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Fluorimetric Assay for Hydrolytic Activity of Cholesterol Esterase in Rat Tissues

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A sensitive fluorimetric method for the assay of the hydrolytic activity of cholesterol esterase in enzyme preparations from rat tissues using cholesterol oleate as a substrate is described. The increase in the amount of cholesterol in the enzyme reaction is measured fluorimetrically by using the cholesterol oxidase–peroxidase–3-(*p*-hydroxyphenyl)propionic acid system. The assay requires only 45—150 μg of protein of preparations from the liver, testis, adipose tissue of the mesentery and adrenal gland.

Keywords——cholesterol esterase; cholesterol ester hydrolysis; fluorimetric assay; cholesterol oxidase–peroxidase–3-(*p*-hydroxyphenyl)propionic acid system; rat tissues; cholesterol oleate; cholesterol

Cholesterol esterase (EC 3.1.1.13; sterol-ester hydrolase) catalyzes the reversible esterification and hydrolysis reaction between cholesterol and its fatty acid ester. The enzyme has principally been assayed by measuring the hydrolytic activity and therefore it has been called cholesterol ester hydrolase (CEH). CEH occurs in mammalian tissues such as liver,[1] kidney,[2] spleen,[3] pancreas,[4] aorta,[5] adrenal gland,[6] testis,[2] brain,[7] lymph-node,[3] adipose tissue of the mesentery[8] and skin fibroblasts,[9] and the optimum pH varies in the acidic and neutral ranges depending on the tissues. No activity of CEH has been detected in normal sera. Decreased activity of CEH which has the pH optimum in the acidic range has been observed in liver, spleen, lymph-node and skin fibroblasts from patients with cholesterol ester storage disease[3] and Wolman's disease.[9] CEH has been found in sera of patients with liver diseases when assayed at neutral pH.[10] In rabbits with atherosclerosis, CEH activity in the aortic wall assayed at pH 8.6 was much less than normal.[11]

CEH has been assayed by radiochemical methods. A biological sample is incubated at 37 °C for 10 min to 6 h with cholesterol ester of ^{14}C-[1b,3,9] labeled fatty acid, or ^{14}C-[1e,2] or ^{3}H-[1a,c,7] labeled cholesterol ester. CEH activity in the sample is assayed by measuring the radioactivity of cholesterol or fatty acid liberated enzymatically after extraction of the lipids followed by thin-layer chromatographic separation.

Recently, we reported a sensitive fluorimetric method for the assay of acyl CoA–cholesterol acyltransferase activity in biological materials, based on the enzymatic assay of cholesterol using the cholesterol oxidase (COD)–peroxidase (POD) system with 3-(*p*-hydroxyphenyl)propionic acid (HPPA) as the highly fluorogenic substrate of POD.[12] This paper describes a sensitive fluorimetric method for the assay of CEH in biological materials. A biological sample is incubated with cholesterol oleate and the increase in the cholesterol concentration is determined fluorimetrically by using the COD–POD–HPPA system. Enzyme preparations from rat liver, testis, adipose tissue of the mesentery and adrenal gland were selected as model preparations to establish the analytical procedure, because CEH activity is

high in these tissues and the enzyme in the adrenal gland shows two pH optima.

Experimental

Reagents and Solutions——All chemicals were of reagent grade unless otherwise noted. Double-distilled water, acetone and isopropanol were used. Cholesterol oleate (Tokyo Kasei, Tokyo, Japan) solution (5 mM) was prepared in acetone. The solution was usable for 1 week when stored at 4 °C. A COD–POD solution (6.9 mU/ml and 1.2 purpurogallin units/ml, respectively) was prepared from COD (30 μl of COD suspension, 23 U/ml, from *Nocardia*; Miles Laboratories, Elkhart, U.S.A.) and 0.4 mg of POD (300 purpurogallin units/mg, from horseradish, Type VI; Sigma, St. Louis, U.S.A.), dissolved in 100 ml of 0.3 M potassium phosphate buffer (pH 7.5). The solution was stable for more than 1 month when stored at 4 °C. An HPPA (Dojindo Laboratories, Kumamoto, Japan) solution (30 mM) in 0.3 M potassium phosphate buffer (pH 7.5) was stored at 4 °C and used within 1 week.

Apparatus——Fluorescence was measured with a Hitachi MPF-4 spectrofluorimeter using quartz cells of 10 × 10 mm optical path length. The spectral band widths were 2 nm in the excitation monochromator and 10 nm in the emission monochromator.

Enzyme Preparations——Male Donryu rats (4 weeks of age, 200—240 g) were killed by decapitation, and the liver, testes, adipose tissue of the mesentery and adrenal glands were rapidly removed. The enzyme preparations were prepared as follows. The tissues were immediately rinsed in ice-cold 0.1 M potassium phosphate buffer (pH 7.5), weighed, homogenized with 10 volumes of the ice-cold buffer in a Potter–Elvehjem homogenizer and centrifuged at 1000 *g* for 5 min. The supernatants were diluted with the phosphate buffer to contain approximately 150 μg of protein for liver and approximately 50 μg of protein for testis, adipose tissue of the mesentery and adrenal gland in 20 μl. The enzyme preparations could be stored at −20 °C for at least 1 month without any loss of CEH activity. Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard protein.[13]

Human Plasma——Normal plasma from ethylenediaminetetraacetic acid (EDTA)-treated blood was used. Plasma could be stored at −20 °C for more than 1 month.

Procedure——To 0.5 ml of McIlvaine's buffer [pH 6.0, containing 0.05% (w/v) Triton X-100 (Rohm & Hass Co., Philadelphia, U.S.A.) for liver; pH 6.4, containing 0.025% (w/v) Triton X-100 for testis; pH 7.4 containing 2 mM sodium taurocholate (Wako, Osaka, Japan) for adipose tissue of the mesentery; pH 5.0 and pH 7.4 for adrenal gland] placed in a glass-stoppered test tube (10 ml), 20 μl each of the enzyme preparation and cholesterol oleate solution were added. The mixture was incubated at 37 °C for 20 h (liver or testis), 1 h (adipose tissue of the mesentery) or 2 or 5 h (adrenal gland, at pH 5.0 and 7.4, respectively), then 3.5 ml of a mixture of methanol–chloroform (1 : 2, v/v) was added to stop the enzyme reaction. The mixture was shaken with a Vortex-type mixer for 10 min and centrifuged at 1000 *g* for 5 min. The lower organic layer (2.5 ml) was evaporated to dryness below 25 °C *in vacuo*. The residue was dissolved in 3.0 ml of 0.3 M potassium phosphate buffer (pH 7.5) containing 0.025% Triton X-100. The COD–POD solution and HPPA solution (0.1 ml each) were then added, and the mixture was warmed at 37 °C for 20 min to develop the fluorescence (mixture A). To determine the initial concentration of free cholesterol in the enzyme preparation, the same procedure was carried out except that the enzyme preparation, McIlvaine's buffer and cholesterol oleate solution were added to the methanol–chloroform mixture, incubation being omitted (mixture B). The fluorescence intensities of the mixtures A and B were measured at 404 nm with excitation at 320 nm. The difference in intensity between A and B was calculated, and the increase in the amount of cholesterol was determined from a standard curve prepared by treating 20-μl samples of cholesterol standard solutions (100—800 nmol/ml, in isopropanol) by the given procedure. The value thus obtained was corrected for the hydrolysis of endogenous cholesterol esters in the biological sample by subtracting the value obtained by treating the enzyme preparation as in the standard procedure in the absence of cholesterol oleate. CEH activity was expressed as pmol of cholesterol increase per min per mg of protein at 37 °C.

Results and Discussion

McIlvaine's buffer was used for the enzyme reaction in the present method, as in the radiochemical methods.[1c,d,14] The optimum pHs for the enzyme in the preparations from liver, testis and adipose tissue of the mesentery were 6.0, 6.4 and approximately 7.4, respectively (Fig. 1). Two pH optima were observed for the preparation from adrenal gland, at 5.0 and 7.4 (Fig. 1). These values are almost identical with the reported data.[2]

CEH was activated by surfactants.[15] The most effective stimulation of CEH activity in the preparations from liver and testis was observed with 0.05 and 0.025% (w/v) Triton X-100 in McIlvaine's buffer, respectively. The enzyme activity in the preparation from adipose tissue of the mesentery was greatly enhanced with 2 mM sodium taurocholate in McIlvaine's buffer.

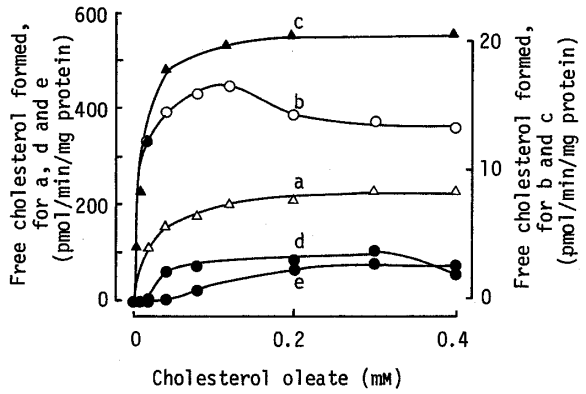

Fig. 1. Effect of pH on the Hydrolysis of Cholesterol Oleate

Portions (20 μl) of the enzyme preparations were treated according to the standard procedure at various pHs.

a, liver (171 μg of protein); b, testis (58 μg of protein); c, adipose tissue of the mesentery (52 μg of protein); d, adrenal gland (62 μg of protein).

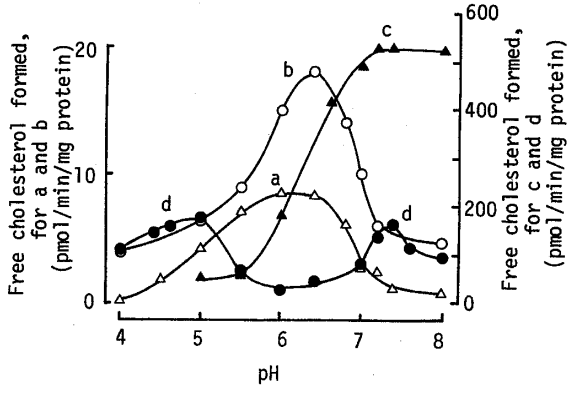

Fig. 2. Effect of Cholesterol Oleate Concentration in the Incubation Mixture on the Amount of Free Cholesterol Formed

Portions (20 μl) of the enzyme preparations were treated according to the standard procedure at various cholesterol oleate concentrations.

a, liver (171 μg of protein); b, testis (86 μg of protein); c, adipose tissue of the mesentery (52 μg of protein); d, adrenal gland (pH 5.0, 62 μg of protein); e, adrenal gland (pH 7.4, 62 μg of protein).

TABLE I. Hydrolysis of Cholesterol Esters and Endogenous Cholesterol Ester in Human Plasma with Enzyme Preparations from Rat Tissues

	Hydrolysis of cholesterol ester (pmol/min/mg protein)				
	Adipose tissue of mesentery pH 7.4	Adrenal gland		Testis pH 6.4	Liver pH 6.0
		pH 5.0	pH 7.4		
Cholesterol palmitate[a]	245	85	0	8	4
Cholesterol oleate[a]	541	88	64	20	9
Cholesterol linolate[a]	577	68	85	21	11
Cholesterol ester in heated human plasma[b]	137	363	14	23	18

a) 100 nmol/assay tube. *b*) Human plasma was heated for 2 h at 60 °C to inactivate lecithin-cholesterol acyltransferase. Cholesterol ester concentration in the plasma was assayed by the previously reported method[16] (16 nmol/assay tube).

However, these surfactants did not stimulate CEH in the adrenal gland. Cholesterol oleate and linolate were enzymatically hydrolyzed more effectively than cholesterol palmitate (Table I). Endogenous cholesterol esters were also well hydrolyzed in the presence of the enzyme preparations (Table I). This is consistent with the finding that human plasma lipoprotein treated with radioisotope-labeled cholesterol ester is hydrolyzed in a CEH-mediated reaction.[1c] Cholesterol oleate was employed in the procedure, as in the radiochemical methods.[1e,2,3,7,9,11]

Cholesterol oleate is insoluble in water, and therefore it was dissolved in acetone. Acetone up to at least 20 μl per assay tube had no effect on the CEH-catalyzed reaction when examined with the enzyme preparation from liver.

Cholesterol oleate afforded almost maximum and constant CEH activity at 0.2 mM or greater in the incubation mixture for the enzyme preparations from liver and adipose tissue of the mesentery (Fig. 2, a and c) with apparent Michaelis constant (K_m) values of 23.5 and 26.7 μM, respectively. The maximum enzyme activity of the preparation from testis was

obtained in the presence of 0.12 mM cholesterol oleate (Fig. 2, b) with the apparent K_m value of 8.7 μM. The enzyme in adrenal gland was most active at approximately 0.3 mM cholesterol oleate in the reaction mixture (Fig. 2, d and e), showing K_m values of 26.0 and 103.1 μM for the enzyme with the optimum pHs at 5.0 and 7.4, respectively. Cholesterol oleate at 0.2 mM in the reaction mixture was used as a saturating concentration in the procedure.

The enzyme activities of the preparations from liver, testis and adipose tissue of the mesentery were linear with time up to at least 48, 48 and 2 h, respectively, when incubated at 37 °C. The enzyme activity in the adrenal gland was linear up to at least 3 h at pH 5.0 and up to at least 10 h at pH 7.4. The amounts of cholesterol formed were proportional to the amounts of protein in the preparations of liver, testis, adipose tissue of the mesentery and adrenal gland up to at least 340, 100, 100 and 150 μg, respectively.

The within-day precision of the present method was examined using the enzyme preparation of liver with a mean CEH activity of 8.5 pmol/min/mg protein. The coefficient of variation was 1.4% ($n=10$). The limit of detection of enzymatic cholesterol increase was 1.6 nmol. The sensitivity permits the assay of CEH in only 150 μg of protein in the enzyme preparations from liver and testis or 45 μg of protein in the preparations from adipose tissue of the mesentery and adrenal gland. The limit was defined as the amount giving a fluorescence intensity twice that of the final reaction mixture obtained according to the standard procedure except that the COD–POD solution was replaced with a POD solution (1 purpurogallin unit/ml).

CEH activities in rat (Donryu male, 4 weeks of age) tissues as determined by the present method were 8 (liver), 19 (testis), 511 (adipose tissue of the mesentery), 88 (adrenal gland, pH 5.0) and 60 (adrenal gland, pH 7.4) pmol/min/mg protein ($n=10$ each). The results are similar to those obtained by the radiochemical method.[2)]

This study provides the first fluorimetric method for the assay of CEH. This method does not require a radiochemical substrate, and should be convenient for biological investigations.

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