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Analytical Methods

Development of the simple and sensitive method for lipoxygenase assay in AOT/isooctane reversed micelles

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ABSTRACT

In this study, we investigated the possibility of reversed micelles, widely used as an enzyme reactor for lipases, for the determination of lipoxygenase activity. Although it is rapid and simple, reversed micelles have some limitations, such as interference by UV-absorbing materials and surfactant. Lipoxygenase activity in the reversed micelles was determined by reading the absorbance of the lipid hydroperoxidation product (conjugated diene) at 234 nm. Among surfactants and organic media, AOT and isooctane were most effective for the dioxygenation of linoleic acid in reversed micelles. The strong absorbance of AOT in the UV region is a major obstacle for the direct application of the AOT/isooctane reversed micelles to lipoxygenase activity determination. To prevent interference by AOT, we added an AOT removal step in the procedure for lipoxygenase activity determination in reversed micelles. The lipoxygenase activity was dependent on water content, and maximum activity was obtained at an *R*-value of 10.

1. Introduction

Lipoxygenases are a family of iron-containing enzymes found in many plants and animals (Brash, 1999; Kuhn & Thiele, 1999). Lipoxygenases are also found in microorganisms such as fungi (Perraud & Kermasha, 2000) and microalgae (Beneytout, Andrianarison, Rakotoarisoa, & Tixier, 1989; Nuñez, Savary, Fogila, & Piazza, 2002). These enzymes catalyse the stereo- and regio-specific dioxygenation of polyunsaturated fatty acids to form fatty acid hydroperoxides (Baysal & Emirdöven, 2007) and convert natural *cis* double bonds to the *trans* double bonds of fatty acids (Maas, Brash, & Oates, 1981).

The roles of lipoxygenases in plants and animals remain unclear. Recent studies (Brash, 1999; Siedow, 1991) suggest that they may be involved in plant physiology related to growth and development, such as pest resistance, senescence, and responses to wounding. In mammals, lipoxygenases are involved in the metabolism of eicosanoids such as prostaglandins, leukotrienes, and non-classic eicosanoids.

Because lipoxygenases catalyse the insertion of oxygen to polyunsaturated fatty acids, lipoxygenases could be negatively related to the development of off-flavours in many plant and animal food systems (Hsieh, 1994). Also, lipoxygenases may catalyse the co-oxidation of carotenoids, resulting in a loss of essential nutrients (Robinson, Wu, Domoney, & Casey, 1995). Lipoxygenases may play an important role in aroma-compound formation, producing hydroperoxides, precursors for the volatile compounds produced by other enzymes (Robinson et al., 1995).

Several methods are available for the determination of lipoxygenase activity, including colorimetric methods (Koch, Stern, & Ferrari, 1958), dye solution bleaching methods (Toyosaki, 1992), and spectrophotometric methods (Axelrod, Cheesebrough, & Laakso, 1981). Among them, spectrophotometry is the most accepted method, determining the absorbance of a lipid hydroperoxidation product such as a conjugated diene at 234 nm (Axelrod et al., 1981). Because this spectrophotometric method can simply and directly measure the reaction product, it is widely used for quantitative kinetics determinations with purified lipoxygenases. However, although it is rapid and simple, this method has two limitations. First, if other UV-absorbing materials are present in the crude enzyme solution, the spectrometric assay loses sensitivity. Second, turbidity resulting from the low water solubility of fatty acid substrates can disturb absorbance readings.

To avoid interference from fatty acid-induced turbidity and UV-absorbing materials, water-in-oil emulsions or reversed micelles have received attention as an enzyme reactor system. Reversed micelles have been widely used as an enzyme reactor system to determine lipase activity (Carvalho & Cabral, 2000).

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Lipoxygenases, like lipases, have catalytic activity with water-insoluble substrates. Conducting the enzyme reaction in reversed micelles has a number of advantages, such as solubilisation of both hydrophilic and hydrophobic substrates and/or products, a low reaction volume, a large hydrophilic/hydrophobic interfacial area, and control over the activity and stability of enzymes (Carvalho & Cabral, 2000; Martinek, Klyachko, Kabanov, Khmelnitsky, & Levashov, 1989; Martinek, Levashov, Klyachko, Khmelnitsky, & Berezin, 1986). Based on previous studies, it appears that AOT/isooctane reversed micelles are the most suitable for the determination of lipase activity (Carvalho & Cabral, 2000). However, this system developed for the lipase assay cannot be used directly for lipoxygenase activity determination because of the overlapping UV-absorbing characteristics of AOT.

Thus, in present study, we investigated the possibility of using AOT/isooctane reversed micelles, developed for the determination of lipase activity, for the determination of lipoxygenase activity. We also assessed which modification(s) to the AOT/isooctane reversed micellar system would be needed to develop a rapid, easy, and sensitive lipoxygenase-activity determination method.

2. Materials and methods

2.1. Materials

Soybean lipoxygenase type I-B (linoleate:oxygen, oxidoreductase, EC 1.13.11.12) with a reported catalytic activity of 50 units/mg solid (one unit is equivalent to the dioxygenation of 0.12 μmol of linoleic acid per min at pH 9.0 and 25 °C) was purchased from Sigma–Aldrich (Milwaukee, WI). HPLC-grade organic solvents (J.T. Baker, Phillipsburg, NJ), isooctane, cyclohexane, *n*-hexane, *n*-heptane, and hexadecane, were dehydrated with molecular sieves (4 Å, Sigma–Aldrich) and filtered through a membrane filter (0.45 μm) prior to use as a reaction medium for the reversed micelles. Linoleic acid ($\geqslant$98.0%) and bis (2-ethylhexyl) sulfosuccinate sodium salt (AOT) were purchased from Sigma–Aldrich, and the latter was purified according to the method of Tamamushi and Watanabe (1980). All other chemicals were of extra pure grade and were used without further purification.

2.2. Preparation of reversed micelles

A typical procedure for the formation of reversed micellar system was carried out as follows. First, an appropriate amount of 50 mM Tris–HCl buffer (pH 9.0) containing lipoxygenase from soybean was added to 5 mL of 100 mM AOT/isooctane to give 5, 10, 15, 20, 25, and 30 of *R*-values, which refer to molar ratio of water to surfactant. To fix enzyme-to-substrate ratio in reversed micellar systems independent on *R*-value, the different lipoxygenase solution was prepared by dissolving various contents of lipoxygenase into 50 mM Tris–HCl buffer (pH 9.0). Subsequently, the mixture was shaken vigorously with a vortex mixer for 60 s until it became clear or optically transparent.

2.3. Procedure for lipoxygenase assay

After formation of AOT/isooctane reversed micelles, the reversed micellar solution was pre-incubated in a water-bath at 25 °C with magnetic stirring (800 rpm). Because of difficult handling of linoleic acid due to its viscosity, linoleic acid stock solution was prepared by dissolving linoleic acid into isooctane to 250 mM in concentration. Then, lipoxygenase-catalysed dioxygenation was initiated by adding linoleic acid stock solution to be 25 μmol linoleic acid/5 mL reversed micellar solution. Each aliquot (200 μL) of reactant was collected at pre-determined intervals and diluted with 1.8 mL of isooctane. An equal volume of 30% ethanol was added to the dilution, and then the mixture was vortex mixed for 60 s. After centrifugation (5000g, 10 min), absorbance of the supernatant (isooctane layer) was measured at 234 nm to quantitatively analyse the conjugated linoleic acids produced. The blank was processed through the same procedure with heat-inactivated enzyme, and the calibration curve (regression equation between molar concentration of conjugated diene and absorbance at 234 nm) had a coefficient of determination above 0.998 (data not shown). For the evaluation of specific activity, quantitative analysis of protein was performed according to the method of Bradford (1976).

2.4. Practical application of the modified lipoxygenase assay in the reversed micellar system

Four kinds of crops in the pea family (*Leguminosae*), harvested in 2010, including soybean (*Glycine max* L. Merr.), black soybean (*Glycine max* L. Merr.), jinuni bean (*Rhynchosia volubilis*), and red bean (*Phaseolus angularis*) were purchased from a local market and applied to the preparation of crude enzyme extracts. The entire procedure for the preparation of crude enzyme from the crops was carried out at 4 °C. The fruiting body of each crop was added into 50 mM Tris–HCl (pH 7.0) in a ratio of 1:10 (w/v) and homogenised using a laboratory blender. After centrifugation at 20,000*g* for 30 min, the supernatant was filtrated though a 0.45-μm membrane filter. Crude enzyme in the filtrate was fractionated by ammonium sulfate precipitation at 80% saturation and subsequent centrifugated at 20,000*g* for 30 min. The crude enzyme was re-dissolved in a minimum volume of 50 mM Tris–HCl (pH 7.0) and then dialysis was conducted against the same buffer for overnight. The resulting crude enzyme solution was applied to further experiments to measure the initial absorbance (referred to as the blank prior to enzymatic reaction) of emulsified or reversed micellar reactant. Emulsion reactant was prepared according to the method of Kuo et al. (2006) with a slight modification for equal reactant volume to that of reversed micellar system. For the comparison of initial absorbances between two different reaction systems (Table 2), protein concentrations of crude enzyme solutions were adjusted to equal level (7.8 mg of protein in 1 mL of crude enzyme solution).

2.5. Statistical analysis

All data are given as mean values and standard deviations of triplicate experiments and were reproducible within ±10%. The initial velocity of lipoxygenase was estimated from the fitted nonlinear regression equation between the reaction time and μmoles of conjugated diene produced, calculated using the SigmaPlot software (ver. 10.0; Systat Software, Inc., San Jose, CA).

3. Results and discussion

3.1. Effects of AOT in reversed micelles on lipoxygenase activity determination

A major limitation on the use of AOT/isooctane reversed micelles as a microreactor for lipoxygenase is the strong UV absorption of AOT used as a surfactant to prepare the reversed micelles (Fig. 1). Fig. 1 shows that AOT alone in isooctane absorbs strongly at 220 nm, in the UV range. The increase in UV absorption was found to be proportional to the amount of AOT. As mentioned, lipoxygenase activity is generally determined spectrophotometrically by the production of conjugated diene, which shows strong absorbance at 234 nm. To overcome the interference caused by AOT absorption in the UV range, Perez-Gilabert, Sanchez-Ferrer, and Garcia-Carmona (1992) suggested an AOT concentration

Table 1
Relative activity of lipoxygenase in reversed micelles formed by AOT in various organic solvents.

Organic solvents	Relative activity (%)
Isooctane	100.00[a]
Cyclohexane	38.61 ± 1.94[b]
n-Heptane	19.15 ± 2.48[c]
n-Hexane	18.80 ± 5.50[c]
Hexadecane	16.75 ± 3.25[c]

The values with different superscripts within a column are significantly different ($p < 0.05$) by Duncan's multiple range test (mean ± SD from independent experiments in triplicate, $n = 2$).

Table 2
Comparison of initial absorbances at 234 nm referred to as the blank prior to enzymatic reaction according to different reaction systems.

Crude enzymes	Analytical methods	
	Emulsion system	Reversed micellar system
Soybean (*Glycine max. L. Merr.*)	0.858 ± 0.046	0.038 ± 0.004
Black soybean (*Glycine max. L. Merr.*)	0.634 ± 0.063	0.033 ± 0.005
Jinuni bean (*Rhynchosia volubilis*)	0.748 ± 0.089	0.040 ± 0.003
Red bean (*Phaseolus angularis*)	0.163 ± 0.002	0.040 ± 0.007

The values represent mean ± SD from independent experiments in triplicate ($n = 2$).

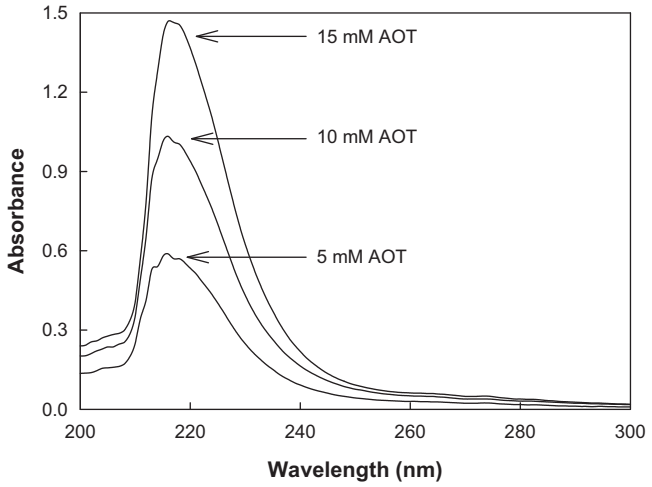

Fig. 1. UV absorption spectra of AOT in isooctane at various concentration.

below 6 mM. However, in our experiment, 5 mM AOT in isooctane still showed fairly UV absorption. Thus, to use an AOT/isooctane reversed micellar system to assay lipoxygenase, removal of AOT is required. The removal of AOT from AOT/isooctane reversed micelles was carried out by addition of an ethanol–water mix. Fig. 2A shows AOT removal from the isooctane layer as a function of the proportion of ethanol in the added ethanol–water mixture. From preliminary experiments for the selection of AOT extraction solvent, we concluded that ethanol was the most suitable solvent for AOT extraction (data not shown). Ethanol is immiscible with isooctane, and it effectively extracts AOT from the isooctane layer. When the absolute ethanol (200 proof) was used as an AOT extraction solvent, not only AOT but also the conjugated diene, the lipoxygenase reaction product, was extracted from the isooctane layer.

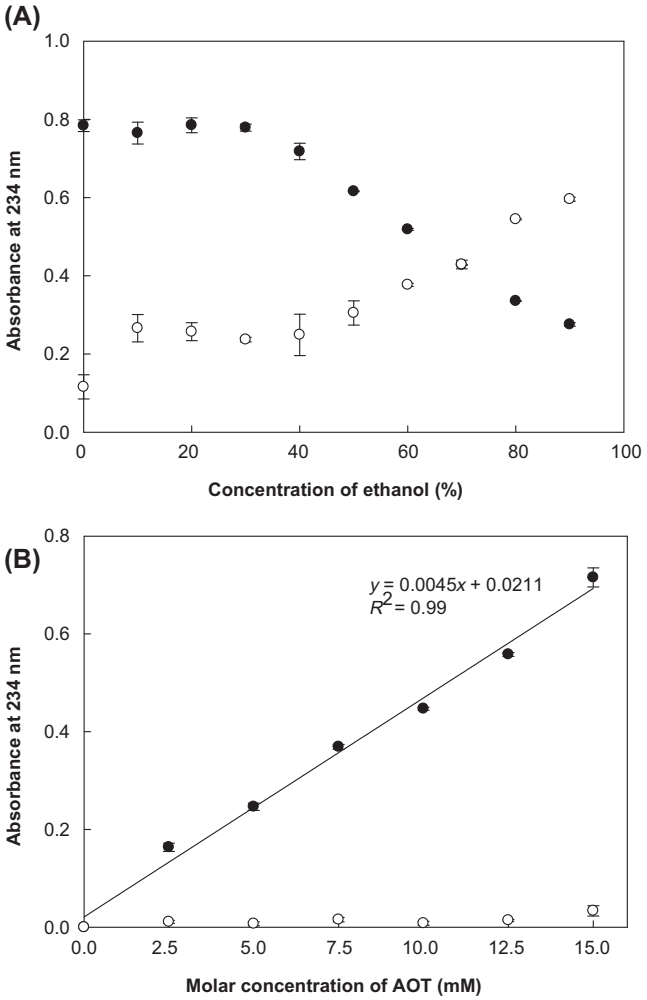

Fig. 2. Effect of ethanol proportion in the ethanol–water mixture added to reaction-terminated reversed micellar system on the content of conjugated diene in isooctane (●) and ethanol layer (○) (A), elimination of AOT from isooctane layer by addtion of 30% ethanol (○) and without treatment (●) (B).

Therefore, the modification in polarity of ethanol was required for the selective AOT extraction from isooctane layer. The modification in polarity of ethanol was attempted by mixing the absolute ethanol with water. Addition of ethanol–water mixture with up to 30% ethanol (v/v) had no effect on the absorbance of the isooctane layer at 234 nm, but addition of more than 30% (v/v) ethanol–water mixture gradually decreased the absorbance. However, unlike the change in the absorbance of the isooctane layer, if the ethanol proportion in the added ethanol–water mixture exceeded 30%, the absorbance of the ethanol layer increased in proportion to the added ethanol in the ethanol–water mixture. These results suggest that the ethanol–water mixture containing up to 30% ethanol selectively extracted AOT from the isooctane layer and that a higher proportion of ethanol may extract not only AOT but also conjugated diene from the isooctane layer. However, the addition of ethanol–water mixture with below 30% ethanol caused turbidity in the isooctane layer, which interfered with the absorbance reading at 234 nm. Thus, for further study, the ethanol proportion of the ethanol–water mixture was fixed at 30% for AOT extraction.

The efficiency of the ethanol–water mixture in AOT extraction was independent of AOT concentration (Fig. 2B). The dependency on AOT concentration was determined by adding 30% ethanol–water mixture to the isooctane layer containing AOT in a range from 2.5 to 15 mM. Addition of 30% ethanol–water mixture

effectively eliminated AOT from the isooctane layer, independent of the AOT concentration in the isooctane layer.

3.2. Selection of organic reaction medium

Five organic solvents, cyclohexane, *n*-heptane, *n*-hexane, isooctane, and hexadecane, were tested because they are frequently used in the determination of lipase activity in reversed micelles (Chang & Rhee, 1990; Han & Rhee, 1986; Kim, Kwon, & Rhee, 1984; Prazeres, Garcia, & Cabral, 1992). Isooctane was the most effective organic solvent tested for the lipoxygenase reaction in reversed micelles (Table 1). This result was consistent with previous reports on lipase activity in reversed micelles (Han & Rhee, 1986; Kim et al., 1984).

In fact, it has been reported that changes in the biocatalytic activity of lipases and lipoxygenases in organic solvents can be explained by the log*P* value, which is the degree of solvent polarity (Leo, Hansch, & Elkins, 1971). Generally, biocatalysis in organic solvents have been reported to be relatively low in hydrophilic solvents having a log*P* < 2, moderate in solvents having a log*P* between 2 and 4, and high in hydrophobic solvents having a log*P* > 4.

However, the hypothesis was proposed before that log*P* of the organic solvents may affect the biocatalysis rate when a microemulsion has a very small amount of water only to solubilise and stabilise enzymes (Laane, Boeren, Vos, & Veeger, 1987). So, following the proposal of Laane et al. (1987), relatively low lipoxygenase activity should be obtained only in reversed micelles prepared with hexadecane (log*P* ≈ 8.8). However, our results were not consistent with this. The highest lipoxygenase activity was obtained with reversed micelles prepared with isooctane (log*P* ≈ 4.1). Although cyclohexane (log*P* ≈ 3.2), *n*-heptane (log*P* ≈ 3.8), and *n*-hexane (log*P* ≈ 3.5) have similar log*P* values to isooctane, lipoxygenase activities in reversed micelles prepared with them were much lower than those in isooctane reversed micelles. Another possible explanation for this observation is the difference in the molecular structure of the organic media used. The organic solvents we tested were straight-chain alkanes, except cyclohexane and isooctane. Hydrocarbons with short chains can embed in the interfacial membrane formed with AOT molecules; then, hydrocarbons can form an additional layer at the interfacial membrane (Hirai, Kawai-Hirai, Sanada, Iwase, & Mitsuya, 1999). Penetration of the mostly saturated hydrocarbons into the surfactant layer of the reversed micelle impedes the contact and/or interaction between lipoxygenase and its substrates, and that could be a reason for the relatively low lipoxygenase activity in *n*-hexane and *n*-heptane. The mostly or fully saturated hydrocarbons, with hydrocarbon chains of C_{9-10}, are able to embed in the surfactant layer of the AOT reversed micelle. The stability of the reversed micelles and of enzymes in the water pool could be governed primarily by the packing pattern of surfactant molecules on the interfacial membrane. Although hexadecane has a too long hydrocarbon chain to penetrate through the AOT interfacial membrane, and cyclohexane does not have the proper structure to penetrate through the AOT interfacial membrane due to its unique ring structure, lipoxygenase activities in these media were quite low compared with those in isooctane. This could be due to a more favourable packing pattern of AOT molecules in isooctane than in hexadecane or cyclohexane. The favourable packing pattern of AOT molecules in isooctane could make the reversed micelles, and the lipoxygenase in them, more stable. In this case, AOT molecules may be packed more appropriately in cyclohexane than in hexadecane.

From these results, we concluded that lipoxygenase activity was not associated with log*P* values of the organic solvents tested but that the biocatalysis of lipoxygenase in reversed micelles had an organic-solvent dependency. In fact, lipoxygenase activity was influenced by the structure of the organic medium rather than simply by its polarity. Thus, isooctane was used as the organic reaction medium for the reversed micelles in further studies.

3.3. Effect of water content in AOT/isooctane reversed micelles

The water content in the reversed micelles is a key factor in biocatalysis using reversed micelles (Avramiotis, Xenakis, & Lianos, 1996; Prazeres et al., 1992), and it may influence the stability of the encapsulated enzyme (Barbaric & Luisi, 1981). Fig. 3 shows the effect of the water content in the AOT/isooctane reversed micelles. The water content in the reversed micelle is expressed as the molar ratio of water to AOT, the *R*-value. The specific activity was strongly dependent on the water content, and lipoxygenase showed a bell-shaped profile of specific activity, with a maximum at an *R*-value of 10. The cores of the reversed micelles are of nanometre size and are able to solubilise proteins, especially enzymes, into them. The water molecules in the reversed micelles may be free or bound. A previous study showed that two or three molecules of water can strongly bind to the hydrophilic head of an AOT molecule, and approximately 13 molecules of water can be trapped in an AOT molecule (Amararene et al., 1997). The properties of water molecules that bind to the surfactant polar head at the interface are significantly different from those of bulk water (Faeder & Ladanyi, 2000). At low *R*-values, meaning that size of the reversed micelles is quite small, most of the water molecules may be directly bound to the hydrophilic heads of AOT surfactants; thus, there may be a lack of water for the hydration of enzymes. The unique internal environment of the reversed micelles with low *R*-values also has effects on the protein folding and unfolding transition and protein–protein interactions. Thus, the low lipoxygenase activity at low *R*-values (<5) could be explained by a lack of water for the hydration of the enzyme and/or by incomplete conformational changes into an active form.

There are several hypotheses for the reduction of lipoxygenase activity with further increases in *R*-value above the *R*-value for maximum lipoxygenase activity. One is that the reduction in enzyme activity when the *R*-value exceeds the optimal value could be due to a decrease in the concentrations of enzyme and substrate in the water pool (Chen & Pai, 1991; Han & Rhee, 1986). Electrostatic interactions between the negatively charged AOT and lipoxygenase could be another explanation for the low activity in the reversed micelles with high *R*-values. According to the lipoxygenase supplier, the pI of lipoxygenase used in this study is in the range of 4.5–6.5. Thus, lipoxygenase in Tris–HCl (pH 9.0) buffer

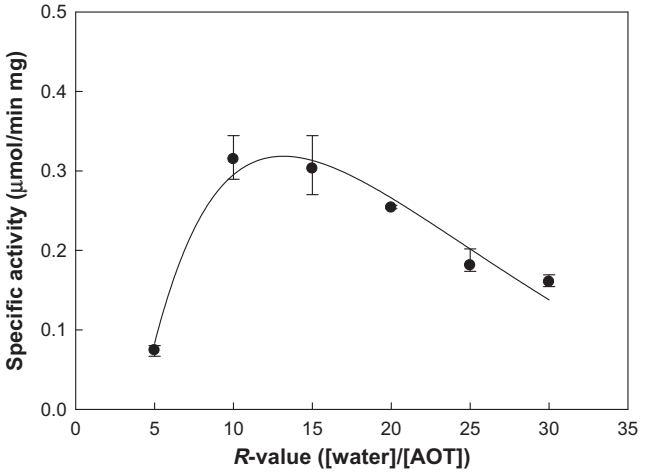

Fig. 3. Effect of *R*-value ([water]/[AOT]) on specific activity of soybean lipoxygenase in reversed micellar system.

should be negatively charged. Because of electrostatic repulsion between negatively charged lipoxygenase and negatively charged AOT, lipoxygenase could be localised to the core of the reversed micelles. The size of the reversed micelles may impact lipoxygenase-membrane and/or lipoxygenase-solvent interactions.

In conclusion, the amount of water molecules in the reversed micelles with an *R*-value of 10 and micellar size could determine the most appropriate conditions for the highest stability and activity of lipoxygenase. The bell-shaped profile of lipoxygenase activity, depending on the *R*-value, is similar to those with lipase (Han & Rhee, 1986; Han, Walde, & Luisi, 1990; Prazeres et al., 1992).

3.4. Verification of lipoxygenase assay in reversed micellar system

To assess the lipoxygenase assay developed here, experiments monitoring changes in initial velocity according to enzyme concentration were performed using commercial lipoxygenase I-B from soybean. Fig. 4 shows that the accumulation of conjugated diene produced by lipoxygenase-catalysed dioxygenation at different enzyme concentration and a fixed substrate concentration (5 μmol/mL reactor). The range of enzyme concentrations was determined from preliminary experiments in which the enzyme concentrations did not reach a saturated level. The fitted equations for a hyperbolic curve at different enzyme concentrations are also given in Fig. 4, which shows non-linear regression between the reaction time and the amount of conjugated diene produced. Initial velocities calculated from the fitted equation of the lipoxygenase-catalysed reaction with 18, 36, 54, and 72 μg/mL were determined as 0.046, 0.114, 0.197, and 0.268 μmol/min, respectively. These values of initial velocities were significantly proportional to the enzyme concentration within the range evaluated. These results agreed with the pattern of fatty acid liberation as a result of lipase-catalysed hydrolysis in reversed micelles reported by Han and Rhee (1986).

When lipoxygenase activity determination by measuring a conjugated diene formation is conducted with the purified lipoxygenase, lipoxygenase assay in emulsion system generally is simple and rapid because of very low contents of other UV-absorbing materials. However, with crude enzyme extracts, water-soluble UV-absorbing materials in the crude enzyme extracts could strongly have an influence on the absorbance at 234 nm (Anthon & Barrett, 2001). Therefore, the lipoxygenase assay with crude enzyme extracts from agricultural products was conducted in emulsion and reversed micellar systems. Table 2 shows the initial absorbances of the reaction mixtures of emulsion system and reversed micellar system. As shown in Table 2, the initial absorbances for emulsion system were always much higher than those for reversed micellar system, independent on the botanical sources of the crude enzyme extracts. We believe that the considerably high initial absorbance value has a negative influence on the entire absorbance reading during time-course lipoxygenase reaction. The high initial absorbances in emulsion system could be due to large amounts of water-soluble UV-absorbing materials, such as pigments and proteins, etc., and these values were high enough to interfere in the quantitative kinetic measurements with accuracy and precision. On the contrary, it was observed that the initial absorbances for reversed micellar system were very low and rarely fluctuated compared with the emulsified system. It suggests that UV-absorbing materials present in the crude enzyme extract did not affect the quantitative analysis of a conjugated diene. It could be mainly due to absence of water-soluble UV-absorbing materials in the isooctane phase to be analysed by UV spectrophotometry. Also, another possible reason is the absence or the very low AOT concentration in the measuring isooctane layer by AOT removal step. These low initial absorbances could not interrupt to monitor the production of the conjugated diene during lipoxygenase reaction. In conclusion, it was determined that the lipoxygenase assay in the reversed micellar system could be useful as a novel method for screening lipoxygenase activity in crude enzyme extracts from various sources in the food industry.

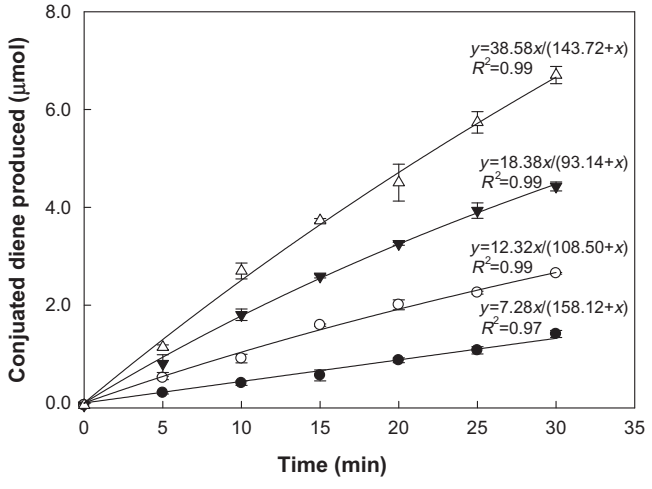

Fig. 4. Formation of conjugated dienes in reversed micellar system, showining the changes in initial velocity of the lipoxygenase-catalysed dioxygenation according to enzyme concentation (●, 18 μg/mL; ○, 36 μg/mL; ▼, 54 μg/mL; △, 72 μg/mL).

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References

Amararene, A., Gindre, M., Le Huérou, J.-Y., Nicot, C., Urbach, W., & Waks, M. (1997). Water confined in reverse micelles: acoustic and densimetric studies. *Journal of Physical Chemistry B, 101*(50), 10751–10756.

Anthon, G. E., & Barrett, D. M. (2001). Colorimetric method for the determination of lipoxygenase activity. *Journal of Agricultural and Food Chemistry, 49*(1), 32–37.

Avramiotis, S., Xenakis, A., & Lianos, P. (1996). Lecithin W/O microemulsions as a host for trypsin. Enzyme activity and luminescence decay studies. *Progress in Colloid and Polymer Science, 100*(286–289).

Axelrod, B., Cheesebrough, T. M., & Laakso, S. (1981). Lipoxygenase from soybeans. *Methods in Enzymology, 71*(441–451).

Barbaric, S., & Luisi, P. L. (1981). Micellar solubilization of biopolymers in organic solvents. 5. Activity and conformation of α-chymotrypsin in isooctane-AOT reverse micelles. *Journal of the American Chemical Society, 103*(14), 4239–4244.

Baysal, T., & Emirdöven, A. (2007). Lipoxygenase in fruits and vegetables: a review. *Enzyme and Microbial Technology, 40*(4), 491–496.

Beneytout, J.-L., Andrianarison, R.-H., Rakotoarisoa, Z., & Tixier, M. (1989). Properties of a lipoxygenase in green algae (*Oscillatoria* sp.). *Plant Physiology, 91*(1), 367–372.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Brash, A. R. (1999). Lipoxygenase: occurrence, functions, catalysis, and acquisition of substrate. *Journal of Biological Chemistry, 274*(34), 23679–23682.

Carvalho, C. M. L., & Cabral, J. M. S. (2000). Reverse micelles as reaction media for lipases. *Biochimie, 82*(11), 1063–1085.

Chang, P. S., & Rhee, J. S. (1990). Characteristics of lipase-catalyzed glycerolysis of triglyceride in AOT-isooctane reversed micelles. *Biocatalysis and Biotransformation, 3*(4), 343–355.

Chen, J.-P., & Pai, H. (1991). Hydrolysis of milk fat with lipase in reversed micelles. *Journal of Food Science, 56*(1), 234–237.

Faeder, J., & Ladanyi, B. M. (2000). Molecular dynamics simulations of the interior of aqueous reverse micelles. *Journal of Physical Chemistry B, 104*(5), 1033–1046.

Han, D., & Rhee, J. S. (1986). Characteristics of lipase-catalyzed hydrolysis of olive oil in AOT-isooctane revesred micelles. *Biotechnology and Bioengineering, 28*(8), 1250–1255.

Han, D., Walde, P., & Luisi, P. L. (1990). Dependence of lipase activity on water content and enzyme concentration in reverse micelles. *Biocatalysis and Biotransformation, 4*(2–3), 153–161.

Hirai, M., Kawai-Hirai, R., Sanada, M., Iwase, H., & Mitsuya, S. (1999). Characteristics of AOT microemulsion structure depending on apolar solvents. *Journal of Physical Chemistry B, 103*(44), 9658–9662.

Hsieh, R. J. (1994). Contribution of lipoxygenase pathway to food flavors. In C.-T. Ho & T. Hartman (Eds.). *Lipids in Food Flavors* (vol. 558, pp. 30–48). Washington, DC: American Chemical Society.

Kim, K. H., Kwon, D. Y., & Rhee, J. S. (1984). Effects of organic solvents on lipase for fat splitting. *Lipids, 19*(12), 975–977.

Koch, R. B., Stern, B., & Ferrari, C. G. (1958). Linoleic acid and trilinolein as substrates for soybean lipoxidase(s). *Archives of Biochemistry and Biophysics, 78*(1), 165–179.

Kuhn, H., & Thiele, B. J. (1999). The diversity of the lipoxygenase family. Many sequence data but little information on biological significance. *FEBS Letters, 449*(1), 7–11.

Kuo, J.-M., Hwang, A., Teh, D.-B., Pan, M.-H., Tsai, M.-L., & Pan, B. S. (2006). Lipoxygenase from banana leaf: Purification and characterization of an enzyme that catalyzes linoleic acid oxygenation at the 9-position. *Journal of Agricultural and Food Chemistry, 54*(8), 3151–3156.

Laane, C., Boeren, S., Vos, K., & Veeger, C. (1987). Rules of optimizing of biocatalysis in organic solvents. *Biotechnology and Bioengineering, 30*(1), 81–87.

Leo, A., Hansch, C., & Elkins, D. (1971). Partition coefficients and their uses. *Chemical Reviews, 71*(6), 525–616.

Maas, R. L., Brash, A. R., & Oates, J. A. (1981). A second pathway of leukotriene biosynthesis in porcine leukocytes. *Proceedings of the National Academy of Sciences of the United States of America, 78*(9), 5523–5527.

Martinek, K., Klyachko, N. L., Kabanov, A. V., Khmelnitsky, Y. L., & Levashov, A. V. (1989). Micellar enzymology: its relation to membranology. *Biochimica et Biophysica Acta, 981*(2), 161–172.

Martinek, K., Levashov, A. V., Klyachko, N., Khmelnitsky, Y. L., & Berezin, I. V. (1986). Micellar enzymology. *European Journal of Biochemistry, 155*(3), 453–468.

Nuñez, A., Savary, B. J., Fogila, T. A., & Piazza, G. J. (2002). Purification of lipoxygenase from *Chlorella*: production of 9- and 13 hydroperoxide derivatives of linoleic acid. *Lipids, 37*(11), 1027–1032.

Perez-Gilabert, M., Sanchez-Ferrer, A., & Garcia-Carmona, F. (1992). Application of active-phase plot to the kinetic analysis of lipoxygenase in reverse micelles. *Biochemical Journal, 288*, 1011–1015.

Perraud, X., & Kermasha, S. (2000). Characterization of lipoxygenase extracts from *Penicillium* sp.. *Journal of The American Oil Chemists' Society, 77*(4), 335–342.

Prazeres, D. M. F., Garcia, F. A. P., & Cabral, J. M. S. (1992). Kinetics and stability of a *Chromobacterium viscosum* lipase in reversed micellar and aqueous media. *Journal of Chemical Technology and Biotechnology, 53*(2), 159–164.

Robinson, D. S., Wu, Z., Domoney, C., & Casey, R. (1995). Lipoxygenases and the quality of foods. *Food Chemistry, 54*(1), 33–43.

Siedow, J. N. (1991). Plant lipoxygenase: structure and function. *Annual Review of Plant Physiology and Plant Molecular Biology, 42*, 145–188.

Tamamushi, B., & Watanabe, N. (1980). The formation of molecular aggregation structures in ternary system: Aeresol OT/water/iso-octane. *Colloids and Polymer Science, 258*(2), 174–178.

Toyosaki, T. (1992). Bleaching of methylene blue as an indicator of lipoxygenase activity. *Journal of the Association of Official Agricultural Chemists, 75*(6), 1124–1126.