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Selective oxidation of enzyme extracts for improved quantification of peroxidase activity

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ABSTRACT

Natural components endogenous to plant material extracts often interfere with traditional peroxidase assays by reducing the oxidized product generated as a result of the peroxidase-catalyzed reaction. This leads to an underestimation of peroxidase activity when the oxidized product provides the signal for enzyme activity quantification. This article describes a relatively simple way to alleviate complications arising due to the presence of such confounding compounds. The method is based on using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as the reducing substrate. The oxidized product of the reaction is $ABTS^{\cdot+}$, the accumulation of which can be followed spectrophotometrically. It is shown here that one can selectively inactivate the endogenous compounds that confound the peroxidase assay by treating the enzyme preparation with the oxidized product itself, $ABTS^{\cdot+}$, prior to initiating the quantification assay. This approach is selective for those compounds likely to interfere with peroxidase quantification. The presented method is shown to alleviate the complications associated with lag phases typical of plant extract peroxidase assays and, thus, to more accurately reflect total peroxidase activity. The presented assay is expected to be applicable to the wide range of biological systems for which the determination of peroxidase activity is desired.

Peroxidase enzymes are common in plants, where they appear to be involved in a wide range of physiological functions, including hydrogen peroxide (H_2O_2)[1] metabolism, formation of lignin and suberin, cross-linking of cell wall components, and plant defense mechanisms [1–6]. Along with their importance for plant vitality, peroxidases also affect the consumer acceptability of plant-based foods through their role in fruit and vegetable coloration/discoloration [7], flavor development [8], nutritional properties [9], and texture [10]. Furthermore, peroxidases are used as indicators of the adequacy of vegetable blanching due to their high thermal stability and wide distribution [11–14]. Selected plant peroxidases, particularly horseradish peroxidase (HRP), are widely used in biotechnological applications, including decolorization of synthetic dyes, chemical syntheses, bioremediation, biosensors, and a range of analytical applications [15–18]. Current understanding of the role of peroxidases in each of the aforementioned areas is incomplete. 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 [19,20], fluorometric [21], chemiluminescent [22], and electrochemical [23] detection. Among these, colorimetric detection is most commonly used because it is simple and economical [24]. 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 H_2O_2. The reporter molecule is the hydrogen/electron donating (reducing) substrate that, after its oxidation, absorbs light in the visible region. Various reducing substrates, including guaiacol, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (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 HRP lists more than 200 substrates for peroxidase activity determinations [25]. As may be expected, commercially available kits for the quantification of peroxidase activity offer a range of reducing substrates. A common drawback with many of the substrates used for colorimetric-based assays, especially when applied directly to biological matrices, is interference

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[1] *Abbreviations used:* H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate); TMB, tetramethylbenzidine; $ABTS^{\cdot+}$, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation; SYPE, sweet yellow pepper extract; YOE, yellow onion extract.

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due to the presence of confounding compounds endogenous to living tissues. An important class of such compounds contains those that react with the assay's reporter molecule; that is, they react with the oxidized product derived from the reducing substrate as a result of the peroxidase-catalyzed reaction [26,27]. These confounding compounds include the broad spectrum of antioxidants common to biological matrices. 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 [26–34]. Disadvantages associated with methods aimed at fractionating confounding compounds may include the need for relatively costly specialized equipment (e.g., chromatographs), difficulty in identifying optimal 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 current study 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. 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-sulfonate) radical cation ($ABTS^{\cdot+}$) as the corresponding reporter molecule. $ABTS^{\cdot+}$ is generated as a result of the peroxidase-catalyzed reaction between ABTS and H_2O_2. 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 $ABTS^{\cdot+}$. During this treatment, $ABTS^{\cdot+}$ 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 $ABTS^{\cdot+}$ 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 on which it is based [35].

Materials and methods

Reagents

H_2O_2 (30 wt%, ACS reagent grade), HRP (type II, 150–250 U/mg solid), ABTS, 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 s 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 number 1 filter paper. The resulting filtrate from sweet yellow peppers was subsequently filtered through 0.45-μm PTFE (polytetrafluoroethylene) 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 $ABTS^{\cdot+}$

$ABTS^{\cdot+}$-containing solutions were prepared as described by Huang and coworkers [36] by incubating an aqueous solution of 7 mM ABTS and 2.45 mM potassium persulfate overnight (12–16 h) in the dark at room temperature. The resulting solution was then adjusted to 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, and 0.1 M sodium phosphate, pH 6.0) to initiate the reaction. Assays were performed at ambient temperature (20–22 °C). The increase in absorbance at 734 nm resulting from peroxidase-catalyzed $ABTS^{\cdot+}$ production was monitored for 30 min. 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 the amount of enzyme that catalyzes the production of 1 μmol of $ABTS^{\cdot+}$ per minute under the defined conditions. The absorptivity of $ABTS^{\cdot+}$ was taken as 15,000 M^{-1} cm^{-1} [37].

Traditional peroxidase assay using guaiacol

A 20-μl aliquot of SYPE was added to 2.18 ml of color-forming reagent (3.6 mM guaiacol, 0.135 mM H_2O_2, and 0.1 M sodium phosphate, pH 6.0) to initiate the reaction. The increase in absorbance at 470 nm resulting from peroxidase-catalyzed guaiacol oxidation was monitored for 30 min. Assays were performed at ambient temperature (20–22 °C).

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/$ABTS^{\cdot+}$ solution, prepared as described above, and allowed to react for 2 min (SYPE) or 30 min (YOE) in the dark at 0 °C. Following this treatment, peroxidase quantification was initiated by adding 0.2 ml of color-forming reagent (9 mM ABTS, 1.5 mM H_2O_2, and 0.1 M 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" section above.

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

Aliquots of SYPE or YOE, ranging from 20 to 100 μl, were added to 1.98 ml of $ABTS^{\cdot+}$ solution having an initial absorbance at 734 nm of between 0.9 and 1.0. The decrease in absorbance at 734 nm resulting from $ABTS^{\cdot+}$ reduction was monitored spectrophotometrically for 30 min. For reference purposes, controls