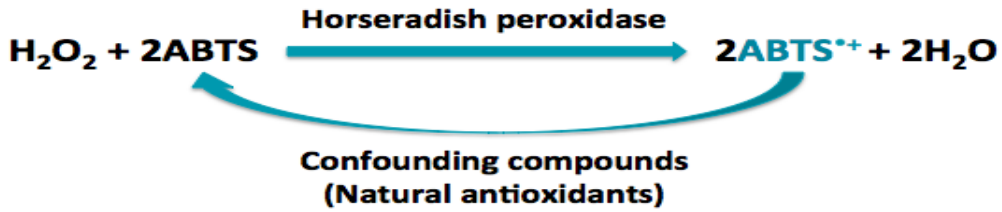

Figure 2.2. A plausible mechanism for assay interference. The concentration of hydrogen peroxide in a sample is based on the amount of $ABTS^{\bullet+}$ formed following initiation of the peroxidase reaction.

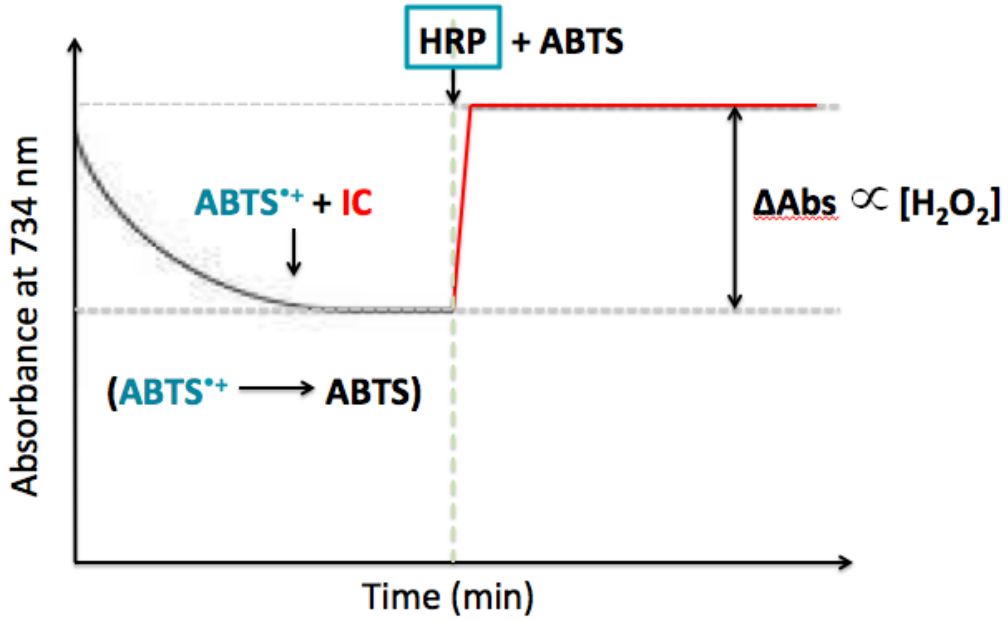

Figure 2.3. Experimental design of modified assay for the determination of hydrogen peroxide in biological matrices. Initially, an aliquot of ABTS and ABTS•$^{+}$-containing solution is added to the hydrogen peroxide containing sample. This step allows confounding compounds to react with ABTS•$^{+}$. Horseradish peroxidase is then added to catalyze for the formation of ABTS•$^{+}$ in proportion to the amount of hydrogen peroxide in the reaction mixture. Hydrogen peroxide is quantified based on the change in absorbance before and after the addition of peroxidase.

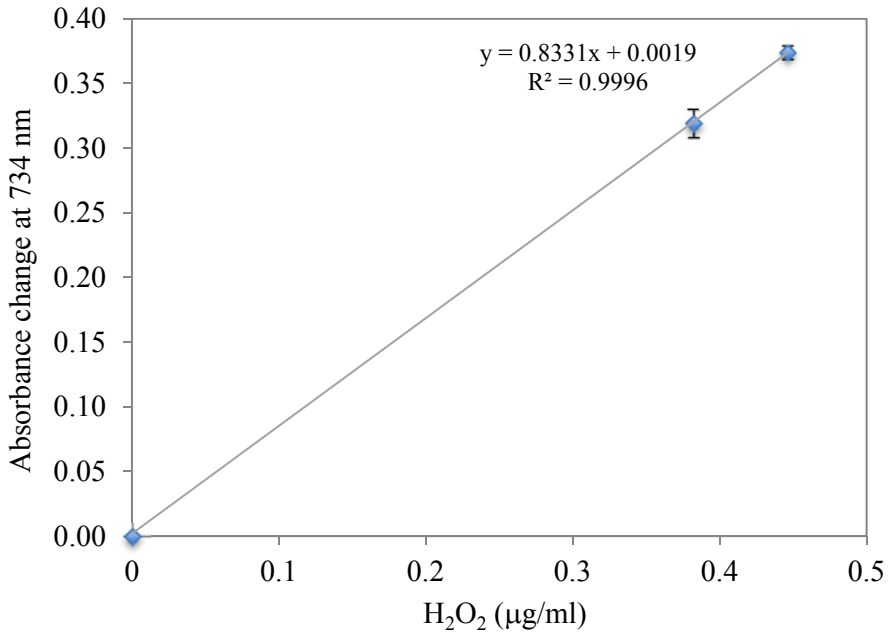

Figure 2.4. Standard curve for hydrogen peroxide determination in ABTS•$^{+}$-treated GPE. Final values are means ± standard deviation from triplicates.

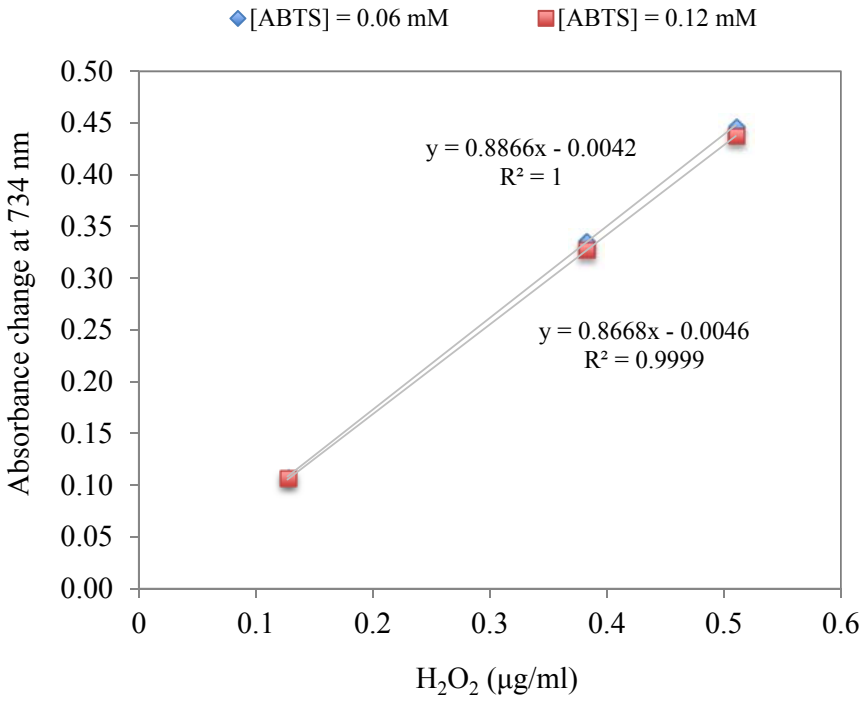

Figure 2.5. Comparison of standard (calibration) curves for hydrogen peroxide determination in ABTS•$^{+}$-treated GPE using different concentrations of ABTS in the reaction mixture. Final values are means ± standard deviation from triplicates.

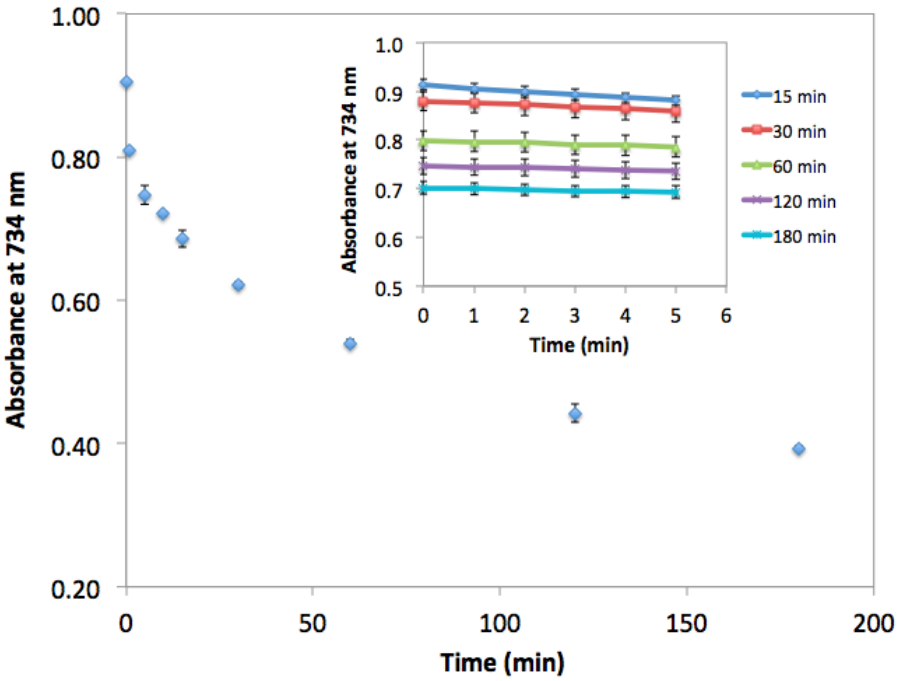

Figure 2.6. Time-course of ABTS•$^{+}$ reduction in GPE. The insert depicts time-courses reflecting rates of ABTS•$^{+}$ reduction following the addition of sufficient ABTS•$^{+}$ to the ABTS•$^{+}$–treated GPE to adjust its ABTS•$^{+}$ concentration back to that amount present at zero time of the initial ABTS•$^{+}$ treatment. The initial ABTS•$^{+}$–treatment was done for different times prior to the addition of the supplemental ABTS•$^{+}$ (initial ABTS•$^{+}$ treatment times, prior to adding supplemental ABTS, are indicated to the right of insert). Rates of ABTS•$^{+}$ reduction (ΔAbs/min), as depicted in the insert, were 0.006, 0.004, 0.003, 0.002, and 0.002 after 15, 30, 60, 120, and 180 min ABTS•$^{+}$ treatments, respectively. Data points represent means ± standard deviation from triplicate samples.

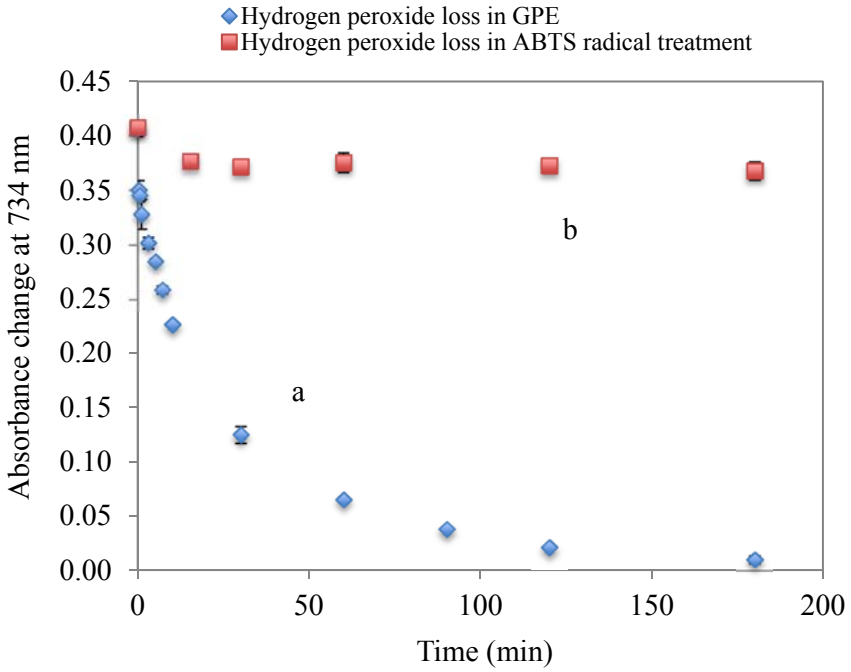

Figure 2.7. Time course of hydrogen peroxide loss, measured as absorbance change due to presence of hydrogen peroxide (y-axis), in GPE. Curve "a" was generated by adding hydrogen peroxide to GPE, allowing it to react for the specified time (as specified on x-axis) before adding $ABTS^{\bullet+}$ to eliminate confounding compounds and terminate hydrogen peroxide reduction. Peroxidase was subsequently added to the $ABTS^{\bullet+}$-containing sample for hydrogen peroxide quantification. Curve "b" was obtained by simultaneously adding hydrogen peroxide and $ABTS^{\bullet+}$ to GPE and allowing the mixture to react for the specified times (as specified on x-axis) prior to quantification of hydrogen peroxide by the addition of peroxidase. Data points are means ± standard deviation from triplicate measurements.

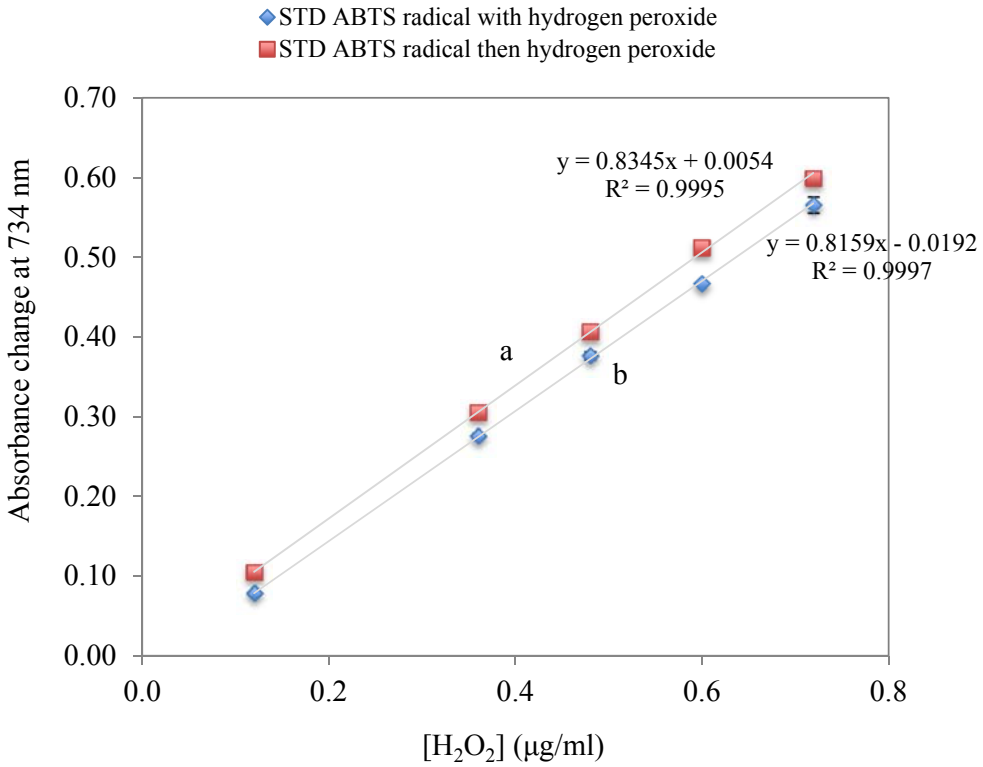

Figure 2.8. Comparison of standard (calibration) curves obtained following spiking known amounts of hydrogen peroxide into ABTS•$^{+}$-treated and untreated GPE samples. Curve "a" was obtained by measuring hydrogen peroxide levels following its addition to 3 hr ABTS•$^{+}$-treated GPE (as in Figure 2.4); Curve "b" was obtained by measuring hydrogen peroxide produced following the addition of hydrogen peroxide and ABTS•$^{+}$ simultaneously to untreated GPE, waiting 15 minutes, and then adding HRP for hydrogen peroxide quantification. Data points are means ± standard deviation from triplicates.

3 Selective Oxidation of Enzyme Extracts for Improved Peroxidase Quantification

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3.1 Abstract

A modified assay that eliminates a source of underestimation of peroxidase activity in plant extracts has been developed. Natural components endogenous to plant materials, such as phenolics and ascorbic acid, may interfere with traditional peroxidase assays by reducing the oxidized product generated as a result of the peroxidase-catalyzed reaction; the problem in such cases is that the oxidized product is typically the reporter molecule that is monitored for enzyme quantification. The reduction of the reporter molecule results in an apparent lag in product accumulation, which is observed as an artificially low enzyme activity. The artificially low activity is thus a consequence of the rate of product accumulation being lower than the rate of product production. This behavior may be reflected in the time course of product accumulation being sigmoidal. This paper describes a relatively simple way to alleviate complications arising from the presence of compounds that confound the peroxidase assay without the need to fractionate the enzyme-containing sample. The method is based on using ABTS as the reporter (reducing) substrate. The oxidized product of the reaction is $ABTS^{\bullet+}$, which can be followed spectrophotometrically due to its relatively high molar absorptivity at 734 nm. It is herein shown that one can selectively inactivate complicating endogenous confounding compounds by treating the enzyme preparation with the oxidized product itself, $ABTS^{\bullet+}$, prior to initiating the assay. This approach is expected to be selective for those compounds likely to interfere with peroxidase quantification via the reductive mechanism described above. The presented method is herein shown to alleviate complications associated with lag phases typical of plant extracts and, thus, to more accurately reflect total peroxidase activity. The presented assay is relatively simple and should be applicable to a range of biological systems.

3.2 Introduction

Peroxidase enzymes are common in plants where they appear to be involved in a wide range of physiological functions, including hydrogen peroxide metabolism,

the formation of lignin and suberin, cross-linking of cell wall components, and plant defense mechanisms (Almagro et al., 2009; Hiraga et al., 2001; Mehlhorn et al., 1996; Fortea et al., 2011; Hamid & Rehman, 2009). Along with their importance for plant vitality, peroxidases also impact the consumer acceptability of plant-based foods through their role in fruit and vegetable coloration/discoloration (Adams & Brown, 2007), flavor development (Burnette, 1977), nutritional properties (Vamos-Vigyazo, 1981) and texture (Manu & Rao, 2011). Furthermore, peroxidases are used as indicators of the adequacy of vegetable blanching due to their high thermal stability and wide distribution (Serrano-Martinez et al., 2008; Goncalves et al., 2010; Ali et al., 2011). Selected plant peroxidases, particularly horseradish peroxidase (HRP), are widely used in biotechnological applications, including the decolorization of synthetic dyes, chemical syntheses, bioremediation, biosensors, and a range of analytical applications (Regalado et al., 2004; Azevedo et al., 2003; Ngo, 2010). Current understanding of the role of peroxidases in each of the aforementioned areas is not complete. It is anticipated that improvements in methods for the quantification of peroxidase activity *in situ* will further such understanding.

A variety of methods exist for the determination of peroxidase activity, including those based on colorimetric (Xianyu et al., 2013, Fortea et al., 2011), fluorometric (Acharya et al., 2013), chemiluminescent (Katsuragi et al., 2000), and electrochemical (Stiene & Bilitewski, 2002) detection. Among these, colorimetric detection is most commonly used as it is simple and economical (Dosoretz & Ward, 2006). Colorimetric quantification is based on the formation of visible light-absorbing products as a result of the peroxidase-catalyzed reaction between a "reporter molecule" and hydrogen peroxide. The reporter molecule is the hydrogen/electron donating (reducing) substrate which, after its oxidation, absorbs light in the visible region. Various reducing substrates, including guaiacol, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), *p*-phenylenediamine, tetramethylbenzidine (TMB), and *o*-dianisidine, have been used as reporter molecules. The flexibility in choosing a reporter molecule is due to the rather broad specificity of these peroxidases. A compendium focusing on the

quantification of horseradish peroxidase lists over 200 substrates for peroxidase activity determinations (Silaghi-Dumitrescu, 2010). As may be expected, commercially available kits for the quantification of peroxidase activity offer a range of reducing substrates. A common concern with many of the substrates used for colorimetric-based assays, especially when applied directly to biological matrixes, is interference due to the presence of confounding compounds endogenous to living tissues. An important class of such compounds are those that react with the assay's reporter molecule, *i.e.* they react with the oxidized product derived from the reducing substrate as a result of the peroxidase-catalyzed reaction (Castillo et al., 1984; Osborne & Metzler, 1984). The net effect of these confounding compounds is a reduction in the observed rate of product accumulation. In such cases the actual rate of product production is less than the rate of product accumulation, leading to underestimates of enzyme activity. To avoid such complications one can separate the confounding compounds from the enzyme prior to quantifying enzyme activity. Approaches toward this end may include a range of separation techniques previously used in peroxidase quantification and/or purification studies (Mall et al., 2013; Valetti & Picó, 2013; Motamed et al., 2009; Fraguas et al., 2004; Magri et al., 2003; Regaldo et al., 1996; Castillo et al., 1984; Osborne & Metzler, 1984). Disadvantages associated with methods aimed at fractionating out confounding compounds include the need for relatively costly specialized equipment (*e.g.*, chromatographs), difficulty in identifying optimum fractionation parameters, difficulty in estimating the extent of confounding compound removal, the time required for such separations, and/or inherent limitations in the extent to which one can minimize changes in the enzyme's environment if interested in *in situ* activity.

The goal of the work presented in this paper was to develop a colorimetric method for the quantification of peroxidase activity that accounts for confounding compounds typical of biological matrices without requiring a fractionation step. Confounding compounds widely present in biological matrices, *e.g.* plant extracts, include a range of natural antioxidants that are capable of reducing the reporter molecules generated during peroxidase quantification. The outcome of this study

is an improved method based on the use of ABTS as the reducing substrate and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) as the corresponding reporter molecule. $ABTS^{\bullet+}$ is generated as a result of the peroxidase-catalyzed reaction between ABTS and hydrogen peroxide. The presented assay effectively eliminates problems associated with confounding compounds inherent in peroxidase-containing samples by selectively oxidizing these compounds prior to peroxidase quantification. Selective oxidation is achieved by treating samples with $ABTS^{\bullet+}$. During this treatment $ABTS^{\bullet+}$ is reduced to ABTS. The ABTS generated in this way later serves in the peroxidase-quantification reaction as the reducing substrate, where the rate at which ABTS is converted to $ABTS^{\bullet+}$ reflects the sample's peroxidase activity. The presented method improves the accuracy of peroxidase activity measurements by eliminating lag phases associated with the presence of confounding compounds. The method retains the simplicity of the traditional ABTS-based colorimetric assay upon which it is based (Childs & Bardsley, 1975).

3.3 Materials and Methods

Reagents.

Hydrogen peroxide (30 wt.%, ACS reagent grade), horseradish peroxidase (HRP, type II, 150-250 units/mg solid), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], guaiacol, potassium persulfate and monobasic sodium phosphate were purchased from Sigma-Aldrich, USA.

Sample.

Sweet yellow peppers (*Capsicum annuum*) and yellow onions were purchased from local markets.

Preparation of Enzyme Extracts.

Vegetables were washed and rinsed with distilled water, cut into small pieces and weighed. Approximately 100 g of cut vegetable weighed to the nearest 0.1 g was homogenized for 30 seconds in 100 mM sodium phosphate buffer (pH 6.0) such that the vegetable:buffer ratio was 1:2 (by weight). The resulting homogenate was filtered through two layers of cheesecloth and then Whatman #1 filter paper. The resulting filtrate from sweet yellow peppers was subsequently filtered through 0.45 μm PTFE syringe-type filters; the resulting filtrate from yellow onions was filtered through 1.2 μm syringe glass fiber filters (Millipore Swinnex). Clear filtrates resulting from these processes were kept in an ice-bath until assayed for peroxidase activity. Enzyme extracts prepared in this way from sweet yellow peppers and yellow onions are hereafter referred to as sweet yellow pepper extract (SYPE) and yellow onion extract (YOE), respectively.

Preparation of ABTS radical cations.

ABTS radical cation ($ABTS^{\bullet+}$) containing solutions were prepared as described by Huang *et al*. (2005) by incubating an aqueous 7mM ABTS, 2.45mM potassium persulfate, solution overnight (12-16 hours) in the dark, at room temperature. The resulting solution is then made 100 mM sodium phosphate, pH 6.0 prior to being used in subsequent experiments.

Traditional peroxidase assay using ABTS.

Aliquots of SYPE (20 μl) or YOE (100 μl) were added to 2 ml of color-forming reagent (0.9 mM ABTS, 0.15 mM H_2O_2, 0.1M sodium phosphate, pH 6.0) to initiate the reaction. Assays were performed at ambient temperature (20-22^{o}C). The increase in absorbance at 734 nm resulting from peroxidase-catalyzed $ABTS^{\bullet+}$ production was monitored for 30 minutes. Initial velocities were calculated from the linear portion of the reaction time course having the highest positive slope. One unit of peroxidase activity, based on initial velocity

determinations, is defined as that amount of enzyme that catalyzes the production of 1 μmole $ABTS^{\bullet+}$ per minute under the defined conditions. The absorptivity of $ABTS^{\bullet+}$ was taken as 15,000 $M^{-1}cm^{-1}$ (Re et al., 1999).

Traditional peroxidase assay using guaiacol.

A 20 μl aliquot of SYPE was added to 2.18 ml of color-forming reagent (0.04% (v/v) guaiacol, 0.135 mM H_2O_2, 0.1M sodium phosphate, pH 6.0). The increase in absorbance at 470 nm resulting from peroxidase-catalyzed guaiacol oxidation was monitored for 30 minutes. Assays were performed at ambient temperature (20-22°C). Initial velocities were calculated from the linear portion of the reaction time course having the highest positive slope. One unit of peroxidase activity is defined as that amount of enzyme that catalyzes the reaction at a rate corresponding to an absorbance change of 0.1 unit per minute under the defined conditions.

Modified peroxidase assay using ABTS.

Aliquots of SYPE (20 μl) or YOE (100 μl) were selectively oxidized by mixing with 2 ml of an appropriately diluted ABTS/$ABTS^{\bullet+}$ solution, prepared as described below, and allowed to react for 2 minutes (SYPE) or 30 minutes (YOE) in the dark at 0°C. Peroxidase quantification was then initiated by adding 0.2 ml of color-forming reagent (9 mM ABTS, 1.5 mM H_2O_2, 0.1M sodium phosphate, pH 6.0) to the extract containing solution. Absorbance changes due to peroxidase-catalyzed ABTS oxidation were monitored at 734 nm. One unit of peroxidase activity in the modified assay is defined as in the "Traditional peroxidase assay using ABTS" (above).

Time course of $ABTS^{\bullet+}$ reduction in SYPE and YOE.

Aliquots of SYPE or YOE, ranging from 20 to 100 μl, were added to 1.98 ml $ABTS^{\bullet+}$ solution having an initial absorbance at 734 nm of between 0.9 and 1.0.

The decrease in absorbance at 734 nm resulting from $ABTS^{\bullet+}$ reduction was monitored spectrophotometrically for 30 minutes. For reference purposes, controls were included which monitored changes in the absorbance of $ABTS^{\bullet+}$ solutions to which aliquots of buffer were added.

3.4 Results and Discussion

Typical time courses depicting product accumulation in the traditional and the modified ABTS-based peroxidase assays applied to SYPE and YOE are shown in Figure 3.1. The time-courses obtained using the traditional assay reflect a lengthy lag period prior to accumulation of product. The lag period can be explained by the presence of confounding compounds common to vegetable extracts that react with the chromophoric oxidized reporter molecule ($ABTS^{\bullet+}$) generated in the peroxidase-catalyzed reaction. This type of interference can be described as follows:

$$2ABTS + H_2O_2 \xrightarrow{HRP} 2ABTS^{\bullet+} + 2H_2O \qquad [1]$$

$$ABTS^{\bullet+} + RA_{reduced} \rightarrow ABTS + RA_{oxidized} \qquad [2]$$

RA = reducing agent, defined as any compound that reduces $ABTS^{\bullet+}$ at a rate which is significant in reference to the measurement of peroxidase.

If the rate of reaction [2] is significant with respect to the rate of reaction [1], then the peroxidase activity of the reaction mixture will be underestimated, at least until RA is depleted to the extent that reaction [2] is insignificant. The underestimate of enzyme activity may appear as a complete lack of peroxidase activity if the rate of [2] is much greater than that of reaction [1], such that $ABTS^{\bullet+}$ is reduced immediately upon being formed. Or it may reflect a fractional underestimate of peroxidase activity if rates of reactions [1] and [2] are similar. One would expect to see an increase in peroxidase activity with time as RA is depleted, provided there is an excess of added substrates (ABTS and H_2O_2) in the reaction mixture.

The time-courses depicted in Figure 3.1 which were obtained with the proposed modified assay do not show a lag prior to product accumulation. This is a result of incorporating into the assay a sample treatment step, prior to peroxidase quantification, to selectively oxidize those compounds likely to reduce $ABTS^{\bullet+}$. The selective oxidation is done by adding the oxidized reporter molecule, $ABTS^{\bullet+}$, to the reaction mixture prior to adding the substrates for peroxidase quantification (*i.e.*, ABTS and H_2O_2). During the pre-quantification period, when the sample is exposed to $ABTS^{\bullet+}$, those components most reactive with $ABTS^{\bullet+}$ are oxidized. The treatment is selective in that it specifically targets those compounds that modify the oxidized reporter molecule, as expected since it is the oxidized reporter molecule itself that is used as the oxidizing agent. The time allotted for the pre-quantification treatment is somewhat flexible; it is dependent on the nature and amount of confounding compounds in the sample and the sample's peroxidase activity. These are major factors dictating the relative importance of reaction [2] in peroxidase quantification. The outcome of incorporating the $ABTS^{\bullet+}$ treatment in the peroxidase assay is a decrease in the error associated with reaction [2] (as depicted in the curves of Figure 3.1 obtained using the modified assay).

The modified assay addresses a problem that has been noted by many researchers (e.g., Fortea et al., 2011; Reszka & Britigan, 2007; Osborne & Metzler, 1984; Celardin et al., 1982; Bruemmer et al., 1976; Castillo et al., 1984). Compounds likely to interfere with the traditional ABTS-based peroxidase assay include a wide range of natural antioxidants. This is not surprising since $ABTS^{\bullet+}$ reduction is the basis of the Trolox equivalent antioxidant capacity (TEAC) assay, which is widely used for determining the antioxidant capacity of biological materials (Huang et al., 2005). Interestingly, the lag phase in the peroxidase/H_2O_2/ABTS system, as depicted in the time-courses obtained using the traditional peroxidase assay, has been suggested as an indicator of the total antioxidant capacity in foods (Arnao et al., 1996). The premise being the length of the lag phase is dictated by the amount of antioxidant in the matrix.

The type of assay interference described by equation [2] is not limited to the ABTS substrate. It is likely to occur with many of the common chromophoric reducing substrates used for peroxidase quantification. This is because the initial reporter molecule-product resulting from the peroxidase-catalyzed reaction is a radical. In many cases it is assumed that these radicals polymerizes to make the final chromophoric reporter molecule. Inhibition of the peroxidase assay by this type of generic mechanism may be described follows:

$H_2O_2 + 2AH \rightarrow 2H_2O + 2A\bullet$ [3]

$2A\bullet \rightarrow A_{2\ (\text{chromophoric reporter molecule})}$ [4]

$A\bullet + RA_{reduced} \rightarrow AH + RA_{oxidized}$ [5]

RA = "reducing agent", here defined as any compound that reduces the initial radical product formed in the peroxidase reaction.

This reaction scheme shows how confounding compounds reacting with the initial product of the peroxidase reaction, A•, will prevent the formation of A_2. This in turn results in an underestimation of peroxidase activity because A_2 is the chromophoric reporter molecule upon which peroxidase quantification is based. A classic reducing substrate used in many peroxidase quantification assays is guaiacol. Assays using guaiacol are susceptible to the type of inhibition described by the above equations. In Figure 3.2 we demonstrate the lag observed when measuring peroxidase activity in SYPE using guaiacol as the reducing substrate. The observed lag is again indicative of the presence of confounding compounds in SYPE. Such lags in peroxidase assays have been noted indirectly before with respect to the behavior of antioxidants (Sanchez et al., 1997; Arnao et al., 1996; Takahama & Oniki, 1997; Demirevska-Kepova & Bakardjieva, 1976). Unfortunately, in contrast to the ABTS substrate, the primary product of guaiacol oxidation, the guaiacol radical, is not sufficiently stable to enable using it in a pre-quantification treatment as proposed for $ABTS^{\bullet+}$ (*i.e.*, the guaiacol radical rapidly dimerizes; see Doerge et al., 1997). $ABTS^{\bullet+}$ is somewhat unique in this respect since it is relatively stable in common buffer systems, thus allowing it to be

prepared and used for the selective oxidation of the peroxidase-containing matrix as described herein.

Investigators using traditional peroxidase assays sometimes report that their initial velocity values, *i.e.* the values used for calculating enzyme activities, were derived from the first linear portion of the time-courses of product accumulation. One assumes the phrase "first linear portion" is not referring to that associated with the lag, but rather the first linear portion that reflects maximum enzyme activity. Hence, the question arises as to whether there is a consequence of measuring enzyme activity in this way. The data of Table 3.1 demonstrate that there is a consequence of measuring enzyme activity following the lag phase. The peroxidase activity was approximately 2-fold higher when determined using the proposed modified assay compared to that obtained using the traditional assay. The slower rate of reaction coming out of the lag phase may be rationalized by at least four mechanisms: (1) slower reacting $ABTS^{\bullet+}$-reducing compounds remain in the reaction mixture following the lag phase when $ABTS^{\bullet+}$ begins to accumulate, thus the rate of accumulation of $ABTS^{\bullet+}$ is decreased to some extent due to a lingering but significant rate of $ABTS^{\bullet+}$ reduction, (2) sufficient hydrogen peroxide is consumed during the lag phase such that the post-lag phase reaction rate reflects the lower hydrogen peroxide concentration (note that in the simplified scheme above the concentration of ABTS would not change during the lag phase since the absence of reporter molecule accumulation is due to $ABTS^{\bullet+}$ produced as a result of the peroxidase/hydrogen peroxide/ABTS reaction being reduced back to ABTS), (3) $ABTS^{\bullet+}$-oxidizable competing substrates remain in the reaction mixture following the lag phase when $ABTS^{\bullet+}$ begins to accumulate, thus the rate of accumulation of $ABTS^{\bullet+}$ is slowed due to the enzyme's interaction with non-chromophoric competing substrates, and (4) the enzyme is inactivated over the course of the lag phase (*e.g.*, suicide inhibition; Valderrama et al., 2002). It is important to recognize that the successful application of the proposed $ABTS^{\bullet+}$ treatment prior to peroxidase quantification should alleviate the above complications.

The key point in considering activities obtained using the traditional versus the modified assay is that the higher activity is expected to more accurately reflect the true peroxidase activity of an enzyme preparation. This reasoning is based on the premise that ABTS$\bullet^+$ activation of peroxidase activity per se is unlikely. The effect of ABTS$\bullet^+$ on horseradish peroxidase (HRP) was directly assessed in this study by doing two minute exposures of HRP to ABTS$\bullet^+$, these treatments are analogous to those used in the SYPE experiments. The results showed no demonstrable change in HRP activity due to ABTS$\bullet^+$ treatments. Assuming this result is applicable to other plant peroxidases, it can be concluded that the higher activity reflected in the modified assay is solely a consequence of removing confounding compounds.

An important parameter with respect to the application of the modified assay is the time required for the pre-peroxidase quantification ABTS$\bullet^+$ treatment. The time required for such treatments will be sample specific. The data of Figure 3.3 summarize the rate of ABTS$\bullet^+$ reduction by SYPE and YOE. The differences in rates are striking. It is well known that antioxidants differ greatly in the rate with which they react with ABTS (Walker & Everette, 2009; Henriquez et al., 2004). Compounds such as ascorbic acid and tocopherol derivatives react with ABTS$\bullet^+$ relatively fast; those such as phenolics react much slower. The composition of the two enzyme preparations used in this work are consistent with the observed time-courses depicting ABTS$\bullet^+$ reduction. Ascorbic acid is the main antioxidant in SYPE (Serrano-Martinez et al., 2008) and thus the majority of its confounding components react relatively fast with ABTS$\bullet^+$ (Tian et al., 2013). In contrast, slower reacting flavonoids (e.g., quercetin, kaempferol) are the predominant antioxidants in YOE (Takahama, 2004; Lanzotti, 2006). Once the kinetics of ABTS$\bullet^+$ reduction are established for a given enzyme preparation, then one can choose an appropriate treatment time. An appropriate ABTS$\bullet^+$ treatment time need not remove all confounding compounds, but the rate of ABTS$\bullet^+$ reduction at the end of the treatment should be negligible relative to the rate of ABTS$\bullet^+$ formation due to the peroxidase quantifying reaction.

3.5 Conclusion

An important complication associated with the measurement of peroxidase activity in plant extracts has been addressed. The complication stems from the fact that plant extracts, like most biological matrixes, often contain confounding compounds that react with reporter molecules generated in typical peroxidase assays. These confounding reactions can lead to false underestimates of enzyme activity. This paper introduces an approach to circumvent this problem. The assay uses ABTS as the reducing substrate in conjunction with hydrogen peroxide. A key piece of the modified assay is the inclusion of a selective oxidizing step, using $ABTS^{\bullet+}$ as the oxidizing agent, prior to peroxidase quantification. This step is selective in that it specifically removes those confounding compounds that interfere with the assay. The modified assay improves the accuracy of enzyme activity determinations while maintaining much of the simplicity of traditional peroxidase assays. The modified assay is better suited for assessing relative activities when working with peroxidases in different biological matrices, such as may occur when applying peroxidase-based technologies in different environments or when fractionating peroxidase-containing preparations. This is because in such cases each sample/preparation/environment will have its own confounding compound profile, thus inhibiting the traditional assay to its own unique extent. This is not an issue with the modified assay since such compounds are inactivated prior to peroxidase quantification. The modified peroxidase assay is expected to be applicable to the wide range of natural and manipulated biological matrixes in which confounding compounds are likely to impact peroxidase measurements.

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