A Simplified Method for Determination of Free Fatty Acids for Soluble and Immobilized Lipase Assay

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ABSTRACT

A simple and rapid method for determination of free fatty acids for soluble and immobilized lipase assay was developed. The free fatty acids could be determined within 10 min with less organic solvent used and the color developed was stable until 60 min. High correlation ($r > 0.97$) between fatty acids content (2 - 10 µmole) and absorbance was observed for fatty acids with carbon number of 6 or higher. Hydrolysis activity of soluble and immobilized lipase could be measured with high sensitivity and reproducibility against incubation time and protein loading. The effect of various substrate concentrations and water against hydrolysis activity could also be measured. The method was suitable for routine analysis such as purification of lipase and continuous hydrolysis of fat and oil.

INTRODUCTION

Methods for determination of free fatty acids and lipase activity have been reviewed by Jensen (1983). Most of the method involved several steps such as extraction of the fatty acids in appropriate solvents, evaporation, centrifugation and used a lot of solvents. In 1963, Duncombe has developed a rapid free fatty acids determination using $Cu(NO_3)_2.3H_2O$ in which fatty acids was converted to the Cu-soaps and then measured spectrophotometrically after reaction with chromogenic reagents. The method was then improved by Lowry and Tinsley (1976) using benzene as solubilizing agents of Copper-soaps complexes. Based on the Lowry and Tinsley's method (1976), Kwon and Rhee (1986) proposed a simple method using isooctane to replace benzene due to the fact that lipase was more stable in isooctane than those of other solvents examined (Kim *et al.*, 1984) and the color development of coppersoaps was stable in a wider pH range. However, the methods still required a lot of isooctane and cupric acetate-pyridine.

The purpose of this study is to simplify the Kwon and Rhee's method by omitting the addition of HC1 and reducing the utilization of isooctane and cupric acetate-pyridine solution for activity determination of soluble and immobilized lipase.

MATERIALS AND METHODS

Chemicals

Lipase from *Rhizopus delemar* was kindly gifted from Amano Pharmaceuticals (Nagoya, Japan). Free fatty acids, isooctane, copper acetate monohydrate, pyridine and olive oil were purchased from Nakalai Tesque (Kyoto, Japan). Cellulose triacetate bead was obtained from Aldrich Chemical Company (Milwauke, USA). All other chemicals used were of analytical grade.

A 5% (w/v) copper acetate-pyridine pH 6.0 was prepared according to the method of Lowry and Tinsley (1976). Five grams of copper acetate was dissolved in 80 ml of distilled water and the pH was adjusted to 6.0 using pyridine then the volume was made to 100 ml using distilled water.

Standard Curve of Free Fatty Acids

The standard curves of each free fatty acid were prepared by dissolving each fatty acid in isooctane to give a series of concentration of 2 - 10 µmole/2 ml in a screw cap vials. In case of stearic acid, the concentration was made between 0.2 - 2 µmole/2 ml. Each concentration of fatty acids was dissolved in 2 ml of isooctane, then 0.4 ml of 5% cupric acetate-pyridine pH 6.0 was added, and mixed vigorously for 90 sec by hand.

The mixture was centrifuged for 2 min, at 2,000 rpm. Fatty acids content in the supernatant phase (isooctane fraction) was measured by spectrophotometer at 715 nm. The centrifugation step could be omitted and replaced by mixing for 5 sec using vortex mixer and allowed for 10 min.

Determination of hydrolysis activity of soluble lipase

Soluble lipase activity was determined in a screw cap vial containing 2 ml of reaction mixture containing 60% (v/v) olive oil in isooctane. The 60% (v/v) olive oil in isooctane was prepared by mixing 60 ml of olive oil with 40 ml of isooctane. The reaction was started by addition of 20 µl of enzyme solution in agitated incubator at 30 °C. After 20 min, the reaction was stopped by placing the reaction mixture in an ice bath for few minutes. Take 200 µl of the aliquots and added to the mixture containing 1800 µl of isooctane and 400 µl of 5% cupric-acetate pyridine pH 6.0. The content of free fatty acids in the mixture was determined as described above by comparing the absorbance of the mixture with standard curve of free fatty acids. One unit of lipase activity was defined as amount of enzyme that produced 1 µmole of fatty acids per min.

Determination of hydrolysis activity of immobilized lipase

Soluble lipase was immobilized in cellulose triacetate beads by soaking the bead (0.5 g) into 1 ml of lipase solution with various concentrations of protein for 24 hours at 24 °C. The beads was separated from the aliquots and washed twice with 1 ml of cold 0.1 M potassium phosphate buffer pH 7.0.

Hydrolysis activity of immobilized lipase was measured by mixing the lipase immobilized beads with 5 ml of 0.1 M potassium phosphate buffer pH. 7.0 and 5 ml of 60% (v/v) olive oil in isooctane in a 100-ml Erlenmeyer flask capped by stopper gum. The mixture was incubated in a shaker waterbath at 30 °C, otherwise indicated in the text. At defined time, take a small volume (50 – 200 µl) of the mixture and added to the mixture containing isooctane (1950 – 1800 µl) and 400 µl of 5% cupric-acetate solution. The free fatty acid content in the isooctane fraction (upper layer) was determined as described above.

Comparison of some lipase activity determination

Sensitivity of modified Kwon and Rhee's method presented in this paper was compared with Lin's method (Lin *et. al.,* 1996) and Amano's method (Anonymous 1998).

RESULTS AND DISCUSSION

Standard curves of free fatty acids

Effect of carbon numbers of fatty acids and their color development was studied spectrophotometrically at 715 nm with various concentration of fatty acids in 2 ml of isooctane (Fig. 1). All fatty acids used in this

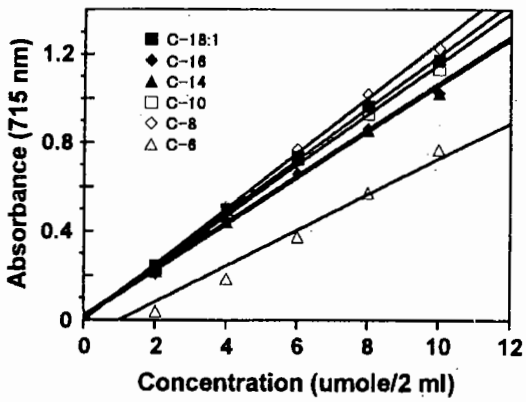

Figure 1. **Standard curves of some free fatty acids in isooctane. The absorbance was measured at 715 nm with cupric acetate-pyridine as color developer. The regression equation of each line of fatty acids was obtained in Table 1. Each point represents the mean value of two determinations and each determination represents µmole fatty acids in 2 ml isooctane system.**

study showed an increase in absorbance with the increase of the concentration, giving a linear lines. Fatty acid with carbon number 4 (butyric acid) was less sensitive compared to the other fatty acids which the color developed at concentration higher than 10 mM. On the contrary, fatty acid having carbon number 18 (stearic acid) was very sensitive in which at lower concentration gave higher absorbance. Among the fatty acids examined in this study, caprylic acid (C-8) gave the highest absorbance (Fig. 2). Fatty acids having carbon number less than 8 showed lower absorbance. Correlation between the concentration of fatty acids and their absorbances is shown in Table 1. All fatty acids examined showed a high correlation with $r > 0.97$. These data suggested that this method was suitable for estimation of free fatty acids having carbon number at least 6. The sensitivity of the method described here has no differences with Kwon

and Rhee's method (1986). Furthermore, the color developed from the complex between fatty acids and copper-acetate pyridine in the form of copper-soap was very stable. This is shown in Fig. 3 in which the color development of oleic acid was stable at least up to 60 min both in low (2 µmole) and high (8 µmole) concentration.

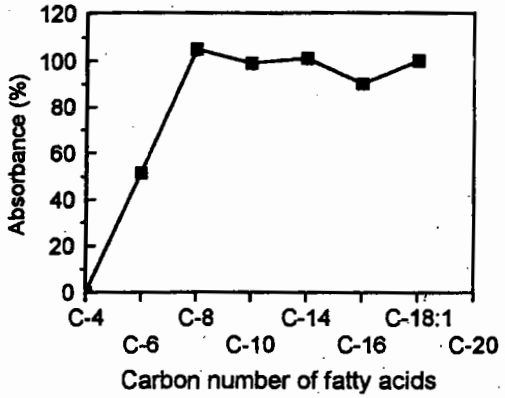

Figure 2. **Effect of carbon numbers of fatty acids on color development. Each point represents relative absorbance at 715 nm in which Caprylic acid (C-8) was used as standard of comparison. The values were obtained from mean of two determinations of 6 µmole/2 ml isooctane system.**

Table 1. **Relationship between concentration of fatty acids and absorbance**

Fatty acids	Coefficient correlation (r) and regression equation (Y)	Concentration range (µmole/2 ml)
1. Stearic acid (C-18)	0.973; Y=-0.0217+0.026 X	0 – 1.8
2. Oleic acid (C-18:1)	0.999; Y= 0.0142+0.118 X	2 – 10
3. Palmitic acid (C-16)	0.997; Y= 0.0126+0.104 X	2 – 10
4. Myristic acid (C-14)	0.986; Y= 0.0239+0.105 X	2 – 10
5. n-Capric acid (C-10)	0.998; Y= 0.0143+0.125 X	2 – 10
6. Caprylic acid (C-8)	0.999; Y= 0.0026+0.125 X	2 – 10
7. n-Caproic acid (C-6)	0.971; Y= 0.0771+0.080 X	2 – 10
8. Butyric acid (C-4)	ND	ND

ND : Not Detected

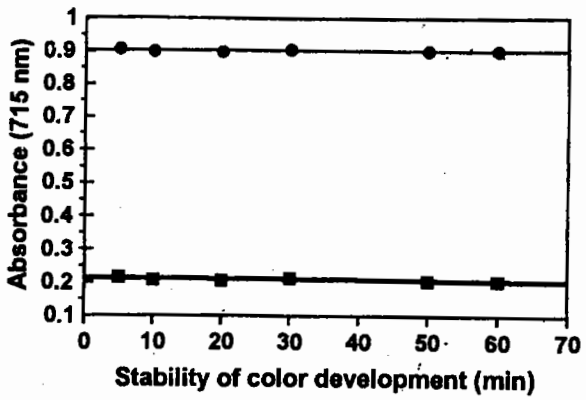

Figure 3. **The stability of color development of oleic acids 2 µmole/2ml (■) and 8 µmole/2 ml (•) isooctane. Each point represents mean of two determinations.**

Hydrolysis activity of soluble and immobilized lipase

Figures 4 and 5 show a hydrolysis activity of both soluble and immobilized lipase in the form of fatty

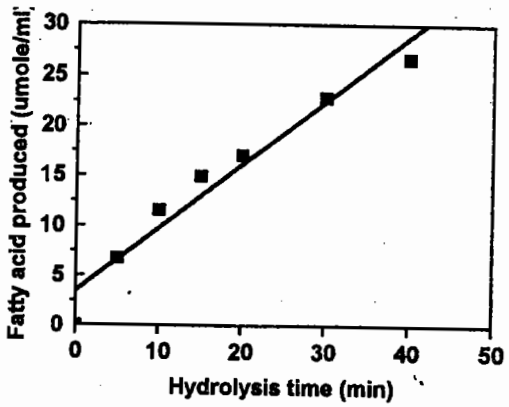

Figure 4. **Effect of incubation time on the hydrolysis activity of soluble lipase in isooctane reaction system. Twenty µl (6.68 µg) of soluble lipase was added to 2 ml of substrate emulsion (60% v/v of olive oil in isooctane) and incubated at 30 °C for various times. The reaction was stopped by placed the mixture in ice bath for few minutes. Two hundreds µl of the aliquots was taken to determine its free fatty acids contents.**

acids (as oleic acid) liberated from olive oil. The fatty acid produced increased gradually with the time incubation. In the first 10 minutes, the activity of soluble enzyme was higher than that of immobilized one. One of the reason is due to the supporting material used for immobilization is hydrophilic which caused difficult contact between substrate in the hydrophobic phase and

enzyme that reside in hydrophilic matrix. From these data, it seems that the method used in this study was suitable for estimation of the lipase activity both for free or immobilized form.

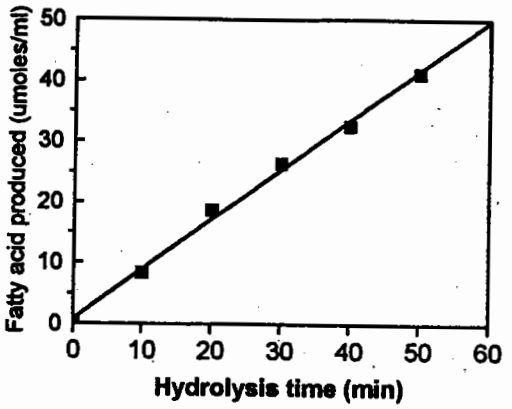

Figure 5. **Effect of incubation time on the hydrolysis activity of immobilized lipase 0.5 g of cellulose triacetate bead containing 801 μg of protein was added to 5 ml of 0.1 M Potassium phosphate buffer pH 7.0 and 5 ml of substrate in a 100-ml Erlenmeyer flask capped with stopper gum. The mixture was incubated in waterbath with shaking at 37 °C. At defined time, a 50 μl of the upper layer was taken to determine its fatty acids content.**

The effect of substrate (olive oil) concentration on the activity of soluble lipase was also examined by this method (Fig. 6). Hydrolysis activity of soluble lipase increased with the increased of substrate concentration (10 - 60% v/v olive oil in isooctane). The data suggest that this method was also appropriate for determination of kinetic parameters (K_m and V-max) of lipase by varying the concentration of substrate.

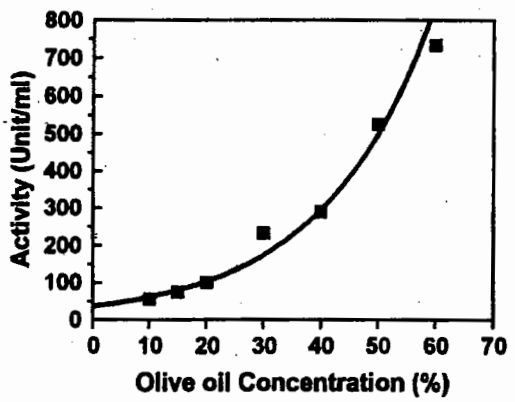

Figure 6. **Effect of substrate concentration on the hydrolysis activity of the soluble lipase. The Substrate was made by mixed the olive oil and isooctane in the volume ratio as mentioned in the figures. The activity was measured as described in Materials and Methods.**

Hydrolysis activity of lipase is greatly affected by the presence of water in microenvironment in which the hydrolysis occurred. In this method, we examined the effect of various concentration of water on the hydrolysis of olive oil by soluble lipase (Fig. 7). The data show that the addition of water increased the hydrolysis activity and even at 5% concentration the increase of activity could be detected. The hydrolysis activity increased about 4 times when the water content reached to 25% in the reaction system.

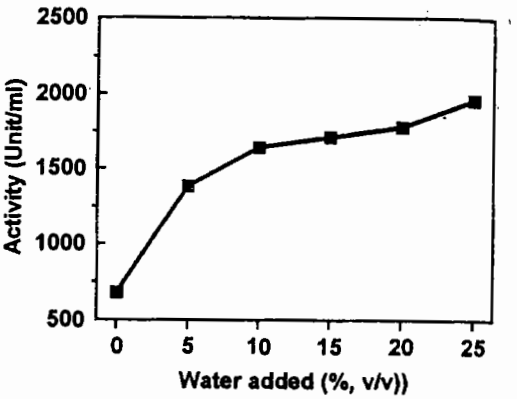

Figure 7. **Effect of water concentration on the hydrolysis activity of soluble lipase. The water was added to the mixture containing olive oil (60%) and isooctane. The concentration of water and isooctane was set at various level to give a total concentration of 40% (v/v) in 2 ml of reaction mixture.**

Comparative study on the hydrolysis activity determination

Figure 8 shows the comparison of three different methods for lipase activity determination at various protein concentrations. The modified method of Kwon and Rhee (Fig. 8-A) was very sensitive compared with the other methods, yielding in high activities. On the other hand, the method of Lin *et. al.*, (1996) (Fig. 8-B) gave the lowest activity. This might result from the different in substrate used. Figure 8 also shows that titration method of Amano (Fig. 8-C) was not sensitive as Kwon and Rhee's method in which yielding in a lower activity. Furthermore, the amount of protein that could be used in the Amano's method was very limited. From these data, it is recommended to use the modified method of Kwon and Rhee to determine lipase activity as presented in this paper.

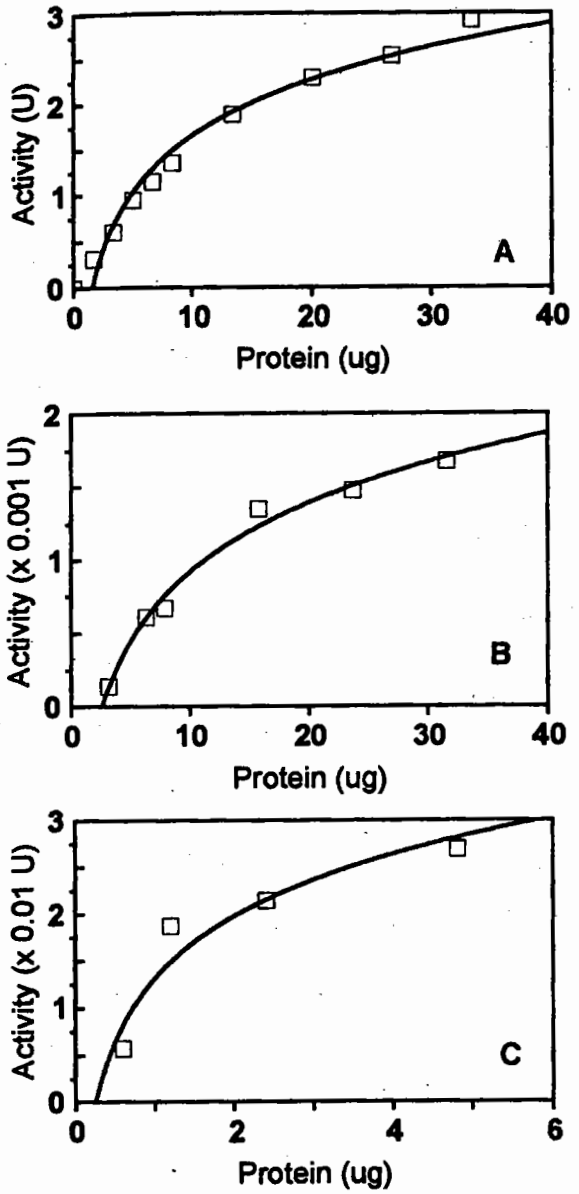

Figure 8. **Comparison of three different methods for lipase activity determination at various protein concentration. One unit of enzyme activity was described as the amount of enzyme which liberate 1 μmole product per min. A Modified Kwon and Rhee's method described in this paper. The enzyme was assayed using olive oil as substrate as described in Materials and Methods. B Method of Lin *et al.* (1996). The enzyme was assayed using p-nitrophenyl miristate as substrate and the amount of p-nitrophenol liberated was determined spectrophotometrically at 410 nm. C. Titration method of Amano (Anonymous, 1998) using olive oil as substrate.**

CONCLUSION

Free fatty acids as a product of enzymatic reaction of soluble and immobilized lipase could be determined rapidly with high sensitivity and reproducibility by the method presented in this paper. The method is capable of measuring the hydrolytic activity of soluble and immobilized lipase that affected by incubation time, protein loading, substrate concentration and water content. It is also suitable for routine analysis such as continuous hydrolysis of fat or oil and detection of lipase activity during purification of lipase.

ACKNOWLEDGEMENT

This work was supported by the Laboratory of Microbial Biochemistry, Faculty of Applied Biological Science, Hiroshima University, Japan. We wish to thank the National Research Council (DRN) of Republic of Indonesia for Research Grant through Riset Unggulan Terpadu VI (RUT-VI) Project.

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