Colorimetric Method for the Determination of Lipoxygenase Activity

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A colorimetic assay for lipoxygenase activity has been developed. The assay is based on the detection of the lipoxygenase reaction product, linoleic acid hydroperoxide, by the oxidative coupling of 3-methyl-2-benzothiazolinone (MBTH) with 3-(dimethylamino)benzoic acid (DMAB) in a hemoglobin-catalyzed reaction. This test reaction is rapid and sensitive, and it offers advantages over other methods for detecting lipoxygenase activity. The assay is capable of detecting activity in a number of crude vegetable homogenates and should be particularly useful where a rapid visual determination of lipoxygenase activity is desired.

Keywords: *Lipoxygenase; colorimetric assay; rapid; vegetable homogenates; hemoglobin*

INTRODUCTION

Lipoxygenase (Linoleate:oxygen oxidoreductase, EC 1.12.11.12) catalyses the dioxygenation of unsaturated fatty acids, yielding a hydroperoxy fatty acid product. The physiological role of lipoxygenase in plants is not certain, although there is considerable evidence indicating its involvement in wounding and other stress responses (*1*). Lipoxygenase activity is also the first step in the pathway leading to the formation of a number of flavor and aroma compounds and has been shown to contribute to development of off-flavors in many vegetables during frozen storage (*2*).

Several methods are available for the quantitative assay of lipoxygenase. The details of these methods and their relative advantages have been extensively reviewed (*3*−*5*). The most commonly employed method makes use of the fact that the hydroperoxy lipid product of the reaction contains a conjugated diene which absorbs strongly at 234 nm. Reaction rates can thus be determined by measuring product formation through the change in absorbance at this wavelength (*3*). For quantitative kinetic measurements, especially with a purified enzyme, this method is preferred because it directly measures the reaction product. Its use with crude enzyme preparations is limited by the presence of other UV-absorbing material in the assay. It is also not suitable for situations where a rapid visual determination of activity is desired.

Assay methods which measure the consumption of the substrates, O_2 and linoleic acid, have also been used. The rate of O_2 consumption is followed with an oxygen electrode (*3*). Although accurate, this method requires specialized equipment and is not suitable for the rapid screening of multiple samples. With crude preparations, it requires a correction for O_2-consuming reactions other than that catalyzed by lipoxygenase. Substrate consumption can also be measured using ^{14}C-labeled linoleic acid followed by chromatographic separation of the radiolabeled products (*6*). This method is neither simple nor rapid.

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For many applications, such as inhibitor screening, thermal inactivation studies, cultivar screening, or protein purification, it is useful to have a method for rapid determination of relative lipoxygenase levels in multiple samples. In addition, in many instances it is desirable to have a method that does not require expensive or specialized equipment such as a UV-spectrophotometer or an oxygen electrode. In such cases a rapid visual assay is desirable. Several such methods exist. An assay based on the bleaching (reduction) of methylene blue was developed by Toyosaki (*7*). This method, although simple and rapid, is not particularly sensitive and is difficult to quantify. It has, nevertheless, been successfully used to follow lipoxygenase inactivation during blanching (*8*) and to differentiate between isozymes in soybeans (*9*). The co-oxidation of carotenoids in the presence of linoleic acid has also been used as a lipoxygenase assay. This carotenoid bleaching reaction was used successfully to distinguish between lipoxygenase isozymes in soybeans (*9*). However, this assay lacks sensitivit, and attempts to adapt it for use as a rapid visual assay in corn and green beans were not successful (*8*).

Rapid visual assays based on the detection of the reactive peroxy group of the lipid product have also been developed. Lipid hydroperoxides react quantitatively with I^- to form I_2, which can then be quantified colorimetrically by the addition of starch (*10*). This method is relatively simple and can detect lipoxygenase activity in crude preparations of a variety of fruits and vegetables. Nevertheless, this method has significant limitations. Activity could not be detected in many highly colored vegetables such as carrots and tomatoes. Furthermore, the reagents rapidly oxidize leading to an increasingly dark background. To quantify activity, spectrophotometric readings must be precisely timed.

One of the oldest methods for measuring lipoxygenase activity involves detection of the lipid hydroperoxide product with ferrous thiocyanate (*25*). This assay is based on the fact that peroxides, including lipid hydroperoxides, oxidize Fe(II) to Fe(III), which then reacts with thiocyanate to form a colored product. This product, however, is unstable, making quantification difficult. A

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much improved method uses xylenol orange for detecting Fe(III) (*11*). In this case the colored product is stable. This method has been used as an assay to screen for inhibitors of human platelet lipoxygenase (*12*).

Hydroperoxides also oxidize a variety of electron donors in a reaction catalyzed by heme compounds such as hemoglobin, hematin, and cytochrome *c*. With an appropriate electron donor, this reaction provides a colorimetric (*13*), fluorometric (*14*), or chemiluminescent (*15*) detection method for measuring lipid hydroperoxides and is suitable for assaying lipoxygenase (*16, 17*).

Our goal was to develop a method that would offer the speed and simplicity of the I_2/starch and methylene blue bleaching methods but with greater quantitative accuracy and applicability. We have developed a spectrophotometric method based on the peroxidase assay of Ngo and Lenhoff (*18*). In their peroxidase assay, added H_2O_2 acts as an oxidant and the peroxidase enzyme acts as a catalyst for the oxidative coupling of 3-methyl-2-benzothiazolinone (MBTH) with 3-(dimethylamino)benzoic acid (DMAB) to form a purple indamine dye. Here, we show that the same oxidative coupling reaction can be used to detect lipid hydroperoxides. In this case, the lipid hydroperoxide acts as an oxidant, and an added heme compound, such as hemoglobin, acts as the catalyst. Detection of lipid hydroperoxides by this method provides a simple, rapid, and sensitive assay of lipoxygenase.

MATERIALS AND METHODS

Chemicals. 3-Methyl-2-benzothiazolinone (MBTH), 3-(dimethylamino)benzoic acid (DMAB), hemoglobin (bovine), hematin (porcine), linoleic acid (free acid), Tween 20, and sodium lauryl sulfate were all purchased from Sigma (St. Louis, MO).

Preparation of Solutions. Stock solutions of 10 mM MBTH and 5 mg/mL hemoglobin (100× their final concentrations) were made by dissolving the reagents in water. These solutions were stable for several weeks in a refrigerator. Hematin at 0.1 mg/mL was prepared by dissolving 2 mg in 1 mL of 0.1 N NaOH, then diluting with 19 mL of water. A solution containing 20 mM DMAB and 100 mM phosphate buffer was prepared by dissolving 330 mg DMAB in 5 mL of 1 N HCl. This was then diluted to about 80 mL with water and 1.42 g of Na_2HPO_4 was added. The pH was adjusted to 6.0 with HCl and the volume brought to 100 mL with water.

Linoleic acid substrate was prepared as described by Axelrod et al. (*3*), except that the ratio of Tween 20 to linoleic acid was increased from 1:1 to 2:1 (w/w). At the assay pH of 6, a 1:1 ratio of detergent to lipid produced a turbid assay solution when linoleic acid was added at 0.5 mM. Increasing the Tween 20 to a 2:1 ratio eliminated the cloudiness. A 25 mM stock solution was prepared by adding 155 μL (140 mg) of linoleic acid and 257 μL (280 mg) of Tween 20 to 5 mL of water. The mixture was emulsified by drawing back and forth in a pasteur pipet, then clarified by adding 0.6 mL of 1 N NaOH. After dilution to the final volume of 20 mL, the solution was divided into 1 mL aliquots which were flushed with N_2 and stored at −20 °C. Linoleic hydroperoxide used as a standard was prepared enzymatically as described in Gibian and Vanderberg (*19*). The concentration of this standard was determined from its absorbance at 235 nm in pH 9 borate buffer assuming an extinction coefficient of 23 000 M^{-1} cm^{-1}.

Reaction of Linoleic Hydroperoxide. The reaction of linoleic hydroperoxide with the colorimetric reagents was carried out in a solution containing 5 mM DMAB, 0.1 mM MBTH, 50 mM phosphate buffer (pH 6.0), and either 50 μg/mL hemoglobin or 1 μg/mL hematin in a final volume of 1.0 mL. Reactions were initiated by the addition of 10 μL of a 4.1 mM solution of linoleic hydroperoxide in ethanol, and absorbance at 590 nm was monitored for 15 min. For the standard curve (shown later in Figure 2), 50 μg/mL hemoglobin was used and the amount of added linoleic hydroperoxide was varied. The reaction was allowed to proceed for 30 min prior to reading absorbances at 590 nm.

Preparation of Plant Extracts. Plant material was purchased at a local market. An enriched lipoxygenase was prepared from red-skinned potatoes by ammonium sulfate fractionation as described by Aziz et al. (*20*). The 45% ammonium sulfate pellet was resuspended in 100 mM phosphate buffer (pH 6) to approximately 5 mg/mL protein, divided into aliquots, and stored at −20 °C. Crude homogenates were prepared by grinding either 20 g of tomato, 10 g of corn, or 5 g of peas, green beans, and potatoes in 50 mL of ice cold water for 30 s in a Waring blender, then filtering the homogenate through 4 layers of cheesecloth.

Lipoxygenase Assay Conditions. All assays were performed at room temperature (23° C). Initially, assay conditions involved incubating a sample in 50 mM phosphate buffer (pH 6.0) and 0.5 mM linoleic acid (plus Tween 20), then adding DMAB, MBTH, and hemoglobin to determine the amount of product formed. Although the latter three compounds could be combined in a single solution and a single addition made, this mixture slowly developed a background color as the mixture aged. To avoid this, the DMAB and MBTH were added separately to samples. Subsequently it was determined that including DMAB during the incubation had no significant effect on enzyme activity. This allowed for a simplified standard assay procedure using two working solutions prepared from the stock solutions described above. Solution A was prepared by mixing 10 mL of the 20 mM DMAB, 100 mM phosphate buffer solution (pH 6), 0.4 mL of the 25 mM linoleic acid stock, and 9.6 mL of water. Solution B was prepared by mixing 0.4 mL of 10 mM MBTH, 0.4 mL of 5 mg/mL hemoglobin, and 19.2 mL water. For the standard two-step assay, the sample, in a volume of 2 to 10 μL, was incubated with 0.5 mL of solution A. After incubation for the specified amount of time (generally 5 min), 0.5 mL of solution B was added. After an additional 5 min, 0.5 mL of 1% (w/v) sodium lauryl sulfate was added to terminate the reaction. Absorbance at 598 nm was then determined.

For samples with low activity, such as the crude homogenates of tomato and corn (shown later in Table 1), the amount of added sample was increased to 0.1 mL and the reaction time increased to 20 min prior to the addition of solution B. In these cases, to avoid changing the final concentrations of the reagents, the amount of water used to prepare solution A was reduced to 5.6 mL, and 0.4 mL of this modified solution A was used such that the final volume of the incubation remained 0.5 mL. To correct for the colored material in crude homogenates, blanks were spiked with the same amount of homogenate as the samples but only after the addition of the sodium lauryl sulfate. Blanks and samples were then centrifuged for 5 min in a tabletop centrifuge to remove any particulate matter or suspended starch prior to determining absorbance at 598 nm.

For the direct comparison of the colorimetric method with the conjugated diene method, 2.0 mL samples containing 100 mM phosphate buffer, 0.2 mM linoleic acid, and 58 μg of potato protein were incubated for 0 to 4 min, then placed in a boiling water bath and boiled for 3 min. After the mixture was cooled to room-temperature, two 0.8 mL aliquots were removed. To one was added 0.4 mL of water, and the absorbance at 235 was determined. To the other was added 0.2 mL of 30 mM DMAB and 0.2 mL of a solution containing 0.6 mM MBTH and 300 mg/mL hemoglobin. After 15 min, absorbance at 590 nm was determined. A blank and standard were prepared in the same way as the samples except the potato enzyme was omitted and a known amount of linoleic hydroperoxide was added to the standard.

In some instances a one-step assay procedure was used. For this procedure solutions A and B were combined prior to the addition of the sample. The reaction was allowed to proceed for times up to 20 min, then the reaction was terminated by the addition of 0.5 mL of 1% lauryl sulfate, and the absorbance at 598 was determined.

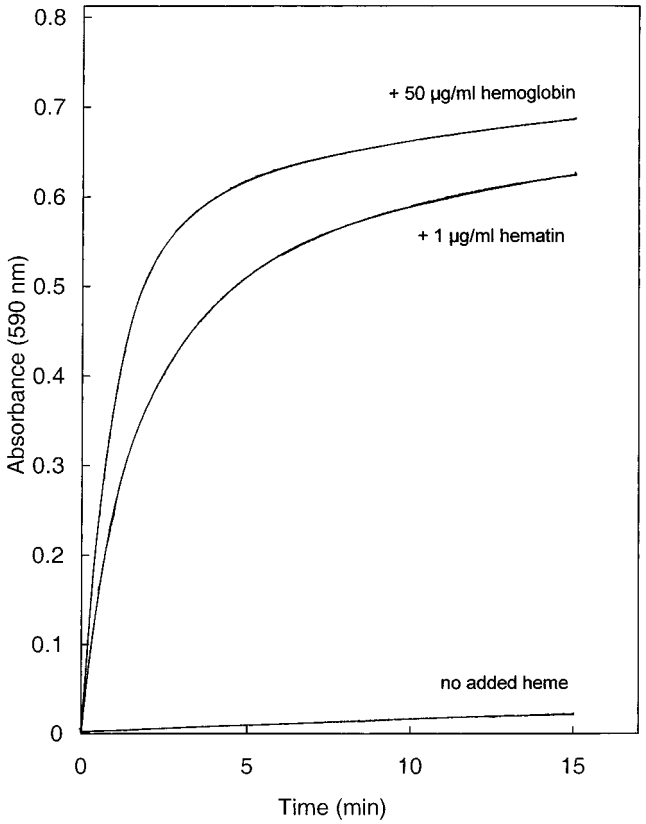

Figure 1. Time course for colored product formation in the reaction of linoleic hydroperoxide with DMAB and MBTH in the presence of heme compounds. Reaction conditions were as described in Materials and Methods with a final linoleic hydroperoxide concentration of 41μM.

RESULTS AND DISCUSSION

Reaction of Linoleic Hydroperoxide. Reaction of linoleic hydroperoxide with MBTH and DMAB in the presence of hemoglobin or hematin formed a purple reaction product (Figure 1). This reaction was rapid, approaching completion in 5 min at room temperature. The reaction was dependent on the presence of heme, with hemoglobin at 50 μg/mL or hematin at 1.0 μg/mL giving similar results. The colored product showed an absorbance maximum at 590 nm suggesting that it is the same indamine dye as reported by Ngo and Lenhoff (*18*). The absorbance at 590 was linear with the concentration of linoleic hydroperoxide up to at least 35 μM (Figure 2); the apparent extinction coefficient was 18 700 M^{-1} cm^{-1}. Color formation showed a broad pH optimum between 5.5 and 7.0. Once formed, the colored product was stable overnight. Other peroxides were much less reactive than linoleic acid hydroperoxide. Essentially no color was produced after 30 min of incubation with *tert*-butyl peroxide at 100 μM. Hydrogen peroxide also produced no color when hematin was used as the catalyst. Hemoglobin, however, catalyzed rapid color formation with H_2O_2. Linoleic hydroperoxide has been shown to be much more reactive than either *tert*-butyl or hydrogen peroxide in a similar heme-catalyzed reaction (*16*).

Sodium lauryl sulfate was added to terminate the lipoxygenase reaction and clarify the solution prior to determining absorbance. The detergent could not be added prior to color development because it interfered with color formation. When added after the colored product had formed, the detergent caused the peak of absorbance to shift from 590 to 598 nm (visually a change from purple to blue), but it left the height of the peak unchanged.

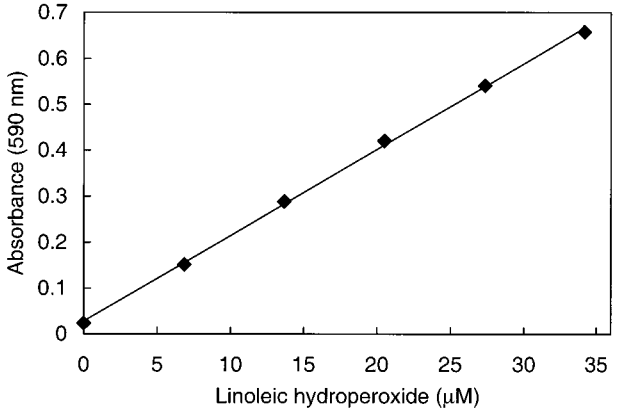

Figure 2. Standard curve for color formation with linoleic hydroperoxide.

Two other types of compounds have the potential to cause significant interferences in this assay. Metal ions such as Cu(II) and Fe(III) may cause the oxidative coupling of MBTH and DMAB (*18*). The presence of these ions would lead to color formation even in the absence of any linoleic hydroperoxide. We confirmed this by adding 50 μM $CuSO_4$ to the colorimetric reagents and observed significant color formation. This effect, however, could be completely blocked by the addition of 100 μM EDTA. We do not believe that the presence of low levels of metal ion contaminants is contributing to color formation observed during the reaction with linoleic hydroperoxide or in a lipoxygenase assay, because the addition of 100 μM EDTA had no effect on either (data not shown). Antioxidants such as ascorbate are a potentially more significant source of interference in plant extracts. Although a detailed characterization of this interference was not undertaken here, the extent of inhibition by ascorbate, and presumably other antioxidants, appeared to depend on the level of hydroperoxide in the assay (data not shown). At low levels of hydroperoxide (less than 20 μM) ,as little as 10 μM ascorbate reduced the extent of color formation. For a quantitative determination of hydroperoxides, the samples would need to be essentially free of this interfering material.

Lipoxygenase Assay. This colorimetric method for linoleic hydroperoxide determination was tested as a lipoxygenase assay using an ammonium sulfate precipitate from potatoes. The assay was directly compared to the conjugated diene method. Samples of the potato preparation were incubated with linoleic acid, then after various amounts of time the enzymatic reaction was terminated by boiling the samples. The amount of linoleic hydroperoxide in these samples was then quantified colorimetrically and this value compared with the value determined by measuring the absorbance at 235 nm. The two methods showed good agreement (Figure 3). Similar results were obtained in experiments where the amount of added enzyme rather than the time of incubation was varied (data not shown).

Because the hemoglobin-catalyzed formation of the colored product occurs at the same pH as the enzymatic reaction, we tested whether the two reactions could be carried out simultaneously in a single coupled reaction. This was possible, but with significant complications. When all reagents were combined and incubated with

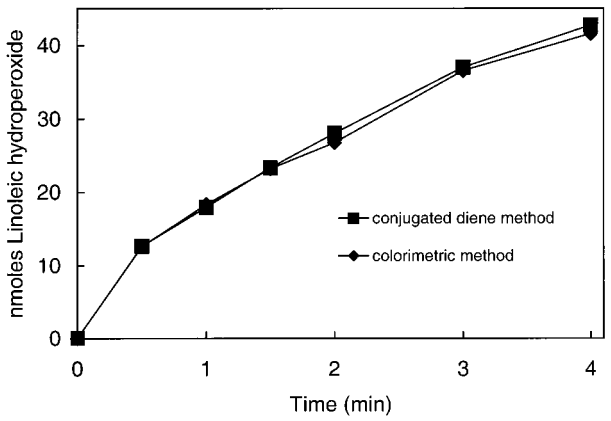

Figure 3. Comparison of the colorimetric and conjugated diene assay methods.

the enzyme, color formation was observed, following a short lag period. The amount of color formed, however, was not proportional to the amount of added enzyme (Figure 4a).

The nature of this nonlinearity was complex and strongly affected by the amount of hemoglobin used in the assay. With a low hemoglobin concentration (5 μg/mL) activity was proportional to the amount of enzyme only at low enzyme levels (Figure 4a). At higher enzyme levels the reaction appears to saturate with increasing amounts of enzyme. This is expected because with low hemoglobin the hemoglobin-catalyzed coupling reactions become limiting as the amount of lipoxygenase activity is increased. However, if higher hemoglobin concentrations were used, activity was still not proportional to the amount of added enzyme. In this case the assay failed to detect activity when the amount of added enzyme was below a certain threshold level. This threshold effect was the result of hemoglobin inhibition of the reaction (Figure 4b). At low enzyme levels, hemoglobin inhibition was much more pronounced than at high enzyme levels. This difference in hemoglobin inhibition at different enzyme levels explains why, at a given hemoglobin concentration, the assay fails to give a linear response with amount of enzyme. A possible explanation for this hemoglobin inhibition would be the well-characterized product activation of lipoxygenase (*21*, *22*). At low levels of lipoxygenase and high levels of hemoglobin the rate of product removal exceeds the rate of product generation, such that insufficient product accumulates to keep the enzyme active. Complete inactivation of the soybean lipoxygenase by product removal has previously been shown (*23*).

For potato lipoxygenase, the inhibition by high hemoglobin levels and the resulting lack of a linear response versus amount of added enzyme in the one-step coupled reaction limits its usefulness for quantifying enzyme activity. However, as explained below, lipoxygenases from other sources, such as corn, are apparently less sensitive to hemoglobin inhibition and can be assayed by the one-step procedure.

Because addition of the colorimetric reagents does not stop the enzymatic reaction, designing a practical two-step assay proved problematic. Adding lauryl sulfate at a concentration as low as 0.015% completely stopped the enzymatic reaction. However, even at this low level some interference with color formation was apparent. Boiling the samples was the simplest and most direct way to stop the enzymatic reaction prior to determining the amount of product formed. This would be the preferred method if a quantitative determination of the product was desired. Our primary interest, however, was in developing a simple and rapid method for determining the relative lipoxygenase activity levels in multiple samples. For such a determination knowing the actual amount of product formed or the true initial rate is not needed. Simply adding the colorimetric reagents and allowing the enzymatic reaction to continue as color developed proved to be satisfactory. After 5 min of color development sodium lauryl sulfate was added to stop further enzymatic activity. Color formation was linear with the amount of added enzyme (Figure 5), indicating that this assay is suitable for measuring relative lipoxygenase activity levels.

Lipoxygenase Activity in Crude Homogenates. Several vegetables known to contain high lipoxygenase activity were assayed to determine whether this method would be suitable for quantifying lipoxygenase levels in crude homogenates. With peas, green beans, and potatoes activity could be detected in volumes of homogenate equivalent to as little as 0.2 mg of plant material. Color formation was proportional to the amount of homogenate added, indicating that this assay can be used to quantify activity levels (Figure 6).

Tomatoes and yellow sweet corn are two vegetables known to contain significant lipoxygenase activity (*8*, *21*) for which the I_2/starch method failed to detect activity in crude homogenates (*10*). We tested homogenates of these two vegetables using the DMAB/MBTH assay, both as a two-step reaction, where lipid hydroperoxide was allowed to accumulate then determined colorimetrically, and as a one-step coupled reaction, where the colorimetric reaction occurred concurrent with the enzymatic reaction. With tomatoes, activity was readily detected in the two-step assay (Table 1), but very little activity was present in the one-step coupled reaction. This relatively low activity was further reduced if a high hemoglobin concentration was used. Tomato lipoxygenase has been shown to be strongly product-activated (*21*), which would explain the low activity in the coupled assay, particularly at high hemoglobin concentrations.

Yellow sweet corn, by contrast, showed activity only in the one-step coupled assay (Table 1). This color formation was completely dependent on the presence of linoleic acid, confirming that it is in fact due to lipoxygenase activity. Activity was much greater at the high hemoglobin concentration, indicating that lipoxygenase from corn is less dependent than the potato or tomato enzyme on the presence of product to maintain activity. The apparent lack of activity in the two-step assay indicates that the product, linoleic hydroperoxide, does not accumulate during the incubation. This may be the result of further reactions which consume the linoleic hydroperoxide occurring rapidly in a crude homogenate. Our attempts to detect the accumulation of linoleic hydroperoxide in the corn assay by the xylenol orange (*11*) or leuco-methylene blue methods (*16*) were also unsuccessful (data not shown). This would also explain the failure of the I_2/starch assay to detect activity in corn because it is a two-step assay which measures accumulated product.

Lipoxygenase has been proposed as an indicator enzyme for determining blanching sufficiency of vegetables for frozen storage (*2*, *10*). To be useful as an indicator enzyme, however, a simple method for the

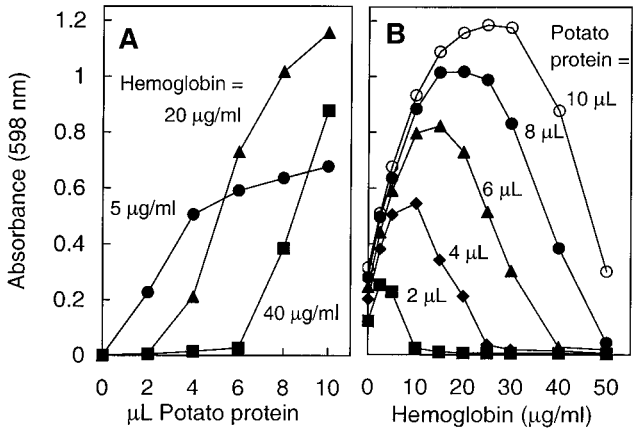

Figure 4. Effect of variable hemoglobin and enzyme levels on the formation of colored product in the coupled one-step assay. (A) Product formation vs amount of potato lipoxygenase at three different hemoglobin concentrations. Lipoxygenase activity was determined by incubating 0−10 μL of a 5.8 mg/mL potato protein solution for 10 min in the one-step assay medium as described in Materials and Methods. Assays were performed at three hemoglobin concentrations as indicated in the figure. (B) Product formation vs hemoglobin concentration at various levels of potato lipoxygenase. As in (A) except product formation is plotted vs the hemoglobin concentration with the amounts of added potato protein indicated in the figure.

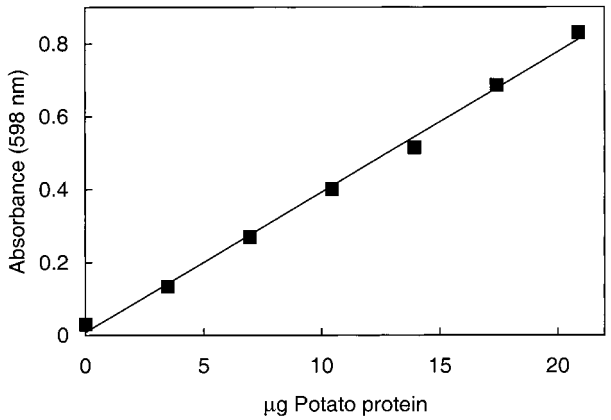

Figure 5. Absorbance vs amount of potato lipoxygenase in the two-step colorimetric assay. Assays were performed under the standard two-step assay conditions as described in Materials and Methods with a final hemoglobin concentration of 50 μg/mL.

rapid visual determination of activity is needed. For this application the DMAB−MBTH method described here offers advantages over existing rapid assays. In this method activity causes the appearance of color against a nearly colorless blank. An absorbance increase as small as 0.1 at 598 nm can easily be detected by eye. By contrast, the methylene blue bleaching method (*7*) is based on the disappearance of color. Activity levels are determined by measuring the time until a color begins to change, a judgment that is visually difficult. With the I_2/starch method the background color in the blank increases rapidly, complicating the visual judgment of activity. This method also fails to detect activity in corn and tomatoes as described above. The DMAB−MBTH method is also considerably more sensitive than either of these. With green beans, for example, more than 100 mg of plant material was used per assay with the methylene blue or I_2/starch methods (*8, 10*). By contrast, a volume of green bean homogenate equivalent

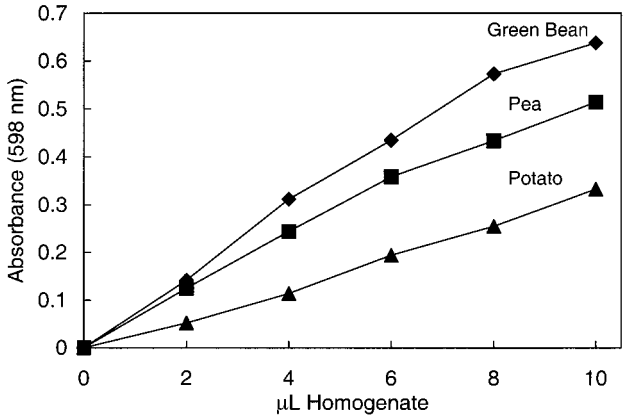

Figure 6. Absorbance vs amount of crude homogenate in the two-step colorimetric assay. Assay conditions were as in Figure 5.

Table 1. Lipoxygenase Activity in Crude Homogenates of Corn and Tomato[a]

		tomato		corn	
assay	hemoglobin concentration	complete	minus LA	complete	minus LA
one-step	5 μg/mL	.091[b]	.005	.082	.000
one-step	50 μg/mL	.020	.007	.346	.004
two-step	50 μg/mL	.771	.000	.007	.011

[a] Assays contained 50 μL of crude homogenate and were incubated for 20 min by either the one-step or the two-step procedure as described in Materials and Methods, with hemoglobin concentrations as indicated. The assay medium contained either 0.25 mM linoleic acid (complete) or no linoleic acid (minus LA). [b] Absorbance at 598 nm.

to 1 mg of plant material yielded an absorbance change of 0.6 in the DMAB−MBTH assay (Figure 6). Because the greater sensitivity of the DMAB−MBTH assay allows for the use of much less plant material, less interfering material is included. This may explain why we are able to detect activity in carotenoid-containing extracts such as tomatoes where the I_2/starch method fails.

The greatest limitation to using this assay with crude preparations is the interference from antioxidants such as ascorbic acid. For material with high lipoxygenase activity, such as potatoes, peas, and beans (Figure 6), activity is readily detected because the amount of crude homogenate needed in the assay is very small and the amount of ascorbic acid included with the homogenate is also small. For other vegetables with low lipoxygenase activity and/or high levels of ascorbic acid this assay may not be suitable for use with a crude homogenate. We were unable to reliably detect lipoxygenase activity in crude homogenates of carrots, broccoli, or green peppers with this assay.

Other rapid lipoxygenase assays exist which also detect linoleic hydroperoxide colorimetrically, but to our knowledge these have not been applied to plant material. The xylenol orange assay (*12*) is not suited for use as a visual assay because it involves a shift in an absorbance maximum rather than the appearance of color against a colorless blank. The leuco-methylene blue assay (*16*) involves a hemoglobin-catalyzed oxidation and may also be useful as a rapid visual detection of lipoxygenase activity. The leuco-methylene blue reagent, however, has low water solubility, and high concentrations of detergent must be included in the assay. This precludes its use in a one-step assay.

In both the DMAB−MBTH assay and the leucomethylene blue assay (*16*), hemoglobin is used as a catalyst in the reaction of linoleic hydroperoxide with the colorimetric reagents. The suitability of these assays for the measurement of lipoxygenase activity may seem somewhat surprising given that hemoglobin itself has quasi-lipoxygenase activity (*24*). The lipoxygenase activity of hemoglobin, however, does not appear to be significant under the assay conditions used here. If substantial hemoglobin lipoxygenase activity was present, significant color formation would occur in the reagent blank. This was not observed. The small and variable amount of color that does appear in the blank (an absorbance at 598 nm generally less than 0.050) is almost certainly from the low level of preformed linoleic hydroperoxide present as a contaminant in the substrate. Our assay conditions (pH 6.0, 0.25 mM linoleic acid) are far from the optimums for hemoglobin lipoxygenase activity (pH 8.5, $S_{0.5}$ linoleic acid of 0.5 mM). If assays were to be performed at higher pH and higher linoleic acid concentrations then this hemoglobin activity may potentially become a complication. A further demonstration that the lipoxygenase activity measured in the DMAB−MBTH assay is not due to hemoglobin lipoxygenase activity is the substrate specificity of the reaction. Potato lipoxygenase, for example, had substantial activity with α-linolenic acid (0.6 times that obtained with linoleic acid) when assayed by either the conjugated diene method or the DMAB−MBTH procedure (data not shown). In contrast, the quasi-lipoxygenase activity of hemoglobin is reported to have no activity with α-linolenic acid (*24*).

If a quantitative measure of the lipoxygenase reaction rate is desired, the O_2 electrode and conjugated diene methods are clearly the methods of choice. These methods allow for continuous measurements and monitor directly the disappearance of substrate or the appearance of product. However, in instances where only relative activity levels are needed, as in cultivar screening, inhibitor screening, or thermal inactivation studies, the DMAB−MBTH method may be a suitable alternative. It would be particularly useful in situations involving large numbers of samples or where the instrumentation required for other methods is not available.

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