Journal of Solid-Phase Biochemistry, Vol. 2, No. 1, 1977

CONTINUOUS NEUTRAL LIPID DETERMINATION WITH LIPASE–COLLAGEN MEMBRANE REACTOR

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Accepted February 11, 1977

Lipase (*Pseudomonas* sp.) was immobilized in collagen membrane and used for the measurement of neutral lipids. The determination system for lipids consisted of a lipase–collagen membrane reactor and glass electrodes. Neutral lipids were hydrolyzed to fatty acids and glycerol by immobilized lipase. The liberated protons were determined potentiometrically by using glass electrodes. A linear relationship was obtained between the logarithm of lipid concentrations and potential differences. The measurement gave results comparable to a conventional assay and was found to be applicable to the assay of neutral lipids in sera. The relative error of the determination by this system was within 4%.

INTRODUCTION

Considerable worldwide interest has arisen in the use of immobilized enzymes as catalyst in analytical chemistry. The electrochemical preparation of enzyme–collagen membrane has been developed in our laboratory. Various enzyme electrodes for hydrogen peroxide (1), uric acid (2), urea (3), lactate (4), ethanol (4), and sucrose (5) have been prepared by using enzyme–collagen membrane. No report has been published on enzyme electrodes for water-insoluble substrates such as lipids. The determination of neutral lipids in serum is very important in clinical field analysis. The quantitative determination of neutral lipids can be made calorimetrically or titmetrically; however, pretreatment such as lipid extraction and separation from other lipid components is needed before the determination. On the contrary, the electrochemical method using enzyme–membrane does not require the pretreatment.

A new sensing system for neutral lipids in sera using lipase–collagen membrane reactor is described in this paper.

MATERIALS AND METHODS

Materials

Lipase (glycerol ester hydrolase E.C. 3.11.3. from *Pseudomonas* sp. 32,000 U/g) was obtained from Amano Pharmaceutical Company. Control serum (Precilip) was purchased from Boehringer Mannheim and human serum was obtained from Japanese Red Cross Central Blood Center (Shibuya, Tokyo). Triton X-100 was purchased from Wako Pure Chemicals Industries. Other reagents were commercially available analytical reagents or laboratory grade materials. Collagen was obtained from bull calf skin, and was purified by a method described earlier (6). Glass distilled water was used in all procedures.

Preparation of Enzyme–Collagen Membrane

Collagen fibril suspension was prepared as described previously (7). Ten milliliters of enzyme solution containing 1 g of crude lipase was centrifuged at $3000 \times g$ for 15 min. The supernatant was dialyzed against a large volume of deionized water for 8 h. Lipase solution (193 mg protein, 7470 U) was added to 84 g of 0.68% collagen fibril suspension. The lipase–collagen membrane was prepared by casting the suspension on a Teflon plate and drying it at room temperature. The lipase–collagen membranes were treated with 0.1% glutaraldehyde for 3 min and dried at 4°C.

Apparatus

The system used for continuous determination of neutral lipids is diagrammatically illustrated in Fig. 1. The pH adjustment of the sample solution was performed with a pH-meter (Model HM-5A, TOA Electronics Company). The reactor was a biocatalytic type (8) (acryl plastic, diameter 1.8 cm × 5.2 cm), with a spacer (glass rod, diameter 1.4 cm × 5 cm) located in the center. Therefore, the inner volume of the reactor was 4.1 ml. The lipase–collagen membrane (5×15 cm^2, thickness 50 μm) was rolled with plastic net (5×20 cm^2, 20 mesh) and inserted into the reactor. The hydrogen ions in the sample solution were determined by glass electrode coupled with calomel electrode (GC-125C TOA Electronics Company) and displayed on a recorder (Model CDR-11A, TOA Electronics Company).

Procedures

The 100μl of serum was added to 1×10^{-4} M tris-HCl buffer containing, per liter, 0.5 ml of Triton X-100 and 0.15 mol of NaCl. The final volume

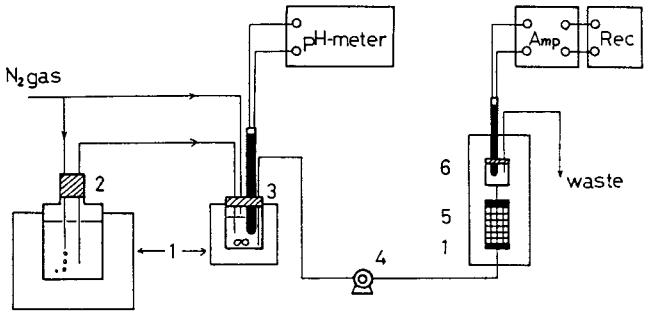

FIG. 1. Block diagram of the measurement of neutral lipid in serum. 1, Incubator (37 ± 0.1°C); 2, buffer solution reservoir; 3, pH-adjustment cell; 4, peristaltic pump; 5, lipase–collagen membrane reactor; 6, sensing chamber (glass electrode and SCE).

was adjusted to 8.0 ml with the buffer and the pH was brought to 7.7 with diluted NaOH or HCl solutions under nitrogen at a temperature equilibrated to 37°C. Then the solution was transferred to the reactor by a peristaltic pump and the hydrogen ion concentration of the sensing chamber was determined.

Enzyme Assay

Lipase activity was determined by the method of Dole (9).

Lipid Determination

Neutral lipids were determined by the colorimetric method using acetylacetone (10).

RESULTS

Figure 2 shows the glass electrode potential–time relationship. Sample solutions containing various amounts of neutral lipids were employed for experiments. The sample solution was transferred to the reactor and the potential in the sensing chamber gradually increased with time until a maximum was reached. As shown in Fig. 2, the response time (time required for the potential to reach the maximum) depends on the lipid concentration, flow rate, and lipase activity of the membrane. A decrease of the lipid concentration shortened the response time. The maximum potential was attained in the case of 4.6 μM lipid concentration, in about 10 min at a flow

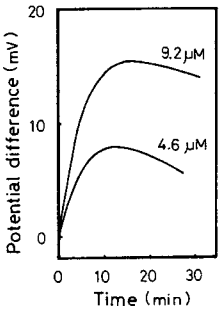

FIG 2. Response time. The reactions were carried out under standard conditions. Neutral lipid concentrations were 4.6 and 9.2 μM, respectively.

rate of 48 ml/h. When the 1×10^{-4} M tris-HCl buffer (pH 7.7) was sent to the reactor, the potential of the glass electrode gradually decreased. The potential returned to its initial level within 10 min at 37°C.

Figure 3 shows the relationship between the concentration and the potential differences. Potential differences were calculated from the potential between the initial state and the maximum state. The lipid concentration was indicated as trioleate. A linear relationship was obtained between the logarithm of the neutral lipid concentration and the potential difference.

The effect of ionic strength on the potential of the glass electrode was examined, using sodium chloride. The potential of the glass electrode was constant below 0.4 M of sodium chloride.

Figure 4 shows the effect of flow rate on the potential of the glass electrode. Three kinds of lipid dispersion were employed for the experiments. As shown in Fig. 4, the decrease of the potential with increase in flow rate was observed above 9.2 μM of lipid. However, no decrease of potential with increase in flow rate was observed at a lipid concentration of 9.2 μM. In this case, the enzyme activity of the collagen membrane was enough for the hydrolysis of the substrate. However, the complete hydrolysis of the substrate was difficult at higher substrate concentration and flow rate.

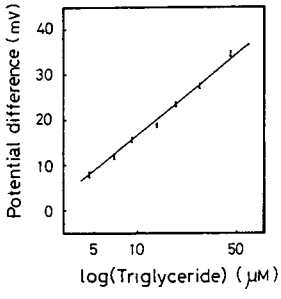

FIG. 3. Triglyceride and potential difference relationship. The reactions were carried out under standard conditions

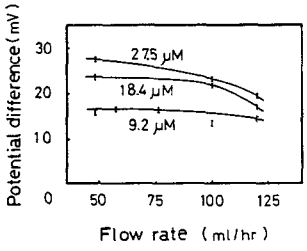

FIG. 4. Flow rate and potential difference relationship. The reactions were carried out under standard conditions. Neutral lipid concentrations were 9.2, 18.4 and 27.5 μM, respectively.

TABLE 1. **The Determination of Human Serum Neutral Lipids by the Lipase–Collagen Membrane and Acetylacetone Methods**[a]

	Serum (μl)	ΔE(mV)	Analytical value (mM)
Lipase–collagen membrane sensor	100	22.4±0.5	1.28±0.06
Acetylacetone method	100	—	1.23±0.06

[a] The reactions were carried out under standard conditions.

Reusability of the reactor was examined with lipid dispersion containing 9.6 μM of neutral lipids. Lipid determination was performed several times a day. However, no decrease of the potential was observed for a 15-day period, and thus this system could be used repeatedly for a long time. This system was applied to the determination of neutral lipids in human serum. Human serum was diluted with 1×10^{-4} M tris-HCl buffer and used for the experiments. Table 1 shows a comparison between the lipid contents of human serum determined by the conventional method (10) and those determined by this method. The relative error of the determination by this system was within 4%. Therefore, this system can be used for the lipid determination of human serum.

DISCUSSION

Neutral lipids were hydrolyzed to glycerol and fatty acids by lipase–collagen membrane and protons were liberated from fatty acids. From Nernst's formula, the membrane potential of the glass electrode is proportional to the logarithm of proton concentration. The concentration of fatty acid is directly proportional to neutral lipid concentration. Therefore, hydrogen ion concentration of the sample solution, i.e., potential difference, is proportional to

the logarithm of lipid concentration (Fig. 3):

$$\Delta E = k \log C$$

where ΔE is a potential difference between the initial state and the maximum state, C is neutral lipid concentration, and k is a constant.

The response time of the lipid determination system depended on the lipid concentration, and reached about 10 min. This low response was caused mainly by the low flow rate of the substrate. In this study, collagen membrane was used as the lipase carrier. Since the molecular weight of the lipase was low, the majority of lipase trapped in the collagen membrane was eluted during the washing procedure. As a result, there was little immobilized lipase in the collagen matrix, and therefore contact between lipase and dispersed large lipid particles was sometimes difficult. Since the activity of the lipase–collagen membrane was low, we could not increase the flow rate of the substrate. This phenomenon was observed at high flow rate (Fig. 4).

The lipase used in this study was a lipoprotein lipase with properties very similar to, if not identical with, those of the lipoprotein lipases of postheparin plasma and other animal tissues in regard to the substrate specificities and behavior against the specific inhibitors (11).

Neutral lipids in serum are solubilized as lipoproteins and remain mainly as chyromycron and low-density lipoproteins (LDL). This lipase–collagen membrane could hydrolyze the neutral lipids in serum (Table 1). Furthermore, the results obtained from the lipid determination system were almost in agreement with those obtained from the conventional method. This lipid determination system could be used repeatedly for a long time. Therefore, this system can be used in clinical analysis.

Further developmental studies in this laboratory are being directed toward increasing the lipase activity of the membrane and decreasing the response time of the system.

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