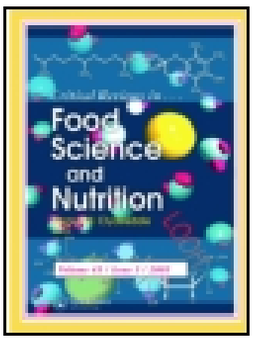

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Lea C. Toledo & Carolina C. Aguirre (2016): Enzymatic browning in avocado (Persea americana) revisited: History, advances and future perspectives, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2016.1175416

To link to this article: http://dx.doi.org/10.1080/10408398.2016.1175416

Accepted author version posted online: 12 May 2016.
Published online: 12 May 2016.

Enzymatic browning in avocado (*Persea americana*) revisited: History, advances and future perspectives

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Abstract

Considering nearly 80 years of research regarding one of the enzymes responsible for catalysing the formation of pigments in higher animals, plants, fungi and bacteria, this review will focus on collecting and categorising the existing information about polyphenol oxidase (PPO) in fruits, with particular emphasis on the information in relation to avocado, which is one of the hardiest species in terms of inactivation, has documented dual activity (EC 1.14.18.1/EC 1.10.3.1), and represents one of the oldest challenges for food science research and fruit processors.

It is expected that this review will contribute to the further development of the field by highlighting the questions that have arisen during the characterisation of PPO, the progress that has been made and the questions that remain today, in addition to new methodologies that are being applied to study this system. Holistic methodologies offer unexplored potential for advancing our understanding of the complex phenomena that govern PPO activity in fruits, because these methodologies will enable the characterisation of this family of enzymes in all of its complexity. Subsequently, it will be possible to develop better techniques for controlling enzymatic browning in this valuable fruit.

Keywords

Avocado, enzymatic browning, polyphenol oxidase, *Persea americana*.

Introduction

Polyphenol oxidases (PPOs), which are also called polyphenolases or phenolases (Knapp, 1965), are a large family of enzymes present in higher animals, plants, fungi and bacteria whose main characteristic is the presence of an active site with two copper atoms. They are capable of catalysing the oxidation of monophenols and polyphenols in the presence of molecular oxygen, leading to the formation of coloured compounds called *o*-quinones, which are subsequently non-enzymatically polymerised producing melanin (Weemaes et al., 1999). In mammals, the function of melanin is to protect against harmful solar radiation (Sugumaran, 2002), and melanin deficiency is associated with the disease called albinism (Oetting and King, 1999). In plants and fungi, PPO enzyme activity seems to be linked to mechanisms of defence against harmful agents (Felton et al., 1989), although its function remains enigmatic (Mayer, 2006).

One of the most interesting behaviours of this enzyme in some plant species is its dual activity. The International Union of Biology and Molecular Biology (IUBMB) has designated its tyrosinase (Tyr) activity (monohydroxylase or phenolase and combined oxidase activity) as EC 1.14.18.1. This activity is responsible for hydroxylation of phenols to form *o*-diphenols, which are not released from the active site and are oxidised to form the final product of the coupled reaction, the *o*-quinone molecule, in a subsequent reaction. The other activity linked to the PPO family is termed catechol-oxidase or catecholase (EC 1.10.3.1), hereafter Cat, which, unlike Tyr, can only catalyse the second step of the Tyr reaction. Cat uses *o*-diphenols as substrate, and they are converted into *o*-quinone (Figure 1). Multiple explanations for this phenomenon have been proposed for different species (Rodriguez-López et al., 1992; Sánchez-Ferrer et al., 1995), and

researchers have realised the complexity of the system and emphasised the many causes of the enzymatic browning phenomenon.

One of the first challenges that the establishment of a comprehensive mechanism for this family of enzymes faces is the difference between those species that only exhibit Cat activity and those in which dual activity is detected. It has been established that in those species in which both activities are presented, the Tyr:Cat ratio is approximately 1:10 to 1:40 (Vámos-Vigyázó, 1981). Another interesting fact is added to this difficult: in those species in which Tyr activity is detected, it has been observed that the lag phase of the hydroxylation reaction is removed in the presence of the reaction product (L-DOPA in the case of tyrosine as substrate); this issue has been addressed for different plant species in the detailed review of Yoruk (Yoruk and Marshall, 2003). Last but not least, is the great variability of this enzyme amongst species in terms of sequence, substrate selectivity and catalytic capabilities (Jaenicke and Decker, 2003; Malviya et al., 2011). It has been suggested that the enzyme could even be coded in different parts in distinct plants (Shaw and Chao, 1991) by diverse genes and appear at disparate times (Gooding et al., 2001).

It is also proposed that the variability may be due to different states of the protein in the cell. One, called the active form (Robinson and Dry, 1992; Steffens and Harel, 1994), can be induced by the use of activating agents, such as the ionic detergent sodium dodecyl sulfate (SDS) (Jiménez and García-Carmona, 1996), or in response to the presence of endogenous proteases. It has also been suggested that these variations could be due to differences in the conditions for determining the activity of Cat and Tyr (Espín et al., 1998; Espin et al., 1995), among many other factors, depending on the species used in studies conducted *in vitro*. Therefore, the

relationship of Cat and Tyr with the *in vivo* mechanisms of different species remains unclear (Yoruk and Marshall, 2003).

In conformational terms, the crystallographic structures of the PPO of bacteria (*Agaricus bisporus*) (Matoba et al., 2006), and three plant PPO (*Iponaeba batata*, *Juglans regia* and *Vitis vinifera*) (Klabunde et al., 1998; Virador et al., 2010; Zekiri et al., 2014) have been characterised, and homology studies have been performed. One such study determined that the sequences of 47 PPO species (from animals, plants, fungi and bacteria) indicated that the large differences amongst PPO species make clustering impossible (Malviya et al., 2011). However, it should be noted that all enzymes that belong to this family have a common characteristic, the high sequence homology at their catalytic site (Decker et al., 2006), thereby, denoting a common evolutionary ancestor; the relevant details will be discussed in a later section.

In food science, the activity of this enzyme is useful in products in which colour is desirable, such as tea, coffee and cocoa (Mazzafera and Robinson, 2000); however, it is undesirable in other vegetable products because pigment formation is associated with nutritional losses linked to damage to DNA, proteins, amino acids or lipids by free radicals generated during the reduction of the *o*-quinone molecules in the chain of redox reactions (Felton et al., 1992). *o*-quinones are highly reactive compounds that are able to react with proteins through interactions with side chains of amino acids that have -SH and -NH_2 groups, thereby decreasing their bioavailability (Matheis and Whitaker, 1984). In addition to nutritional losses, consumer perceives the presence of browning as an indicator of poor quality or not fresh products (Mayer and Harel, 1979). In summary, in some products, enzymatic browning reactions produce damage that invalidates the invested efforts along the production chain, thus wasting money, time and

valuable natural resources, which in the case of the avocado, according to global statistics from the World Organisation for Food and Agriculture, the worldwide avocado production present a systematically increased since 1993 to reach 4,717,102 million tons in 2013, with 69.3% of the production occurring only in America (FAO).

Many technological efforts for controlling PPO in avocados have been published since 1937 (Samisch, 1937). These works have focused on PPOs characterisation and controlling the phenomenon of enzymatic browning, because it has been difficult to achieve lasting inhibition over time, which is necessary to preserve the freshness and quality of the pulp, because this fruit contains particularly active PPOs that are resistant to common inhibition treatments (Weemaes et al., 1998; Vanini, Kwiatkowski and Clemente, 2010).

Progress towards a targeted solution indicates that a detailed understanding of the phenomena involved in catalysis is the only solution. Thus, the present review, in addition to gathering the various sources of information regarding PPOs in avocados, identifies new fields of research that could help to define many of the questions that remain about the mechanism and explain the dominance of EC 1.14.18.1, relative to EC 1.10.3.1, and the PPOs' high resistance to inhibition treatments, while recognising the implications that this information might have for other fruits or related systems.

History of its characterisation

One of the first attempts to characterise the enzyme responsible for avocado browning was documented by Samisch in 1937. Working with Spinx variety, Samisch concluded that this PPO is able to oxidise monophenols and that the presence of oxygen, apparently, limits the reaction. Samisch questioned whether the observed differences amongst the PPOs of different carrot

varieties are related to enzymes or the environment, among other questions (Samisch, 1937). In 1965, Knapp characterised the PPO content of the Lula avocado (Knapp, 1965). However, it is possible that another variety (a Guatemalan-West Indian hybrid fruit) was used because at that time, it was unknown that PPOs of different varieties had different behaviours, as reported by the same author five years later (Dizik and Knapp, 1970). Since 1949 it has been clear that plant PPOs correspond to metalloproteins with copper in their active sites and are capable of transporting and participating in the metabolism of molecular oxygen (Arnon, 1949). Later, thanks to advances in spectroscopy, these copper metalloproteins were classified into three large families. The first one is the Type I family, or blue copper centre, which contain copper atoms at their active sites and are responsible for electron transfer. The Type II family, including dopamine-β-monooxygenase among others, do not have a blue copper centre and exhibit oxidase and galactose oxidase activities. Members of the Type III family contain two copper atoms at their active sites, and their functions are oxygenation/oxidation and transport of molecular oxygen. The PPO family is found within this last group, which is composed in turn by hemocyanins (Hcn) and the enzymes with Tyr and Cat activity (Solomon et al., 1996; Gerdemann et al., 2002). Recently, a fourth member was added. This enzyme was detected in *Streptomyces murayamaensis* and is capable of hydroxylating *o*-aminophenols, leading to the formation of *o*-iminoquinone, but nevertheless is unable to hydroxylate monophenols, unlike the enzymes with Tyr activity (Noguchi et al., 2010).

Obtaining the crystal structures of enzymes with Tyr and Cat activity has been a significant step in the elucidation of their enigmatic behaviour. Comparative studies of these structures have demonstrated that both activities are catalysed by the same structure; nevertheless,

conformational changes induced by the environment (substrates, products and co-factors) can apparently favour one activity over another, under a dynamic projection of the mechanism.

In 1975, the EC 1.10.3.1 activity was evaluated in three varieties of avocado (Fuerte, Horishim and Lerman); the results indicated that Fuerte activity was significantly greater than Horishim and Lerman activities, and also that PPO activity was directly related to the level of browning (Kahn, 1975). However, the possible association between hydroxylase activity and Tyr was ignored. In 1983, it was explicitly stated that the PPO activity in avocado is neither EC 1.10.3.1 nor EC 1.14.18.1 (Van Lelyveld et al., 1984). The latest report regarding avocado PPO activities indicates that Hass variety exhibits both activities, Tyr and Cat, thereby highlighting that it is necessary to use Triton X-114 and a nucleophilic trailer molecule, such as 3-methyl-2-benzothiazolinona (MBTH) during the procedure (Espín et al., 1997).

The fact that previous studies have not been able to define both activities, is mainly attributed to the Tyr hydroxylase activity being modified during the usual purification procedures (Walter and Purcell, 1980). Nicolas et al. provided evidence that the proportion of activity hydroxylase:oxidase is 1:10 to 1:40 when both activities coexist in plants (Nicolas et al., 1994). To these facts is added that during the reaction of hydroxylation (the first step of the activity Tyr), and according to the previously presented general mechanism (Figure 1), the *o*-diphenol remains attached to the active site, making it difficult to detect with standard colorimetric methods.

Based on this information, the history of the characterisation of PPO enzyme system in avocado continues with attempts to control browning reaction using various methods, which are reviewed in a later section.

Role of PPOs

Discussing the role of PPOs in avocados necessarily involves reviewing what conclusions have been reached in studies of their roles in plants and other fruits. There are multiple interesting reviews (Yoruk and Marshall, 2003; Constabel and Barbehenn, 2008); therefore, this section aims to provide only general guidance and consider what is currently unknown even though relevant studies regarding avocado are scarce.

Regarding the Fuerte variety, it was reported that PPO was located in the intracellular tissue, demonstrating its presence in the tilakoides plastids in normal tissues (Engelbrecht, 1982). In other fruits, it has been reported to be anchored to cell membranes, which has been associated with the latent form of the enzyme, as opposed to its postulated active form, which would be in solution (Mayer and Harel, 1979). If it is further considered that the location of the substrates is in the cell vacuoles, this would indicate that the access of the enzyme to its substrate appears to be limited to situations of damage or cell disruption in the system (Constabel and Barbehenn, 2008).

One of the main roles attributed to the presence of PPO in fruits is protection against parasites (Li and Steffens, 2002). Different studies have pointed out that cell damage triggers a signal that indicates attack by parasites and induces the formation of methyl-jasmonate, a molecule that activates PPOs and also seems to promote their synthesis at mRNA level, the latter having been evaluated in tomato (Constabel et al., 1995). In other studies, it has been suggested that there is a correlation between increases in the activity of the enzyme and decreases in pest attack, but the mechanism that blocks pest activity in the presence of PPOs remains unclear (Li and Steffens, 2002). This raises the following question about the defensive role of PPOs: does an external

agent trigger overexpression of the enzyme, or is there some property of the species itself that promotes or inhibits the activity (Mayer, 2006)?

This theory remains a subject of active study, because of the major differences that are evident amongst the PPO family and the lack of informatio of its effect on pests (intake and metabolism of *o*-quinone by parasites).

Isoforms and related proteins

The presence of isoforms of PPO in fruits has been documented since 1965 (Robb et al., 1965), being first reported in a Guatemala-West Indian hybrid avocado variety. In this study, five isoenzymes of varying molecular weight, ranging from 14 to 112 kDa, were identified. Their activity was evaluated by considering the detection of the products of Cat activity, and it was proposed that the fraction with the lowest response time at 420 nm, was that responsible for browning in the fruit (Dizik and Knapp, 1970). This study also highlights the fact that different isoforms appeared to correspond to multiples of the molecular weight of a basic unit, it was additionally suggested that this behaviour may be associated with a high concentration of enzymes in a polymeric state that increases in the ionic strength of the solution (due to the presence of dodecyl disulfate sodium, heat and/or ethylenediaminetetraacetic acid (EDTA)) could dissociate. In 1975, a new study of the varieties Fuerte, Horishim and Lerman detected six active isoenzymes with differentiated activity that depended on the substrate (Kahn, 1975). Subsequently, Van Lelyveld (Van Lelyveld et al., 1984), studying the Fuerte variety, detected five fractions with Cat activity.

Multiple isoforms are not only an issue regarding the PPOs in avocados but rather seem to be a common feature among fruits and vegetables, having also been documented in apple (Barrett et

al.,1991), banana (Oba et al., 1992), grape (Harel et al., 1973), kiwi (Park and Luh, 1985), lettuce (Heimdal et al., 1994), mushroom (Constantinides and Bedford, 1967), potato (Constantinides and Bedford, 1967) and spinach (Angleton and Flurkey, 1984) using a electrophoretic mobility-based method. Various proposals to explain the diversity of forms in the PPOs of the same species have been presented, among which are counted isoforms that are associated with different tissues in the fruit (Shaw and Chao, 1991), isoforms that depend on the ripeness of the fruit (Gooding and Bird, 2001), and environmental agents that could affect transcription of the same gene. Other research regarding isoforms in fruits suggests that this phenomenon corresponds to the association and/or dissociation of a single molecule anchored to another cellular structure (e.g., membranes); upon its release, a number of structural changes could occur in the enzyme (Meyer and Biehl, 1980), which would lead to the active conformation (Tolbert, 1973). The detection of different forms has also been suggested to be due to different extraction procedures, which may favour the presence of a specific form, in addition to their association or interconversion (Harel and Mayer, 1968).

Other studies suggest that the variability is due to post-translational effects, which could explain why in some PPOs, binding to carbohydrates has been detected (Raffert and Flurkey, 1995). Transcriptional mutations are another possible explanation because protease activity has been observed in some Type III enzymes (Lieberei et al., 1981). In this regard, a recent study identified two enzymes with the same genetic origin as Tyr in mammals that led to the appearance of the tyrosinase-related proteins, hereinafter TyrRps, identified as TyrRp1 and the TyrRp2 (Jackson, 1994). TyrRp1 DHICA causes *in vitro* oxidisation of the corresponding quinone. TyrRp2, which is also called Dct dopachrome tautomerase, and catalyses the

rearrangement of dopachrome to DHICA without the step of decarboxylation, hence the name Dopachrome tautomerase (Dct) (Jiménez-Cervantes et al., 1994). These enzymes also exhibit different behaviour depending on the species; variations between humans and mice have been detected (Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994). These new species represent an open field of research amongst this large family of enzymes.

These results leave open the following questions for future research: with what does the presence of polymers with enzymatic activity correlate? Do isoforms of the enzyme work together to achieve the active form? Conversely, do these polymers correspond to a basic unit that is capable of polymerising until the active form is obtained?

Proposed mechanism for PPO activity

Based on the facts that the same structure is responsible for catalysing both Tyr and Cat activity (Mayer, 2006) and that both coopers atoms have the same catalytic potential, it has been suggested that other factors could modulate the reaction; some species can catalyse the coupled reaction of hydroxylation and oxidation, whereas others catalyse only the oxidation of *o*-diphenols. Without going into detail regarding the great diversity observed among species, it is useful to define a mechanism to account for the divergent results in this family of enzymes using copper; the principal components are presented below.

During catalysis, the active site of the enzyme undergoes structural changes (Klabunde et al., 1998), which are associated with changes in their electronic nature, that define three states of oxidation for the copper (*deoxy*, *oxy*, and *met*) during the two coupled catalytic cycles of Tyr activity. Figure 2 shows a diagram of these configurations.

Both the *oxy* state and inserted, the copper atoms are in oxidation state +2, but only in the state configuration *oxy* copper atoms have been reported type *side-on* copper $(II)_2$ (μ-η^2: η^2-O_2^{-2}) (Solomon et al., 1996).

While there are various schematic representations of the two activities coupled (Tyr and Cat) (Lerch, 1995; Espín et al., 1997), it is presented in the Figure 3 the diagram proposed by Solomon (Solomon et al., 1996), because as well as integrating both activities, it provides a clear view about structural reactions and the structure of the probable states of transition.

The cycle begins with the enzyme activation from a pre-catalytic state (*deoxy*) to *oxy*; this process is mediated by incorporation of molecular oxygen (Mason et al., 1955). The union of one molecule of monophenol in the active site of the enzyme, imposes to the substrate the need to adopt a conformation that maintains the trigonal pyramidal geometry for the copper atom (axial union) in addition to establish additional relationships based on the nature of the remainder of the cavity structure (Solomon et al., 1996), which has not been described accurately for all species. Upon reaching the transition state (proposed to be *met*-D, which is not detectable by EPR), catalysis proceeds with formation of the product of the hydroxylation reaction, which has been suggested to acquire an equatorial configuration at the active site, emulating movement of butterfly wings from the income of the ligand to the transition state, a process which should be accompanied by some residues in the active site but that is still not fully defined. It has been postulated that the coupled mechanism leads to the following reaction, without the release of the substrate, and is the reason why it is difficult to assess the hydroxylase activity via the product formed. In the second stage of the reaction catalysed by Tyr (also called Cat activity), the molecule *o*-diphenol bidentate fixes to copper atoms in the active site and reaches the transition

state of the reaction, the moment at which electron transfer occurs, and an *o*-quinone molecule is released as product.

In most of the species with Tyr activity, it has been found that hydroxylase activity has a lag phase in the presence of its characteristic substrate (L-tyrosine), fact also observed in avocado PPOs (Varda et al., 1980), which is removed by the presence of product (L-DOPA) during the reaction and would act as a co-substrate for this first stage. According to studies of other fruits, the *o*-diphenol molecule in this case would neither act as a competitive substrate for Cat activity nor alter the reaction rate of Tyr (Martínez-Cayuela et al., 1989; Sánchez-Ferrer at al., 1993; Sánchez-Ferrer et al., 1994 ; Espín et al., 1997). According to the mechanism proposed by Solomon (Solomon et al., 1996), this may explain why only monophenols in the active species react with the *oxy* form, allowing axial entry to the substrate, and subsequent to final accompaniment until an equatorial conformation is reached in the transition state. In the absence of diphenol, it has been reported that PPO enzymes were present in an *oxy*-detectably form between 1 and 15% (Jolley et al., 1974); moreover, *o*-diphenols may react both with the *oxy* and *met* forms, and therefore, in the presence of diphenols, the present *met* form is converted to the *oxy* form after releasing *o*-quinones. This process could increase this initial 15% by eliminating the lag phase for the hydroxylation reaction (the first stage of the Tyr activity).

Then, this proposed mechanism seems then to answer a series of questions about the dual activity of the enzyme; however, it raises new questions about whether it is effective in defining a general mechanism that is governed only by some residues of the catalytic site, which would ultimately determine the differentiated activity between species. Considering a report in which a single 10-kDa peptide exhibited Tyr activity (Mayer, 1966), is it appropriate to explore potential

allosteric effects that are remote-modulated by other molecules (e.g., other monomers) that eventually act in a coordinated fashion? This suggestion is based on the active polymeric forms Hcn detected in crustaceans (Gerdemann et al., 2002), which could appear *in vivo* on plants and could be linked to the number of isoforms present in fruits, as mentioned above.

Relationship between structure and function

Even though neither the structure nor the sequence of avocado PPOs have been published, there is much structural information regarding the Type III enzyme family, and the information that is presented below is intended to provide context for future research in this area.

Using crystallographic structures of four species (*Ipomoea batata, Agaricus bisporus, Juglans regia and Vitis vinifera*) published in the Protein Data Bank, with codes 1BT1, 1BT2, 1BT3, 1BUG, 2Y9W, 2Y9X, 5CE9 and 2P3X, it can be attempted to advance the understanding of the complex mechanism underlying the formation of pigments in nature mediated by Tyr and/or Cat activity. Structural studies generally include the third member of the group of enzymes of Type III, Hcn, because although it does not possess Tyr or Cat, according to the reports, its study enables a deeper understanding of the binding of molecular oxygen to the active site, considering that this binding is the main function of Hcn.

Despite the differences in sequence, folding and coordination geometry of copper atoms, there is remarkably high sequence homology in the first coordination sphere of the copper atoms of the active site, a fact observed amongst the entire family (Gerdemann et al., 2002). Detailed structural characterisation and understanding the relationship of the structures to enzymatic activity by determining the factors that modulate this phenomenon have been suggested to be the solution for controlling the enzymatic-browning phenomenon.

Regarding the study of structure/function in this family of enzymes, two approaches are used, as mentioned at the end of the previous paragraph; the first states that before the great variability among – and even within a single – species can be understood, it is best to focus efforts on the description of the active site (Mayer, 2006). The second indicates that an overview of the molecule, its relationships and interactions would ultimately yield an understanding of this phenomenon (García-Borrón and Solano, 2002). Considering the latter view, one study evaluated the possibility of detecting a pattern of amino acid sequence homology of 47 PPOs available sequences and stresses that the presence of six coordinated histidine residues, two phenylalanine, two arginine and two aspartic acids, in the active site cavity is common to all species. Their role in the reaction mechanism remains speculative, and it was concluded that the low clustering achieved during the procedure prevented the authors from making any inferences regarding the relationship between the studied sequences and the published crystallographic structures (Malviya et al., 2011).

One of the facts that complicates the study of PPOs is that their active sites are constituted by two copper atoms (called CuA and CuB), each of which may play a role during catalysis, and whose oxidation states change along the catalytic cycle in response to the agents that can interact with them, in addition to the coordination histidines (Lerch, 1983) mentioned above.

From spectroscopic and theoretical studies, it was determined that copper atoms are stabilised symmetrically in the following parallel configuration (*side-on*): μ-η^2: η^2 (Solomon et al., 1996). Then, working on the structure of the PPO *Ipomoea batata* using EXAFS, a copper-copper distance equal to 2.9 Å in the *met* state, with three histidines coordinating each copper atom and an oxygen per metal atom, was determined; a value of 3.8 Å was obtained for the distance in the

oxy state and coordination of five atoms (N_ε y O) (Eicken et al., 1998). In another study, from the crystallographic structure of the same species, it was confirmed that the distances for the *met*, *oxy* and *deoxy* states were 2.9, 4.2 and 4.4 Å, respectively (Klabunde et al., 1998), and that the changes associated with boundary residues (first coordination sphere) were negligible, results which were interpreted as evidence of the rigidity of the active site during catalysis (Gerdemann et al., 2002). The mechanisms, which are possibly differentiated by incorporation of copper atoms at the catalytic sites of the enzymes, are not included in this revision of the enzyme system, which considers the avocado PPOs in their pre-catalytic states, e.g., in their states of activity or latency.

Continuing the analysis of the role they might have residues in the dual activity of the enzyme, it has been suggested that the presence of a thioether bond in the second coordination sphere of copper (CuA), which is present in enzymes with Tyr and Hcn, could have a reactive role during catalysis, but its role in the structure remains under discussion (Gerdemann et al., 2002). In this regard, a recent comparison study that included new crystallographic structures for Type III enzymes suggested that the presence of such links could affect the flexibility required for adapting to the substrate in the active site in the transition state during hydroxylation reaction of monophenols (Kanteev et al., 2015).

Moreover, of special interest is the presence of a residue present in enzymes that exhibit Cat but not Tyr activity, which appears to block access to CuA (F216 in *Ipomoea batata*); some studies have proposed that this residue would modulate the access of monophenols to CuA and thus determine the hydroxylase activity. In this sense, the oxidase activity would be defined by the union of diphenols to CuB (Olivares, García-Borrón and Solano, 2002; Decker et al., 2006). This

hypothesis is supported by the fact that access to CuA is also locked in the crystal structure of Hcn, which, as has been mentioned, does not exhibit hydroxylase or oxidase activity (Volbeda and Hol, 1989; Gerdemann et al., 2002). Otherwise, experimental studies of bacterial Tyr demonstrate that mutations to the residue V218 can lock CuA in one of the bulkier phenylalanines, thus increasing Tyr activity and decreasing Cat activity (Goldfeder et al., 2014). Other studies suggest that the differential activity could be related to the presence of amino acid patterns N and E in the active site (Goldfeder et al., 2014), not only linked to the residues that lock CuA. Thus, considering the overall mechanism proposed by Solomon (Solomon et al., 1996), as described above, it is observed that the intermediate in the transition state of the hydroxylation reaction of the Tyr activity has an apparent steric barrier to the movements necessary to achieve the pre-catalytic state upon insertion of oxygen in the monophenol molecule; thus, it is likely that one of the factors to consider in the elucidation of the mechanism of this reaction is the degree of flexibility in the interaction between the enzyme and the substrate. Therefore, the dynamics of the molecule upon interaction with the ligand is critical in determining its activity. In this sense, the presence of a thioether in the second coordination sphere bond may also play a role in the support needed to reach the transition state in the mechanism of hydroxylation, although it has also been noted that its presence could influence the oxidation potential of Cat activity more than the structural mechanisms for recognition and substrate binding (Gerdemann et al., 2002). Given these observations, in such a diverse family, the following question arises: can both catalytic sites work collaboratively through asynchronous movements, depending on the availability of oxygen and the nature and size of the substrate, without necessarily being one of the copper atoms (CuA or CuB) that develop one of the key

functions, considering the observation in the previous section that both have the same catalytic potential (Solomon et al., 1996)?

Based on the structures and information presented and the lack of secondary structures and/or sequences for avocado, one could infer that in the structure of avocado PPOs, some of the mentioned structural factors may be present and correlate with the avocado's high resistance to different inhibition treatments compared with other fruits.

Accordingly to the published by Bates (Bates, 1970), the first attempt to control browning in avocado was documented in 1931 by Harrold, and considering more than eighty years of work and numerous attempts to inhibit browning activity, the next section highlights the advances and results regarding this subject.

Attempts to inhibit PPO activity in avocado pulp

Avocado PPO stands out as one of the most active and most resistant to inhibition treatments in fruits (Weemaes et al., 1998); thus, it is conceivable that if we learn how the variables interact and influence the browning phenomenon observed in this system, we could develop targeted methods to control the stages (harvesting, processing and storage (Weemaes et al., 1998)) in which PPO activity is exhibited in other species.

Although various methods have been employed to inhibit PPO in fruits and vegetables (Soliva-Fortuny et al., 2002), this section will focus on the results related to avocado PPOs.

Six types of inhibitors have been proposed:

1. Reducing agents of the *o*-quinones, such as ascorbic acid, whose role is to react with the *o*-quinones to form the corresponding diphenol, the reverse of the reaction catalysed by Cat.

2. Entrainers for the *o*-quinone molecules with a thiol group in their structure, which achieve interaction with the *o*-quinone, preventing further polymerisation. However, when they are exhausted, the reaction is not prevented from proceeding.

3. Alternative substrates of the enzyme, which prevent the formation of *o*-quinones, and whose reaction products absorb a different wavelength. However, they should not be classified as inhibitors.

4. Nonspecific scavengers, which denature and thus non specifically inhibit enzyme activity.

5. Scavenger-based mechanisms: these specific molecules react with the enzyme and bind covalently to the active site, thereby inducing suicide catalysis; these are also called suicide substrates.

6. Specific inhibitors that bind reversibly to the enzyme, thus reducing its catalytic activity.

The molecules of types 5 and 6 are also referred to as "true inhibitors" (Chang, 2009).

Inhibition studies regarding avocado PPOs have included almost the complete spectrum of the abovementioned inhibitors, except for type 5, mainly because the mechanism of catalysis in avocado has not been fully defined.

The research related to the methods used to achieve inhibition of avocado PPOs has been divided into three groups for clarity: chemical inhibition (which includes inhibitor types 1, 2 and 6), physical (type 4), and combined (chemical and physical) inhibition methods.

Chemical methods

There is much interest in inhibiting Tyr because of its role in the genesis of pigment formation in humans. However, beyond its topical use, inhibitors application in food must be weighed against the regulations of each country because of severe controls regarding the matter. Naturally

occurring inhibitors are presented as a solution to this and, in the case of Tyr activity, the most-studied are kojic acid, arbutin, catechin, hydroquinone and azelaic acid (Parvez et al., 2007). The use of additives, exclusively as inhibitors to the appearance of pigments, in avocado pulp began with the use of citric acid in combination with salt in cool pulp (Maefle, 1955), continuing it use in combination with physical techniques (with ascorbic acid, citric acid, EDTA and sodium metabisulfite), finding that despite their use, avocado PPO was more resistant to the inhibition (Almeida and Nogueira, 1995). Is important to mention that the effect of some inhibitors on Tyr activity has been investigated, but many of these studies have been conducted with mushroom Tyr (*Agaricus bisporus*) because this is the purest species that is marketed at present (Chang, 2009). The results obtained for other species may vary considerably.

Physical methods

When markets do not allow addition of synthetic additives (chemical methods) (Earnshaw et al., 1995), physical methods constitute one of the alternative treatments, and in the next sections are covered the main results of these studies are covered.

Treatment with high temperatures

Inhibition of browning in avocado pulp through thermal treatments was one of the first methods used and has been declared as the most effective method for control of PPO activity (Golan-Goldhirsh et al., 1984; McEvily et al., 1992). Knowing the diversity among enzymes of different varieties, a study in the year 2010 evaluated the thermal resistance of the PPOs in three varieties of avocado (Choquete, Fortuna and Quintal) activity measured as Cat (catechol as substrate measured at 395 nm), concluding that regardless of the state of maturity and heat treatment temperature (60-80°C), after ten minutes of treatment, an enzyme activity of 50 to 60% on

average was achieved in the samples, suggesting the presence of thermo-resistant isoenzymes in the matrix of the fruit. However, the use of high temperatures has been discarded as method of inhibition because the results demonstrated that while it was the best method (in comparative terms), bitter flavours developed in the final product (Bates, 1970); thus, this technique is not useful for preserving avocado pulp.

High hydrostatic pressure

Another physical method used to inhibit PPO activity in avocado pulp is the application of high hydrostatic pressure, which is a promising emerging technology because it destabilises the secondary structure of proteins (enzymes) and inactivates microorganisms without altering sensory and nutritional food properties (Weemaes et al., 1998). This result is achieved because only the hydrophobic interactions of biological structures are affected, without affecting their covalent bonds (Hayashi, 1989; Knorr, 1993). As reported by Weemaes et al. (Weemaes et al., 1998), who studied the inhibition of PPO in avocados, apples, grapes, pears and plums at 20 °C, a treatment of 900 MPa (maximum assessed value) was necessary to inhibit the PPO of a South African avocado variety. They hypothesised that avocados are characterised by a so-called isokinetic temperature, above which the thermal inactivation is delayed at elevated pressures (exponentially in the case of avocado). Avocado's stability to pressure treatment does not necessarily correspond with thermal resistance, and each PPO responds differently to high pressure. Therefore, extrapolating these results to other varieties is risky.

Whereas work with pure extracts of avocado PPOs has attempted to characterise the simplest enzymatic system, a study has aimed to analyse PPO activity in the pulp of Hass avocado, thus giving the context to the *in vivo* enzyme matrix during storage, and after being to subjected

treatment with a high hydrostatic pressure of 600 MPa for 3 minutes. The main result is that the enzyme maintained a residual activity on the order of 50.72% and that its activity recovered approximately 10 to 15 days after the treatment, thus suggesting that the inactive enzyme corresponded to a treatment-sensitive isoenzyme but a resistant isoenzyme became prevalent over time (Jacobo-Velázquez and Hernández-Brenes, 2010). Another study observed a residual activity of PPO in guacamole (a preparation based on avocado) of approximately 15% with a treatment of four cycles at high pressure (689 MPa at intervals of five minutes each) (Palou et al., 2000).

Recently, a study of the behaviour of pieces of avocado (of the Hass variety) compared in response to treatment with high pressure (200 to 600 MPa for 3-6 minutes) noted that there was no inhibition of PPO (measured as Cat activity) and reported a 30% increase approximately in its activity, which was attributed to disruption of the membranes that separate the enzyme from its substrate and first observed in this species by electron micrograph, as presented in Figure 4. Despite this disruption, no changes in product colour detectable to the human eye were observed (Woolf et al., 2013).

In summary, treatment with high pressure has been demonstrated to be an effective method for inhibiting pure extracts of avocado PPO, but nevertheless presents as a disadvantage the fact that depending on the variety studied, there exists residual activity in the pulp and pieces. Additionally, it is necessary to strictly control the variables of the process (time, temperature, and pH) to prevent activation of PPO, as it has been reported to occur in pears, at a pressure of 450 MPa (Asaka and Hayashi, 2014). Finally, the implementation of this technique in industrial applications is costly (Gómez-López, 2002).

Ultrasound

Application of ultrasound is another method that has been used as a natural alternative for inhibiting avocado PPO. The principle underlying this method is the phenomena of cavitation of the liquid matrix in which enzymes are suspended. The high frequency leads to formation, growth and implosion of small gas bubbles in the liquid, which affect the activity of enzymes during their collapse (Raviyan et al., 2005). However, in a study using avocado mashed pulp (of the Hass variety) an increase in the PPO activity after treatment, measured as Cat activity, was recorded. The authors observed that the percentage increase in PPO activity was related to the level of dilution of the samples (1:2 (25.1%) 1:5 (36.9%) 1:9 (187.8%)) and that the combined effect of the particle size and cavitation caused disruption of the cell membranes, favouring the release of the substrates and putting them in contact with the enzyme (Bi et al., 2015). It is possible that the effect of this method was masked by this activity. The study did not mention the probable latent state of the enzymes previous to the treatment, and it is possible that the treatment activated these latent enzymes affecting the structures that were in suspension.

Modified atmosphere

Whereas application of modified atmospheres did not inhibit the catalytic activity of avocado PPOs, the absence of oxygen in the reaction medium stops the progression of PPOs, at least until the time when the system reincorporates oxygen. Many studies have reported the use of modified atmospheres for treating avocados, mainly from Hass and Fuerte varieties (Chaplin and Hawson, 1981; Gerdes and Parrino-Lowe, 1995; Meir et al., 1997; Yahia and Gonzalez-Aguilar, 1998); however, these studies did not evaluate the direct impact of the method on the PPO activity.

Combined methods

Inactivation of PPO enzyme via combined methods was one of the first attempts to preserve the quality by preventing the enzymatic browning of the fruit as a processed product.

In 1995, with information regarding the PPO activity in avocado, studies began to assess the degree of inhibition of PPO. Almeida et al. measured the combined effect of temperature (2 minutes at 70°C) and 4 additives (ascorbic acid, citric acid, EDTA and sodium metabisulfite) on PPO activity of eight fruits and vegetables (avocado, banana, apple, pear, peach, potato, mushroom, and palm), finding that avocado PPO was the most resistant to the inhibition treatment (Almeida and Nogueira, 1995). Subsequently, Weemaes et al. the combined use of high pressure and temperature. They observed that near neutral pH, the optimal temperature for inhibition of avocado PPO was close to 65°C, and changes in the pH (acid) caused the PPO to be sensitive to the high-pressure treatment, in contrast with other varieties (Weemaes et al., 1998). In 2002, a combination of modified atmosphere (air/N_2/vacuum), antioxidant (without antioxidant/ascorbic acid/EDTA) and antimicrobial agent (agentless/sorbic acid) was used to establish the kinetics associated with the deterioration in the preservation of mashed Hass avocado. It was concluded that the best correlation of the kinetics with all variables was of order 1 and that complete inactivation of the enzyme by this model could happen after three months in the presence of EDTA; moreover, there was no inactivation after the addition of ascorbic acid (Soliva-Fortuny et al., 2002).

Following with physicochemical methods, Weemaes et al. (Weemaes et al., 1999) proposed the use of high pressure as a physical agent in combination with five chemical agents (EDTA, NaCl, benzoic acid, 4-hexylresocinol and glutathione) at 25°C and pH 5.0 to inhibit avocado PPO.

Based on their results, the authors suggested the presence of two fractions of PPO in avocado matrix, one of them resistant to treatment with high pressure, which would correspond to the 10-20% of total enzymes, and the other one sensitive to the treatment. Additionally, they suggested that perhaps the isoenzymes had different isoelectric points and this might explain their different behaviour during the treatment.

Finishing with this section, the presence of Tyr inhibitor observed in mushroom was detected in the pulp of avocado; according to these results, the molecule has been claimed to belong to the family of proanthocyanidins (Chai et al., 2015). No further information about the variety of avocado used was given. Based on these results, it would be interesting for future studies to evaluate the role of these endogenous inhibitors in the activity of avocado PPO.

New disciplines and perspectives in the understanding of avocado PPO

Avocado PPO belongs to a family of enzymes that has been intensively characterised throughout its history. However, questions regarding the type and cause of their behaviour (differentiated activity (Tyr/Cat), isoforms, quaternary structures, differences between species, different degrees of inhibition, differential expression in time and plant parts or their role in plants) have not been completely resolved.

At present, new methodologies are being combined to characterise this complex system, yielding information that has not been obtained using traditional methods (Mayer, 2006).

Molecular studies of natural phenomena have emerged as a new tool for understanding the basis of enzymatic browning, contributing with additional information from genomics (study of the genome), proteomics (proteome studies) and metabolomics (metabolites studies and relationships), with excellent reviews that clearly demonstrate the progress in knowledge and

benefits offered by these new disciplines (Bonghi and Trainotti, 2006; Chen and Harmon, 2006; Boschetti and Righetti, 2008; Gstaiger and Aebersold, 2009) providing a holistic approach to the layout of directed solutions in the future.

In the field of genomics, last year was the complete genome of avocado (*drymifolia* variety) was published. Using transcription techniques, the sequence of avocado in three states of maturation (pre-climacteric/climacteric/post-climacteric) in roots, stems, leaves, seeds, fruit, buds and air flowers was obtained. This information will be the cornerstone for molecular characterisation of the species, from which a high percentage of genes (47.40% of the sequences) that have roles in metabolic and cellular processes have been identified. Moreover, genes associated with the decline in redox functions over time, along with genes related to the response to damage have been determined. Approximately, 51% of the genes were classified as novel; within them, 17% were classified as unknown, 19% without similarity, and 16% with some similarity. Finally, it was reported that there were similarities between the genes of the seed and fruit, but there were major differences between them and those of the root; these differences could be connected with the observed differences in the PPOs of different tissues and flowers in this species (Ibarra-Laclette et al., 2015).

Proteomic studies of this species have determined that a total of 1012 proteins are present in the pulp of avocado, where 64% (648 proteins) correspond to new species (Esteve et al., 2012); this result highlights the fact that a vast field of research remains. Ending with advances in the 'omics' disciplines, metabolomics have also taken steps in the characterisation of metabolites associated with pre- and post-harvest changes in avocado (Pedreschi et al., 2014; Donetti and Terry, 2014), analysing, for example, the evolution of fatty acids or C7 sugars depending on the

harvest area, state of maturity and storage conditions, information which manages to prioritise the relevance of an external agent (weather and agronomic management conditions, among others) correlated manner with the metabolic processes of a type. A more recent study (2015) included the use of chemiometric methods (Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA)) for the characterisation of metabolic profile in 13 varieties of avocado at two different ripening degrees. Demonstrating that mannoheptulose, pentadecylfuran, aspartic, malic, stearic, citric and pantothenic acids, mannitol, and β-sitosterol, were the most influential metabolites in the ripening process. These results prove the value of metabolomics as a key tool in fruit ripening mechanism characterisation (Hurtado-Fernández et al., 2015).

In absence of further information at the molecular level regarding the details of the mechanism of the reaction catalysed by avocado PPO, there is a possibility that characterisation of the metabolites that may be increased by browning depends on other variables, considering the multi-faceted nature of the phenomena.

From a predictive approach, based on the atomic scale behaviour of matter, which is described by the quantum mechanical laws of classical physics, computational chemistry can likewise contribute to a detailed definition of the mechanisms that govern natural phenomena, reaching unattainable information using traditional testing methods. While the complexity of the active site of such enzymes is a great barrier to these studies, mainly due to the multiconfigurational electronic character of the two copper atoms (occupation of orbitals d), progress has been made in the characterisation of adducts in the reaction of PPO. There are no publications related to *in silico* studies on avocado PPO, mainly because not even the sequence or crystallographic

structure is available. These characterisation studies are focused on the investigation of biomimetic copper, resembling the states of biological coordination of both copper atoms in an enzyme, specifically addressing the description of the complexes with molecular oxygen (oxygen activation) from the electronic point of view, defining the most probable conformations of the structures with low energy and comparisons between different methods of calculation that achieve an effective description of the core without losing the sensitivity of the results. The *superoxide* state stands out as the dominant character of oxygen and formation *side-on* for bidentate copper cores (Zapata-Rivera et al., 2011b; Zapata-Rivera et al., 2011a) and therefore provides relevant information regarding the complex catalytic cycle in which this family of enzymes participates.

In a recent study that combined both experimental and computational methods, the transition state of an enzyme associated with Tyr, NSpF, was characterised in the *oxy* state, which was mentioned in a previous section, using *Density Functional Theory* (DFT) B3LYP method, with a base Ahlrich triple-ξ with polarisation (TZVP) (Ginsbach et al., 2012). Nonetheless, the implementation of the method for this system has been questioned because of its monoreference character, present sensitivity depending on the functional use and the presence of spin contamination (Zapata-Rivera et al., 2011b). However, it is interesting that it has been postulated that although all the copper atoms in the active site of all PPOs are identically reactive, the different substrates can establish different interactions with residues of the second coordination sphere, which ultimately determines the role of each different subclasses or PPO activities, including the latter integrant to the family (NSpF) (Ginsbach et al., 2012).

One of the most recent studies dynamically characterised the entire structure of mushroom Tyr (PDB code 2Y9W) using classical molecular dynamics simulations (Ioniţă et al., 2014). Although such simulations can serve as a good approximation to the movements of the structure (from a classical point of view), this method does not enable the system reactivity evaluation. To overcome this difficulty, multiscale methods (Warshel and Levitt, 1976), which combine molecular dynamics simulations (to treat the dynamical behaviour of the biological macromolecules) with the electronic description of the reactivity for the atoms in the active site, have been developed. These methods have been applied in the elucidation of the first step in the hydroxylation reaction, catalysis by Tyr, revealing some differences in the results (Siegbahn and Borowski, 2011); these differences seem to be linked to the use of crystallographic structures as pre-catalytic complexes.

There are excellent reviews about these methods (Warshel, 2003; Kamerlin and Warshel, 2011), which have been applied to other metalloenzymes (Saura et al., 2014), and in general to other enzyme systems (Monard et al., 2003; Gómez et al., 2012), providing valuable information regarding the detailed description of the mechanism and the phenomena associated with it; hence contributing to complete the landscape of information that seems to be necessary to elucidate the complex phenomena underlying enzymatic browning triggered by PPO.

Conclusions

With almost 80 years of work focused on the reaction catalysed by different species PPO´s, and with various targets ranging from diseases treatment of to beauty products, and technology solutions for agribusiness, this review seeks to highlight the complexity of this system. Although

enzymatic browning has been partially characterised in a number of species, the phenomenon continues being uncontrollable.

Although there have been great advances in the characterisation of this enzyme using traditional experimentation methods, non-traditional research methods -such as those evaluated in the last section of this document- will surely provide new information about this phenomenon. This knowledge accruing will enable future research advances in this field, and the development of new technologies for controlling enzymatic browning in avocado pulp. It is hoped that the knowledge generated from this holistic perspective can be applied effectively in other systems.

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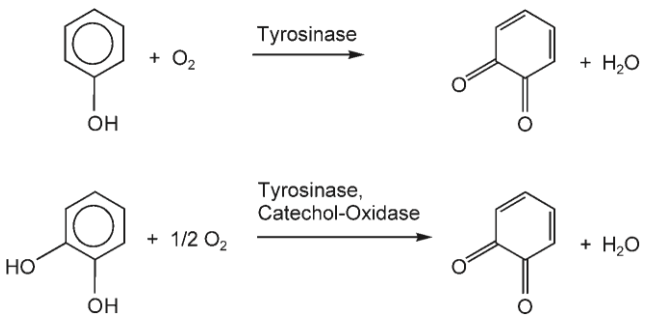

Figure 1: Top: Tyrosinase catalyses the conversion of monophenols to *o*-quinones via *o*-diphenols. Bottom: Catechol oxidase catalyses only the second step (*o*-diphenol to *o*-quinone) (Decker et al., 2006).

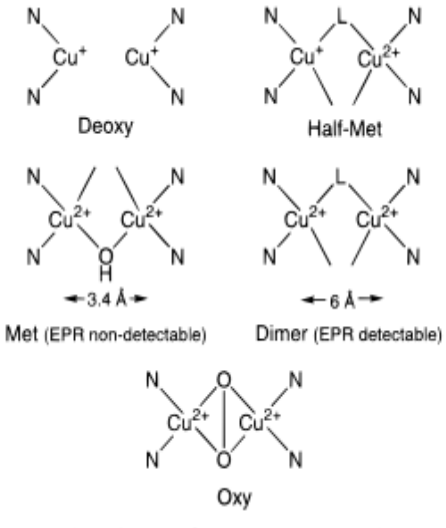

Figure 2: Derivatives of the coupled binuclear copper active site (L) exogenous ligand. Note that a third histidine is present on one or both of the coppers, but this is not shown for clarity (Solomon et al., 1996).

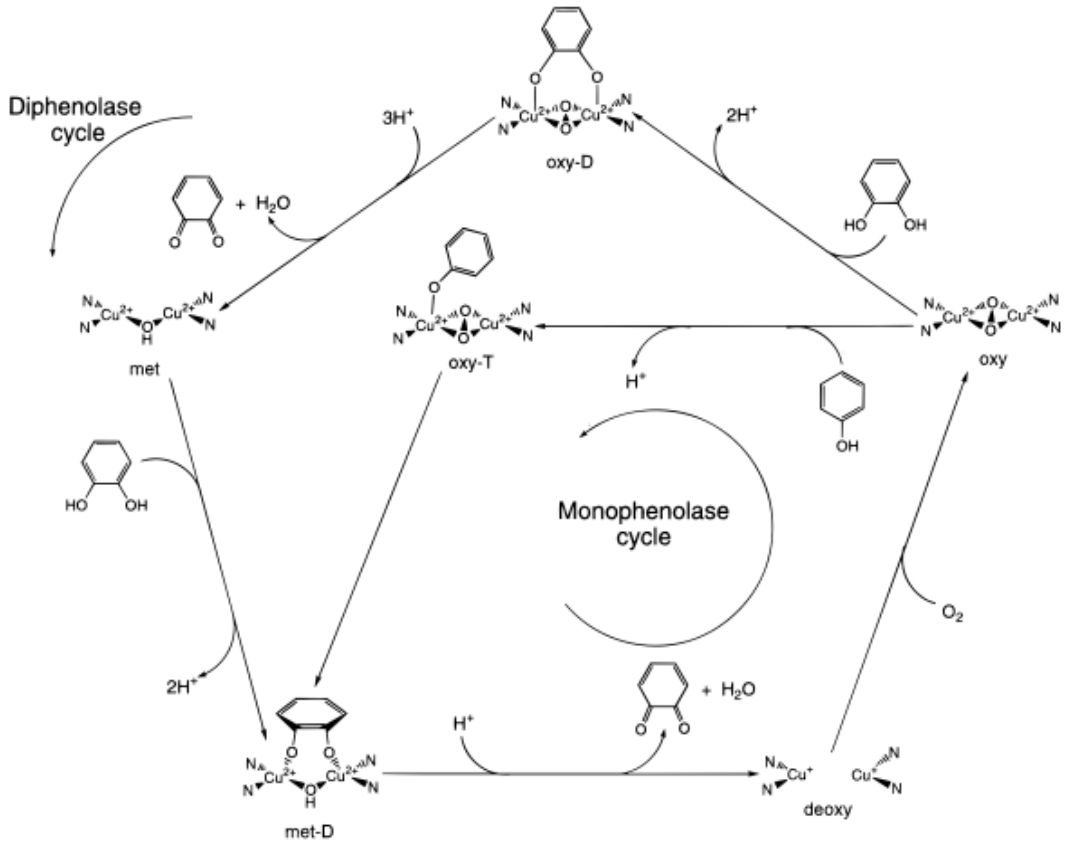

Figure 3. Catalytic cycle for monooxygenation of monophenols and oxidation of *o*-diphenols to *o*-quinones by tyrosinase. The axial ligands at copper are not included for clarity. T) Tyrosine and D) DOPA-bound forms (Solomon et al., 1996).

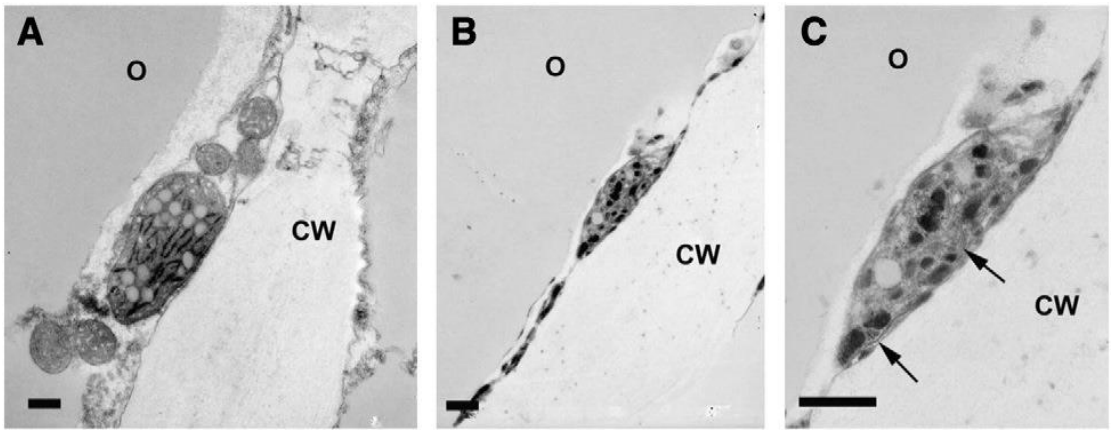

Figure 4. Detailed structure of avocado cytoplasm. a) Untreated tissue with easily distinguishable cell organelles and membranes. b) and c) Tissue treated for 6 min at 600 MPa; the cytoplasm is grainier, pressed to the cell wall and contains many electron-dense spots. However, some membrane integrity is retained (arrows). All images were obtained using transmission electron microscopy. Bars = 0.1 μm, O — oil deposit, CW — cell wall (Woolf et al., 2013).