Determination of Lipase Activity by High Performance Liquid Chromatography with Precolumn Fluorescence Derivatization Using 9-Bromomethylacridine

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A simple and highly sensitive method for the measurement of lipase activity has been developed using a fluorescent labeling reagent, 9-bromomethylacridine (9-BMA). With an organic solvent-soluble lipase prepared with synthetic detergent, triglycerides were hydrolyzed in a homogeneous solvent system (such as aqueous tetrahydrofuran). The enzyme reaction was terminated by the addition of DMSO containing 9-BMA; then the liberated fatty acid was labeled in the following derivatization reaction at room temperature for 40 min. The 9-acridinylmethyl derivative of the fatty acid was separated and determined by high performance liquid chromatography (HPLC). The sensitivity of the method was so high that several nmol level of fatty acid in 200 µl of the reaction mixture could be determined. The effects of the pH and of the concentration of buffer on the fluorescent labeling reaction were examined. This method was found to be useful for studying the kinetic properties of lipase.

Keywords 9-Bromomethylacridine, high performance liquid chromatography, lipase assay, precolumn derivatization

Many studies have been carried out to estimate the hydrolytic activity of carboxylesterases such as lipase. Most of the procedures are based on titrimetric or colorimetric methods. For the determination of the lipase activity, the content of fatty acids or glycerol can be measured by titrimetry, copper soap calorimetry, chromophore spectrophotometry, isotopic method, gas chromatography, enzymatic method, and immunological method. The conventional titrimetric and calorimetric methods lack sensitivity and/or simplicity for a routine analysis, requiring large sample volume and long incubation time. The fluorometric method using 4-methylumbelliferyl oleate (4-MUO) as a substrate is so sensitive that 0.1–5 nmol of the product could be detected. However, 4-MUO is unstable in aqueous solutions, especially under alkaline conditions, and is easily subject to nonenzymatic hydrolysis. Furthermore, 4-MUO is not a specific substrate for the lipase.

Recently, several fluorescent labeling reagents for fatty acids or glycerol have also been used to determine the lipase activity. The labeling with these reagents was employed after enzymatic hydrolysis of triglycerides. We determined the produced fatty acid or glycerol by fluorometry.

Previously, we reported on a sensitive method for the determination of fatty acids using 9-bromomethylacridine (9-BMA), one of the fluorescent labeling reagents of carboxyl group, by high performance liquid chromatography (HPLC). In this paper, the HPLC method for the determination of the lipase activity is described; here fatty acids liberated from triglycerides by lipase were labeled with 9-BMA and determined quantitatively by HPLC. An organic solvent-soluble lipase which was modified with synthetic detergent was employed as a model enzyme, because the labeling reaction by 9-BMA can proceed in various organic solvents. The use of the modified lipase in organic solvents enables the observation of the kinetics of hydrolysis in a homogeneous solvent system without the influence of emulfying reagents. The sensitivity and precision of the determination by this method were compared with those of other fluorometric methods. The new method was found to enable the measurement of the lipase activity.

Materials and Methods

Reagents

Lipase from *Pseudomonas fragi* 22. 39B was purchased from Wako Pure Chem. Co. (Osaka, Japan). 9-Bromomethylacridine and tetraethylammonium carbonate (TEAC), a catalyst for the labeling reaction, were prepared according to the method of Akasaka *et al.* Oleic acid used as a standard was supplied by
Nihonyushi Co. Ltd. (Tokyo, Japan). Triolein and other triglycerides were obtained from Wako Pure Chem. Co. Ethanol and acetonitrile for HPLC were purchased from Wako Pure Chem. Co. Other chemicals were of reagent grade and were used without further purification. An organic solvent-soluble lipase was prepared with a synthetic detergent, didodecyl glucosyl glutamate, according to the method of Okahata et al. \(^\text{23}\) with modifications as follows. Ten milligrams of lipase in 2 ml of water was added to 50 mg of didodecyl glucosyl glutamate in 4 ml of THF and the mixture was stirred vigorously at 4°C for 24 h. After evaporation of the solvent, the precipitates were collected by centrifugation. They were dispersed in water to remove water-soluble unmodified lipase and then collected by centrifugation and lyophilized overnight. The powder thus obtained, which contained the organic solvent-soluble lipase and free didodecyl glucosyl glutamate, was stored at 4°C until use.

**Determination of lipase activity**

The assay was performed using the organic solvent-soluble lipase. A standard reaction mixture for lipase assay consisted of 20 µl of organic solvent-soluble lipase THE solution, 100 µl of triolein THE solution (1 mM), 40 µl of THE and 40 µl of Britton-Robinson buffer (0.04 M, pH 8.0). The enzyme solution added to the mixture was diluted appropriately. The mixture was incubated at 37°C for 30 min for the routine assay.

**Fluorescent labeling of fatty acid**

After hydrolysis by lipase, 200 µl each of 9-BMA (5 mM in dimethylsulfoxide, DMSO) and TEAC (2.5 mM in DMSO) was added to the reaction mixture, which was then allowed to stand at room temperature for more than 40 min. Then, a 20 µl aliquot was injected into the HPLC. Free oleic acid was also labeled by the same procedure.

**HPLC conditions**

The HPLC apparatus used was an EYELA Model PLC-5D equipped with a loop type sample injector (Tokyo Rika Kikai Co. Ltd. Tokyo, Japan). The separation column was TSK-gel ODS 80Tm (Tosoh Co. Tokyo, Japan) with a solvent system containing ethanol:acetonitrile:water=95:95:10, under the flow rate of 0.8 ml/min. For the detection, a JASCO Model FP-820 spectrofluorometer was used by monitoring the fluorescent intensity at 425 nm (excitation at 365 nm). The data were processed with a Chromatopack C-R1A integrator (Shimadzu Seisakusho, Kyoto, Japan) and the peak area was used for quantification.

**Results and Discussion**

**HPLC separation**

9-Acridinylmethyl derivatives of fatty acid were separated and determined by HPLC. Figure 1 shows the chromatograms of fluorescent labeled solution after hydrolysis of triolein without (A) or with (B) the organic solvent-soluble lipase (0.1 mg/ml). The derivative of oleic acid was eluted at 10.6 min. When trilaurin or tristearin was hydrolyzed, the corresponding peak of lauric acid or stearic acid was eluted at 6.3 min or 14.7 min, respectively. This method thus appears useful routine lipase assay.

**Fluorescence derivatization**

To investigate the time course of the fluorescent labeling reaction, standard oleic acid dissolved in 200 µl of a mixture of THF and Britton–Robinson buffer (0.04 M, pH 8.0) (4:1 v/v), and the enzymatic reaction mixture were labeled with 9-BMA (Fig. 2). Both of the labeling reactions with 9-BMA were completed within 40 min.
30 min; the intensity was stable for more than 4 h.

The conditions of the labeling with 9-BMA were studied using 200 ng of oleic acid dissolved in a mixture of THF and the buffer (4:1 v/v). When the concentration of 9-BMA was set at twice of TEAC, the labeling reaction progressed most rapidly. Five mM of 9-BMA and 2.5 mM of TAEC were sufficient. Though the speed of labeling reactions increased with an increase in temperature, the labeling was performed at room temperature for convenience. The optimal conditions were almost similar to those in the previous report. Due to the ionic properties of the 9-acridinylmethyl derivatives of fatty acid, the pH of the buffer in the enzyme reaction mixture affects markedly the rate of fluorescence reaction (Fig. 3). The optimum pH for the enzyme reaction (pH 8.0) is also suitable for the labeling reaction.

The peak area of its 9-acridinylmethyl derivatives increased proportionally to the concentration of oleic acid over the range from 0.2 to 4.0 nmol per assay tube. The detection limit (S/N=10) was as low as 0.1 nmol per assay tube within a relative standard error of 5%. The sensitivity is one thousand times higher than that of spectrophotometric methods and close to that of 4-MU. These results show that the new method can obtain accurate and reproducible results for the measurement of oleic acid even at a low concentration.

**Enzyme reaction**

The enzyme was denatured and the hydrolysis reaction was terminated by the addition of DMSO solution of 9-BMA and TEAC. The fluorescent intensity was studied against the amount of organic solvent-soluble lipase in the range of 10 - 50 ng per assay tube. After hydrolysis, the peak area of 9-acridinylmethyl derivatives of oleic acid increased linearly as a function of the increase of the concentration of the modified lipase. As a result, only 20 µg of the organic solvent-soluble lipase was sufficient for the reaction for the routine assay.

As shown in Fig. 4, the fluorescent intensity of the reaction mixture increased proportionally for an incubation time up to 100 min, when 20 µg of the organic solvent-soluble lipase was used. Thus, the reaction time for the routine assay was determined to be 30 min.

In this method, the effect of the components in the reaction mixture on the fluorescent labeling should be taken into account, since the fluorescent labeling reagents were directly added to the reaction mixture after the enzyme reaction.

Water in the enzyme reaction inhibited the formation of the 9-acridinylmethyl derivatives of fatty acids (Fig. 5(A)). The influence of the concentration of water in the enzyme reaction on the activity of the organic solvent-soluble lipase was also examined (Fig. 5(B)). The lipase activity increased remarkably with the increase of the concentration of water. A significant increase in lipase activity was also recognized when 4-MUO was used as a substrate instead of triolein. When the concentration of water in enzyme reaction mixture increases, some triglycerides which have long side chains of fatty acid, for example tripalmitin and tristearin, cannot be dissolved at concentrations of more than several mmol/l. If the enzyme reaction was...
conducted in a heterogeneous solvent system, it would be impossible to investigate the direct interaction between the enzyme and the substrate. Thus, the concentration of water was set up at 20% in this method.

As mentioned above, the high sensitivity of detection and simplicity of the procedure are the major advantages of the new HPLC fluorometric assay measurement for lipase activity compared with the conventional colorimetric and titration methods. The enzymatic properties and kinetic analysis, therefore, can be studied with a small amount of lipase by this method. Though one of the disadvantages of the titration method is that the use of some activators, such as bile salts, leads to a high background reading, the HPLC fluorometric assay alleviated these shortcoming. Since analytical, kinetic and other data are easily and rapidly obtained, the use of 9-BMA for the lipase assay should facilitate studies on the enzymatic properties of the lipase, as well as applications to the purification of the enzyme.

The authors wish to thank Nihonyushi Co. Ltd. for providing the samples of standard fatty acid and Tosoh Co. for supplying the HPLC column. We are also grateful to Dr. Osamu Katayama for his valuable suggestions. This work was supported partly by the fund "Biomaedia Project" from the Ministry of Agriculture, Forestry and Fisheries.

References


(Received October 30, 1990)
(Accepted January 21, 1991)