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ADAPTING OF SPECTROPHOTOMETRIC ASSAY OF PARAOXONASE ACTIVITY WITH 4-NITROPHENYLACETATE FOR MURINE PLASMA AND LIVER

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Abstract. Paraoxonase 1 (PON1) is the most studied enzyme of the paraoxonase family, which are mammalian enzymes with aryldialkylphosphatase activity. Paraoxonase 1 was first described in the 1940s as an enzyme found in mammalian tissues capable of hydrolyzing organophosphate pesticides. However, recent studies have shown that PON1 also plays a protective role in diseases related to inflammation and oxidative stress, and exhibits anti-inflammatory, antioxidant, anti-atherogenic, and detoxifying properties. In particular, an important function for PON1 is to protect against vascular diseases by metabolizing oxidized lipids in blood lipoprotein complexes. Some studies show that reduced PON1 activity is associated with the risk of developing cardiovascular disease, as well as obesity, metabolic syndrome, and cancer. Thus, PON activity can be an important biomarker in the diagnosis of the above-mentioned diseases. Paraoxonase 1 is synthesized in the liver, then released into the bloodstream and is found mainly in high-density lipoproteins. Because PON1 has broad substrate specificity, different activities of PON1 can be assessed. In this study, we optimized the conditions for the spectrophotometric determination of paraoxonase activity in the blood and liver of mice. The PON1 arylesterase activity protocol was adapted using nitrophenylacetate as a substrate. A concentration of 3.2 mmol/l nitrophenylacetate was chosen for the analysis of PON in mouse tissues. Specific PON activity was different in mouse tissues – 210±17 mU/mg in blood plasma and 66.5±8.5 mU/mg in liver. Therefore, the supernatant volume recommended for PON determination is 5 µl for plasma and 10 µl for liver homogenate in a total reaction mixture of 1.25 ml.

Keywords: paraoxonase 1, spectrophotometric assay, 4-nitrophenylacetate, plasma, liver, mice.

1. INTRODUCTION

Paraoxonases are a group of mammalian enzymes possessing aryldialkylphosphatase activity. Aryldialkylphosphatase activity means that these enzymes are able to hydrolyse the triester bond that is present in organophosphate compounds, particularly insecticides. In mammals, there are three isoenzymes of paraoxonase: paraoxonase 1 (PON1, EC 3.1.1.2, 3.1.1.81, 3.1.8.1), paraoxonase 2 (PON2, EC 3.1.1.2, 3.1.1.81), and paraoxonase 3 (PON3, EC 3.1.1.2, 3.1.1.81, 3.1.8.1), which have been identified so far (Hussein et al., 2012; Meneses et al., 2019). The most studied isozyme among them is PON1 form. Paraoxonases are very similar in their amino acid composition but differ in

their expression in mammalian tissues and perform different functions. PON1 and PON3 bind to high-density lipoprotein (HDL) in the blood and hydrolyze organophosphates and statin lactones, whereas PON2 is found in the lungs, liver, intestines, heart, brain, and kidneys, and functions as an intracellular protector against reactive oxygen species (ROS) (Taler-Verčič et al., 2020; Garrick et al., 2022). Paraoxonase PON1 is a protein with a molecular weight of 43 kDa and consists of 354 amino acids (Harel et al., 2004; Hussein et al., 2012; Shunmoogam et al., 2018). Paraoxonases have been found in all mammals, some vertebrates and nematodes, but they have not been found in bacteria (Bar-Rogovsky et al., 2013).

Paraoxonase PON1 is synthesized in the liver and then released into the bloodstream, where it associates mainly with HDL and partially with very low-density lipoproteins (Deakin et al., 2012). Myeloperoxidase (MPO) and paraoxonase 1 (PON1) bind to high-density lipoproteins, which are associated with inflammation, oxidative stress, and atherosclerosis. During inflammation, MPO is a source of ROS and can oxidize HDL apolipoprotein A1 (APOA1). PON1 possesses antioxidant properties. That is, a complex of MPO, PON1, and HDL is formed, in which PON1 inhibits MPO activity, while MPO inactivates PON1 (Huang et al., 2013). Free PON1 has a lower enzymatic activity than HDL-bound one. After synthesis, PON1 is transported from the liver to some tissues, where it binds to cell membranes and prevents lipid peroxidation (Huang et al., 2013). It is also known that PON1 prevents oxidation of low-density lipoproteins (LDL) and is a anti-inflammatory protein of the acute phase, its concentration decreases during inflammatory conditions, that is, PON slows down inflammatory processes (Deakin et al., 2012; Taler-Verčič et al., 2020). HDL-associated PON1 inhibits monocyte differentiation in macrophages. That is very important because macrophages play an important role in the development of arterial foam cells and atherosclerotic lesions (Farid & Horii, 2012).

Recent studies show that PON1 prevents the oxidation of LDL and HDL both in vivo and in vitro by hydrolyzing oxidized lipids. Early atherogenesis is characterized by the accumulation of cholesterol in the arterial walls with subsequent development of the lesion but the main cause of this is oxidative damage to LDL. Many beneficial effects of paraoxonase have been found, namely protective effects on myocardial infarction (Ayub et al., 1999), diabetes (Kulka, 2016), hypercholesterolemia (Ikeda et al., 1998), tumors (Witte et al., 2012; Mogarekar & Chawhan, 2013). Also, PON1 is capable to metabolize drugs, so it has been proposed therapeutically for use in drug inactivation (Camps et al., 2011).

Paraoxonase 1 has two calcium binding sites, one responsible for the stability of the enzyme and the other required for its catalytic activity. Since PON1 is a calcium-dependent enzyme, it is recommended for studies to add Ca salts to ensure correct determination of enzyme activity (Ceron et al., 2014).

Paraoxonase 1 catalyzes the hydrolysis of some xenobiotics, namely organophosphorus compounds (paraoxon), unsaturated aliphatic esters, and aromatic carboxylic esters (phenylacetate, carbamates) (Bassu et al., 2022). Since PON1 has a broad substrate specificity, different PON1 activities can be assessed, such as paraoxonase activity (if paraoxon is used as a substrate), arylesterase activity (if phenylacetate or 4(p)-nitrophenyl acetate is used as a substrate), or lactonase activity (5-thiobutylbutyrolactone or dihydrocoumarin, are used as substrates) (Mogarekar & Chawhan, 2013; Ceron et al., 2014).

Different methods are used to determine PON activity, namely spectrophotometric and fluorimetric methods, and it can also be quantified by immunological methods using specific antibodies against PON1 (Costa et al., 2005). Unfortunately, fluorometric methods of analyzing PON1 activity have drawbacks, as the research uses highly toxic reagents that have low sensitivity, which does not allow determining the lactonase activity of PON1. For spectrophotometric analysis of PON1, such substrates as phenylacetate, paraoxon, dihydrocoumarin, and homocysteinethiolactone are most often used (Fang et al., 2022).

2. MATERIALS AND METHODS

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), KH2PO4, ethylenediamine tetraacetic acid (EDTA), 4-nitrophenylacetate were from Sigma-Aldrich (St. Louis, USA); Tris from Carl Roth (Karlsruhe, Germany); CaCl2 was obtained from local supplier (Ukraine).

2.2. Blood and liver tissue samples

In the experiments, PON1 activity was measured in blood plasma and liver tissue of 9-month-old male C57Bl/6J mice. Mice were kept in a 12-hour light/dark cycle at a temperature of 22 ± 2°C and a humidity of 50-60%. Mice were fed standard rodent chow (10 kcal% fat) with free access to water. Mice were euthanized using anaesthesia induced by carbon dioxide gas. Blood samples were taken from the retro-orbital sinus and then liver was dissected and frozen in liquid nitrogen (−196°C) followed by transfer to −80°C for storage. Blood samples were collected in tubes with heparin and centrifuged (1500 g, 15 min, 4 °C) to get plasma. The resulting blood plasma was used to run enzyme activity measurements and were kept on ice prior to analysis.

Liver tissue samples were homogenized in a Potter-Elweheim homogenizer in the medium containing 50 mM potassium phosphate buffer (KPi, pH 7.0), 0.5 mM EDTA, and 1 mM PMSF. The homogenates were centrifuged (13200 rpm, 15 min, 4°C) in an Eppendorf 5415 R centrifuge (Hamburg, Germany) at 1:10 ratio. The supernatant was used for measurements.

All mouse protocols were approved by the Animal Experimental Committee of Vasyl Stefanyk Precarpathian National University (Ukraine) and were conducted in accordance with the European Union for the protection of animals used for scientific purposes of 22 September 2010 (2010/63/EU).

2.3. Paraoxonase assay

The procedure for paraoxonase assay was adapted from the method described in (Mogarekar & Chawhan, 2013) for the human enzyme. The original technique was developed for arylesterase activity of PON. Arylesterase PON activity was determined with p-nitrophenyl acetate as substrate and formation of p-nitrophenol was monitored at 405 nm.

Paraoxonase activity was determined in the plasma and liver of mice. The components of the mixture were added to it in the following sequence (in final concentrations): 50 mM Tris-HCl (pH 7.0), 1 mM $CaCl_2$, distilled water and supernatant to a total volume of 1.25 ml. The reaction was initiated by adding 25 μl of 160 mM 4-nitrophenylacetate (to a final concentration of 3.2 mM) as a PON substrate. Since the reaction can be occurring slowly non-enzymatically, the blank should be measured in parallel, using distilled H_2O instead of the supernatant. Enzyme activity was measured at a wavelength of 405 nm for 90 seconds with an interval of 10 seconds in a plastic UV cuvette with using spectrophotometer Spekol 211 (Jena, Germany).

To optimize the conditions for determining the enzyme in the liver of mice, nitrophenyl acetate was used in the range of concentrations from 0.1 mmol/l to 4 mmol/l. Since the linearity of the reaction is an important indicator, we determined the linearity of product formation for different amounts of supernatant - 1 μl and 5 μl in a reaction mixture volume of 1.25 ml. Next, to determine K_M for p-nitrophenyl acetate, we determined the dependence of the reaction rate on the concentration of the substrate.

Soluble protein concentrations were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as the standard.

The PON activity was expressed as mU/mg protein is based on the molar absorbance (14,000) of p-nitrophenol at 405 nm. Enzyme activity was calculated according to the Formula 1 :

$$A = \frac{\Delta OD/min \times Vpr}{\varepsilon \times V_{sup} \times [pR]} \times 1000 \quad (1)$$

A – specific enzyme activity, mU/mg protein;
$_{\Delta}OD/_{min}$ – change in optical absorption per min;
V_{pr} – total volume of reaction mixture, mL;
ε – the molar extinction coefficient for p-nitrophenol at 405 nm; ε = 14000 $M^{-1}cm^{-1}$
V_{sup} – volume of supernatant, mL;
[pR] – the protein concentration in the supernatant (mg/mL).

2.4 Calculation of the Michaelis constant

The Michaelis constant (K_M) was calculated in the Kinetics program from the building of the graph dependency of ΔD/min from enzyme content (namely volume of supernatant). The Kinetics program calculates Vmax and $S_{0.5}$ (half-maximal saturation constant). Vmax is the maximum speed of reaction (this is the asymptote to which the graph is directed). It is necessary to choose for assay of enzyme activity a substrate concentration range that is close to saturation, usually in the order of 5-10 K_M values, which is about 95% of Vmax.

3. RESULTS AND DISCUSSION

Paraoxonase 1 is mainly synthesized in the liver and transported into the bloodstream, where it forms a complex with high-density lipoproteins (Deakin et al., 2002; Camps et al., 2009). One of the main functions of PON1 is the protection of serum HDL and LDL particles from oxidation of lipids and inhibition of N-homocysteinylation of proteins. N-homocysteinylation is characterized by unfolding pf protein, loss of their functions and amyloid formation. Accumulation of lipid peroxides and homocysteinylated proteins results in arterial inflammation, which can ultimately lead to atherosclerosis, ischemic stroke, and myocardial infarction. Based on this, changes in PON1 activity are considered a clinical biomarker and are used to identify hepatic and systemic oxidative stress. Also, measurement of PON1 activity is considered as a test for evaluating of liver function and the risk of development of cardiovascular diseases (Mackness et al., 1993). In this study, we optimize experimental conditions for assay of mouse PON1 using protocol for human arylesterase PON1 activity (Mogarekar & Chawhan, 2013). In enzymology, there are four procedure rules used for the correct determination of enzyme activity. These rules are as follows:

1) Dependence of the reaction rate (V) on the substrate concentration (S)
2) Dependence of the amount of product (substrate) on the reaction time course.
3) Dependence of the reaction rate on the concentration of the enzyme (supernatant).
4) For the time of activity measurement less than 10% of substrate can be used.

The abovementioned rules determine that (1) substrate concentration virtually should not limit the reaction rate, (2) amount of product of enzymatic reaction should be increased linearly with time, (3) reaction rates should linearly depend on the concentration of the enzyme preparation; (4) reaction is not limited by substrate concentration.

First of all, we determined the dependence of the reaction velocity (V) on the substrate concentration (S). As a substrate, p-nitrophenol acetate was used. The amount of blood plasma was 5 μl. Fig 1 shows that velocity of reaction rate increased linearly to 3 mM p-nitrophenol acetate and then reached a plateau. This curve corresponds a typical curve of dependency of velocity of enzymatic reaction on substrate concentration which is described by Michaelis' kinetics.

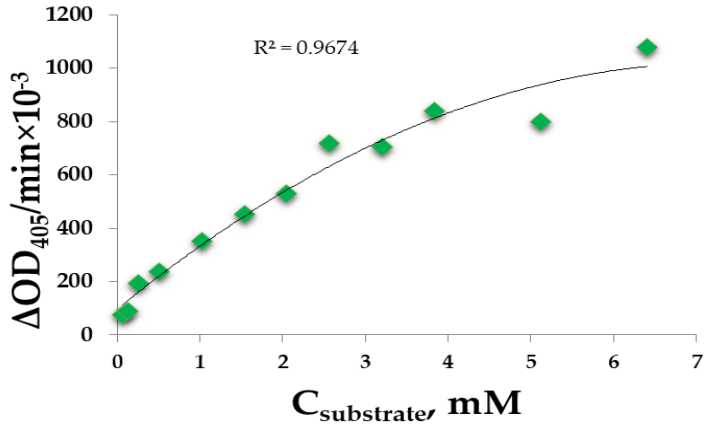

Fig. 1. Dependence of the reaction rate (V) on the substrate concentration (S) in blood plasma of mice. Volume of plasma was 5 μl

To calculate Vmax (maximal velocity) and K_M, we used the program Kinetics. K_M is formally defined as the substrate concentration at which the velocity of reaction is half the maximum (Vmax/2). According to calculations, Michaelis constant for p-nitrophenyl acetate was about 2 mM. To correct assay, it is necessary to choose a substrate concentration range close to saturation, usually in the order of 5-10 K_M, which is equal to 95% of Vmax. Therefore, for the next determinations, we had to use p-nitrophenyl acetate at concentration of 10 mM. But it should be noted that at adding to reaction mixture p-nitrophenyl at the concentrations higher 4 mM the reaction mixture became muddy, may be due to phenol-induced denaturation of proteins, whose content was high in blood plasma (62 mg/ml). In order to avoid protein denaturation and density interference, we chose a concentration of 3.2 mM p-nitrophenyl acetate for next measurements (in original protocol the concentration of 2.5 mM p-nitrophenyl acetate was used) (Mogarekar & Chawhan, 2013). We also calculated the amount of substrate used the time of activity measurement. Less than 4% of substrate was used per a minute of the measurement. It is in a line with the fourth rule of for the correct determination of enzyme activity. So, this allow to use nitrophenyl acetate at the concentration of 3.2 mM for the assay of PON activity.

As was mentioned above, for adequate measurement of paraoxonase activity, we should choose the range of enzyme concentrations in which the amount of the used substrate / (formed product), S/P, is changed linearly over time. Therefore, in the next stage, we checked whether the amount of product of the PON reaction depended linearly on the reaction time at used concentrations of enzyme, namely on supernatant volumes. For testing, we chose different amounts of supernatant - 1 and 5 μl, and the amount of product formed was measured registering optical density increase every 10 sec for one min. Figure 2(A-B) shows that at both amounts of supernatants the initial rate of the reaction demonstrated a linear dependence of the amount of product formed with time.

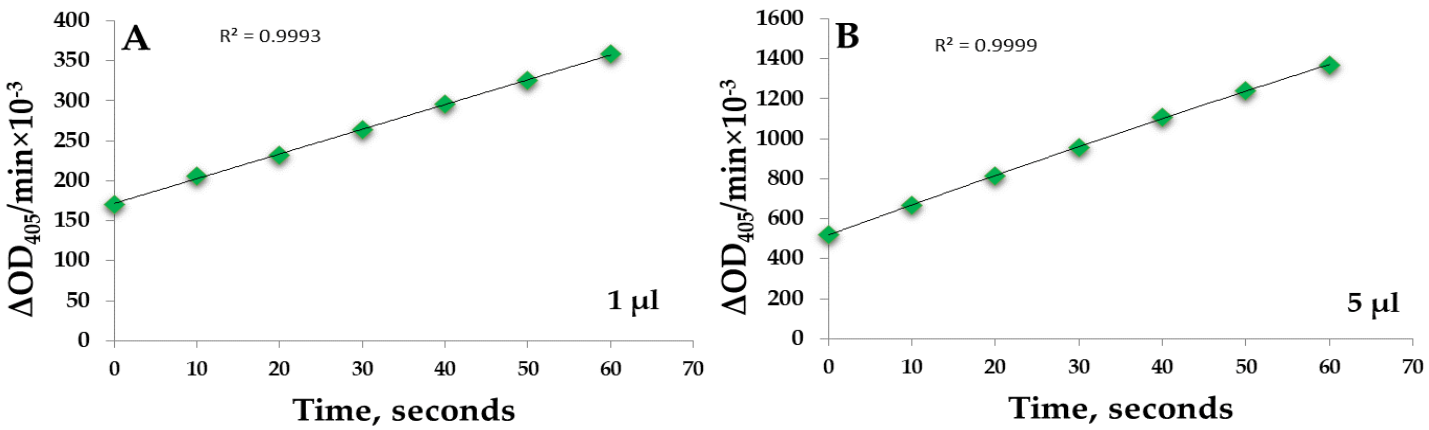

Fig. 2. Dependence of the amount of product formed in PON catalyzed reaction on the reaction time at 1 µl (A) and 5 µl (B) of blood plasma from mice

The last stage of optimization procedure was the determination of the dependence of the reaction rate on the amount of the supernatant (the concentration of enzyme). Figure 3 shows that at used range of supernatant volumes, this dependence was linear and velocity of the reaction increased linearly to the amount of supernatant that we added. This was true for supernatant from liver tissue (Fig. 3A) and blood plasma (Fig. 3B). It should be noted that reaction rate was higher in blood than in plasma at the same amount of supernatant and by calculating of specific PON activity. In particular, specific PON activity was 66.5±8.5 mU/mg and 210±17 mU/mg protein in liver homogenate and blood plasma, respectively. It is expected, because paraoxonase is predominantly blood enzyme despite it is synthetized by liver (Meneses et al., 2019; Milaciu et al., 2019).

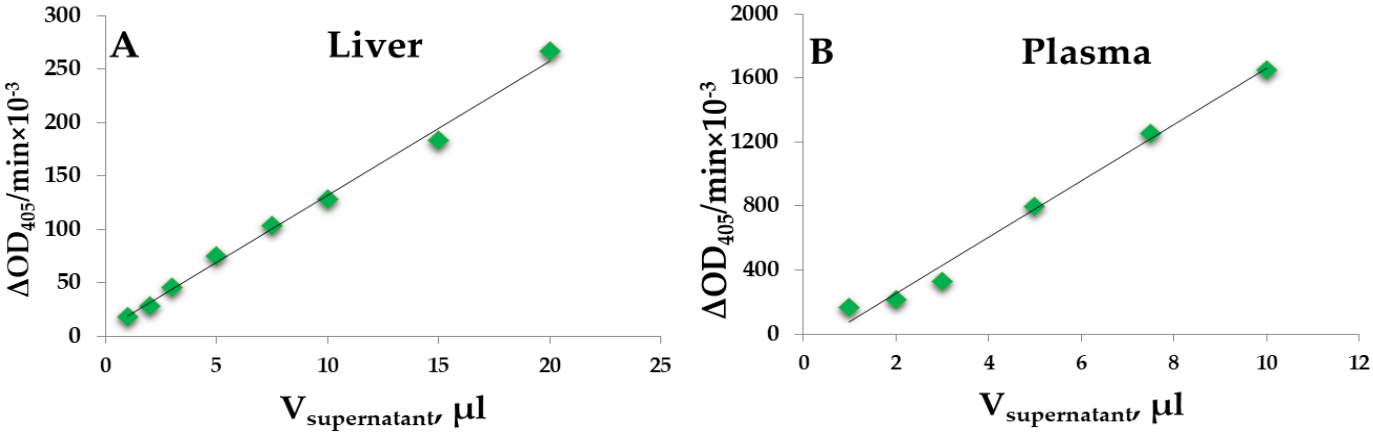

Fig. 3. Dependence of the reaction velocity on the concentration of the enzyme - amount of supernatant from the mouse live r(A) and blood plasma (B)

4. CONCLUSIONS

Paraoxonase 1 (PON1) is synthesized by the liver and found in the blood circulation being associated with high-density lipoproteins. In this study, we optimized conditions for spectrophotometric determination of paraoxonase activity in blood and liver of mice. Protocol for arylesterase activity of PON1 was adapted with using p-nitrophenylacetate as a substrate. The concentration of 3.2 mM of nitrophenylacetate was chosen for PON assay in mouse tissues. The supernatant volume recommended for PON determination is 5 µl for plasma and 10 µl for liver in

a total volume of 1.25 ml because of different specific PON activities in these tissues (210±17 mU/mg in plasma and 66.5±8.5 mU/mg in liver).

Conflict of interest

The authors declare that they have no conflict of interest.

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Ватащук Мирослава, Гурза Вікторія, Байляк Марія. Підбір умов для спектрофотометричного визначення активності параоксонази з 4-нітрофенілацетатом у плазмі та печінці мишей. *Журнал Прикарпатського університету імені Василя Стефаника*, **9** (4) (2022), 6–14.

Параоксонази - це група ферментів ссавців з арилдиалкілфосфатазною активністю, і найбільш вивченою з праоксоназ є параоксоназа 1 (PON 1). Параоксоназа 1 вперше була описана у 1940-х роках як фермент, виявлений у тканинах ссавців, здатний гідролізувати фосфорорганічні пестициди. Однак останні дослідження показали, що PON 1 також відіграє захисну роль при захворюваннях, пов'язаних із запаленням і оксидативним стресом та виявляє протизапальні, антиоксидантні, антиатерогенні, антидіабетичні, антимікробні та детоксикаційні властивості. Зокрема, PON 1 важлива для захисту від судинних захворювань, оскільки метаболізує окислені ліпіди у складі ліпопротеїдних комплексів крові. Деякі дослідження показують, що знижена активність PON 1 пов'язана з ризиком розвитку серцево-судинних захворювань, а також ожиріння, метаболічного синдрому та раку. Тому визначення активності параоксонази можна вважати важливим біомаркером у діагностиці вищевказаних захворювань. У цій роботі ми оптимізували умови спектрофотометричного визначення активності параоксанази в крові та печінці мишей. Адаптовано протокол для визначення арилестеразної активності PON1 з використанням нітрофенілацетату як субстрату. Концентрація нітрофенілацетату 3,2 ммоль/л була обрана для визначення параоксонази в тканинах мишей. Питома активність PON 1 у цих тканинах була різною - 210±17 мОд/мг у плазмі крові та 66,5±8,5 мОд/мг у супернатанті печінки. Рекомендований об'єм надосадової рідини для визначення PON 1 становить 5 мкл для плазми та 10 мкл для печінки в реакційній суміші загальним об'ємом 1,25 мл.

Ключові слова: параоксоназа 1, спектрофотометричний метод, 4-нітрофенілацетат, плазма, печінка, миші.