ORIGINAL ARTICLE

An improved method to characterize crude lipoxygenase extract from wheat germ

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Abstract

Introduction Spectrophotometric assay is commonly applied in measuring activity of lipoxygenase (LOX). But the accuracy of this method is greatly affected by transparence of the substrate solution and the purity of enzyme solution; it is especially difficult to directly measure the activity of LOX in crude enzyme solution in the condition of pH value lower than 7.0. *Objectives* To provide a convenient, efficient, accurate and reproducible assay of LOX activity from wheat germ (WG), an improved method to characterize crude lipoxygenase extracted from wheat germ was established. *Methods* First the stability of substrate at different pH values was studied. Then a transparent substrate solution was obtained by mixed solution of 'absolute ethanol – linoleic acid – Tween 20'. When determining LOX activity, we took substrate solution mixed with completely inactivated enzyme solution as the blank, which effectively eliminated the assay error and the complicated purifying process. Detection wavelength was 234 nm at 25 °C. In order to study the enzyme characteristics of crude LOX from WG, we used this modified method to determine the optimum pH and temperature of LOX. The kinetic parameters including Michaelis constant (Km) and the maximum rate (Vmax) were determined as well. *Results* The optimum conditions of assaying LOX activity of WG were: a mixed solution of 2.0 mL 0.1 M phosphate buffered solution (pH = 6.5); 200 μL substrate solution [the concentration of linoleic acid and Tween 20 are 1.25–2.53 mM and 0.08–0.12% (w/v)]; and the temperature of 25 °C. When LOX activity was determined at the optimum condition, Km value and Vmax of the crude LOX extract were 2.64 mM and 256.41 unit per mg protein per min, respectively. *Conclusion* The method is convenient, efficient, accurate and reproducible for the determination of LOX activity in WG.

Introduction

Wheat germ (WG) contains lots of lipoxygenase (LOX) and lipases. LOX can catalyze the oxidation of linoleic acid, which takes up 50% of all oil contained in WG, and produces linoleic acid hydroperoxide, and the POV of WG rises rapidly and further decomposes into volatile aldehyde and ketone. WG can deteriorate within 2 weeks at room temperature (Srivastava *et al.*, 2007). In order to fully apply the precious resource to food industry, many flour manufacturers implement a heating step to inactivate LOX and lipase, and extend shelf life. But excessive temperature and time of the heat treatment may cause undesirable browning, damaging appearance and can also accelerate its oil oxidation.

Therefore, to choose the right temperature and time length of WG heat treatment is very important to inactivate the lipase sufficiently and to maintain as many nutrients as possible at the same time. The study of enzymatic characteristic of WG and accurate measurement of enzyme activity is the base of research on technology of WG stabilization.

Spectrophotometric assay and oxygen electrode method are the top two measurements of LOX activity, of which the former is applied more commonly. Present research shows that the activity assay of LOX can be affected by factors like source of LOX, reaction temperature, concentration of substrate and pH value of reaction solution, etc. When determining LOX activity using spectrophotometric assay, the following four points are the main points that we should pay attention to.

(1) Transparent substrate solution is a premise of the accuracy of spectrophotometric method (Surrey, 1964), which can be obtained by adding Tween 20 to the substrate and increasing pH value to 9.0. The solubility of linoleic acid decreases gradually and the substrate solution becomes turbid if the pH value is lower than 7.0 (Gökmen et al., 2002). In addition, Tween 20 can inhibit LOX activity with high Tween-substrate ratio (Aziz et al., 1970).

(2) Furthermore, determining both the stability of substrate and activity of LOX, pH value is the decisive factor to the accuracy and sensitivity of LOX activity assay (Grossman & Zakut, 1979).

(3) It is also observed that the pH and temperature optima of different LOX vary greatly (Baysal & Demirdoven, 2007). The optimum pH of barley germ LOX is between 6.0 and 6.3 (Yang et al., 1993), while the optimum temperature of LOX from mung bean and horse bean is 30 °C (Gökmen et al., 2002). So, the optimum pH and reaction temperature of buffered solution should be determined according to the type of source.

(4) The accuracy of spectrophotometric assay is significantly affected by both the transparence of the substrate solution and the purity of enzyme solution. Present method of assaying LOX activity involves complicated purifying process (Anese & Sovrano, 2006). So far, no other method specialized in assaying LOX of WG has been found in relevant studies; all of them are about other botanic sources (Gökmen et al., 2007).

This study is an attempt to modify the spectrophotometric measurement of LOX activity to give better precision and accuracy by preventing the turbidity of the reaction medium just before the absorbance reading. The modified method was then used to determine the pH and temperature optima of crude LOX from WG and to determine the Michaelis constant (K_m) and maximum rate (V_{max}) in the crude enzyme extract.

Materials and methods

Reagents

Linoleic acid (99% pure) and bovine serum albumin were purchased from Sigma (St Louis, MO, USA). Tween 20, KH_2PO_4, Na_2HPO_4, NaOH, glacial CH_3COOH and CH_3COONa were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of crude enzyme extract

Samples of WG flakes, milled from Chinese hard white spring wheat by Fada Flour Co., Ltd. (Xiajin, Shandong, China), were blended to provide a uniform sample. A portion of the flakes was ground to pass a 300-μm screen to obtain full fat flour.

LOX extracts were prepared daily, before assay, according to the method of Bhirud and Sosulski (1993). Ground sample was extracted for 30 min at 4 °C with 10 times volume of cold acetate buffer (pH = 4.5, 0.1 M). The slurry was centrifuged at 12 000 g for 30 min. Then the supernatant containing LOX was diluted with 20 times volume of cold acetate buffer (pH = 4.5, 0.1 M) and stored at 5 °C as a stock solution A.

The protein determinations were carried out using the dye-binding method of Bradford (1976). A standard curve was constructed using bovine serum albumin in the concentration range 50–1200 μg mL^{-1}, in which a linear response was observed.

Preparation of substrate solution

The linoleic acid emulsion used as the lipoxygenase substrate was prepared by a modified method of Aziz et al. (1970). Linoleic-absolute ethanol solution was prepared in an atmosphere of nitrogen by dissolving 111 μL of pure linoleic acid in 10-mL absolute ethanol. Then, 0.08 mL Tween 20 was added to 7.1 mL of the prepared solution. The ethanol was removed by rotary evaporator and the residues were dissolved in 100 mL 0.05 M Na_2HPO_4, then the solution was brought to pH = 9.0 by adding a few drops of 1 N NaOH. The resulting substrate was approximately 2.53 mM in linoleic acid and 0.08% (w/v) in Tween 20, which was sealed under nitrogen in a syringe bottle and stored at 5 °C in the dark as a stock solution B.

Spectrophotometric assay of LOX activity

LOX-catalyzed hydroperoxidation of linoleic acid at 25 °C was measured as an increase in absorbance at 234 nm on DU 800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) according to the method of Aziz *et al.* (1970). The reaction was initiated by injecting 50 μL of working enzyme solution into a thermally equilibrated cuvette containing 0.20 mL of substrate solution and 2.0 mL of phosphate buffer. The blank solution was prepared by mixing 0.2 mL of substrate solution with 2 mL of buffer and 50 μL of inactive enzyme solution. The reaction mixture was stirred for 10 s after which absorbance was recorded by computer at intervals of 1 s for 300 s. The initial reaction velocity was calculated by linear regression using an Excel spreadsheet on data set between 30 and 210 of the reaction curve. One unit of LOX activity was defined as an increase in absorbance of 0.01 at 234 nm per minute per mg of protein under assay conditions.

Substrate pH optimum

The substrate solution was diluted to 10 times volume by buffer solutions of pH = 4.5, 6.0, 7.0, 8.0, 9.0, respectively, and the absorbance change at 234 nm was observed within 2 h.

Substrate concentration optimum

Substrate solutions of seven different concentration were prepared, in which the concentrations of linoleic acid were 0.313, 0.625, 1.25, 2.5, 5.0, 7.5, 15 mM, respectively, then the substrates were diluted with 10 times of volume of phosphate buffer (pH = 7.0), and scanned from 215 to 375 nm against the phosphate buffer (pH = 7.0). Phosphate buffer (2.0 mL 0.1 M; pH = 7.0) and 50-μL crude enzyme solution were added to the substrates. Then the LOX activity of crude enzyme in each solution could be measured, respectively.

Tween-20 concentration optimum

Three different concentration solutions were prepared, in which concentrations of linoleic acid are all 2.53 mM; Tween-20 concentrations are 0.08%, 0.16% and 0.24% (w/v), respectively.

pH optimum for LOX activity

To determine the pH optimum, the activity of crude LOX was determined spectrophotometrically in the range of pH = 4.5–9.0. The buffer systems were prepared by mixing appropriate amounts of 0.1 M KH_2PO_4 and Na_2HPO_4 in the range of pH = 5.0–8.0.

Temperature optimum for LOX activity

The optimum temperature for LOX activity could be determined in the mixture of 200 μL substrate solution buffered with 0.1 M sodium phosphate (pH = 7.0) and 25 μL crude enzyme solution at 15 °C, 25 °C, 35 °C, 45 °C, 55 °C, 65 °C and 75 °C, respectively.

Kinetic study of LOX

Substrate solutions were prepared by mixing linoleic acid and Tween 20 together. The consequent concentrations of linoleic acid were 0.312 mM, 0.469 mM, 0.625 mM, 0.937 mM and 1.250 mM, while the concentration of Tween 20 was always 0.08% (w/v). Subsequently, for the measurement of LOX activity at 25 °C, 2.0 mL 0.1 M (pH = 7.0) phosphate buffer and 25 μL crude enzyme solution were added to 200 μL substrate solutions with various concentrations, respectively.

Then the K_m and V_{max} of lipoxygenase from WG could be calculated using the method of double reciprocal and linear regression as well.

Results and discussion

Modified spectrophotometric method for LOX activity

Firstly, the stability of substrate was studied at different pH. In spectrophotometric studies of the LOX-catalyzed reaction, the low solubility of fatty acids was a common problem. Transparent solution could be obtained by adding Tween 20 to the substrate and adjusting its pH to 9.0 and if pH was lower than 8.0, the solubility of linoleic acid would decrease gradually while the turbidity of substrate solution would increase.

The absorbance of substrate solution of pH = 4.5, 6.0, 7.0 increased in the first 10 min after mixing with buffer due to the light-scattering effect. The changes of absorbency per minute were 0.19%, 0.17% and 0.13%, respectively. The lower pH value of the solution, the greater change would be. The substrate solutions of pH = 8.0 and pH = 9.0 remained stable, with almost no absorbance change.

According to the method of Grossman and Zakut (1979), the substrate was diluted to the needed pH by adding buffer
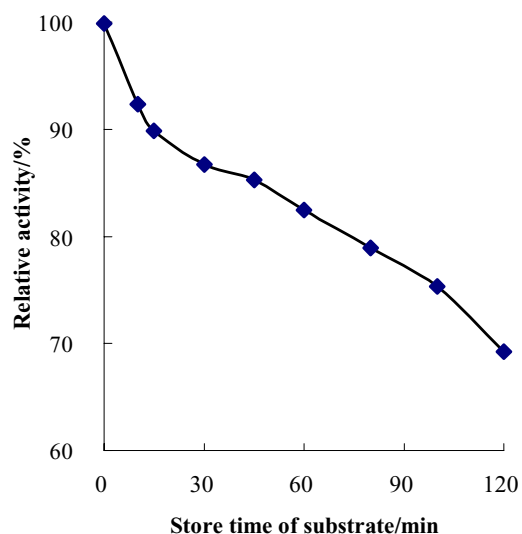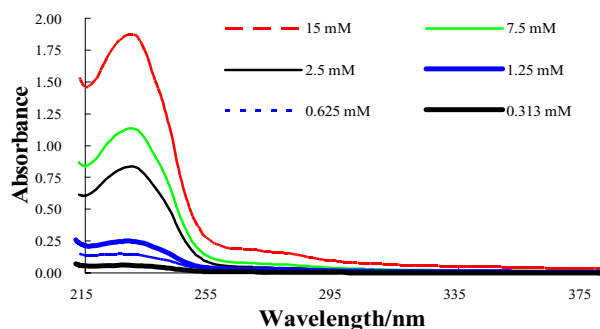
Figure 1 Influence of store time of substrate solution on initial velocity of linoleate oxidation: 200 µL substrate [linoleate 2.5 mM, Tween 20 0.08% (w/v)] +2 mL 0.1 M phosphate buffer (pH = 7.0) + 50 µL crude lipoxygenase.

Figure 2 Ultraviolet scan of substrate solution at different concentration: 200 µL substrate [linoleate 2.5 mM, Tween 20 0.08% (w/v)] +2 mL 0.1 M phosphate buffer (pH = 7.00).

Figure 3 The typical data for spectrophotometric measurement of crude lipoxygenase (LOX) activity at different concentration of substrate solution: 200 µL substrate [linoleate 2.5, 1.25 and 0.625 mM, respectively, Tween 20 0.08% (w/v)] + 2 mL 0.1 M phosphate buffer (pH = 7.0) + 50 µL crude LOX.
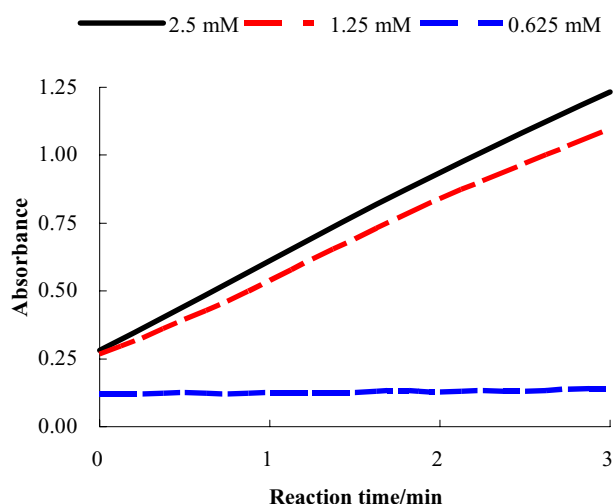
solution, and then enzyme solution was added to the diluted solution before measuring it. The activity of enzyme would decline if the pH of the reaction system was lower than 8.0. Figure 1 shows that, for example, due to the decrease of solubility of linoleic acid and the amount of substrate involved in catalyzed reaction, the LOX activity at pH = 7.0 was only 69% of its initial level after 2 h.

In order to overcome that problem, in this study we used a mixed solution of 'absolute ethanol – linoleic acid – Tween 20' as the substrate solution, in which linoleum acid could be completely dissolved in Tween 20, and by adding proper amount of Na_2HPO_4 and NaOH, a transparent substrate solution could be obtained very conveniently and efficiently. The method introduced in the above (spectrophotometric assay of LOX activity) was applied to assay the activity of LOX. More specifically, buffer solution of the needed pH was added to the reaction medium at first, and then the enzyme solution was added 5 min later. The measurement error caused by the decrease of solubility of linoleic acid in the condition of pH value lower than 7.0 could be effectively eliminated through this means.

Effect of substrate concentration is shown in Figure 2. The absorbance increased gradually as the amount of linoleic acid in the substrate rose. When the substrate concentration went up to 5 mM, its absorbance was 0.77 against the blank solution of 0.1 M phosphate buffer. During the period of measuring LOX activity, the absorbance of mixed solution rose rapidly to 2.0 within 5 min, which means its transmittance
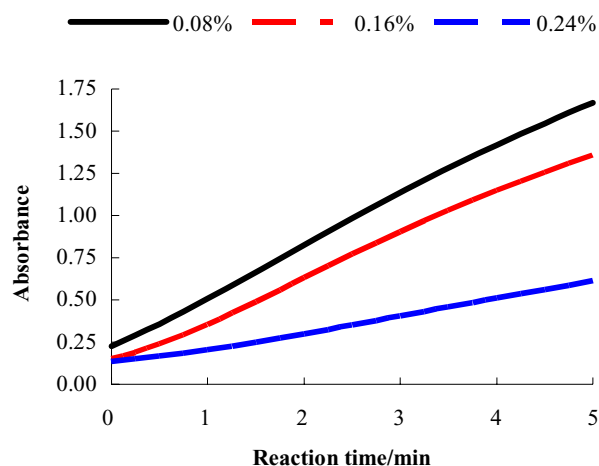
was only 1% and might lead to great error. Therefore, the concentration of mixed solution must not exceed 5.0 mM.

Figure 3 shows that optimum concentrations of substrate solution were 2.5 mM or 1.25 mM. Because their initial absorbance readings against the blank solution of phosphate buffer were 0.23 and 0.13, then the absorbance of the mixed solutions rose to 1.23 and 1.10, respectively, within 3 min. Such a result corresponded to the theory that the best linearity of spectrophotometry appeared with absorbance reading from 0.1 to 1.0. Meanwhile, activity assay could be finished within 3 min. When the concentration of substrate solution was lower than 0.625 mM, the activity of enzyme failed to be measured due to the poor linearity and the insufficient quantity of substrate.

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Figure 4 The typical data for spectrophotometric measurement of crude lipoxygenase (LOX) activity at different concentration of Tween 20: 200 μL substrate [linoleate 2.53 mM, Tween 20 0.08, 0.16, 0.24% (w/v), respectively] + 2 mL 0.1 M phosphate buffer (pH = 7.0) + 50 μL crude LOX.

To sum up, the optimal concentration range of linoleic acid in substrate solution was 1.25–2.5 mM.

Lastly, the concentration of Tween 20 was optimized. Tween 20 could increase the solubility of linoleic acid in water, especially in alkaline environment (pH higher than 8). But when Tween 20 in the substrate was at a concentration of 0.24%, the inhibitory effect on LOX was observed (Figure 4). Thus, the optimum range of Tween concentration was 0.08–0.16% as the concentration of linoleic acid in substrate was 2.5 mM.

Effect of catalyst concentration on LOX activity

Crude LOX solutions of different volumes (5 μL, 10 μL, 20 μL, 30 μL, 40 μL, 50 μL, 60 μL and 70 μL) were added to the reaction medium, and the optimum quantity of catalyst was determined by its activity in the solution. The reaction rate and the enzyme concentration had a good linear relationship (the volumes of crude LOX solutions ranging from 5 μL to 70 μL):

$$\geq OD_{234/min} = 0.0053V - 0.0075, R^2 = 0.9984 \quad (1)$$

Effect of pH on LOX activity

The optimum pH for activity of LOX from WG was 6.5, but LOX was also found to have noticeably high activity in a broad pH range of 4.5–8.5 (Figure 5). Such a result corresponds to Nicolas's report, but higher than Shiiba's. Nicolas
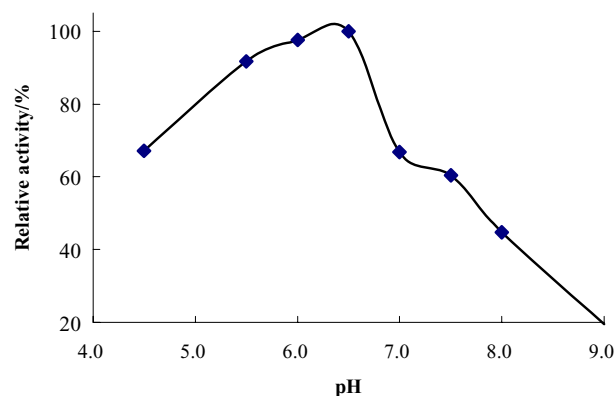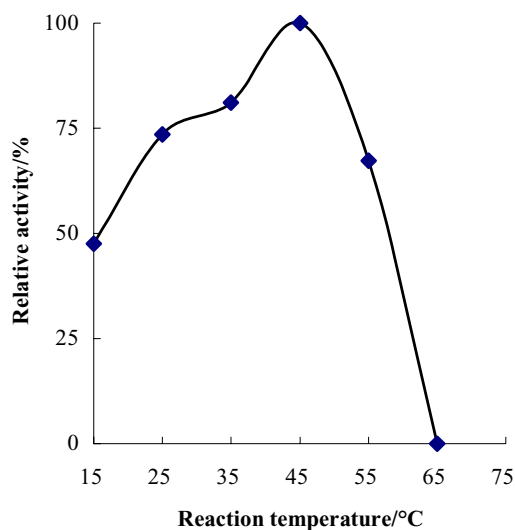

Figure 5 pH activity profile for crude lipoxygenase (LOX) extract from wheat germ: 200 μL substrate [linoleate 2.53 mM, Tween 20 0.08% (w/v)] + 2 mL 0.1 M phosphate buffer (pH = 4.5–9.0) + 50 μL crude LOX.

separated three LOX isozymes (L-1, L-2, L-3). These three isozymes and crude enzyme solution had the same optimum pH in the range of 6.0–6.5 (Nicolas *et al.*, 1982). In Shiiba's report, LOX from WG contained four isozymes (L-1, L-2, L-3, L-a), in which L-3 took up 50% of the total amount. The optimum pH of L-1, L-2, L-3 were all 5.5, and L-2 had the broadest pH range of 4.5–6.0. L-3 catalyzed linoleic acid effectively, but L-1 and L-2 were relatively weak catalysts to linoleic acid (Shiiba *et al.*, 1991).

Effect of temperature on LOX activity

The optimum temperature for LOX activity was 45 °C; LOX activity was also determined to be high in a broad range of temperatures between 15 °C and 45°C. But LOX activity decreased rapidly at a temperature higher than 55 °C, and completely passivated at a temperature higher than 65 °C (Figure 6). Such a result corresponds to Shiiba's; it shows that L-1, L-2 and L-3 LOX isozymes have similar sensitivity to temperature, have the highest activity at 45 °C, and completely passivated at 75 °C.

LOX have similar activity in a broad range of temperatures between 25 °C and 55°C, at 25 °C reaction time, and the reaction solution shows a linear relationship. However, at 55 °C, the curve dropped rapidly after 2-min reaction. The cause must be that at a relatively higher temperature, LOX activity was improved in the starting stage, and as the reaction went on, part of LOX lost their activity, which reflected on the decrease of total activity. Based on the said analysis, the optima temperature range is between 25 °C and 35°C in LOX activity assay.

Figure 6 Temperature activity profile for crude lipoxygenase (LOX) extract from wheat germ: 200 μL substrate [linoleate 2.53 mM, Tween 20 0.08% (w/v)] + 2 mL 0.1 M phosphate buffer (pH = 6.5) + 50 μL crude LOX.
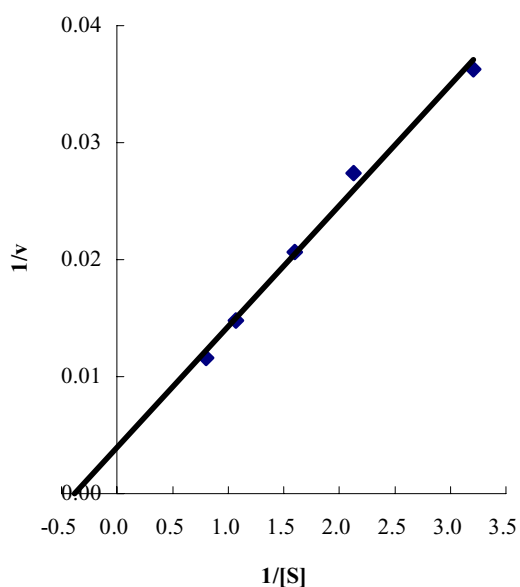
Figure 7 The Lineweaver–Burk plot of crude lipoxygenase (LOX): 200 μL substrate [linoleate 0.312–1.25 mM, Tween 20 0.08% (w/v)] + 2 mL 0.1 M phosphate buffer (pH = 7.0) + 25 μL crude LOX.

Kinetic study of LOX

According to the Lineweaver–Burk method, a curve was drawn on Figure 7 to describe the relationship between initial velocities of linoleate oxidation by crude LOX extract from WG (v) and linoleic acid concentrations (S) ranging from 0.312 mM to 1.25 mM. It is obvious that the linearity of that curve is good, and the equation of it is:

$$1/v = 0.0103 \times 1/[S] + 0.0039, R^2 = 0.9920 \qquad (2)$$

By some simple geometric calculation on the Lineweaver–Burk plot, K_m value and V_{max} of the crude LOX extract were found to be 2.64 mM and 256.41 units per mg protein per min, respectively.

Conclusions

To conclude, a modified spectrophotometric method was used to characterize crude LOX from WG in terms of thermal stability, pH and temperature optimum, and kinetic parameters. We applied mixed solution of 'absolute ethanol – linoleic acid – Tween 20' and obtained a transparent substrate solution conveniently and efficiently. To be specific, with the optimum pH = 7.0 in buffered solution, the high stability of substrate solution was acquired. In addition, the optima of temperature and proportion of linoleic acid, Tween-20 and enzyme insured that the absorbance of the reaction mixture within 5 min is around 1.0. Furthermore, the blank sample applied in this study was a mixture of substrate solution, buffer solution and completely inactivated enzyme solution, through which the assay error caused by crude enzyme solution can be effectively eliminated and the complicated purifying process can also be prevented. By examining thermal stability of crude enzyme solution, optimum pH value and temperature, and kinetic parameter of LOX from WG with a modified spectrophotometric method, the activity of crude LOX can be measured directly, and the kinetic study on thermally inactivated enzyme in WG can be conducted conveniently.

Acknowledgements

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