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A simple method for the determination of the cholesterol esterase activity

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The proposed method determines the activity of cholesterol esterase (CEH) and takes advantage of its ability to catalyze the hydrolysis of cholesterol esters naturally present in human serum. The assay is based on Allain's method of spectrophotometric determination of cholesterol by means of cholesterol oxidase, peroxidase, but using 3,5-dichloro-dihydroxybenzenesulfonic acid (DHBS) as phenolic chromogen and human serum as a source of substrate for the CEH as a novelty. Furthermore, it is characterized by low costs and high precision. It can be employed to control the activity of CE preparations used for the preparation of enzymatic kits for the determination of cholesterol or for screening of potential bacterial enzyme producers.

Key words: cholesterol esterase, cholesterol, activity determination, human serum

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INTRODUCTION

Cholesterol esterase (CEH, EC 3.1.1.13) is present mainly in pancreas, its secretory liquid as well as in such tissues as: liver, epithelial cells, and in blood. It is essential in lipoprotein metabolism catalyzing the hydrolysis of cholesterol esters to free cholesterol and fatty acids (Cook *et al*., 1983). ORF (Tanaka *et al.* 1999) coding for cholesterol esterase is localized on human chromosome 10 (locus 10q23.2-q23.3). This gene (*LIPA*, lysosomal lipase gene), also referred to as lysosomal acid lipase gene (*LAL* or *LIPA*) or acid cholesterol ester hydrolase gene, has a variable number of tandem repeats (VNTR). Mutations in the LIPAgene result in disorders of esterase synthesis. Deficiency of this type of lipase that takes part in cleaving some cellular lipids, leads to the accumulation of cholesterol esters and triglycerides in the cells and tissues of spleen, liver, bone marrow, small intestine, adrenal gland, or lymph nodes. Such kinds of disorders are defined as cholesterol ester storage disease (CESD) and Wolman disease (WD) (Anderson *et al*., 1993; 1994). The clinical profile includes multi-organ disorders caused by cholesterol metabolic transformation failures. The activity level of cholesterol esterase in blood serum in CESD equals to 2.7±0.7 pmol·min^{-1}·mg^{-1} and in WD is equal to 2.8±0.9 pmol·min^{-1} ·mg^{-1}, and is very low as compared to the standard level of 141±16.9 pmol·min^{-1} ·mg^{-1} (Anderson *et al*., 1999).

CEH is produced by macrophages in order to defend the human organism against introduced foreign matter, e.g. medical implants (Christenson *et al*., 2006). Using polymers as biomaterials is limited by their sensitivity to enzymatic degradation in an organism. It is known that polymers containing in their structure the ester bond, such as PLA, undergo enzymatic degradation of cholesterol esterase having the capacity to hydrolyse these bonds (Liu *et al*., 2001).

The availability of a simple and valid method to determine cholesterol esterase activity will enable the selection of the best biomaterials resistant to enzymatic degradation. The study of biodegradation of biocompatible polymers by different enzymes *in vitro* is very important to predict their potential impact on implants.

Different methods for the estimation of the enzyme activity have been developed. Loginov *et al.* (1987) determined the activity of CEH (5 mU/ml), present in human duodenum, using a fluorometric approach because of the low value of CEH activity. They used cholesterol ester of *o*-coumaric acid as a substrate.

Other scientists (Tanga *et al*., 2003 and Christenson *et al*., 2006) in their study of CEH isolated from cattle pancreas (Sigma-Aldrich product) used its ability to hydrolyze the *p*-nitrophenyl acetate in the presence of phosphate buffer with pH 7.0.

Determining the CEH activity, Pioruńska-Stolzmann and others (2001) focused on human blood. They isolated the enzyme with the activity of 7.2±0.9 mU/mg using the ester of oleic acid and cholesterol as substrates.

Cholesterol esterase is mainly used in clinical studies to determine the level of cholesterol in human blood (Uwajima *et al*., 1975). The determination is based on the method of Allain and Trinder (Allain *et al*., 1974), by monitoring generated free cholesterol in the reaction catalyzed by cholesterol oxidase. Finally, cholesterol is converted to cholest-4-en-3-one and hydrogen peroxide, which in reaction with phenol and 4-aminoantipyrine in the presence of peroxidase forms a red colored chinoimine derivative. Colour intensity is directly proportional

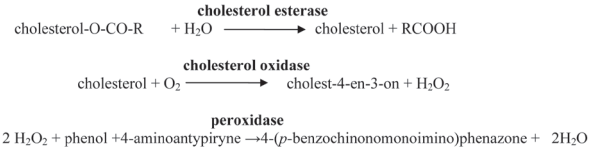

Figure 1. Enzymatic reactions in the determination of cholesterol using the method of Allian *et al.* (1974)

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Abbreviations: CEH, Cholesterol esterase; CESD, cholesterol ester storage disease; LIPA, lysosomal lipase gene; VNTR, variable number of tandem repeats; WD, Wolman disease.

to the amount of cholesterol having a maximum absorption at a wavelength of 500 nm.

In numerous studies, the protocol for cholesterol esterase activity determination proposed by Sigma company is used. It is based on the method of Allain with some modifications (Gallo *et al*., 1978; Singh *et al*., 2006).

The aim of the current study was to develop a method for the determination of cholesterol esterase activity (on the model enzyme from *Pseudomonas sp*.) and for testing its stability in polymer's biodegradability tests. The method is based on fundamental reaction sequences developed by Allain (Allain *et al*., 1974) using a natural substrate of cholesterol ester, which is present in human serum and phenolic chromogen as ingredients of the reaction mixture — 3,5 dichloro-dihydroxybenzenesulfonic acid (DHBSA) with the molar extinction of the final dye ($15.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$).

MATERIALS AND METHODS

The reaction mixture. 0.6% m/v $(NH_4)_2HPO_4$, 0.334% (m/v) $NH_4H_2PO_4$, 0.017% (m/v) 4-aminoantypiryne, 0.0086% (m/v) sodium cholate, 0.002% (m/v) cholesterol oxidase (Roche, 17.7 U/mg), $0.46 \cdot 10^{-3}$% (m/v) horseradish peroxidase (Fluka, 150 U/mg), 0.066% (m/v) DHBSA (Sigma-Aldrich) water solutions and 0.02% (v/v) detergent Triton X-100 (20%).

The composition of the reaction mixture was developed in the Institute of Cell Biology (Lviv) for the determination of cholesterol in human blood. The choice of DHBSA as a chromogen was made taking into account its excellent solubility and better stability (a low autooxidation rate) when compared to phenol.

Cholesterol esterase solution (50 U/ml). Cholesterol esterase (5 KU/42 mg, Roche) was dissolved in 50 mM phosphate buffer, pH 7.0 to the concentration 0.42 mg/ml and sterilized by filtration though filter (Millipore, 0.22 μm).

Analytical procedures. **Depletion of free cholesterol in the serum**. 5 μl of sterile human serum was added to 1 ml of reaction mixture to deplete a free cholesterol. The incubation was carried out for 10 min at 37°C, simultanously monitoring the coloured product at the wavelength of 516 nm in comparison with a control sample containing only distilled water.

Determination of exogenous activity of cholesterol esterase. 10 μl cholesterol esterase analyzed the appropriate dilution of 10, 12.5, 25, 100, 500-foldCEH solution (50 U/ml) was added to 1 ml of the reaction mixture after cholesterol depletion. The incubation was continued at 37°C for 10 min, measuring the amount of coloured product at the wavelength of 516 nm.

All the measurements were taken using UV-VIS spectrophotometer SpectroLAB.

The activity of CEH was determined from the slope of linear fragment of absorbance dependence on time after adding CEH aliquot to the reaction mixture (Fig. 2).

Calculations of CEH activity. The linear range of the plot was treated with Origin 8.00 program to calculate the rate of the reaction. Milimolar extinction coefficient ε_{mM} for oxidized DHBSA was accepted as 15.6 $mM^{-1} \cdot cm^{-1}$, as determined by calibration with cholesterol.

$$\text{Activity}(\text{U/ml}) = \frac{1.015 \cdot slope}{0.01 \cdot 15.66}\ \mu\text{mole/(min·ml)}, \text{ where}$$

μmole/(min·ml), where 1.015 — total volume of assay (ml); 0.01 — volume of enzyme used (ml); 15.6 — milimolar extinction coefficient ε_{mM} for oxidized DHBSA ($mM^{-1} \cdot cm^{-1}$).

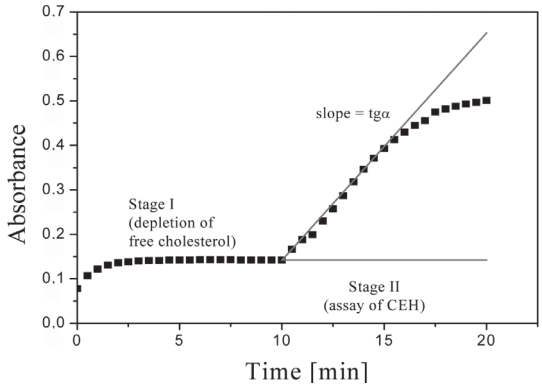

Figure 2. Principle of the CEH activity assay.

Incubation conditions in polymers' biodegradability test: temp. 30°C, time: 1 month.

RESULTS AND DISCUSSION

Figure 2 presents two stages of the proposed analytical procedure: I — oxidation of free cholesterol present in human serum and II — determination of exogenous CEH activity. It is clearly shown that depletion of free cholesterol in serum used as a source of cholesterol esters is finished in the 3rd min. After the addition of CEH, a well expressed linear range in kinetics profile suitable for reaction rate calculation it is observed .

The stable saturation effect in the absorbance at stage 1 of the reaction (Fig. 2) is an evidence of a negligible endogenous CEH activity. This proved the possibility to use a human serum as a natural substrate for the exogenous CEH activity assay.

The values of the CEH activity after 1 month of incubation at 30°C (Table 1) of the enzyme in solution are rather comparable to the initial values. These results demonstrate a relatively high stability of the enzyme at the conditions required for testing the biodegradability of polymers used for the production of medical implants. The biodegradability test includes an evaluation of the CEH activity after the incubation of polymer samples such as polyurethanes in solutions of known initial enzyme activity of this enzyme.

Table 1. Cholesterol esterase activity after incubation for 1 month at 30°C.

CEH activity of the prepared solutions before, U/ml	Determined CEH activity after, U/ml
0.1 ± 0.01	0.08 ± 0.01
0.48 ± 0.05	0.46 ± 0.04
0.93 ± 0.05	1.87 ± 0.03
3.85 ± 0.05	3.80 ± 0.11
4.78 ± 0.01	4.68 ± 0.01

CONCLUSIONS

The proposed method based on the usage of a new phenolic chromogen DHBSA (as an ingredient of the reaction mixture) and human serum (as a natural source of cholesterol esters) was presented as a sensitive and valid assay for measuring the exogenous CEH activity.

Interestingly, it can be employed in order to test CEH commercial preparations as well as to monitor changes in enzyme activity (e g., in polymers' biodegradation

tests). The biodegradability test involves the evaluation of the degradative activity of CEH to polymers. After a period of incubation of polymers in solution of the enzyme with a known initial activity we assessed the possible change.

REFERENCES

Allain CC (1974) Enzymatic determination of total serum cholesterol. *Clin Chem* **20**: 470–475.

Anderson RA, Byrum RS, Coates PM, Sando GN (1994) Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc Natl Acad Sci USA* **29**: 2718–2722.

Anderson RA, Rao N, Byrum RS, Rothschild CB, Bowden DW , Haywotrh R, Pettenati M (1993) *In situ* localization of the genetic locus enconding the lysosomal acid lipase/cholesteryl esterase (LIPA) deficient In Wolman disease to chromosome 10q23.2-q23.3. *Genomocs* **15**: 145–147.

Anderson RA, Bryson GM, Parks JS (1999) Lysosomal acid lipase mutations that determine phenotype in wolman and cholesterol ester storage disease. *Mol Genet Metab* **68**: 333–345.

Cook KG (1983) The identity of the cholesteryl ester hydrolase of bovine corpus luteum. *Biochem Soc Trans* **11**: 703.

Cook KG (1983) Cytosolic cholesterol ester hydrolase from bovine corpus luteum. Its purification, identification, and relationship to hormone-sensitive lipase. *Biochim Biophys Acta* **752**: 46–53.

Christensona EM, Patel S, Anderson JM, Hiltnera A (2006) Enzymatic degradation of poly(ether urethane) and poly(carbonate urethane) by cholesterol esterase. *Biomaterials* **27**: 3920–3926.

Gallo LL, Atasoy R, Vahouny GV, Treadwell CR (1978) Enzymatic assay for cholesterol ester hydrolase activity. J Lip Res **19**: 913–916.

Loginov A S, Shibaeva LO, Planutis KS, Sadoko VM (1988) Determination of cholesterol esterase activity in human duodenal contents. *Bulletin Experim Biol Med* **106**: 1366–1367.

Liu J W, Zhao Q, (2001) Research progresses on degradation mechanism *in vivo* and medical applications of polylactic acid. *Space Med Eng (Beijing)* **14**: 308–312.

Pioruńska-Stolzmann M, Iskra M, Majewski W (2001) The activity of cholesterol esterase and ceruloplasmin are inversely related in the serum of men with atherosclerosis obliterans. *Med Sci Monit* **7**: 940–945.

Singh S, Solanki PR, Pandey MK, Malhora BD (2006) Cholesterol biosensor on cholesterol esterase, cholesterol oxidase and peroxidase immobilized onto concducting polyaniline films. *Sensors and Actuaors B* **115**: 534–541.

Sigma quality control test procedure http://www.sigmaaldrich.com/technical-documents/protocols/biology/assay-procedure-for-cholesterol-esterase.html

Tanaka H (1999) Purification and characterization of bovine pancreatic bile salt-activated lipase. *J Biochem* **125**: 883–890.

Tanga YW, Labow RS, Santerre JP (2003) Enzyme induced biodegradation of polycarbonate-polyurethanes: dose dependence effect of cholesterol esterase. *Biomaterials* **12**: 2003–2011.

Taylor AK, Zambaux JL, Klisak I, Mohandas T, Sparkes R S, Schotz MC, Lusis AJ (1991) Carboxyl ester lipase: A highly polymorphic locus on human chromosome 9qter. *Genomics* **10**: 425–431.

Uwajima T, Terada O (1976), Production of cholesterol esterase by *Pseudomonas fluorescens*. *Agric Biol Chem* **40**: 1605–1609.

Uwajima T (1975) Purification and properties of extracellular cholesterol esterase ester hydrolase of *Pseudomonas flourescens*. *Agric Biol Chem* **39**: 1511–1512.