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Abstract: Crude tomato lipoxygenase (LOX) from ripe Florida47 tomatoes was used in this study. Extracted crude LOX was assayed spectrophotometrically. The effects of different buffers and pHs, substrate preparations, temperature and tissue location on LOX activity were evaluated. The enzyme was most active with MES buffer at a pH of 6.0 and a temperature of 25ºC. The best substrate was the first preparation with linoleic acid/Tween 20 at a ratio of 1:1.5. The kinetic parameters determined under the best conditions were a Km of 4.198 mM and a Vmax of 0.84 mM/min. The enzyme was heat labile, and most activity was located in the fruit pericarp tissue. These results may help in employing crude tomato LOX as a biotechnological source.

Key Words: tomato, lipoxygenase, crude, kinetics, substrate

Ham domates Lipoksigenazıyla Kinetik Çalışmalar

Özet: Bu çalışmada, Florida47 domateslerinden elde edilen ham domates lipoksijenazı (LOX) kullanılmıştır. LOX ölçümü spektrofotometrik metotla yapılmıştır. Faklı tampon ve pH'ların, hazırlanmış substratların, sıcaklığın ve doku bölgesinin LOX aktivitesi üzerine etkisi belirlenmiştir. Enzim, pH'sı 6.0 olan MES tamponu ile 25 ºC'de en yüksek aktiviteyi göstermiştir. En iyi substrat, linoleik asit / Tween 20 oranının 1:1.5 olduğu birinci preperattır. En iyi koşullarda ölçülen kinetik parametreler, 4.198 mM Km ve 0.84 mM / min Vmax değerlerini vermiştir. Sıcağa dayanıklı olmayan enzim aktivitesinin çoğu meyvenin perikarp dokusunda toplanmıştır. Bu çalışmanın sonuçları, domates LOX' unun biyoteknoloji alanında kullanımına yardımcı olabilir.

Anahtar Sözcükler: domates, lipoksigenaz, ham, kinetik, substrat

Introduction

Lipoxygenase (LOX, EC 1.13.11.12, linoleate:oxygen oxidoreductase) refers to a group of enzymes that catalyze dioxygenation of fatty acids by use of molecular oxygen which contain one or more 1,4(Z)-pentadiene systems, yielding chiral (E,Z) conjugated hydroxy fatty acids (Veldink *et al.* 1998, Riley *et al.* 1996). Multiple forms or isozymes exist in many tissues showing differences in regiospecificity, pH optimum, pI and enzymic properties. Most are composed of a single polypeptide chain of approximately 850 amino acids with an average 96 kDa molecular weight (Grechkin 1998).

Although the functional role of plant LOX is still largely unknown, metabolites of unsaturated fatty acids have beeen implicated in growth and development, plant senescence and response to diseases and wounding. Furthermore, in fruits and other plant food products, LOX plays a role in the formation of volatile flavor compounds (Veldink *et al.* 1998, Riley *et al.* 1996, Ealing 1994).

LOX activity in tomato fruits was reported both in membraneous systems (Thompson *et al.* 1990, Riley *et al.* 1996) and in soluble form (Ealing 1994, Veldink *et al.* 1998). Enzyme activity has been assayed by colorimetric, manometric, polarographic, spectrophotometric and radiolabeled substrate monitoring assays (Thompson *et al.* 1990, Surrey 1964, Riley *et al.* 1996, Veldink *et al.* 1998). Values of Km and Vmax vary due to purity and assay differences (Thompson *et al.* 1990, Veldink *et al.* 1998). Product analysis has revealed a 96% product specificity of 9-S-hydroperoxy-trans-10,cis-12-octadecanoic acid (9S-HPOD) formation from linoleic acid with an 82% S enantiomeric excess. The 13-HPOD formation was 1% and racemic (Veldink *et al.* 1998).

In the present study, crude tomato LOX was used to investigate the effect of different substrate forms and assay buffers on enzyme activity and also the thermal stability of the enzyme.

Materials and Methods

Materials

Tomatoes of the cultivar Florida47 were grown following standard commercial practices, and were supplied by a local grower in Tifton, Georgia. Pink tomatoes were harvested and transported to the University of Georgia, Athens, on the same day for the study. Tomatoes were ripened (2 days) at room temperature (20ºC) to a table ripe stage before homogenizing.

Tween 20, EGTA (ethylene glycol tetraacetic acid), linoleic acid, sorbitol, $MgCl_2$, PVPP (polyvinylpyrrolidone), DTT (dithiothreitol), PMSF (phenylmethylsulfonyl fluoride), benzamidine and aminocaproic acid were purchased from Sigma Chem. Co. (St. Louis, MO). Glycerol, boric acid, sodium-phosphate, KOH, NaOH and glycine were from J.T. Baker Inc. (Philipsburg, NJ). Tris (N-tris hydroxymethylmethyl glycine), MES (morpholinoethane sulfonic acid), EPPS (N-2-Hydroxyethylpiperazine-N'-3-propanesulfonic acid), MOPS (3-(N-Morpholino)propanesulfonic acid), Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and sodium-acetate were purchased from Aldrich Chem. Co. (Milwaukee, WI).

Enzyme Extraction

The composition of homogenizing buffer was 150 mM Tris-HCl (pH 8.0), including 250 mM sorbitol, 10 mM $MgCl_2$, 1% glycerol (v/v), 0.2% PVPP (w/v), 5 mM DTT, and the following protease inhibitors: 0.1 mM PMSF, 0.1 mM benzamidine, and 5 mM aminocaproic acid. Isolated and cube cut pericarp tissue was blended at high speed with cold homogenizing buffer (1 g/ml) at a setting of 4 for 30 sec with 10 sec cooling intervals. After filtration through a nylon cloth, the homogenate was centrifuged for 20 min at 12,000 x g at 4ºC, and the supernatant was collected as a crude enzyme source containing both TomloxA and B isozymes (Grechkin 1998) and assayed immediately.

Protein Determination

Protein determination of the extract was carried out according to the Bradford (1976) method (protein assay kit, Biorad, Hercules, CA), using a microtiter plate assay equipped with a computer and software, according to the instructions of manufacturer. The calibration curve was determined with IgG (bovine plasma gamma globulin) as the standard.

Preperation of Substrate

Linoleic acid substrate was prepared in three different forms. In the first preparation, 0.5 ml of Tween 20 was dissolved in 10 ml of 0.1 M Borate buffer (pH 9.0). Then 0.5 ml of linoleic acid was added drop by drop during mixing. Excess air was avoided by closing the flask. Finally, 1.3 ml of 1 N NaOH was added and agitated until a clear transparent solution was obtained. An additional 90 ml of Borate buffer was added and the total volume was made up to 200 ml with water. The second substrate preparation was based on dissolving an equal amount of linoleic acid (0.5 ml) in 200 ml of methanol without the addition of detergent or buffer. In the third preparation, 0.5 ml of linoleic acid was dissolved in 20 ml of methanol and added to 180 ml of Borate buffer Tween 20 mixture with the same ratios. Also, for the first substrate preparation, the linoleic acid to Tween 20 ratio was evaluated (1:0, 1:1, 1:1.5, and 1:2).

LOX Assay

A HP 8451A Diode Array Spectrophotometer (190-820 nm range with 2 nm bandwith) was used to determine LOX activity. The assay method was adapted from Riley *et al.* (1996). An increase in absorbance at 234 nm was followed for 5 min at 20ºC following the addition of 1ml substrate solution into a cuvette containing 1ml of the crude enzyme extract and 1ml of assay buffer (0.1 M Na-Acetate, pH=5.0; 150 mM MES, pH=6.0; 50 mM MOPS, pH=7.0; 0.2 mM Hepes-KOH, pH=7.5; 150 mM EPPS-KOH, pH=8.0; 150 mM Tris-HCl, pH=8.5; 0.1 M Borate, pH=9.0; and 50 mM Glycine-KOH, pH=9.6). Enzyme specific activity is defined as the amount of enzyme producing one unit change in absorbance in one minute. By using the same method of assay with the first substrate preparation of 1:1.5 LA:Tween 20 ratio, the values of Vmax and Km were also determined with varying concentrations of the substrate (1.6-72.4 mM).

Thermal Stability Determination

The enzyme extract was kept in a pre-set thermostatic water bath at 60, 70, and 80ºC for 1, 2, 3, and 5 min (after 13, 16, and 18 min come-up times, respectively), and cooled to 20ºC before assay to determine the thermal stability. In the other set of experiments, enzyme extract was incubated for 10 min at 20, 25, 30, 35, and 40ºC and immediately assayed at that temperature to determine the temperature optimum.

Tissue Localization of LOX Activity

Crude enzyme extract was prepared from isolated pericarp tissue and used for all the above assays. In addition, tomato skin, locular gel material and whole fruit were used to compare tissue activity distribution.

Statistical Analysis

Statistical procedures were performed using the Statistical Analysis System (SAS Institute, Cary, NC). The effect of LOX specific activity by different substrate preparations was determined by ANOVA with means seperation by Duncan's Multiple Range test. Regression Analysis on the double-reciprocal plot determined the best fitting straight line. All measurements were made twice and the result reported was the mean of the two. The level of significance was 5% for all tests.

Results and Discussion

In this study, isozyme separation was not achieved and the crude LOX included both TomloxA and B isozymes. Of the assay buffers evaluated, the MES buffer (pH 6.0) was the best in terms of crude LOX specific activity (Figure 1). MOPS (pH 7.0) and Tris-HCl (pH 8.5) were also sufficiently good for the enzyme, while Borate (pH 9.0) and Glycine-KOH (pH 9.6) buffers yielded the lowest enzyme activity. An optimal pH value of around 6.0-6.5 has been reported in several studies (Thomson *et al.* 1990, Lauriere *et al.* 1993, Ealing 1994). The very low value with Borate buffer might be due to its pH (9.0) and nature (inorganic buffer). The eight buffers with different pH values evaluated for the specific LOX activity all showed different values (Figure 1), and MES buffer was selected for the subsequent analyses.

The effectiveness of the different substrate preparations for crude tomato LOX is shown in Table 1. The first preperation with the 1:1.5 linoleic acid:Tween 20 ratio was the best of all evaluated, with a 0.865 mean LOX specific activity (Units/mg protein) value. On the other hand, the poorest substrate preparation was the third, with a 0.405 U / mg protein specific activity value. The second substrate preparation was equally effective as a 1:0 ratio subclass of the first preparation. These observed differences can be explained by the availability of the linoleic acid in the substrate preparations. It thus can be inferred from the results that when it forms micelles with sufficient amount of Tween 20, linoleic acid becomes the most suitable for the reaction, while very high and low amounts of the detergent (Tween 20) can cause solubility and diffusion problems. Similarly, for the third preparation, substrate diffusion barriers can be a problem since the detergent and substrate are in different phases of the mixture. From these results, the first substrate preperation with the 1:1.5 linoleic acid:Tween 20 ratio was selected for the rest of the analyses.

Figure 2 shows the Lineweaver-Bulk (double-reciprocal) plot of the crude tomato LOX. Data were analyzed by the Regression Analysis procedure of the SAS to determine the best fitting theoretical straight line. The kinetic parameters were estimated from the slope and intercept of the line. The Km of the enzyme is 4.198 mM with a 0.84 mM/U Vmax value. Previous studies have reported Km values ranging from 0.015 x 10^{-4} M to 4.1

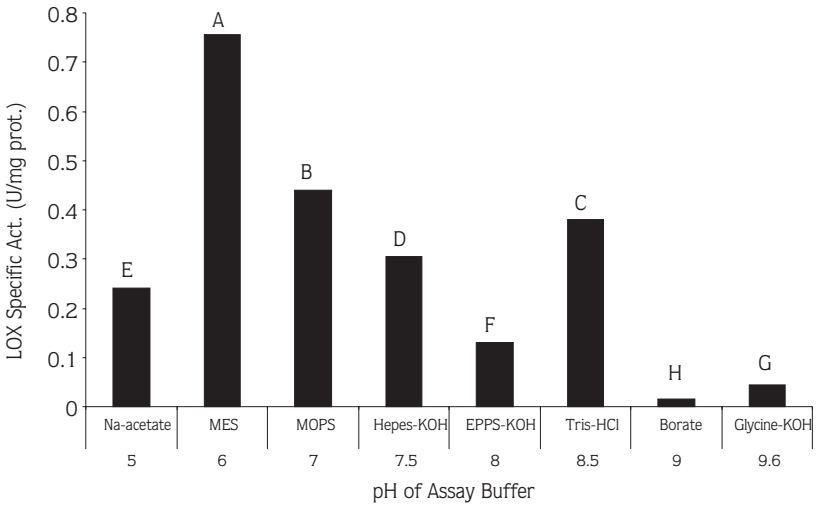

Figure 1. Effects of the different assay buffers (and pHs) on the activity of crude tomato LOX.

Table 1. Effect of substrate preparation methods on the crude tomato LOX specific activity.

Substrate Preparation Used	Mean LOX Specific Activity* (U/mg Protein)
First preparation	
LA/Tween 20 = 1:0.0	0.515 d
LA/Tween 20 = 1:1.0	0.730 b
LA/Tween 20 = 1:1.5	0.865 a
LA/Tween 20 = 1:2.0	0.605 c
Second preparation	0.515 d
Third preparation	0.405 e

* Means separation was done by Duncan's test with $p<0.05$
Same letters show no statistical difference among the values.

Table 2. Tissue distribution of crude tomato LOX activity.

Tissue Part	Mean LOX Specific Activity (U/mg Protein)	Yield* (%)
Whole pruit	0.834	100
Pericarp only	0.856	102.6
Locular gel	0.02	2.4
Fruit skin	0.03	3.6

* Yield calculation is based on whole fruit LOX activity being 100%.

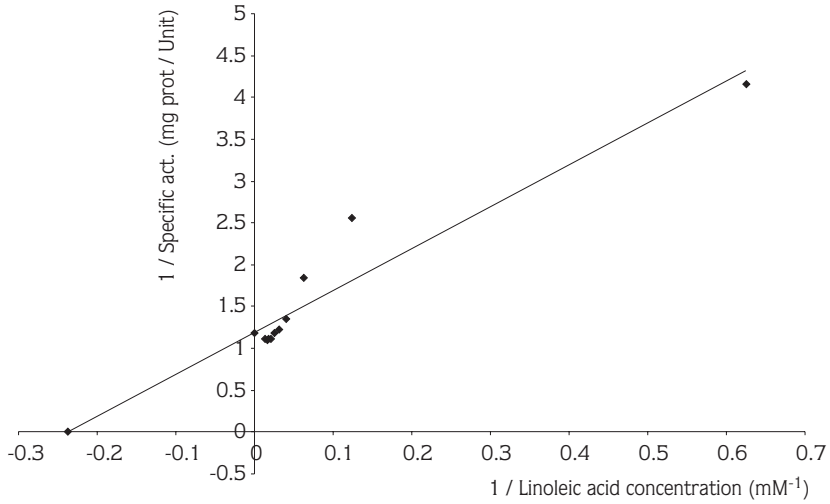

Figure 2. Lineweaver-Bulk plot of crude tomato LOX with first substrate (1:1.5 ratio) and MES buffer (pH 6.0). The linear model was Vo = 4.998(So) + 1.187 with a R^2 = 0.925.

mM, and Vmax values ranging from 0.186 mM /U to 7.4 mM /U (Thompson *et al.* 1990, Veldink *et al.* 1998, Biacs and Daood 1987). The values vary with the level of enzyme purity and difference in assay method. The enzyme in this study was crude, and the assay was done with the best buffer (pH) and substrate selected at room temperature.

The heat stability of the enzyme at different temperatures is illustrated in Figure 3. The enzyme was rapidly inactivated at 80ºC, and in 3 min almost all activity diminished. When the enzyme was kept 1 min at 70ºC after 16 min come-up times, only 20% of the initial activity was retained, but when the time was increased to 5 min, all of the activity was lost. On the other hand, keeping for 3 min at 60ºC caused only 65% activity loss. At 5 min, however, only 5% of the activity remained at the same temperature. The optimum temperature for activity is shown in Figure 4. It was shown that crude tomato LOX is fully active at room temperature (20-30ºC), while the best activity (100%) occurred at 25ºC. At 40ºC, only 38% and at 45ºC only 14% of initial activity is achieved. Henceforth, the enzyme is active at room temperature range where it naturally acts on substrates, but activity up to 35ºC can successfully be measured.

Tissue distribution of crude tomato LOX activity is shown in Table 2. With whole fruit chosen as the 100% base, pericarp tissue showed the highest activity (102.6%). Fruit skin with some pericarp tissue peeled with it yielded only 3.6% of activity, while locular gel itself does not contain any LOX activity. Locular gel was assayed without fruit seeds. The highest yield of the pericarp tissue may be explained by two factors. First, most of the active enzyme should be placed within the

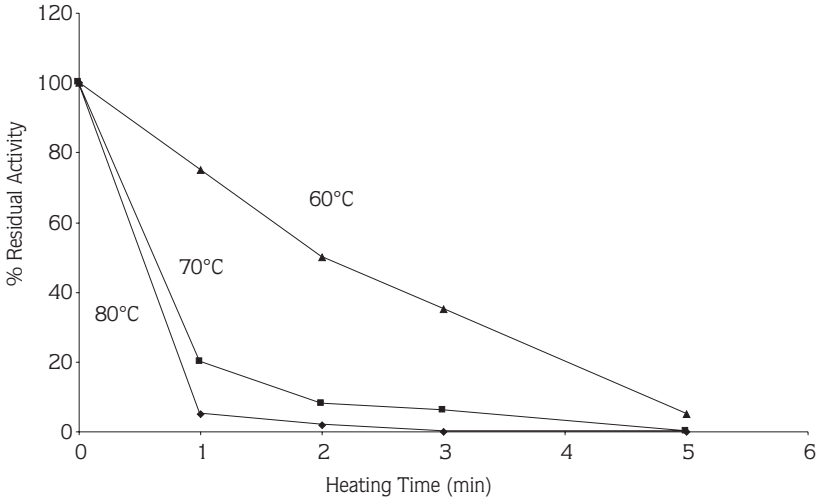

Figure 3. Thermal stability of crude tomato LOX.

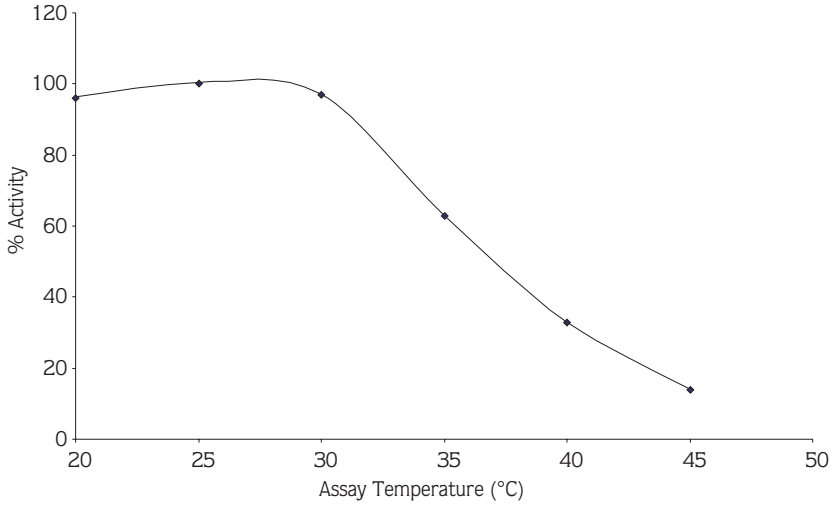

Figure 4. Optimum temperature range for activity of crude tomato LOX.

tissue, and secondly, the enzyme might best extracted from the pericarp tissue. The whole fruit contains some other components (locular gel, seeds and skin) which can cause enzyme loss by protein precipitation or complexation during the isolation prosess.

Conclusion

Overall, crude tomato LOX is best active at pH 6.0 in the MES buffer at a temperature of 25ºC. Similarly, the best substrate was first preparation with linoleic acid:Tween 20 = 1:1.5 ratio. The kinetic study with the best buffer and substrate revealed a Km of 4.198 mM and a Vmax of 0.84 mM/min value. The enzyme activity was optimum at room temperature range (20-30ºC), and at 80ºC at 2 min almost all activity was diminished. Most of the activity occurred in the pericarp tissue. The results of this study might be very useful when tomato pericarp tissue is considered as a LOX source in biotechnological applications.

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