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Note

Chemiluminescence detection of free fatty acids by high-performance liquid chromatography with immobilized enzymes

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Recently, the assay of free fatty acids (FFA) has become important in biochemical and clinical investigations. Although high-performance liquid chromatography (HPLC) is widely used for this purpose, most of these methods involve the formation of derivatives having ultraviolet or fluorescent groups[1,2]. Fatty acids themselves generally do not show strong absorption in the ultraviolet region, so that direct detection is not suitable for trace analysis. In the clinical field, an enzymatic method has been used for the determination of total fatty acids in serum[3,4] by spectrophotometry, using an acyl-CoA synthetase (ACS) and acyl-CoA oxidase (ACO) reaction system:

$$\text{RCOOH} + \text{CoA} + \text{ATP} \xrightarrow{\text{ACS, } Mg^{2+}} \text{acyl-CoA} + \text{AMP} \quad (1)$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACO}} \text{2,3-}\textit{trans}\text{-enoyl-CoA} + H_2O_2 \quad (2)$$

Chemiluminescence detection:

$$H_2O_2 + \text{luminol} \xrightarrow{\text{mPOD, } OH^-} \text{aminophthalate} + N_2 + \text{light} \quad (3)$$

of the hydrogen peroxide formed by the same reaction system has also been reported[5]. As these methods are carried out in a test-tube reaction system, the differential analysis of constituents of the fatty acids is difficult. However, this problem can be approached by coupling the HPLC system with immobilized enzyme reactors in the similar manner to that described by Koerner and Nieman[6], in which the glucosides were determined by HPLC using immobilized enzyme reactors and a chemiluminescence detector. Lawrence and Charbonneau[7] reported a simple HPLC method with a post-column ion-pair extraction and detection system; however, the enzymatic method has the advantage of selectivity. Therefore, in this paper, we describe a novel HPLC method

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for the detection of individual fatty acids without the labelling procedure, using the immobilized ACS–ACO system, coupled with chemiluminescence detection of hydrogen peroxide catalysed by microperoxidase (mPOD).

EXPERIMENTAL

Materials

Luminol was of analytical-reagent grade from Tokyo Kasei Kogyo (Tokyo, Japan) and microperoxidase (MP-11) was a product of Sigma (St. Louis, MO, U.S.A.). N,N′-Dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (for peptide synthesis) and standard fatty acids were products from Nacalai Tesque (Kyoto, Japan). Acyl-CoA synthetase (type III) and acyl-CoA oxidase (type II) were purchased from Toyobo (Osaka, Japan). Coenzyme A (CoA) and adenosine 5′-triphosphate (ATP) were products of Khojin (Tokyo, Japan). Carboxyl-controlled-pore glass (carboxyl-CPG, CBX-500, 120–200 mesh) was obtained from Electro-Nucleonics (Fairfield, NJ, U.S.A.) and polyoxyethylene(10)octyl phenyl ether (Triton X-100) was obtained from Wako (Osaka, Japan). Other reagents were of analytical-reagent grade. Desalted, distilled water was used throughout.

High-performance liquid chromatography

The high-performance liquid chromatograph used was a Shimadzu (Kyoto, Japan) Model LC-6A and the high-pressure sample injector was a Shimamura (Tokyo, Japan) Model EIE-005. The separation column was LiChroCART Superspher 60 RP-8 (25 mm × 4 mm I.D.) (Merck, Darmstadt, F.R.G.). The post-column reactor consisted of double plunger pump (Simamura Keiki, Tokyo, Japan) for reagent delivery and an immobilized enzyme column (50 mm × 3 mm I.D.) (Omnifit minicolumn). The flow cell of the detector was a laboratory-made spiral-shaped PTFE tube (290 μl). The light emitted was measured with a Niti-On (Tokyo, Japan) photometer. The recorder was a Shimadzu Chromatopak C-R3A. The arrangement of the manifolds used is shown in Fig. 1.

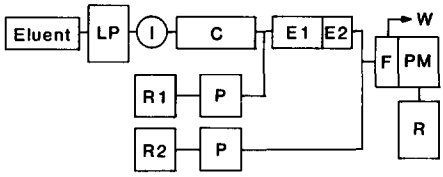

Fig. 1. Schematic diagram of the HPLC system and the manifold for chemiluminescence detection. LP = Liquid chromatograph pump; I = injector; C = LiChroCART Superspher 60 RP-8 (HPLC column); E1 and E2 = immobilized ACS and ACO column, respectively; F = flow cell; R1 = reagent R1; R2 = reagent R2; P = pump; PM = photometer; R = recorder–integrator; W = waste.

Immobilization of enzymes

Immobilization of enzymes was performed as follows. Carboxyl-CPG (0.2 g) was added to 10 ml of a dioxane solution of 0.1 *M* N-hydroxysuccinimide and 0.1 *M* N,N′-dicyclohexylcarbodiimide and the mixture was stirred for 120 min at room temperature. The CPG was washed with 20 ml of dioxane, 10 ml of methanol and 10 ml

of dioxane successively under suction and then dried. The imidoester-CPG thus obtained was reacted with ACO (10 mg, 10 units/mg) dissolved in 5 ml of phosphate buffer solution (pH 5.5) below 4°C. After 6 h it was washed extensively with 0.1 *M* phosphate buffer solution (pH 7.4), the same buffer solution containing 1 *M* NaCl and water for three cycles, and was kept below 4°C. ACS (5 mg, 1.47 units/mg) was added to 5 ml of 0.1 *M* phosphate buffer solution (pH 8.0) and treated in a similar manner to ACO. The immobilization yields of the enzymes were calculated from the protein concentration of the solution determined by the Lowry method[8] before and after the reaction.

Separation and detection of fatty acids

The HPLC mobile phase was methanol–phosphate buffer solution (pH 8.0) (1:1), and was degassed by ultrasonication for 10 min prior to use. The flow-rate was 0.3 ml/min. Reagent R1 (flow-rate 0.45 ml/min) was 20 m*M* phosphate buffer solution (pH 7.6) containing 0.5 m*M* $MgCl_2$, 0.05 m*M* CoA, 0.1 m*M* ATP and 0.25% Triton X-100. Reagent R2 (flow-rate 0.75 ml/min) was 50 m*M* carbonate buffer solution (pH 10.5) containing 10 μM luminol and 0.8 μM microperoxidase. The lengths of the immobilized ACS and ACO columns were 35 and 15 mm, respectively. The sample solution of fatty acids injected was 10 μl of a methanol–phosphate buffer solution (pH 7.6) (1:1) containing 1 nmol of each fatty acid. The output from the photometer was recorded with the C-R3A.

RESULTS AND DISCUSSION

The chemiluminescence detection system with a flow cell unit was devised and reported previously[9]. In this work, the system was used with the enzyme reactor inserted in front of the detection unit and with a slightly modified flow cell, in which the volume was increased from 75 to 290 μl so as to detect as much of the light emitted from the samples as possible. The whole apparatus for fatty acid detection is shown schematically in Fig. 1. Each fatty acid eluted from the HPLC column was mixed with reagent R1 and subjected to enzymatic reaction in the immobilized ACS–ACO column to form hydrogen peroxide, which was mixed with the chemiluminescence reagent R2 and the light emitted in the flow cell was detected by the photomultiplier.

Optimization of the reaction conditions for the detection of hydrogen peroxide was carried out by a flow-injection method without the HPLC column. The effect of the concentrations of luminol and microperoxidase in reagent R2 on the chemiluminescence intensity was first examined by injecting 450 pmol of hydrogen peroxide. The concentrations examined for luminol and microperoxidase were in the ranges 10 n*M*–100 μM and 0.2–2.0 μM, respectively, and maximum intensity was obtained at 10 μM for luminol and 0.8 μM for microperoxidase. The variations in chemiluminescence intensity and background noise level with changes in the pH of reagent R2 were examined in the pH range 5–10.5, and the best result was obtained at pH 10.5. Under the optimized conditions, a linear calibration graph for hydrogen peroxide was obtained in the range 12.5–200 pmol. The limit of detection was 5 pmol (10-μl injection, signal-to-noise ratio = 2, relative standard deviation = 1.6%, n = 5).

The immobilization of ACS and ACO has not been reported previously, so the methods for immobilization of these enzymes were examined first. As the detection

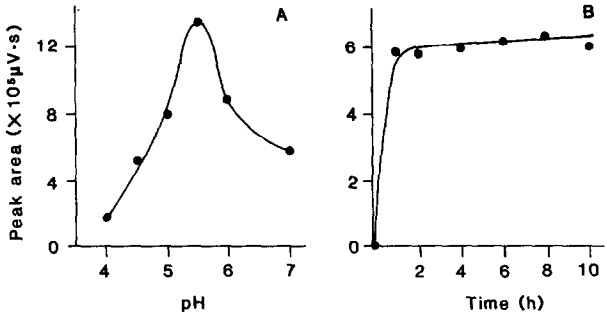

Fig. 2. (A) Optimum pH for immobilization and (B) time course of immobilization reaction of acyl-CoA oxidase. Experimental conditions as described in the text.

system was operated under high pressure, controlled-pore glass beads were used as the support. First, immobilization of the enzymes with the glutaraldehyde method was attempted, but this method did not give a reproducible activity of the immobilized enzymes. However, the use of DCC and N-hydroxysuccinimide gave good results. Therefore, the conditions for the immobilization of enzymes were investigated further using this method. The optimum pH for immobilization of ACO was examined in the range 4.0–7.0, and the maximum activity was obtained at pH 5.5 (Fig. 2A). The activity of immobilized ACO was measured with the flow-injection system, injecting 0.2 μmol of palmitoyl-CoA as a sample. The immobilization reaction was allowed to proceed for 6 h at 4°C, which was adequate for the reaction (Fig. 2B). The immobilization yield was 67%. The optimum pH for immobilization of ACS was examined in the range 5.5–8.5, and pH 7.6 was found to give the best results. As ACS was labile during the immobilization reaction, even if the enzyme was treated at the optimum pH, the yield obtained was only 7.6%.

The enzyme reactor consisted of two columns, packed with the immobilized ACS and ACO. The flow-injection method with these columns was used for optimization of the reaction conditions. The effect of the concentration of CoA in reagent R1 on the chemiluminescence was examined up to 0.075 m*M*, injecting 5 nmol of palmitic acid as a sample. As shown in Fig. 3A, 0.01 m*M* gave the maximum response, and it decreased slowly as the concentration was increased further. This is

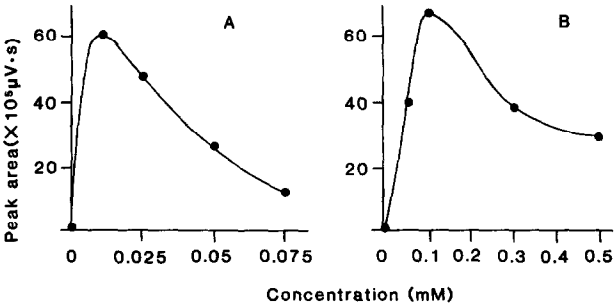

Fig. 3. Effect of the concentration of (A) coenzyme-A and (B) ATP on the immobilized enzyme reaction. Experimental details as described in the text.

probably due to the effect of the SH group of CoA remaining, but this effect can be avoided by the use of N-ethylmaleimide if the remaining CoA extremely interferes with the detection. Therefore, 0.05 m*M* was chosen as an excess amount for the enzyme reaction. Fig. 3B shows the effect of ATP concentration between 0 and 0.5 m*M*, the maximum response being obtained at 0.1 m*M*. The concentration of Mg^{2+} had little effect on the reaction of ACS, so 0.5 m*M* $MgCl_2$ was used.

The ratio of ACS to ACO was also examined, and it was found that the sensitivity was constant at ratios above 3:2. In view of sample dispersion, a shorter column affords compact and sharp peaks, *i.e.*, higher sensitivity. Therefore, the two immobilized enzymes were packed in a single column of length 50 mm; a 35-mm length of ACS followed by a 15-mm length of ACO showed the highest sensitivity.

Under the optimized conditions specified above, the relationship between the chain length of saturated fatty acids and the chemiluminescence response was examined. Fatty acids from C_8 to C_{10} showed a strong response, and as the carbon number increased further a lower response was obtained. However, fatty acids up to C_{17} were definitely detected (Fig. 4).

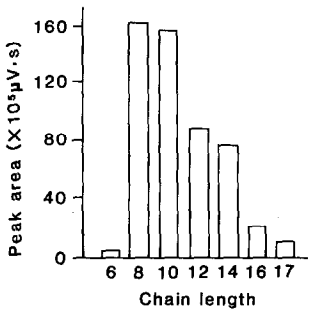

Fig. 4. Specificity of immobilized acyl-CoA synthetase and acyl-CoA oxidase on chain length of fatty acids. The samples were methanol–phosphate buffer solutions (pH 7.6) (1:1) of *n*-caproic acid (C_6), *n*-caprylic acid (C_8), *n*-capric acid (C_{10}). lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}) and margaric acid (C_{17}). The sample volume was 10 μl, containing 5 nmol of the fatty acid.

The HPLC separation of fatty acids was examined with RP-18, RP-8 and RP-4 reversed-phase columns. On the RP-18 column, fatty acids were so strongly retained that the retention time was impractical for analysis. The RP-4 column did not show the features of a reversed-phase packing but of ion exclusion at higher pH. Of these columns examined, LiChroCART Superspher 60 RP-8 gave the best separation of the fatty acids. The effect of the pH of the eluent on retention times was examined between 6 and 8.5, and it was found that fatty acids were more retained as the pH was decreased, but there was almost no effect above pH 7.6. An eluent pH of 8.0 was chosen. The methanol concentration in eluent was also examined in the range 40–55%, and 50% was chosen as 55% methanol lowered the luminescence strength.

Fig. 5 shows the separation and detection of fatty acids in 3 h under the above conditions. Each fatty acid was well separated, except parmitoleic ($C_{16:1}$) and linolenic ($C_{18:3}$) acids. The retention times of oleic ($C_{18:1}$) and stearic ($C_{18:0}$) acid were so long that their detection was difficult because of the broadening of the peaks. The

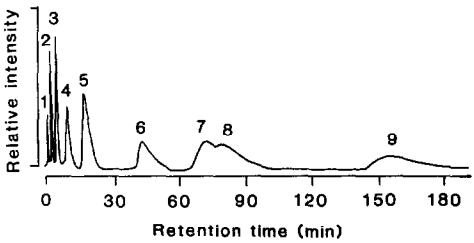

Fig. 5. Chromatogram of the authentic fatty acids. HPLC conditions as described in the text. Peaks: 1 = *n*-caprylic acid ($C_{8:0}$); 2 = *n*-capric acid ($C_{10:0}$); 3 = dodecenoic acid ($C_{12:1}$); 4 = lauric acid ($C_{12:0}$); 5 = myristoleic acid ($C_{14:1}$); 6 = myristic acid ($C_{14:0}$); 7 = linolenic acid ($C_{18:3}$); 8 = palmitoleic acid ($C_{16:1}$); 9 = linoleic acid ($C_{18:2}$).

calibration graph for *n*-capric acid was linear in the range 0.4–2.0 nmol. The resolution of the RP-8 column was maintained during 2 months of these experiments (more than 100 injections), provided that the column was filled with methanol between experiments. Usually higher concentrations of methanol (80–100%) are employed in the separation of fatty acids by reversed-phase HPLC for a faster separation. However, high concentrations of methanol inactivate the enzymes immobilized in the column and so could not be used in the present method.

In conclusion, fatty acids were selectively detected by a chemiluminescence method without labelling using an immobilized ACS–ACO column. Unfortunately, the method requires a long chromatographic separation, has a limited linear range, is not suitable for $C_{18:0}$ and $C_{18:1}$ fatty acids and the organic content of the mobile phase is restricted.

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