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Properties and Applications of Plant Peroxidases

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Abstract

Peroxidases are proteins that contain heme and are widely active in plants, microorganisms, and animals. In this review study on plant peroxidases, in addition to introducing them, how to measure the activity of the enzyme, checking the number of isozymes, and tracking the structural changes of the enzyme have also been examined. This two-substrate enzyme, which converts hydrogen peroxide into water, oxidizes many organic and inorganic substrates during its catalysis, all of which can be used to measure enzyme activity. However, its specific substrate is still hydrogen peroxide. The presence of calcium and at least four disulfide bonds in the structure of peroxidases have been proven to help the formation and strength of the three-dimensional structure of the molecule. Peroxidases of plants have several roles, including involvement in lignin biosynthesis, auxin metabolism, cell growth, creation of transverse connections of the cell wall, and especially response to environmental stresses. It has been proven that in all physiological processes and phenological changes of the plant, hydrogen peroxide is produced in tissues, and peroxidase catalase cleans the cell from this toxic substance in two different ways. Therefore, peroxidases are considered a good option to follow the path of the cell's confrontation with stress-causing factors and to face situations such as oxidative stress. Due to the advances in biological sciences and the production of pure samples of peroxidases, this molecule is also used for ligand-protein studies in pharmaceutical research.

Keywords: Peroxidases, Plant peroxidases, Enzyme, Oxidative stress

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Introduction

The large peroxidase family (EC:1.11.1.7) that exists in plants, animals, and microorganisms is a set of enzymes that oxidize various organic and inorganic compounds by consuming hydrogen peroxide (Conesa *et al*., 2002; Ahmed *et al*., 2022; Al Hamazani *et al*., 2022; Kumbhar *et al*., 2024). Louis-Anthony Planche first introduced this enzyme in the early 19th century; however, it was named peroxidase in the late 19th century, and scientists such as Robert Cudat, Nikolaevich Bach, and David Collin from the beginning of the 20th century, began extensive studies on the biochemical properties of peroxidase using plant peroxidases (Lewis & Yamamoto, 1990; Zagade *et al*., 2022). In this way, the first peroxidase to be obtained in pure form was horseradish peroxidase, which was purified in 1982 and gradually paved the way for structural studies on peroxidases (Silva *et al*., 2024). During about 25 years of study (1920-1945), the presence of a carbohydrate molecule in the structure of horseradish peroxidase was reported, and shortly thereafter, the reaction was analyzed using hydrogen peroxide. Then, peroxidases, including plant peroxidases, were among the important enzymes in biochemical, physiological, and pharmaceutical studies (Hiraga *et al*., 2001; Akhtanin *et al*., 2022; Al Hussian *et al*., 2022; Ewuzie *et al*., 2024). The superfamily of plant peroxidases, which is the second superfamily of the three groups defined by Wallinder in 1992 (this scientist divided peroxidase into three superfamilies of animal peroxidases, plant peroxidases, and catalases), includes three general classes. Class I: prokaryotic peroxidases, the most important member of which is ascorbate peroxidase. Yeast and mitochondrial cytochrome C peroxidase is also included in this group. The similarity of the first protein structure in this group is high and there are no disulfur, calcium, and carbohydrate bridges in their structure; Class II: fungal peroxidases, from this group lignin peroxidase and manganese peroxidase are well identified. These enzymes have two calcium ions, four protected disulfide bridges, and carbohydrates in their structure (Alic *et al*., 1997; Al Neimat *et al*., 2022; Zreiq *et al*., 2022); Class III: classical plant peroxidases, which are mostly secreted, and in terms of the first structure of the protein, there is at least 50 and at most 95% structural similarity between them. This family includes four protected disulfide bridges, two calcium ions, and between 10-25% carbohydrates. In addition, all peroxidases of this large group have histidine and arginine in the distal region in the protected amino acid and a protected histidine in the proximal region (Gajhede *et al*., 1997; Abd Elmeged & Alshehri, 2021; Al-Lbban, 2022). Classes I and III have a common bacterial ancestor, in class 1, its bacterial sequence is preserved in all respects, but in class III, it is

only superficially similar to this common ancestor (Schuller *et al*., 1996; Al-Jaloud *et al*., 2022).

Building and Sustainability

Yeast cytochrome c peroxidase was the first three-dimensional structure of peroxidases, which was obtained about twenty-five years ago with the help of X-rays (Poulos *et al*., 1980). The presence of a large amount of carbohydrates in this molecule made it difficult to carry out structural studies on it. Recently, using a recombinant method, non-glycosylated horseradish peroxidase C has been obtained, which has provided significant progress in the structural studies of peroxidases. In this way, we know that classical plant peroxidases in class III have a single polypeptide chain that contains at least 300 amino acids and there is a group as a prosthetic root in them. Two structural calcium atoms and four disulfide bridges between four pairs of cysteines and one aspartate-arginine salt bridge can be seen in the structure, and most plant peroxidases have many N-glycosylated groups that make to 25% of the structure (Smith *et al*., 1990). The presence of calcium binding sites and disulfide bridges ensures the stability of the enzyme, and the loss or binding of calcium affects the active site of the enzyme due to its role in positioning. Finally, peroxidase activity is dependent on the presence of calcium or a similar ion in terms of ionic radius. Separation of calcium due to the removal of the protein from its natural folds causes the thermal stability of the enzyme to decrease. However, the presence of carbohydrates in the structure does not have much effect on the thermodynamic stability and it mostly provides the kinetic stability of the enzyme (Tams & Welinder, 1998). In higher organisms, there is usually more than one isoform of peroxidase, which is generally different in catalytic and structural terms. For example, 25 isozymes are known in rice and 12 in tobacco, and of course, most of the diversity is related to plant class III peroxidases. However, an independent and specific biological role for each of these isoforms has not yet been defined. Some of these isozymes are more expressed in a specific tissue and others are more expressed in a certain stage of the plant's growth path. In addition, environmental stresses play a role in the more or less expression of a specific isozyme. The presence of all these isoforms of peroxidase and the diversity of expression indicates their presence in a wide range of physiological processes in a living system (Gaspar *et al*., 1985; Manjunatha *et al*., 2003).

Mechanism of Action

After many studies on the mechanism of action of peroxidases, scientists finally proposed a single mechanism for all peroxidases (Welinder & Gajhede, 1993; Smith, 2001). In the first step of this pathway, hydrogen peroxide binds to iron in heme as the sixth ligand. In this step, the peroxide is oxidized to two electrons, and thus the first intermediate compound is formed (Sivaraja *et al*., 1989). In this compound, first, an electron is separated from the ferric present in heme, and then by receiving an oxygen atom separated from the peroxide, a center is formed. The second electron is sometimes provided from an amino acid (in cytochrome peroxidase or from porphyrin in horseradish peroxidase) (Veitch, 2004). The first intermediate compound is reduced by one electron in two steps. In this way, the reducing substrate is transformed into a radical form by donating an electron to an amino acid or porphyrin (at this stage, the second intermediate compound is formed). At the end of the catalytic cycle, with the second stage of reduction, the single electron is completed. In this way, first, a molecule is transformed into a radical by transferring an electron to the second intermediate compound, finally, the second intermediate compound opens to the resting state, which is the form before the reaction starts (Hayashi & Yamazaki, 1979). Active site amino acids that help in substrate binding to this site are constant or highly conserved among plant peroxidases. A wide variety of compounds, including aromatic amines, indoles, phenols, and phenic acids, are oxidized by peroxidases and eventually, dimers or oligomers are formed, which can sometimes be oxidized again by peroxidases (Veitch, 2004). Usually, peroxidases have a prosthetic group in their active site, which is generally coordinated to a histidine amino acid as a proximal (front) ligand in the fifth iron position. The length of the 5-coordinate bond of iron depends on the increase and decrease of the oxidation and reduction potential of different peroxidases. For example, the weaker the bond, the more alkaline the imidazole group will be, which ensures the stability of intermediate compound 1 and increases its oxidation and reduction potential (Hayashi & Yamazaki, 1979; Banci *et al*., 1991). Through its proton, it establishes 247 hydrogen bonds with the side chain carboxylate in aspartate, and this bond the playing power of the imidazole ring has a positive effect. In the distal region, the residues of arginine 38, histidine 42, and asparagine 70 are important. The side chain nitrogen of histidine 42 is located at a further distance from iron compared to the proximal histidine, and in this way, the sixth coordination position remains open for molecules such as cyanide, azide, and fluoride, which bind in the form of protons. In addition, there is a hydrogen bond between the carbonyl oxygen in the side chain of asparagine 70 and the distal histidine proton, which is necessary for the stability of the active site (Valderrama *et al*., 2002). These cases are true for most plant peroxidases, and only rare cases of difference have been reported, one of which can be mentioned: The orientation of phenylalanine 41 and histidine 42 residues in the distal position of peanut and soybean peroxidase is slightly has changed and this change is due to the replacement of a bulky isoleucine residue in peanut and soybean peroxidase instead of alanine 174 (Candeias *et al*., 1997).

Reaction and Role of Peroxidases

The reaction catalyzed by peroxidases is mainly oxidative dehydrogenation, but in addition to oxygen transfer, hydrogen peroxide destruction is also performed (Valderrama *et al*., 2002). Kinetically, the Km of peroxidases is usually an unsaturation constant. Therefore, their Km is usually large and the enzyme-Swistra complex is weak. It used to be said that the rate of peroxidase reaction increases depending on how easily the substrate is oxidized, but today it is known that the rate of peroxidase catalysis depends only on the regeneration potential of the radical substrate (Job & Dunford, 1976; Candeias *et al*., 1997). Peroxidases can participate in a wide range of physiological processes due to their participation in both peroxidative and hydroxylic cycles. In addition, the diversity of peroxidase substrates and the production of substances such as lignin and suberin or auxin catabolism is a decisive reason for the non-negligible function of this enzyme in the secondary metabolism of plant cells and fungi (Scialabba *et al*., 2002). In addition,

peroxidases are also effective in cell elongation and defense against pathogens, and in general, the set of functions of peroxidases leads to physiological and developmental changes in the plant during its growth path from germination to senescence, which includes the following:

Cell Growth and Destruction of the Cell Wall

Peroxidases directly participate in the cell growth process by controlling the amount of hydrogen peroxide inside the cell by peroxidative and hydroxylic cycles. The HO radical produced by peroxidases can cleave cell wall polysaccharides such as pectin and xyloglucan (Cosgrove, 2001).

Making Transverse Connections in the Cell Wall

Peroxidases can control the amount of hydrogen peroxide in the cell wall, which is necessary for the cross-linking of phenolic groups, and with this work, they play a role in cell elongation (Welinder, 1979).

Germination of Oxygen-Free Radicals (ROS)

Germination of free oxygen radicals (ROS) plays a role in the protection of seeds against pathogens and the expression of the hyperoxidase gene starts with germination. The production of HO radicals in this stage has a protective role (Morohashi & Matsushima, 2000).

Formation of Lignin and Suberin

Oxidative coupling of lignin subunits as a part of lignin biosynthesis is accompanied by the formation of cell walls and the reduction of cell growth. By using hydrogen peroxide as an oxidant, peroxidases produce phenoxy, which spontaneously combine to form lignin polymers. On the other hand, the crosslinking of phenolic monomers produces suberin (Alexander & Grierson, 2002).

Growth and Ripening of Fruits

Fruit growth is associated with cell enlargement, and cell enlargement is completely dependent on the loss of the cell wall. Therefore, the role of peroxidases in this process is completely dependent on the balance that these enzymes can establish in the loosening and tightening of the plant cell wall (Alexander & Grierson, 2002).

Response to Tensions

The production of oxygen free radicals (ROS) is a natural occurrence in the cell, which is generally the result of normal metabolic pathways, including respiration and oxidation of molecules. But the amount of production of these compounds increases under environmental stress, these free radicals which include superoxide radicals, hydroxyl radicals plus oxygen derivatives with unequal electrons (such as singlet oxygen and hydrogen peroxide) are the most important factors leading to oxidative stress. are considered to have irreparable destructive effects on cell structure and components and are considered to be one of the main factors in inducing programmed cell death by apoptosis (Khavari-Nejad *et al*., 2007; Khavari-Nejad *et al*., 2016). Therefore, a set of non-enzymatic and enzymatic antioxidants such as superoxide dismutases, catalases (Khavari-Nejad *et al*., 2007; Khavari-Nejad *et al*., 2016), and peroxidases (Khavari-Nejad, 2015) start to remove oxygen free radicals in the cell and try to save the cell from entering the apoptotic pathway. In addition, peroxidases prevent the penetration of these factors by producing strong physical barriers such as the production of lignin and suberin, and glycoproteins rich in hydroxyproline (Bowles, 1990).

Application of Peroxidases

Due to the ability to transfer electrons from an electron-donating substrate to an accepting molecule, peroxidases are used in a variety of industries (An *et al*., 2002). In addition, in recent decades, for the preparation of electrochemical biosensors, electrodes based on peroxidases have been produced, which have wide applications for determining hydrogen peroxide and organic hydroperoxides in analytical systems (Jia *et al*., 2002). However, what has recently caused more attention to these enzymes is the theory of inducing apoptosis through direct damage to vital cell enzymes (Hadizadeh *et al*., 2009). In addition, the recombinant horseradish peroxidase C, which does not have bulky carbohydrate parts and whose structure and function are well known, has proposed peroxidases as a suitable model for investigating ligand-protein binding in the path of pharmaceutical studies, which previously was done with the help of albumin (Keyhani *et al*., 2003; Khavari-Nejad *et al*., 2016).

Common Laboratory Methods for Studying the Function and Structure of Activity Assays and Determining the Number of Isoenzymes

Peroxidase enzyme is a two-substrate enzyme that uses hydrogen peroxide and a reducing substrate for enzyme catalysis. According to this article, usually the concentration of one of the two substrates according to the laws of Michaelis and Menten is considered so high that the reaction is independent of it and the activity of the enzyme increases depending on the concentration of the other substrate. In most references, the conditions that the researcher designs for the enzyme are called false single substrate (Tayefi-Nasrabadi *et al*., 2006). One of the substrates is hydrogen peroxide, which is usually prepared daily in the laboratory, and the reducing substrate is usually odianizidine (Khavari-Nejad & Attar, 2015). In the case of odianizidine, the enzyme assay is performed at a wavelength of 460 nm, and for guaiac, the assay is usually performed at 470 nm (Suchy & Jurkowski, 2024). Considering that several isoenzymes of peroxidase are expressed in different stages of growth in different organs of the plant and with different environmental conditions in the cell, usually, to determine the isoenzymes present in the cell, a gradient sample of Hp is considered as Hp profile. Usually, this gradient is provided with the help of 0.1 M citrate-phosphate-borate, which tolerates a wide range of Hp between 3 and 12. By measuring the activity of the enzyme in this range of Hp, the optimal activity level is determined, and the obtained peaks represent the isoenzymes of peroxidase in the sample cell (Babakhani *et al*., 2014). Peroxidase kinetic parameters such as Km and Vmax can be calculated at this stage (Graefen *et al*., 2024; Sahu *et al*., 2024; Shrestha *et al*., 2024).

Gel Electrophoresis

It is possible to confirm the number of peroxidase isoenzymes or increase and decrease the amount of enzyme in the sample cell with the help of gel electrophoresis. Usually, natural polyacrylamide gel without SDS (non-denaturant) and specific staining, specific for peroxidase enzyme activity, are used for this test. The use of hydrogen peroxide and odianizidine in this staining method reveals the peroxidase bands specifically on the gel (Paknia, 2005). On the other hand, if the project is defined with pure enzyme, agarose gel, and Coomassie blue staining can be used to determine peroxidase bands.

Studying Structural Changes of Peroxidases Using Visible-Ultraviolet Spectrophotometry

Peroxidase is a hemoprotein containing a prosthetic root heme, which makes it sensitive to changes in the surroundings heme. Therefore, changes in the surrounding environment also cause changes in the electron absorption of the enzyme. The electron absorption spectrum for peroxidase has been reported in Hp 4 and 7 and at a distance of 400-700 nm, which are very similar to heme (Kelly & Price, 2000). This spectrum includes a sort band (related to hem) at 403 nanometers, a beta band at 500 nm, and a band called TC at 642 nm. The Soert band is very sensitive to the structural changes of the heme envelope so any change in the surrounding heme envelope affects the intensity of the Soert band and the displacement of its absorption maximum. As a result, this area is an acceptable indicator for examining the surrounding structure (Feng *et al*., 2024).The TC band that appears at a wavelength above 600 nm is known as the charge transfer band from the porphyrin ring to iron heme. The shift of this band to the left and right (wavelengths less than or greater than 642 nm) indicates the effects of hydrogen bonding (formation or breaking of a hydrogen bond). So if the ligand attached to iron heme is a hydrogen bond donor, due to the strength of the bond and more charge transfer to the iron atom, the CT band appears at a lower wavelength, and if the ligand attached to iron heme is a hydrogen bond acceptor Therefore, this band appears at a higher wavelength. Additionally, if the ironbound ligand is a hydrogen bond acceptor, when this hydrogen bond is broken, a shift to the shorter wavelength region occurs. In addition to the information mentioned above, the determination of the kinetic parameter K_d or the apparent dissociation constant for the ligand-protein complex can be calculated using the data of this technique for peroxidases (Khavari-Nejad & Attar, 2015).

Examining Structural Changes of Peroxidases Using Fluorescence Spectroscopy

Peroxidases have inherent fluorescence due to having tyrosine and tryptophan residues in their structure. The intensity of this intrinsic fluorescence can be measured in the emission distance of 300 to 600 nm at two excitation wavelengths of 280 nm (related to hydrogen and tryptophan) and 295 nm (specific to tryptophan) (Tayefi-Nasrabadi *et al*., 2006; Khavari-Nejad & Attar, 2015). In this way, the emission spectrum of peroxidase gives the required information about the structural changes of the molecule under environmental stress conditions and during ligand-protein binding. On the other hand, many structural differences between peroxidase isoenzymes in the same tissue can be traced in this way. Determining the type of fluorescence quenching by the Stern-Volmer method, which determines the quenching of the fluorophore near the quenching molecule, is one of the important parameters that can be measured in ligand-protein projects for peroxidases, which can be achieved by this method.

Peroxidases and Rotational Telescoping

For peroxidases, like many proteins, it is possible to study the second structure using two rotational spectra in the distance from 190 to 260 nm (which is the absorption region of the peptide bond). Estimating the percentage of each component of the second protein structure in peroxidase, in addition to determining the differences and similarities of isozymes, is also used during their purification to estimate the purity of the sample and study the structural details of the protein (Kelly & Price, 2000).

Conclusion

In general, antioxidants are molecules that, by transferring electrons to free radicals, convert them into their stable form, and in this way, they are considered to provide cell survival. On the other hand, the production of free radicals, especially oxygen free radicals, is an inevitable and natural issue in the metabolic pathways of the cell, but the effect of environmental stress on growth and development, the structure of proteins, respiration, and cell metabolism causes an increase in reactive oxygen species. Finally, it becomes oxidative stress. Damage to the cell membrane, changes in the structure and function of vital proteins, DNA damage, and finally the initiation of programmed cell death are the events that occur in the continuation of oxidative stress in the cell. The first defensive reaction of the cell against oxidative stress is to increase the amount of antioxidative activities and antioxidants, which ultimately lead to an increase in the plant's tolerance to environmental stress. Meanwhile, the big family of peroxidases generally catalyze the oxidation and reduction reaction between hydrogen peroxide as an electron acceptor, and various types of phenolic substances, ascorbic acid, and aromatic amines. The conversion of hydrogen peroxide into water molecules by peroxidase prevents the production of oxygen free radicals and is considered the first defense barrier of the cell against oxidative stress. Fields of studying peroxidases as the first defense barrier of the cell against oxidative stress imposed by environmental factors, in the growth path of very diverse plants and due to the presence of pure and well-known examples such as horseradish peroxidase in the field of changing protein structure and function is Common. The second case, which is mainly due to Smith's success in producing the recombinant type of this enzyme in 1995, especially brought the boundaries of plant science and cellular and molecular science closer to each other and connected both of them to the realm of pharmaceutical science.

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