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OPTIMIZATION OF PARAOXONASE ORGANOPHOSPHATASE ACTIVITY ASSESSMENT IN MOUSE PLASMA USING PARAOXON-ETHYL

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Aim. To select optimal conditions for determining the organophosphatase activity of paraoxonase in mouse blood plasma using paraoxon-ethyl as a substrate.

Materials and Methods. The method was modified from a protocol developed for rats. Enzyme activity was assessed by measuring the formation of 4-nitrophenol at 405 nm. The optimal pH was determined within the range of 6.0–10.5 using 50 mM buffer solutions. The reaction mixture contained fixed concentrations of $CaCl_2$, NaCl, and paraoxon-ethyl (1 mM). The reaction was initiated by adding 20 µL of blood plasma. For kinetic analysis, Tris-HCl buffer (pH 8.0) and varying concentrations of paraoxon-ethyl (0–2 mM) were used. Absorbance was measured over 90 seconds using a ULAB-102 spectrophotometer at 405 nm. Enzyme activity was calculated using a molar extinction coefficient of 18290 $M^{-1}cm^{-1}$, and protein concentration was determined by the Bradford method. Kinetic parameters (K_m, V_{max}) were calculated using the "KINETICS" software.

Results. The highest paraoxonase activity was observed at pH 8.0–9.0. Thus, pH 8.0 was chosen for further assays. The determined K_m was 229 ± 22 µM, and V_{max} was 0.070 ± 0.009. A paraoxon-ethyl concentration of 1.5 mM (5–10×K_m) ensured substrate saturation. Time-dependence analysis confirmed a linear increase in product formation within the range of 1–50 µL of plasma. A working volume of 15–20 µL was considered optimal.

Conclusions. The assay conditions for paraoxonase activity were optimized: pH 8.0, K_m = 229 µM, V_{max} = 0.070, and a working plasma volume of 15–20 µL.

Keywords: paraoxonase, atherosclerosis, paraoxon-ethyl, kinetics.

Paraoxonase (PON) is a hydrolase enzyme with three isoforms (PON1, PON2, PON3) involved in the detoxification of xenobiotics and protection against oxidative stress. PON1 is the most extensively studied isoform, known to metabolize oxidized lipids, and is considered a protective factor in the development of inflammatory and vascular diseases, particularly atherosclerosis. Paraoxonase exhibits three main types of activity—organophosphatase, arylesterase, and lactonase — depending on the substrate used [1]. These activities are viewed as potentially informative markers for diagnosing metabolic disorders, cardiovascular diseases, and conditions associated with oxidative stress [2]. To improve the accuracy of biochemical analysis, conditions for determining paraoxon-ethyl-dependent (organophosphatase) activity in mouse blood have been optimized. The results may be of scientific value for advancing the understanding of antioxidant defense mechanisms, as well as of practical importance for developing new diagnostic and preventive strategies.

Aim. To select conditions for the determination of organophosphatase activity of paraoxonase using the substrate paraoxon-ethyl in mouse blood plasma.

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Methods. The procedure was adapted from a technique described for rat paraoxonase [3]. The assay was designed to determine the organophosphatase activity of the enzyme using paraoxon-ethyl as a substrate. Enzymatic activity was assessed by the formation of 4-nitrophenol, with absorbance measured at 405 nm.

The optimal pH of the reaction was determined within the range of 6.0–10.5 using appropriate buffer systems at a constant concentration of 50 mM. The reaction mixture contained fixed concentrations of $CaCl_2$, NaCl, and paraoxon-ethyl (1 mM). The reaction was initiated by adding 20 µL of blood plasma.

For kinetic analysis, Tris-HCl buffer (pH 8.0) and varying concentrations of paraoxon-ethyl (0–2 mM) were used. To assess the dependence of reaction rate on enzyme amount, a fixed substrate concentration (1.5 mM) and different volumes of plasma (0–50 µL) were applied. Absorbance was recorded over 90 seconds using a ULAB-102 spectrophotometer at 405 nm. Enzyme activity was expressed in mU/mg of protein and mU/L of plasma, using a molar extinction coefficient of 18290 $M^{-1}cm^{-1}$. The protein concentration was determined by the Bradford method.

Kinetic parameters, including the Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}), were calculated using the "KINETICS" software based on plots of ΔA/min versus substrate concentration. The program also estimates S0.5 (half-saturation constant) and V_{max}. For accurate enzyme activity determination, substrate concentrations close to saturation (typically 5–10 K_m, corresponding to ~95% of V_{max}) were selected. Statistical analysis was performed using Microsoft Excel and GraphPad Prism 8.0.1. Results were presented as mean ± standard error of the mean (SEM).

Results. There is no consensus in the scientific literature regarding the optimal pH for assessing paraoxonase organophosphatase activity using paraoxon-ethyl as a substrate. Depending on the study, activity has been measured at pH values ranging from 8.0 to 10.5. Therefore, the first step of our study was to determine the pH optimum. The highest PON activity was observed between pH 8.0 and 9.0, with a decline at higher pH levels. For subsequent experiments, pH 8.0 was selected as it is closest to physiological blood pH (Fig. 1).

Kinetic parameters were also established: the Michaelis-Menten constant (K_m) was 229 ± 22 µM, and the maximum reaction rate (V_{max}) was 0.070 ± 0.009. For further measurements, a paraoxon-ethyl concentration of 1.5 mM was used, corresponding to 5–10×K_m, ensuring substrate saturation (Fig. 2).

Time-dependence analysis confirmed a linear increase in reaction product formation using 20 µL of blood plasma. The reaction rate remained linear across a range of 1–50 µL of plasma. Based on these results, a working volume of 15–20 µL was chosen to ensure reliable measurements while minimizing sample consumption (Fig. 3).

Conclusions. This study optimized the paraoxonase activity assay, determining the optimal pH (8.0), kinetic parameters (K_m = 229 µM, V_{max} = 0.070), and plasma volume (15–20 µL) for reliable measurements.

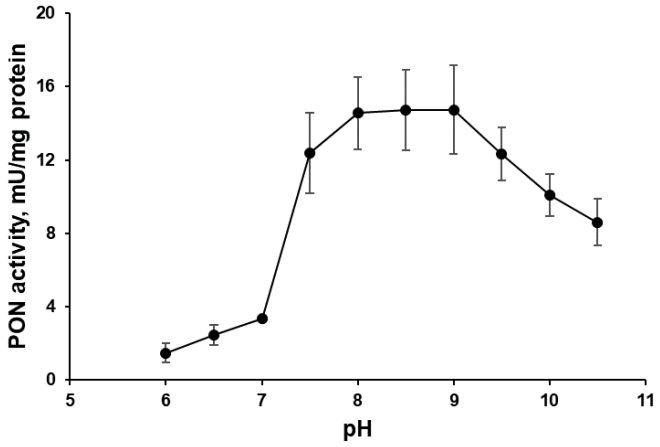

Fig. 1. **Dependence of the specific activity of PON in mouse blood plasma on pH (with the addition of 1 mM paraoxon-ethyl and 20 µl of plasma)**
Values are presented as mean ± standard error of the mean (SEM), n = 3

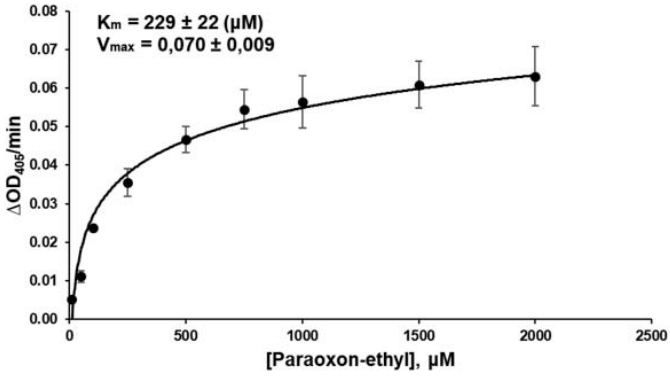

Fig. 2. **Dependence of the reaction rate on the concentration of paraoxon-ethyl in the blood plasma of mice (volume of added blood plasma 20 μL)**
Values are presented as mean ± standard error of the mean (SEM), $n = 3$

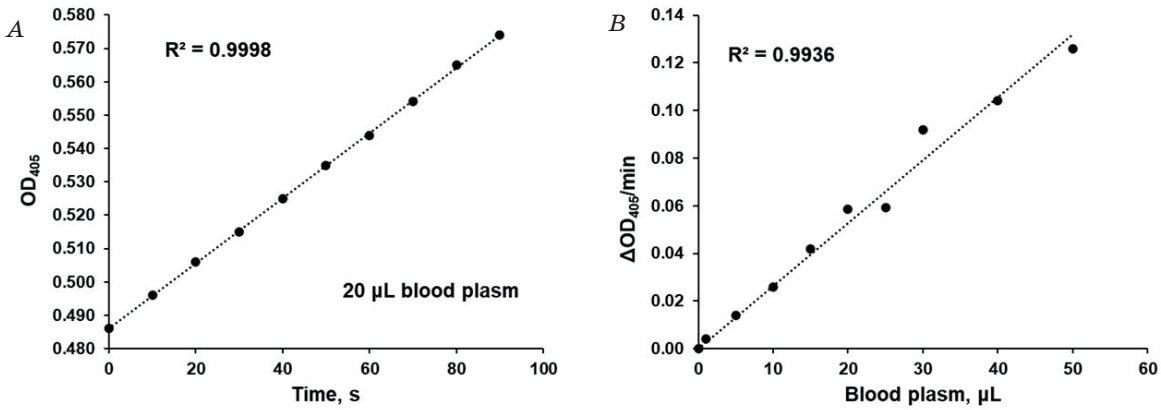

Fig. 3. A **— Dependence of the optical density of the reaction product formed on time (with the addition of 1.5 mM paraoxon-ethyl);** *B* **— Dependence of the reaction rate on the amount of added blood plasma (with 1.5 mM paraoxon-ethyl**

Authors' Contribution

ARO performed spectrophotometric determination of PON activity, processed and statistically analyzed the data, wrote the original draft of the abstract; OID assisted in the experiments; MMB conceptualization and supervision, reviewed and edited the abstract.

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