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A Bioreactor Used for the On-Line Determination of Cholesterol Distribution in Human Serum Lipoprotein Classes

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On-line detection of cholesterol in each human serum lipoprotein class could be performed by combining a bioreactor system prepared by immobilizing cholesterol ester hydrorase and cholesterol oxidase on the same matrix with a metal free size-exclusion chromatographic system separating different classes of lipoproteins.

Determination of cholesterol in human sera is important in diagnosis of hyperlipidemia directly related to heart-attack diseases.[1,2] The methods used for this purpose can be devided into two types. The one is total cholesterol determination and the other analysis of cholesterol of separated lipoprotein classes.[3] The latter is more informative for the diagnosis. For the separation, electrophoresis,[4] precipitation method,[5] and ultracentrifugation[6] have been used. However, these methods can not be a breakthrough for the difficulty in incorporating into automated conventional systems. Recently, size-exclusion chromatography of human serum lipoprotein has been combined with a post-column staining using cholesterol ester hydrorase (CEH) and cholesterol oxidase (COD).[7-9] Although automatic analysis of human sera could be performed by this method, a large quantity of expensive enzymes is consumed for staining such that the method has not been useful in clinical medicine. In the present work, therefore, to establish practically automated

clinical analysis of cholesterol in human serum lipoprotein classes, a bioreactor consisting of immobilized enzymes was used in the detection system. Human serum samples were obtained from healthy male volunteers after 12 h of fasting. CEH (EC 3.1.1.13, from Pseudomonas fluorescence) and COD (EC 1.1.3.6, from Brevibacterium sterolium) were obtained from Kyowa Medex (Tokyo, Japan) and peroxidase (EC 1.11.1.7, from horseradish) (POD) was from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan). G6000PW size-exclusion chromatographic gel used as a matrix of immobilization of CEH and COD was obtained from Toyo Soda (Tokyo, Japan) and subjected to amination by propylenediamine through p-toluenesulfonyl-chloride activation. 4-Aminoantipyrine (4-AA) and N-ethyl-N-(2-hydroxy-sulfopropyl)-metha-toluidine (TOOS) were purchased from Dojin Kagaku (Kumamoto, Japan) and polyoxyethylene-(10)-octylphenylether (Triton X-100) was from Wako Junyaku Kogyo (Osaka, Japan). Determiner TC-555 was obtained from Kyowa Medex. All other chemicals were purchased from Nakarai Chemicals (Kyoto, Japan) and used without further purification. Water was purified by a Milli R/Q water purifier (Millipore, Bedford, MA, USA).

G6000PW gel (0.5 cm^3, wet), aminated with propylenediamine, was activated in a 2.5% aqueous glutaraldehyde solution (10 cm^3) for 1.5 h under the atmospheric conditions.[10,11] After rinsing with water, to the activated gel, was added CEH (0.6 mg, 62 unit) and COD (9.4 mg, 119 unit) dissolved in 3 cm^3 of 150 mM phosphate buffer (pH 7) containing 2 mM ethylenediaminetetraacetic acid (EDTA). This mixture was allowed to stand for 1.5 h at 5 °C. After the coupling reaction, excess enzymes were washed out with the same phosphate buffer. Thus prepared immobilized enzyme was kept wet at 5 °C and was found to maintain its initial activities for more than a month while on keeping dry, only 60% of the initial activities remained in the same period. This result is favorable for its use in a flow system. Coupling yield of enzymes immobilized was determined by monitoring the absorbance at 280 nm using bovine serum alubmin as a standard for calibration. The value obtained was 5.4 mg-enzyme/1 cm^3 of wet gel. Assay for the enzymic activities of thus prepared coimmobilized enzyme was performed by incubating at 40 °C with human serum (total cholesterol being 2.32 $g \cdot dm^{-3}$), staining released H_2O_2 with 0.5 mM 4-AA, 0.3 mM TOOS, and 6.7 unit$\cdot cm^{-3}$ POD, and measuring the absorbance at 550 nm. This coimmobilized CEH and COD, determined to be active, was packed in a glass-column (2 mm I.D. and 10 cm long), which was connected in tandem to the excit of a size-exclusion

column array (G5000PW + G3000SW, Toyo Soda, both columns being 8.0 mm I.D. and 30 cm long). To the inlet of the enzyme column, staining fluid composed of Triton X-100 (1%, v/v), 4-AA (0.5 mM), TOOS (0.3 mM), and POD (6.7 unit·cm^{-3}), was also introduced. To the exit, Teflon tubing (0.4 mm I.D. and 1 m in length) used as a reaction coil was attached. The effluent was monitored at 550 nm.

On the system, using human serum as a sample, requirement for the total system was explored. When a conventional system made of stainless steel was used, no signal was produced. On replacing all the parts by those with polymer (mainly Teflon) surface, chromatographic peaks of separated lipoprotein classes appeared. In this experiment, however, by use of a poorly controlled pump, strong dip was produced in the chromatogram disturbing the main peaks. This became worse at low flow rate (i.e. 0.2 $cm^3 \cdot min^{-1}$) at which better resolution of the peaks should be expected. When an accurately controlled pump (polymer coated CCPM, Toyo Soda, announced lowest flow rate being 1 X 10^{-6} $cm^3 \cdot min^{-1}$) was

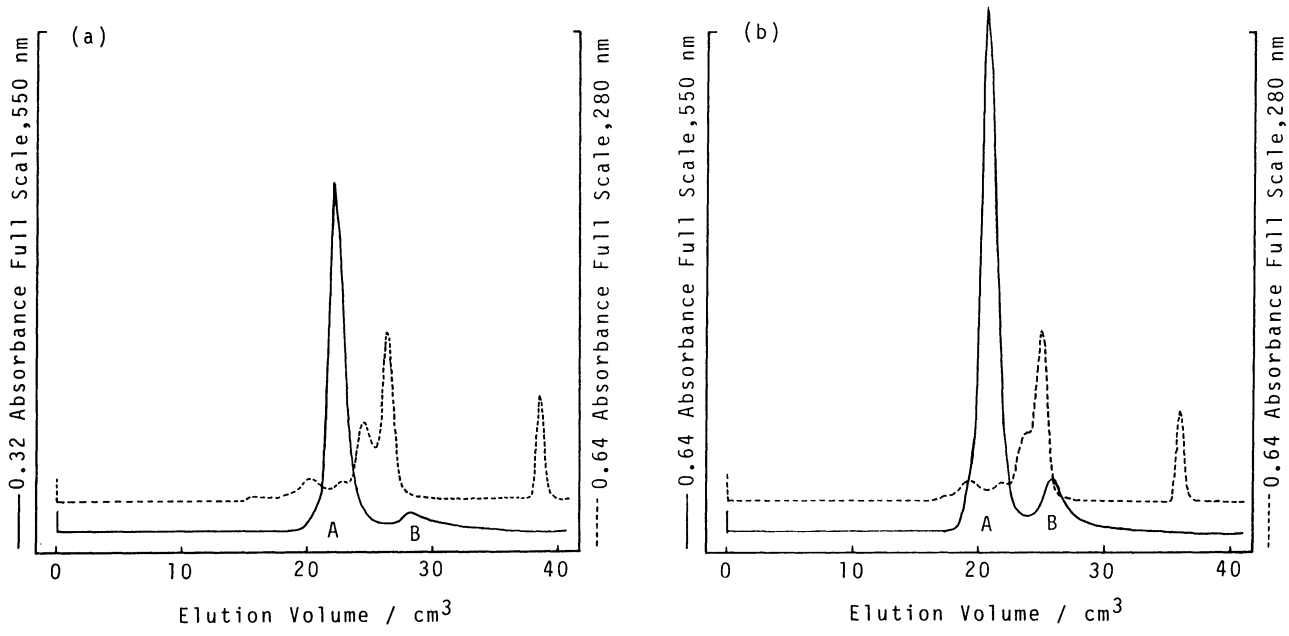

Fig.1. Chromatograms obtained by the methods using CEH and COD immobilized (a) and in solution (b). The solid and dot lines were obtained by monitoring at 550 nm and 280 nm, respectively.
Conditions: Column, G5000PW + G3000SW; Sample, 10 times diluted human serum (cholesterol content being 2.32 g·dm^{-3}; Sample volume, 0.1 cm^3; Main path eluent, (a) 150 mM phosphate buffer (pH 7.0) containing 2 mM EDTA and b) 150 mM Tris buffer (pH 7.0); Flow rate of main path, 0.2 $cm^3 \cdot min^{-1}$; Staining eluent, (a) 150 mM phosphate buffer (pH 7.0) containing 0.5 mM 4-AA, 0.3 mM TOOS, 6.7 unit·cm^{-3} POD, 1% Triton X-100, and 2 mM EDTA and (b) Determiner TC-555; Flow rate of staining path, 0.08 $cm^3 \cdot min^{-1}$; Temperature, 40°C; Detection, 550 nm.

used, this dip could be eliminated producing the peaks due to the sample. The final system was composed of a polymer coated CCPM pump, a polymer coated injector, glass separation columns (G5000PW + G3000SW), a glass immobilized enzyme column, Teflon tubing, and polymer parts.

Shown in Fig.1(a) is a chromatogram obtained on the above system. This result was comparable with that (Fig.1(b)) produced by the method demonstrated previously, in which CEH and COD were used in solution as staining reagents. By use of standard samples, peak A and B in the chromatograms were assigned to low density (LDL) and high density (HDL) lipoproteins, respectively. Since the concentration of LDL in serum is indicative in diagnosis of hyperlipidemia, the result implies the usefulness of this method in clinical medicine. The lowest detectable quantity of cholesterol was determined to be 2 μg and 20 times repeated injection of the same sample produced the peaks with unchanged intensities showing good reproducibility by this method.

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