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ARTICLE

High-Throughput Assay for Detection of Soybean Lipoxygenase-1

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A high-throughput assay was developed to detect soybean lipoxygenase 1 (LOX-1) using a multilabel plate reader. The assay was also adapted to a single cell fluorometer. Fluorescein is degraded by linoleic hydroperoxide produced from soybean lipoxygenase and linoleic acid. The decrease in fluorescence is measured over time, and the area-under-the-curve (AUC) is used to quantify the LOX-1 content of soybean extract. A dose-dependent response is seen with varied dilutions of pure LOX enzyme or soybean extracts. Percent recovery was between 97% and 108%, and relative standard deviation was 4.3%. Advantages of the assay include the reduced preparation time of samples and reduced use of reagents in the high-throughput assay. Multiple samples can be measured in a single run with a multilabel plate reader.

KEYWORDS: Soybean; lipoxygenase; fluorescein; high-throughput

INTRODUCTION

Soy foods are recognized for their health beneficial properties. Soy food consumption has been associated the reduced risk of cardiovascular disease, osteoporosis, and some types of cancer (*1*). Replacing some dietary animal protein with soy protein has been recommended for lower risk of chronic disease (*2*). Although soy foods are widely consumed in many Asian countries, they are less popular in the United States. One possible reason for the dislike of soy products is the distinct "beany" flavors that they possess (*3*). These undesirable flavor components are primarily the peroxidation products of polyunsaturated fatty acids catalyzed by lipoxygenase and hydroperoxide lyase.

Soybean lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a hydroperoxidase and catalyzes the oxidation of unsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene moiety. Conventional soybean seeds generally have three LOX isozymes including LOX-1, LOX-2, and LOX-3. The resulting products of the oxidation are converted to the undesirable odors and flavors associated with soy foods through a number of other reactions (*4*). Processing treatments at high temperatures are used to inactivate the lipoxygenase (LOX) enzyme; however, the heat may denature proteins and destroy other nutrients or health components such as isoflavonoids (*5*, *6*). Through selective breeding, soybean lines have been developed that are null for one or more LOX isozymes. The goal for cultivation of these soybeans is to develop soy food products with reduced fatty acid oxidation and therefore improved flavor.

Rapid detection of the LOX enzymes is important to soybean breeding programs. Several colorimetric methods that can be measured visually or spectrophotometrically have been developed to detect the presence of lipid hydroperoxides that result from the LOX enzyme activity in soybeans or other plants (*7*−*9*). One of the earliest methods involves the spectrophotometric measurement of conjugated diene generated from the LOX catalyzed peroxidation reaction of unsaturated fatty acid substrates at 234 nm (*10*). A colorimetric method described by Suda et al. (*7*) uses the bleaching of methylene blue or β-carotene as a spectrophotometric or visual indicator to determine the three isozymes of LOX. The methylene blue and β-carotene bleaching method was modified by Narvel et al. (*11*) for use with small chips of soybean sample, which reduces sample preparation time. This modification of the method allows more rapid analysis but can provide only a qualitative measure of the enzyme through a visual color change. The iodine-starch method and 3-(dimethylamino)-benzoic acid−3-methyl-2-benzothiazolinone (DMAB−MBTH) method allow for relative quantification of LOX among samples. The iodine-starch method and DMAB−MBTH methods can measure activity of LOX in crude vegetable homogenate, and absorbance is read once after a specified time (*8*, *12*). However, the presence of the LOX enzyme is measured by color change after a predetermined time, which does not account for the benefits of the measuring reaction, and may not be as accurate as those accounting for reaction thermodynamics and kinetics such as the quantification using area-under-the-curve (AUC). Measurement of blood platelet 12-lipoxygenase with ferrous oxidation of xylenol orange has been adapted to a high-throughput format (*9*), and could possibly be adapted as a method for LOX detection in plants. However, one drawback of this method is that it may result in a false positive if the sample is able to chelate iron. This problem is more likely when using antioxidant-containing material such as soybeans. The presence of pigments may also interfere with the estimation of LOX using these colorimetric methods. In addition, measurement of O_2 consumption by electrode is another method

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that can quantify the LOX activity, although special equipment is required (*10*). To date, the possibility to develop a fluorescence assay for LOX determination has not been investigated.

Rapid and efficient measurement of LOX activity is desirable for screening LOX-null soybean cultivars and will be useful for soybean breeding programs. Despite the number of available detection methods, there is still a need for an efficient and quantitative method for measurement of LOX activity in soybeans. The purpose of this study was to develop a fluorescence assay for measurement of soybean LOX that can be adapted for high-throughput analysis and therefore improve the screening and quantification of LOX in modified soybeans.

MATERIALS AND METHODS

Materials and Chemicals. Whole soybeans were provided by Dr. William Kenworthy in the Department of Plant Science and Landscape Architecture at the University of Maryland, College Park. Varieties included Manokin, Japan 123 (Null for LOX-1, LOX-2, and LOX-3), and experimental lipoxygenase-null lines. Fluorescein, methylene blue, linoleic acid, and Tween 20 were obtained from Sigma Aldrich (St. Louis, MO). 15-Lipoxygenase Type 1 (from soybean) was obtained from Cayman Chemical (Ann Arbor, MI). All other reagents were of highest commercial grade.

Soybean Extract. Soybeans were ground to 20-mesh using a household coffee grinder. For the methylene blue assay and single-cell fluorometric assay, 0.10 g of soy meal was combined in a 15 mL test tube with 10 mL of ultrapure water and vortexed twice for 15 s. For the 96-well high-throughput fluorescein assay, 0.020 g of soy meal was combined with 10 mL of ultrapure water in a 15 mL test tube and vortexed twice for 15 s. Test tubes were held at 10 °C for 1 h and then centrifuged at 1500 rpm for 5 min. The supernatant was collected and used immediately.

Lipoxygenase-1 Assay with Methylene Blue. The methylene blue assay for soybean lipoxygenase-1 was conducted according to the method previously described by Suda et al. (*7*) and Narvel et al. (*11*) with minor modification. Commercially purchased 15-lipoxygenase Type 1 (from soybean) was diluted in 200 mM pH 9.0 sodium borate buffer to five concentrations and held on ice until use. Sodium linoleate substrate was prepared with 90 mg of linoleic acid, 40 μL of Tween 20, 875 μL of 0.5 M sodium hydroxide, and ultrapure water in a final volume of 25 mL. The final reaction mixture contained 500 μL of 200 mM pH 9.0 sodium borate buffer, 100 μL of 200 mM methylene blue solution, 100 μL of 12.6 mM sodium linoleate solution, 200 μL of ultrapure water, and 100 μL of soybean extract or pure enzyme in a 1.5 mL cuvette. Absorbance was read at 660 nm and recorded at 10-s intervals for 6 min.

Lipoxygenase-1 Assay with a Single Cell Fluorometer Using Fluorescein as the Probe. Fluorescein stock solution was prepared at a concentration of 0.1 mM in pH 9.0 sodium borate buffer and stored at 0 °C. Secondary stock solution was prepared by diluting the original stock solution to 0.01 mM. The secondary stock solution was diluted to 100 nM in pH 9.0 buffer to make the working solution. Pure soybean lipoxygenase was diluted in pH 9.0 sodium borate buffer to five concentrations and held on ice until use. The final reaction mixture contained 600 μL of 100 nM fluorescein working solution, 225 μL of pH 9.0 buffer, 100 μL of 12.6 mM sodium linoleate solution, and 75 μL of soybean extract or pure enzyme in a 1.5 mL cuvette. A blank was prepared with buffer in place of fluorescein to determine the baseline fluorescence of the mixture. The fluorescence was read in a single-cell fluorometer and recorded at 10-s intervals for 6 min. The excitation and emission wavelength was 485 and 515 nm, respectively. Fluorescence of the blank was subtracted from the fluorescence of the standard or sample to obtain net fluorescence. The AUC was calculated for net fluorescence as described by Moore et al. (*13*):

$$\mathrm{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_i - 1/f_0 + 0.5(fi/f_0)$$

where f_0 is the fluorescence at 0 min and f_i is the final reading. The concentration of the commercially purchased enzyme dilutions was plotted against the calculated AUC and a standard curve was determined by linear regression. The concentration of LOX-1 in soybean extract was calculated using the equation derived from the standard curve.

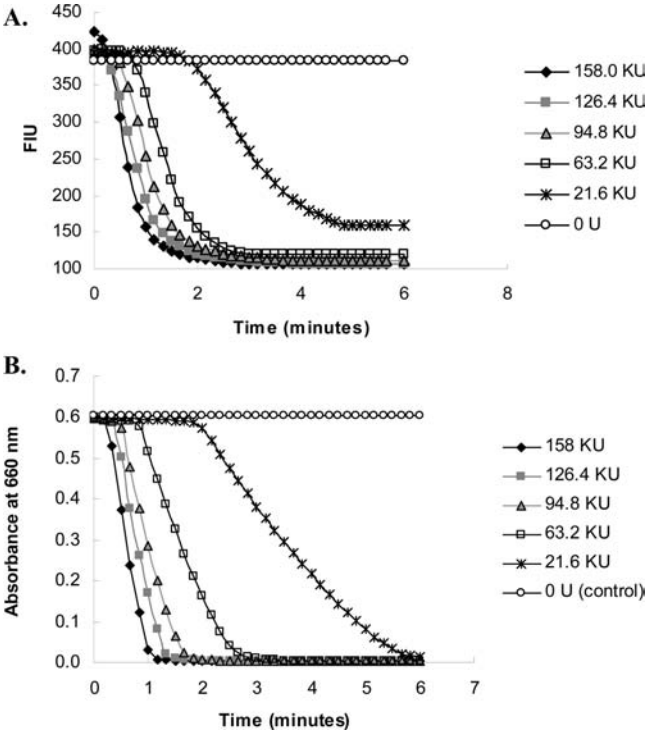

Figure 1. Comparison of (**A**) fluorescein LOX-1 assay and (**B**) methylene blue LOX-1 assay. The reagents were (**A**) 225 μL of 200 mM pH 9.0 sodium borate buffer, 600 μL of 100 nM fluorescein, 100 μL of 12.6 mM sodium linoleate solution, 75 μL of 15-lipoxygenase (varied concentrations); (**B**) 500 μL of 200 mM pH 9.0 sodium borate buffer, 100 μL of 200 mM methylene blue, 100 μL of 12.6 mM sodium linoleate solution, 100 μL of 15-lipoxygenase (varied concentrations), and 200 μL of ultrapure water. Absorbance or fluorescence was measured for 6 min, and each assay was measured in triplicate. FIU = fluorescence intensity units.

High-Throughput Lipoxygenase-1 Assay Using Fluorescein as the Probe. 15-Lipoxygenase Type 1 (from soybean) was diluted with pH 9.0 sodium borate buffer to five concentrations between 5 and 25 kilounits (KU). The dilutions were prepared immediately before use and held on ice. 200 nM fluorescein working solution was prepared from the 0.01 mM secondary stock solution (described above). Sodium linoleate was prepared as described above. 100 μL of 200 nM fluorescein working solution or pH 9.0 buffer (blank) was added to each well using a multichannel pipet. 120 μL of pure LOX-1 enzyme, soybean extract, or buffer (control) was added. Finally, 80 μL of 12.6 mM sodium linoleate solution was added to each well using a multichannel pipet to initiate the enzyme reaction. Fluorescence was recorded continuously for 6 min using a Victor^3 multilabel plate reader (Perkin-Elmer). Excitation and emission wavelengths were 485 and 515 nm, respectively. AUC calculations were performed as described above.

Statistical Analysis. SPSS (version 10.0.5, 1999, SPSS Inc., Chicago, IL) was used to conduct statistical analysis. Means were compared by one-way analysis of variance with Tukey's posthoc test. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Development of LOX-1 Assay Using Fluorescein as the Probe. The first step in developing a high-throughput method was determining the appropriate probe for LOX activity detection. Lipoxygenase has both dioxygenase and hydroperoxidase activity. Many assays currently used for LOX measurement were developed based on the lipid peroxidation reaction catalyzed by LOX. Peroxide radicals are generated during the oxidative chain reaction with or without LOX. Fluorescein ($C_{20}H_{12}O_5$) has been used as a probe for free radical scavenging capacity assays, as it is

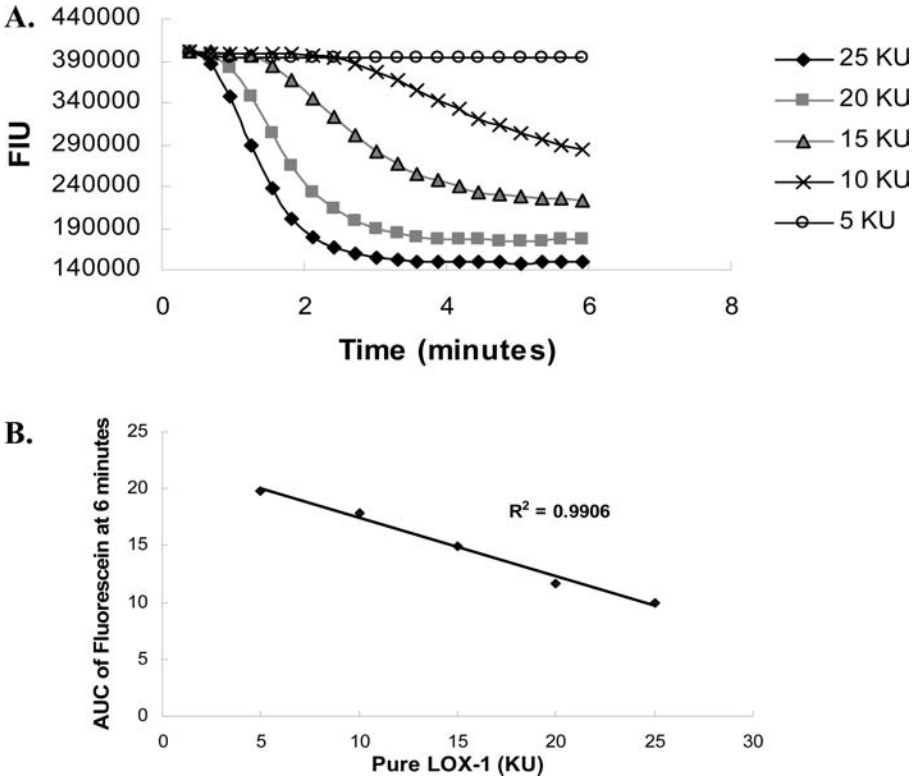

Figure 2. (**A**) High-throughput fluorescein LOX-1 assay. The reagents were 100 μL of 200 nM fluorescein in pH 9.0 buffer, 120 μL varied concentration of 15-lipoxygenase, and 80 μL of 12.6 mM sodium linoleate solution. Fluorescence was measured for 6 min. Each lipoxygenase concentration was measured in triplicate. (**B**) Standard curve of high-throughput fluorescein LOX-1 assay. Concentration of 15-lipoxygenase (from soybean) is plotted against the area under the curve of fluorescence at 6 min of reaction time. All measurements were conducted in triplicate. FIU = fluorescence intensity units.

degraded by peroxyl radical (ROO•). This reaction is used as a probe in the oxygen radical absorbance capacity (ORAC) assay (*14*) and hydroxyl radical scavenging capacity (HOSC) assay (*13*). According to Ou et al. (*14*) and Moore et al. (*13*), fluorescein is stable and resists degradation by light in the plate reader, so it may serve as an ideal probe for use in a fluorometric assay of LOX activity.

Figure 1A shows the dose−response of fluorescein at different levels of the pure LOX-1 enzyme. The pattern of **Figure 1A** is very similar to that of **Figure 1B**, which shows the dose−response of methylene blue at the selected pure LOX-1 concentrations, indicating that fluorescein may be used as a quantitative probe for LOX-1. Another reaction was conducted with fluorescein and LOX-1 without the sodium linoleate substrate, and no change in fluorescence was observed over time (data not shown). This indicated that fluorescence was not affected by LOX-1 in the absence of the PUFA substrate. There are multiple LOX isozymes known, and LOX 1, 2, and 3 are most prevalent (*10*). These three isozymes are active at different pH levels. Assays to detect the presence of all enzymes must therefore be conducted at the three pH levels. Linoleic acid is commonly used as a substrate for LOX-1. LOX-2 and LOX-3 are most active at pH 6−7, and it can be difficult to distinguish them based on pH alone (*10*).

The use of fluorescein for detection of soybean LOX-1 was modeled using the previous methylene blue bleaching method (*7*). The methylene blue LOX assay measures the bleaching of methylene blue by hydroperoxides formed by the radical-mediated enzymatic oxidation of linoleic acid (*15*). The new fluorescein assay measures the decrease in fluorescence of fluorescein as it is degraded by hydroperoxide radical attacks. This test was conducted at pH 9.0 to measure the LOX-1 isozyme. This isozyme was chosen because it was available commercially while LOX-2 and LOX-3 were not. LOX-1 also has stronger catalytic activity than the other isozymes according the observations of Suda et al. (*7*). Ou et al.(*14*) stated that a fluorescein solution will lose its fluorescence intensity below pH 7. Therefore, detection of the LOX-2 and LOX-3 isozymes with this fluorescein probe may have less definitive results than detection of LOX-1. Because lipoxygenase also has dioxygenase activity, a measurement of conjugated diene formation was performed according to the spectrophotometric method of Axelrod et al. (*10*) and compared to the fluorescein lipoxygenase assay (figure not shown). The increase in absorbance at 234 nm coincided with the decay of fluorescein over time and confirmed the LOX-1 activity.

Interestingly, it was discovered during assay development that Tween 20 contributed to fluorescence, and therefore the amount added to the substrate was decreased to the point where there was sufficient emulsification in the solution but little fluorescence. Fluorescence of compounds in the blank solution was accounted by subtracting the blank reading from the sample readings.

Five minutes is the time recommended for bleaching of methylene blue by LOX-1 (*7*). Six minutes was an appropriate

Table 1. Linearity of LOX-1 Fluorescein Assay[a]

	R^2
day 1	0.9906
day 2	0.9652
day 3	0.9494
day 4	0.9675
day 5	0.9872
day 6	0.9915

[a] Measurements were conducted under conditions for the high-throughput fluorescein LOX-1 assay in a 96-well plate. Measurements were conducted in triplicate.

time for the degradation of fluorescein when using the quantities of reagents described above. After the initial degradation of fluorescein with peroxide radicals, products of lipid oxidation are formed which can increase the fluorescence of the solution. Therefore, the fluorescein assay has a cutoff point at which the measurement was stopped. Higher enzyme concentrations begin to produce lipid oxidation products more quickly, and the assay measurement was ended when the fluorescence of the strongest concentration reached its lowest level, which occurred after 6 min.

High-Throughput Fluorescein LOX-1 Assay. A high-throughput quantitative assay is needed to rapidly screen the large soybean seed samples from the breeding program. The assay was modified for use in a Victor^3 multilabel plate reader. **Figure 2A** shows the dose−response of fluorescein at different levels of the pure LOX-1. An excellent linear relationship (R^2 = 0.9906) between LOX-1 concentration and the AUC of the FIU-time plot was observed (**Figure 2B**), indicating that the high-throughput fluorescence assay may be used to quantify LOX-1 activity. This facilitates the measurement of multiple soybean samples as well as the creation of the standard curve using the pure LOX-1 enzyme, since 4−5 dilution levels of the LOX-1 standard as well as 27−28 soybean samples can be tested in triplicates using one 96-well plate within 6 min. This is an advantage over previous methods that use a single-cell spectrophotometer, as they require up to 5 min for the measurement of each sample. These previous methods may take hours when measuring multiple samples. The high-throughput method also reduces the quantity of sample and reagents used. More importantly, the high-throughput assay is more sensitive and could detect a lower level of LOX-1 (**Figures 1** and **2A**).

The addition of additional buffer solution and water was eliminated for the high-throughput assay. This reduced the reagents to only fluorescein (200 nM) in pH 9.0 buffer, pure enzyme or soybean extract, and substrate solution. The substrate solution must be the last addition to the wells as the reaction will begin as soon as the substrate is added. The plate must then be placed in the plate reader quickly so that readings can begin before fluorescence begins to decrease. Six minutes was determined as the appropriate time for measurement in the multilabel plate reader. This allowed substantial degradation of fluorescein at the selected LOX-1 dilution levels.

Quantification. The use of the area under the curve (AUC) calculation for fluorescence allows for quantification of the LOX-1 enzyme. The AUC of fluorescence of the samples over time was compared to a standard curve based on the fluorescence of pure LOX-1 enzyme of known quantity. There are other methods that quantify LOX activity. For example, the methylene blue assay quantifies the enzyme by measuring time until bleaching begins (*15*). Measuring the AUC of fluorescence allows for more objective and accurate quantification. A potential difficulty in this quantification method is the instability of the pure LOX-1 enzyme that is used as a standard. The commercially purchased pure enzyme can lose activity quickly with time, even when held on ice. The enzyme was stored at −80 °C and used immediately after thawing in order to obtain a consistent result. The aqueous extract of soybean meal was less sensitive to changes in temperature and time.

Linearity and Range. For the high-throughput assay, the linear range of soybean LOX-1 (plotting concentration versus AUC) was determined at 5−25 KU (**Figure 2B**). The results of six repeats of this assay are shown in **Table 1**. Soybean extract should be diluted to fall within this range if quantification is desired. Soybean extract from the Manokin line (normal LOX) at a concentration 2 mg/mL was appropriate to fit within this range upon measurement with the high-throughput fluorescein assay.

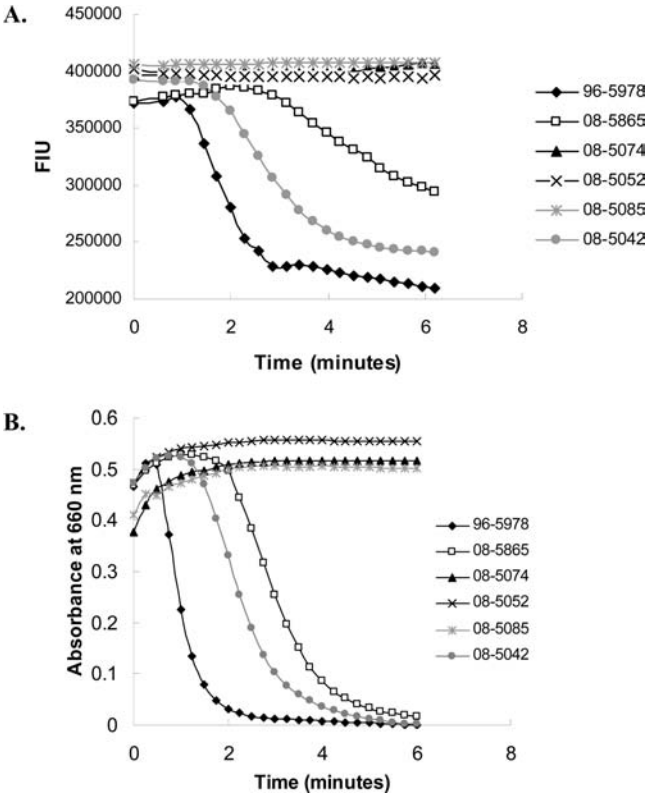

Figure 3. Comparison of the (**A**) high-throughput fluorescein LOX-1 assay and (**B**) methylene blue LOX-1 assay using the extracts of six soybean lines. The reagents were (**A**) 100 μL of 200 nM fluorescein in pH 9.0 buffer, 120 μL of soybean extract, and 80 μL of 12.6 mM sodium linoleate solution, and (**B**) 500 μL of 200 mM pH 9.0 sodium borate buffer, 100 μL of 200 mM methylene blue, 100 μL of 12.6 mM sodium linoleate solution, 100 μL of soybean extract, and 200 μL of ultrapure water. Fluorescence or absorbance at 660 nm was measured for 6 min. Each extract concentration was measured in triplicate. FIU = fluorescence intensity units.

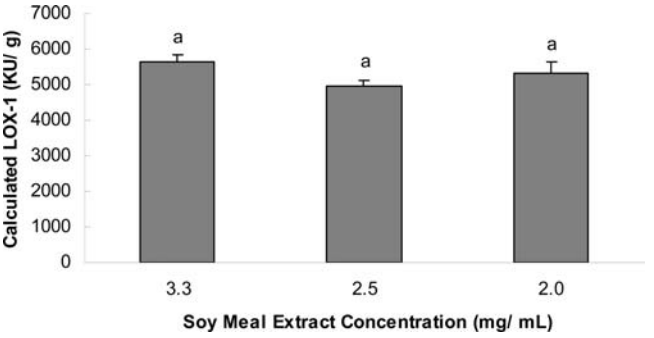

Figure 4. Effect of the ratio of solvent to soybean in extraction on LOX-1 activity estimations. The LOX-1 of Manokin soybean was estimated using three ratios of solvent volume to soybean weight. The reagents were 100 μL of 200 nM fluorescein in pH 9.0 buffer, 120 μL of soybean extract, and 80 μL of 12.6 mM sodium linoleate solution. Fluorescence was measured for 6 min. Each extract concentration was measured in triplicate. Columns with the same letter indicate no statistical difference at P = 0.05.

Validity. Figure 1 demonstrates the validity of the fluorescein LOX-1 assay compared with the traditional methylene blue LOX-1 assay when using the pure LOX-1 enzyme. In **Figure 3**, the selected LOX-1-null soybeans as well as nonmodified soybeans were tested and compared using the high-throughput fluorescein assay and the methylene blue assay. The results demonstrated the validity of the high-throughput fluorescein

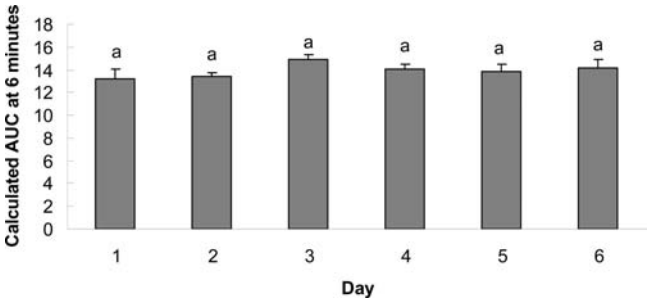

Figure 5. Reproducibility of the high-throughput LOX-1 fluorescein assay. 0.020 g of Manokin soy flour was extracted in 10 mL of distilled water and measured daily for 6 days. Measurements were conducted under conditions for the high-throughput fluorescein LOX-1 assay in a 96-well plate. Measurements were conducted in triplicate. Columns with the same letter indicate no statistical difference at $P = 0.05$.

assay for measuring LOX-1 in soybean extract in comparison with the methylene blue LOX-1 assay. It is clear that each soybean extract shows a similar reaction in the two assays.

Additionally, the assay has shown to be specific for the LOX-1 enzyme. In **Figure 1A**, fluorescence does not decrease in the control reaction where there was no LOX-1 enzyme present in the mixture. In **Figure 3**, the LOX-null soybean extracts do not have an effect on fluorescence when compared to the normal-LOX lines. Although experimental LOX-null lines are demonstrated in **Figure 3**, this reaction was also tested with the Japan 123 soybean that is known to be null for LOX (data not shown). This demonstrates that it is the LOX-1 enzyme and not another component of soybean extract that causes the degradation of fluorescein.

Figure 4 shows the calculated LOX-1 concentration per gram of flour when multiple concentrations of extract from one soy meal sample were analyzed. The calculated LOX-1 content of the soybean was not significantly affected by the concentration of the enzyme. Calculated values had a relative standard deviation (RSD) of 9.4%. These data indicated that the ratio of extraction solvent to soybean sample weight may not be very critical for LOX-1 activity estimation using the high-throughput fluorescein assay, and the high potential of this assay for practical utilization.

Reproducibility. The interday reproducibility of the high-throughput fluorescein LOX-1 assay was determined by measuring the AUC (at 6 min) of the reaction of an aqueous extract of soybean meal from the Manokin line (2 mg/mL). This test was repeated six times within 6 days (**Figure 5**). Fresh soybean extract and sodium linoleate were freshly prepared each day. The RSD of 4.3% demonstrates an excellent reproducibility of the assay.

Accuracy and Precision. Soybean LOX-1 was analyzed at three different concentrations (10, 15, and 20 KU) on 3 separate days to determine the accuracy of the high-throughput assay (**Table 2**). The measured concentration was compared to the expected concentration to determine the percent recovery. Average percent recovery varied from 97 to 108%, which demonstrates good accuracy. The RSD of pooled samples was between 4.62 and 7.35% which indicates excellent precision.

Advantages and Limitations of the Fluorescein LOX-1 Assay. The purpose of the described method is to quickly measure the presence of LOX-1 in soybean extract. This method requires less preparation time than some other frequently used spectrophotometric methods. The direct soybean extract can be used without additional filtration and purification steps. Using a multilabel plate reader, multiple soybean samples can be tested within 6 min. The time is much reduced from the previous methylene blue method where each individual sample will require at least 15 min to be measured in triplicate. Sample extracts may be compared against each other, or quantified using the standard curve of the purified enzyme, which makes the comparison of cross-laboratory data possible. No expensive reagents are required, and the high-throughput assay reduces the amount of reagents used. Additionally, only a small amount of ground soybean is required for the tests. This method can be used as a screening tool for soybean breeding programs as they select for seeds that are low in LOX-1. The method can potentially be modified for use with other soy foods and may assist in quality control during food processing. However, it needs to be pointed out that the assay depends on the peroxide radical attack to fluorescein, and the presence of antioxidants such as reducing agents and radical scavengers in the sample may lead to underestimation of LOX-1 activity or level.

Table 2. Accuracy and Precision of Quality Control (QC) Samples[a]

	QC1	QC2	QC3
nominal lipoxygenase concentration (KU)	10	15	20
run 1			
intramean	12.75	15.94	20.92
SD	1.11	1.50	0.33
% RSD	8.68	9.43	1.61
% Rec	127.5	106.3	104.6
N	3	3	3
run 2			
intramean	9.69	14.70	18.85
SD	0.85	0.42	0.85
% RSD	8.78	2.87	4.51
% Rec	96.1	98.0	94.3
N	3	3	3
run 3			
intramean	10.03	15.31	18.83
SD	0.46	0.70	1.63
% RSD	4.58	4.59	8.64
% Rec	100.3	101.9	94.1
N	3	3	3
pooled runs			
intermean	10.82	15.15	19.53
SD	0.82	0.87	0.94
% RSD	7.35	5.63	4.92
% Rec	108.0	101.8	97.67
N	9	9	9

[a] Measurements were conducted under conditions of the high-throughput fluorescein LOX-1 assay in a 96-well plate in triplicate.

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