis *de novo*, changing original PPO activity[37] and activating latent PPO enzyme. According to the distribution range of induced PPOs enzymes in plants, induction of PPO is divided into systemic and local induction. The systematically induced PPO is distributed in not only directly the induced parts but also the non-induced parts, and the locally induced PPO is distributed only in the induced parts. For example, in tomato, a *cis*-acting element in promoter responds to wounding and PPO is generated systematically[30,37,73]. However, in the coffee

bean, the PPO is induced only in the wounded branch. Induced PPO is most likely to function as a defensive anti-nutritive protein, and the constitutive PPO may function in the defense against insects[76].

3.2 Activation of latent PPO

In many plants, the PPOs enzymes usually are inactive or latent. They can be activated by a variety of treatments such as salt treatment, wounding, acid and basic shock, urea, exposure to fatty acid, detergents (SDS)[77], proteases, trypsin[40, 78], pancreatin[3], acidic pH[79] and others. Solubilization, interconversion of isozymes, chemical modification like intermolecular disulfide bridge, glycosylation, phenolic glucosides[80, 81], proteolytic activation[30] and the dissociation of an enzyme-inhibitor complex have all been proposed to account for the activation of latent PPO by changing limited conformation. It seems that nearly all inducible factors activate latent PPO through wiping off the shield of extension peptide. And the model of the precursor catechol oxidase from *Ipomoea batatas* may help interpret the activation mechanism of latent PPO. In this model, the C-terminal extension peptide consisting of 32 amino acids may shield the active site and make the precursor peptide of PPO inactive or latent, so, proteases, trypsin, pancreatin may cleave the extension peptide and cancel its shield function, then make the latent PPO active ultimately. The following mechanism[3] is proposed for the proteolytic activation of latent peach PPO by trypsin: The trypsin treatment of PPO generates an active enzyme form with a slightly smaller molecular weight than the native enzyme. Latent peach PPO is activated by the proteolytic action of trypsin, yielding the second product of the proteolytic reaction as active peptide. SDS might also change the orientation of the shield region and enable access of substrate to the active site[42].

pH value can also lead to activation of PPO. This conclusion is previously drawn according to 3 facts: (1) PPO activity varies as the pH changes; (2) PPO activation kinetic characteristic changes with the change of pH; (3) the enzyme activation process by pH is accompanied by changes in the electrophoretic mobility of the enzyme, as well as changes in the Stokes' radius of the protein. Although these 3 facts imply the involvement of conformational changes in the enzyme during activation[82], there is no sufficient direct proof until the percentages of β-turn and random coil of PPO II separated from tobacco leaves by a series of chromatography techniques increase rapidly and the percentages of α-helix and anti-parallel β-sheet of PPO II decrease sharply at higher pH (pH 10.0) compared with those of native PPOII at pH 3[83] on the basis of the data of the FT-IR spectrum, Fourier self-deconvolution (FSD) spectrum and second-derivative spectrum. This phenomenon further provides the direct proof to support the idea that the optimal pH may also activate the latent PPO by changing the PPO secondary structure and cancel the shield effect.

In tomato, a *cis*-acting element in promoter of one of seven PPO genes responds to wounding signal and PPO is generated systematically[30, 37, 73], which implies that wounding can lead to the synthesis of PPO *de novo* and increase of PPO activity. Therefore, the increase in PPO activity in plants after being induced by wounding may be attributed to either the synthesis of PPO *de novo*, or the activation of latent PPO, or both of these two types, as may be judged from the varieties of plants and types of wounding[76]. The hypothesis that wounding can increase the PPO activity by changing the conformation of latent PPO is still not supported by direct proof, and so the related mechanism is unclear.

The processes of separating and purifying sometimes also have the positive effect on the activation of latent PPO in some plants[84]. On the other hand, when the purity of PPO increases the latency degree of PPO becomes higher[82]. This superficial contradictive conclusion can be interpreted as that during extraction and purification, partial latent PPO is activated by some inducible factors, the increase in PPO activity is due to both constitutive PPO and newly activated PPO, but once the inducible factors and constitutive PPO are separated from each other, the latent PPO with high degree of purity indicates more obvious characteristics of latent PPO and stronger typical hysteresis of latent PPO rather than gradual activation of latent PPO.

3.3 Kinetic characterization of latent PPO

Kinetic properties of latent PPO have not been investigated for a long time because of the disturbing phenomenon that both constitutive active PPO and newly activated PPO emerging together during purification have an influence on PPO activity. The inves-

tigation of influence of SDS and pH value on the kinetic behavior of latent PPO, purified from Iceberg lettuce (*Lactuca sativa* L) indicates that kinetic behavior, the latency and the lag phase of latent PPO varies at different steps of the purification process.

For initial extract, the kinetic plot of reaction velocity against concentration of substrate is hyperbolic. Product accumulation curves do not show a lag phase. There is no hysteresis, in the range of lower concentration of substrate. However, while the degree of PPO purity becomes higher, the kinetic plot is sigmoid. Product accumulation curves do gradually show lag phase showing the obvious hysteresis even in the range of lower concentration of substrate. Meanwhile, in a certain range of substrate concentration, PPO affinity to substrate increases with the increasing of substrate concentration after the PPO purity increases to some extent. Substrate can induce slow conformational change of the oligomeric enzyme toward a more active enzyme form with higher affinity for the substrate. We can define this type of conformational change as positive cooperativity, and this change can be favored by acidic pH and SDS[82]. In the defined range of substrate concentration, as the pH value increases, the hysteresis of PPO becomes obvious. Furthermore, conformational change induced by the substrate is more sensitive to change of pH; the substrate would induce the enzyme transition to a catalytically more active form and the $[H^+]$ increment would favor this isomerization. Changes in the concentration of hydrogen ions usually result in modifications in the regulatory properties of allosteric enzymes, especially in their sensitivity to allosteric effectors and in the positive kinetic cooperativity toward allosteric ligands, so that hydrogen ions may be regarded as a particular type of allosteric effector. SDS can affect the cooperativity of the highly purified enzyme. The degree of positive cooperativity decreases or even disappears as SDS concentration increases, suggesting that SDS can also favor the enzyme transition and provoke a conformational change in the enzyme and make the latent PPO active and then it is impossible to observe the characteristic of cooperativity of latent enzyme. At the different step of purification, in the different pH value and/or different concentration of SDS of solution, kinetic behavior of latent PPO is different. Therefore it is necessary for investigators to obtain high purity of PPO and eliminate inducible factors for further study on latency of PPO and inhibition of PPO activity.

4 Some catalysis mechanisms and catalysis theory about PPO

4.1 The mechanism of browning inhibition by L-cysteine

The mechanism of browning inhibition by L-cysteine has been controversial for a long time. There are two main opinions: (1) a colorless conjugate between cysteine and quinone[85] rather than polymerized pigment substance of cysteine, quinones, amino acids and proteins results in the disappearance of browning reaction without negative influence of cysteine on PPO activity; and (2) cysteine may directly inactivate the PPO by combining irreversibly with copper at the active site[86] and resulting in structural modifications of the active site. The second opinion holds true to the fact that cysteine can affect the PPO directly, because SH groups of cysteine have a strong affinity to copper, and possibly displace histidine residues liganded to the copper of the active site of PPO even completely remove the copper from the enzyme[87, 88].

However, the results from the recent experiment[8] suggest that the inhibition of browning reaction is mainly caused by the formation of colorless thiol-conjugated reaction products (Fig. 1) rather than direct enzymatic inhibition of protein. The relevant experiment result about loquat PPO shows that the degree of browning reaction decreases while L-cysteine concentration increasing in a range of lower concentration of cysteine (< 0.3 mmol/L), although the residual substrate chlorogenic acid level was relatively stable regardless of the concentration of L-cysteine. When the concentration of cysteine increases to more than 0.3 mmol/L, the chlorogenate solution does not turn brown, but the 61% of chlorogenic acid is oxidized. On the other hand, chromatographic analysis of solutions shows that many oxidized products are

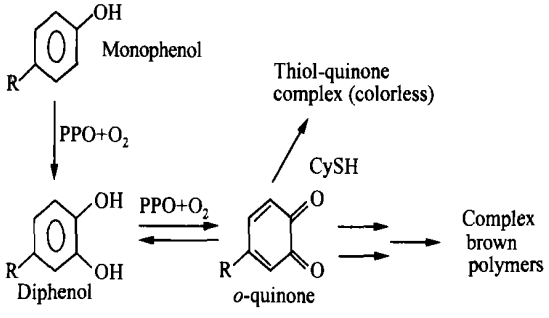

Fig. 1. Possible effect of L-cysteine on the enzymatic oxidation of chlorogenic acid[8].

observed in the absence of cysteine. Therefore the newly formed colorless SH-compound complex between quinone and cysteine avoids browning reaction at least in most plants, rather than the cysteine directly inhibiting toward PPO enzyme.

4.2 Mechanism of suicide inhibition of latent PPO

The polyphenol oxidase in some plants can oxidize the *o*-diphenols to the corresponding *o*-quinones, whereas, in other varieties of plants, the activity of PPO decreases gradually within several minutes, and finally becomes inactive, which is found in the study of the latent polyphenol oxidase from iceberg lettuce[89]. Normally, PPO can catalyze oxidization of phenols to quinines as catalytic enzyme without loss of activity. However, in the presence of some detergent such as SDS, the PPO enzyme can react with the primary substance acting on a substrate, which results in inactivation of PPO enzyme. This phenomenon termed as suicide inhibition takes place in some PPOs enzymes, and the involved substrates are defined as suicide substrates. In a scheme of suicide inhibition, there is a branched mechanism consisting of a catalytic route and an enzyme inactivation route (Fig. 2).

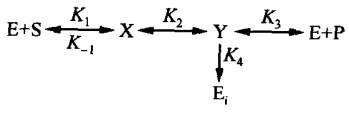

Fig. 2. The simplest reaction mechanism about the kinetic behavior of a suicide substrate of PPO[89]. E, S, X, Y, P and E_i denote enzyme, suicide substrate, first intermediate, second intermediate, product and inactivated enzyme, respectively.

In the browning tissues of many plants, the occurrence of excessive browning reaction in the condition of normal PPO activity can be prevented by keeping the balance between the suicide inhibition and enzymatic browning reaction.

4.3 Kinetic synergism about PPO

The phenomenon termed "kinetic synergism" (Fig. 3)[90], found by Mar in the banana pulp PPO, is one kind of collaboration of oxidation in which one substrate speeds up the oxidation of another substrate, instead of the competitive inhibitor. For example, banana pulp PPO can oxidize SAL to its corresponding SAL-*o*-quinone and furthermore, this oxidation is enhanced by the presence of small amounts of dopamine.

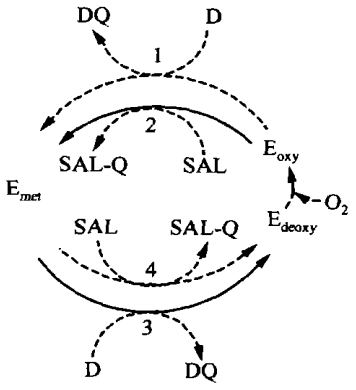

Fig. 3. Model for the kinetic synergism found in the oxidation of dopamine in the presence of SAL by PPO[90]. D, dopamine; DQ, dopaminequinone; SAL, salsolinol; SAL-Q, salsolinol-*o*-quinone; E_{met}, enzyme in met-form; E_{deoxy}, enzyme in deoxy-form; E_{oxy}, enzyme in oxy-form.

Meanwhile, SAL also activates 1.4-fold the oxidation of dopamine to dopaminequinone by acting directly on the PPO catalytic cycle (Fig. 3). Only the oxy- and met-forms of the enzyme are capable of binding the diphenol, whereas the deoxy-form can bind molecular oxygen. These new combinations (step1 plus step4 or step2 plus step3) mean that SAL opens up a faster way for the turnover of dopamine. It is suggested that the browning reaction of banana pulp should be attributed to not only co-oxidation but also a kinetic synergism, both of which might serve as a regulatory mechanism together during postclimacteric stages.

5 Future work

Inhibition of enzymatic browning reaction mediated by PPO has been adopted to increase profits of some crop plants. Although physical control method and chemical inhibitory method can control PPO activity to a certain degree, in some cases, they have their individual disadvantage. Application of antisense RNA and transgenic technique to modify PPO gene expression then becomes more and more important. Theoretical research on location of PPO genes can help with the construction of recombinant gene, which can be used to control PPO activity and is helpful to discover expression discipline of PPO genes and physiological functions of PPO enzymes. However, the initial studies on the location of PPO gene on the chromosome in the plants have just been carried out. Until now, it is only reported that the PPO gene of the recombinant inbred (RI) lines of wheat[91] is mapped on the long arm of the chromosome 2A, and

some genes responsible for high PPO activity in durum wheat are also located on chromosome 2A[92]. Therefore, in the future, the studies of location of PPO gene on the chromosome and its regulatory genes will be more and more important events.

Furthermore, practical application of PPO is implemented as a result of the more complete understanding in theory. For example, in the course of the industry production, laccases occurring widely in fungi have been used to delignify woody tissues, clarify wine, oxidize alkenes, produce ethanol, and distinguish drugs. Meanwhile, in the environment protection, laccases have been used in elimination of industrial wastes, decoloration of dyes, degradation of herbicide and biomediation[19]. The exploitation of high sensitivity biosensor made of PPOs in higher plants is getting more and more interesting. For example, the biosensor depending on the affinity of mushroom PPO toward gentisic acid has been exploited[93] and the amperometric bioelectrode for the quantification of phenols and catechols basing on the incorporation of iridium microparticles and PPO within carbon paste matrices will be useful in clinical, pharmacological and environmental area[94]. In one word, the practical application of PPOs in higher plants will be expected to explore continuously in the future.

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