DOI 10.1007/s10517-018-4219-2
Bulletin of Experimental Biology and Medicine, Vol. 165, No. 4, August, 2018 ERRATUM

ERRATUM

Correction to the article "A Method of Measuring Glutathione Peroxidase Activity in Murine Brain in Pharmacological Experiments,"

by A. V. Razygraev, A. D. Yushina, and I. A. Titovich, Vol. 165, No. 2, pp. 292-295, June 2018

The article text should be read as follows.

A Method of Measuring Glutathione Peroxidase Activity in Murine Brain: Application in Pharmacological Experiment

A. V. Razygraev, A. D. Yushina, and I. A. Titovich

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 165, No. 2, pp. 261-264, February, 2018
Original article submitted July 25, 2017

A method of measuring of glutathione peroxidase activity using H_2O_2 was adapted for homogenates of murine brains. If the amount of reduced glutathione was at the constant level of 0.55 mM, the concentration of H_2O_2 of 0.192 mM was saturating for glutathione peroxidase of murine brain and was selected as an optimal concentration for the estimation of enzyme activity in tris-HCl buffer with addition of NaN_3 and EDTA (pH 8.5) at the incubation temperature of 37°C. The homogenates were diluted with the reaction mixture by 10.4 times. The duration of incubation did not exceed 60 sec, if 13% homogenate was used. The experiment, in which this method has been applied, showed increased activity of glutathione peroxidase in the brain of mice treated with a derivative of acetaldehyde ammonia during long-term intermittent normobaric hypoxia. These data might reflect activation of glutathione peroxidase.

Key Words: *glutathione peroxidase; quantitative analysis; brain; hydrogen peroxide; Ellman's reagent*

The enzymes of glutathione peroxidase (Gpx) family catalyze the thiol-dependent reduction of H_2O_2 and organic hydroperoxides. This property determines the protective role of Gpx under conditions of oxidative stress. The cytosol isoenzyme glutathione peroxidase-4 (Gpx-4), one of the main neuroprotective enzymes, plays an important role in brain cells [7]. Thus, estimation of Gpx activity is an important stage for studies of substances with possible neuroprotective properties.

St. Petersburg State Chemical Pharmaceutical Academy, St. Petersburg, Russia. ***Address for correspondence:*** alexeyrh@mail.ru. A. V. Razygraev

There are several easily acceptable methods of estimation of Gpx activity based on the analysis of concentration of residual reduced glutathione after interaction with hydroperoxide. Glutathione concentration is estimated by the product of its reaction with Ellman's reagent [1,4,5]. However, the search for the easiest method for the estimation of Gpx activity in brain tissues of laboratory rodents using H_2O_2 as a substrate has not been performed previously. The required parameters of this method include constant rate of the enzyme reaction, proportionality of reaction rate to the protein amount, and saturation with H_2O_2 at the constant thiol level.

Here we adapted the method of estimation of Gpx activity based on H_2O_2 and Ellman's reagent for homogenates of murine brain. We used this method for testing of the substance with neuroprotective properties.

MATERIALS AND METHODS

Mature male and female white outbred mice were kept under standard vivarium conditions [5,10]. The animals were narcotized with trichloromethane and chloral hydrate. The isolated brain was washed in physiological saline (0.9% NaCl), homogenized in 0.9% NaCl in a glass homogenizer, and centrifuged at 10,000-12,000*g* for 10-15 min at 4°C. The supernatant with Gpx activity was used as a sample material. Pooled samples from at least 3 animals were used in each experiment for establishing the method. Reagent A containing 2.55 ml 0.1 M tris-HCl buffer (pH 8.5), 0.34 M EDTA, 0.15 ml water solution of NaN_3 (26 mg/ml), and 0.0105 ml of water solution of reduced glutathione (51.1 mg/ml) was used for preparation of the reaction mixture I. The reaction was initiated by the addition of 3-6 mM H_2O_2 (water solution). The reaction was stopped by the addition of 30% (w/v) trichloroacetic acid.

The reaction was conducted in Eppendorfs at 37°C in a water bath. The reagents were added in accordance to 4 following schemes (the sequence of addition of reagents to the reaction mixture is described):

- SUM: reagent A, simultaneously H_2O_2 and biomaterial, incubation, trichloroacetic acid.
- NEO: reagent A, simultaneously H_2O_2 and biomaterial solvent, incubation, trichloroacetic acid.
- B: reagent A, biomaterial, incubation, simultaneously H_2O_2 and trichloroacetic acid.
- ST: reagent A, biomaterial solvent, incubation, simultaneously H_2O_2 and trichloroacetic acid.

The reagents were not preliminary mixed during simultaneous addition (Fig. 1). The following volumes were used: 0.18 ml reagent A, 0.02 ml biomaterial, 0.008 ml H_2O_2 solution, and 0.04 ml water solution of trichloroacetic acid. After addition the reagents were mixed by pipetting.

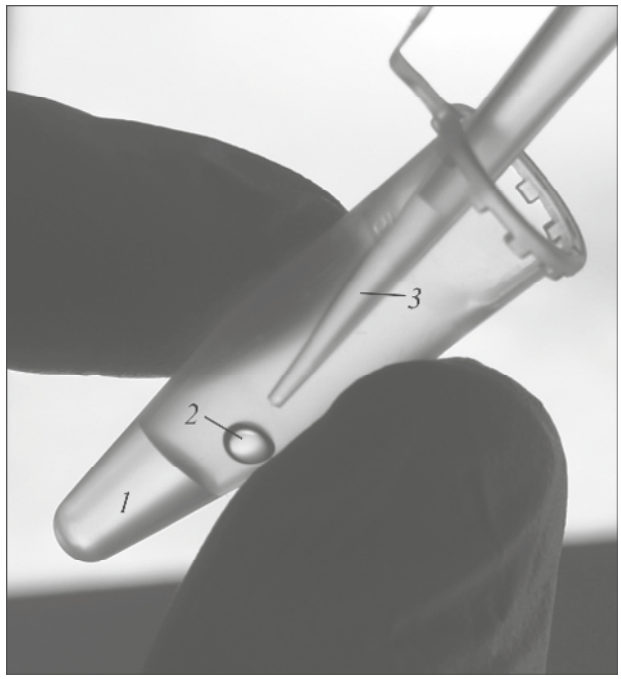

Fig. 1. Simultaneous addition of hydrogen peroxide and biomaterial to the reaction mixture. *1*) Reagent A (0.18 ml); *2*) 5 mM H_2O_2 (0.008 ml); *3*) biomaterial (supernatant of 13% homogenate of murine brain, 0.02 ml).

After the termination of reaction, the samples were centrifuged at room temperature at 1000*g* for 10 min. Reaction mixture II consisted of 0.108 ml of supernatant containing glutathione, 1.16 ml 0.1 M tris-HCl buffer with 0.34 mM EDTA (pH 8.5), and 0.0075 ml Ellman's reagent (4 mg/ml of absolute methanol). The absorption maximum was registered at 412 nm. 1 absorption unit (1.000) of the reaction mixture II corresponded to 1 mM reduced glutathione in the reaction mixture I. These measurements were based on the previously described method [5] also used in this study.

Enzyme activity was calculated by decrease in concentration of reduced glutathione (recalculated per 1 min of incubation) using the following formula:

$$\Delta A_{Gpx}=(A_{ST}-A_{SUM})-(A_{ST}-A_{NEO})-(A_{ST}-A_B),$$

where A with subscript refers to the absorption level at 412 nm after the corresponding reaction scheme; ΔA_{Gpx}, difference between absorption levels and the corresponding decrement in glutathione level during enzymatic oxidation within 1 min expressed in milimol per 1 liter.

Thus, the non-enzymatic decrease of reduced glutathione concentration and the decrease in reduced glutathione concentration during glutathione oxidation by biomaterial without Gpx contribution were subtracted from the total decrease in reduced glutathione concentration.

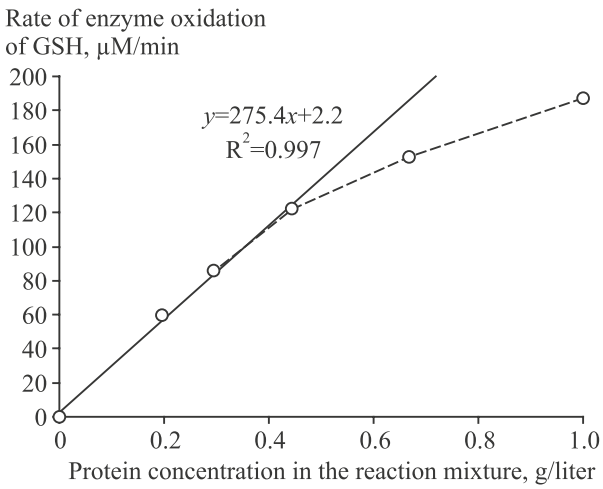

Fig. 2. Dependence of rate of enzymatic oxidation of glutathione (GSH) and protein concentration in the reaction mixture I. Regression coefficient (275.4) equals to the relative activity in the used pooled sample (nmol of GSH/(min mg of protein).

Protein concentration was estimated by the turbidimetric method [6].

Long-term intermittent normobaric hypoxia (LINH) was modeled in the BIO-NOVA-204 hypoxia chamber (Bio-Nova). Hypoxia gas mixture containing 6% O_2 was daily blown to the chamber for 6 h for 2 weeks. Animals not exposed to LINH (control-1) were placed in the chamber without it being pressurized and hypoxia being modeled (non-process allowing correction for stress factor). The study substance synthesized in the Department of Organic Chemistry of the St. Petersburg State Chemical Pharmaceutical Academy on the basis of bis[2-[(2E)-4-hydroxy-4-oxobut-2-enoyloxy]-N,N-diethylethanaminium]butandionate (FDES) [3] was administered intragastrically 10 min before the experiment in doses of 10 and 75 mg/kg. The animals of control-1 (without LINH) and control-2 (LINH) groups received 0.9% NaCl in the same volume.

TABLE 1. Analysis of Effects of the Study Substance FDES on Gpx Activity in the Brain of Mice Exposed to LINH (Me (Q1-Q3)

Group	Specific activity of Gpx, nmol glutathione/(min×mg protein)
Control-1 (*n*=13)	378 (334-434)
Control-2 (*n*=16)	320 (252-415)
FDES, 10 mg/kg (*n*=7)	425 (261-490)
FDES, 10 mg/kg under LINH conditions (*n*=9)	515* (394-579)
FDES, 75 mg/kg (*n*=7)	214 (188.5-347.5)
FDES, 75 mg/kg under LINH conditions (*n*=11)	593** (427.5-707.5)

Note. **p*=0.0275 and *p*=0.0066 in comparison with the control-1 (without LINH) and control-2 (LINH) groups, respectively; **p*=0.0149 and *p*=0.0012 in comparison with the control-1 (without LINH) and control-2 (LINH) groups, respectively (without corrections for multiple comparisons).

The coefficients of linear regression equation were calculated using the software environment R (version 2.13.1) [9]. Comparison of independent samples was also conducted in the software environment R using the non-parametric Mann—Whitney test (with correction for continuity at the presence of repeated measurements). The results for each experimental group were presented as median and quartiles (Me(Q1-Q3)). The estimation of Gpx activity in the groups was performed to investigate the pharmacological substance during hypoxia in a complex way (the investigation included big number of experimental groups and estimated parameters). As the experiment was pilot, the correction for multiple comparisons was not used for the probability of type I error (*p*) during estimation of Gpx activity.

RESULTS

Enzyme activity is estimated at saturating concentration of the substrate. This concentration is significantly higher than the Michaelis constant (K_M). Thus, a slight decrease or increase in substrate concentration does not affect the rate of enzyme reaction. Glutathione peroxidase reaction involves two substrates — H_2O_2 and glutathione — interacting with the enzyme via the ping-pong mechanism. In this case, K_M and maximum rate are unlimited [8]. The kinetics of these reactions is analyzed using the constant concentration of one of the substrates and various concentrations of the second substrate. The second substrate is used to check for saturation. Variation in the final concentration of H_2O_2 in the reaction mixture was followed by saturation by H_2O_2 in the range of 0.154-0.231 mM. This allowed selecting the same final concentration as described previously (0.192 mM) [5]. Thus, 5 mM H_2O_2 was added to the reaction mixture.

The rate of enzyme reaction (after subtraction of the rate of non-enzymatic glutathione oxidation and rate of glutathione oxidation by biomaterial) was proportional to the protein amount in the reaction mixture until this level reached 0.444 mg/ml. This level corresponded to addition of supernatant of 14.8% homogenate to the reaction mixture (Fig. 2). That means that specific activities estimated at various levels of protein in one sample corresponding to the linear dependence

would be identical. We further used the homogenate in a concentration of 13% and lower not to use the threshold level.

The rate of the summary reaction was constant within the first 60 sec after addition of the supernatant of 13% homogenate to the reaction mixture. The regression equation for the summary reaction was:

$$y=181.5x+9.0,$$

where x is the time of incubation (min) before addition of trichloroacetic acid and y is reduced glutathione consumed (μM).

Coefficient of linear dependence R^2 was 0.99.

The percent of non-enzyme oxidation of glutathione by peroxide and by biomaterial was 26.4 and 8.1%, respectively. The use of 5% biomaterial was associated with constant reaction rate remaining for the longer time, up to the maximum incubation time (80 sec). Thus, incubation time until reaction termination should not exceed 60 sec for 13% brain homogenate. This parameter can be increased up to 80 sec for 5% of the homogenate.

The results of a pilot study of Gpx activity in the murine brain exposed to FDES and LINH are presented in the Table 1. The complex analysis of anti-hypoxic effects of this potential pharmacological substance was performed. Gpx activity was increased after FDES treatment under hypoxic conditions. Low levels of type I error indicate that the observed changes might serve as a dependence, which can be reproduced in further experiments [2].

Therefore, the method of estimation of Gpx activity is applicable for the new biomaterial. The enzyme is saturated by H_2O_2 at constant thiol level; reaction rate is stable and proportional to protein concentration. We have shown the possibility of usage of this method for estimation of Gpx activity in the brain of laboratory rodents under experimental conditions. The results of the pilot study indicate the neuroprotective properties of FDES.

The authors are grateful to K. M. Pats (St. Petersburg State Chemical Pharmaceutical Academy) and M. O. Matrosova (Peter the Great St. Petersburg Polytechnic University) for their help in the study.

REFERENCES

1. Arutyunyan AV, Dubinina EE, Zybina NN. Methods for Assessing Free-Radical Oxidation and Antioxidant System of the Body. Methodical Recommendations. St. Petersburg, 2000. Russian.
2. Glantz SA. Primer of Biostatistics. Moscow, 1999. Russian.
3. Okovityj SV, Shustov EB, Bolotova VT, Titovich IA. Patent RU No. 2588365. Neuroprotective agent based on bis{2-[(2E)-4-hydroxy-4-oxobut-2-enoyloxy]-N,N-diethylethanaminium} butanedionate. Bull. No. 18. Published June 27, 2016.
4. Petushok NE, Tarasov YuA, Pekhovskaya TA, Evkovich IN, Shevalye AA, Chumachenko SS. Effects of organic selenium substance, selenomethionine, in acute alcohol intoxication. Biomed. Khimiya. 2011;57(2):180-186. Russian.
5. Razygraev AV, Tumasova ZN, Pats KM, Pitukhina NN, Alexeeva PA, Baranova NI. Determination of glutathione peroxidase activity in serum of laboratory mice using hydrogen peroxide and Ellman's reagent. Butlerov. Soobshch. 2016;45(2):123-130. Russian.
6. Chesnokova LS, Voinova NE, Komkova AI, Lyanguzov AYu. Protein quantification methods. Enzymes and Nucleic Acids. Vladimorov VG, Lyzlova SN, eds. St. Petersburg, 1997. P. 5-25.
7. Brigelius-Flohé R, Maiorino M. Glutathione peroxidases. Biochim. Biophys. Acta. 2013;1830(5):3289-3303.
8. Flohé L, Brigelius-Flohé R. Selenoproteins of the glutathione peroxidase family. Selenium: Its Molecular Biology and Role in Human Health. Yatfield DL, Berry MJ, Gladyshev VN, eds. New York, 2012. P. 167-180.
9. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, 2011.
10. Razygraev AV, Taborskaya KI, Volovik KY, Bunina AA, Petrosyan MA. Monoamine oxidase activity in the rat pineal gland: comparison with brain areas and alteration during aging. Adv. Gerontol. 2016;6(2):111-116.