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Brief communication

Effect of multiple freeze–thaw cycles of cytoplasm samples on the activity of antioxidant enzymes

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Abstract

Introduction: In this paper the activity of cytoplasmatic antioxidant enzymes after multiple freeze–thaw cycles of samples of cytoplasm was assayed in order to characterise the limits that would be necessary to impose on experimental design. **Methods:** The activities of antioxidant enzymes in samples of rat liver cytoplasm were evaluated after 1, 2, 3, 4, 5, 10 and 15 freeze–thaw cycles. The activity of antioxidant enzymes was determined by standard methods developed for this purpose. **Results:** The most resistant against freeze–thaw stress was catalase; even 15 freeze–thaw cycles did not change its activity. The remaining enzymes tested revealed a significant loss in their activity after 4–5 freeze–thaw cycles. The most sensitive was glutathione *S*-tranferase; its activity was significantly decreased after 3 thaw cycles. **Discussion:** The data presented may be helpful in designing experiments in which assay of antioxidant enzymes is a part.

Keywords: Catalase; Freeze–thaw stress; Glutathione peroxidase; Glutathione reductase; Glutathione *S*-transferase; Superoxide dismutase

1. Introduction

Reactive oxygen species (ROS) are a class of highly reactive molecules derived from the metabolism of oxygen playing important role in various degenerative disorders, such as cardiovascular disease, mutations, cancer, ageing and neurodegenerative diseases (Schoneich, 1999). Additionally ROS are produced during inflammation and ischaemia/reperfusion processes (Toledo-Pereyra, Lopez-Neblina, & Toledo, 2004). Certain toxicants and ionizing radiation are also a source of ROS in living organisms (Bauer & Bauer, 1999). Living organisms have developed defence mechanisms for radical detoxification. Key metabolic steps are: catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, by the superoxide dismutase (SOD) and conversion of H_2O_2 into water and oxygen by catalyse (CAT) or glutathione peroxidase (GPx), which can also destroy toxic peroxides. Moreover, glutathione *S*-transferases (GSTs) and glutathione reductase (GR) are considered as ancillary antioxidant enzymes. GSTs catalyze metabolic detoxification of xenobiotics, drugs and carcinogens, thus, protect the cells against redox cycling and oxidative stress, while GR reduces glutathione, oxidised by GPx and restores this important component of intracellular redox system (Mates, 2000). Therefore, antioxidant enzymes play a key role in redox balance and their activity is often tested as a marker of pathophysiological status of a cell (Alissa, Bahijri, Lambs, & Ferns, 2004; Cristiano, de Haan, Iannello, & Kola, 1995; Dandekar, Nadkarni, Kulkarni, & Punekar, 2002). They are therefore assayed in experiments dealing with oxidative stress (Coskun, Kanter, Korkmaz, & Oter, 2005; Koruk et al., 2004; Mohamadin, El-Beshbishy, & El-Mahdy, 2005), as well as cancer treatment and development (Hussain, Hofseth, & Harris, 2003; Iynem et al., 2004; Khanzode, Muddeshwar, Khanzode, & Dakhale, 2004) or life span extension (Cristiano et al., 1995; Sanz, Diez-Fernandez, Andres, & Cascales, 2002). Most often however, antioxidant enzymes are assayed as factors of toxic liver injury

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in experiments performed with the use of "model hepatotoxins" (e.g acetaminophen, galactosamine, or carbon tetrachloride) and hepatoprotective compounds or plant extracts (O'Brien et al., 2000; Sreepriya, Devaki, Balakrishna, & Apparanantham, 2001; Wang, Liu, Tseng, Wu, & Yu, 2004; Yanpallewar et al., 2003; Zavodnik et al., 2004). Such experiments involve a large number of animals, therefore, a large number of samples (most often liver cytoplasmatic fraction is used) must be prepared and stored at −80 °C. Usually, for enzyme activity determination the samples are thawed, small amounts of cytoplasm are drawn and the rest of the sample is frozen again. If the cytoplasm is used for evaluation of several kinds of enzymes, samples are thawed and frozen repeatedly; therefore, the question arises: to what extent does the effect of freeze–thaw affect antioxidant enzyme activity in samples of cytoplasmatic fractions. In order to clarify this problem we have isolated cytoplasm from rat liver and assayed the activity of antioxidant enzymes after multiple freeze–thaw cycles.

2. Methods

2.1. Isolation of cytosolic fraction

The rats used in the experiment were housed in an animal facility at 22±1 °C with 12 h light–dark cycle, controlled humidity and circulation of air. Five untreated male Wistar rats used as control group for a separate experiment were killed by decapitation. The livers were removed and perfused with ice-cold 1.15% KCl. The cytoplasmatic fraction was prepared by differential centrifugation according to the standard procedure. Briefly, the livers were homogenized on ice by four strokes, in a motor-driven Potter–Elvehjem homogenizer with three volumes of ice-cold buffered saccharose solution (150 mM KCl, 50 mM Tris, pH 7.4). The crude homogenate was centrifuged at 8750 ×*g* for 15 min at 4 °C. Microsomes were sedimented from the 8750 ×*g* supernatant by centrifugation at 105,000 ×*g* for 60 min at 4 °C. Supernatant–cytosolic fraction was aliquoted into ten vials and stored at −80 °C.

The experiment was performed according to the Local Animal Ethics Committee Guidelines for Animal Experimentation.

2.2. Biochemical assays

For each type of enzyme the following protocol was used. The sample of cytoplasm (200 μl) was thawed in a waterbath at 20 °C, initial activity of the enzyme tested was measured and the sample was placed in ultrafreezer at −80 °C for 30 min. Then the sample was thawed again, the activity of the enzyme was measured and the sample was frozen again. The enzyme activity was measured after 1, 2, 3, 4, 5, 10 and 15 freeze–thaw cycles.

The methods used in the experiment included the following. Superoxide dismutase (SOD) activity was determined by the method of Sun and Zigman (1978). SOD activity was measured by inhibition of spontaneous epinephrine oxidation. In the calculations the standard curve of SOD activity was used.

Catalase (CAT) activity was determined according to Beers and Sizer (Beers & Sizer, 1952). CAT activity was measured by the rate of H_2O_2 reduction. Molar extinction coefficient E^{M}(230 nm)=81 was used to calculate activity. One unit of CAT reduces 1 μM of H_2O_2. The results were expressed in units per minute per milligram protein.

Glutathione peroxidase (GPx) activity was determined according to Mohandas, Marshall, Duggin, Horvath, and Tiller (1984). Cumene peroxide was used as a substrate. The disappearance of NADPH at 340 nm was a measure of enzyme activity and extinction coefficient E^{mM}(340 nm)=6.22 was used for calculations. Activity was expressed in nmol NADPH oxidized per minute per milligram protein.

Glutathione reductase (GR) was assayed by measuring NADPH oxidation at 340 nm using oxidized glutathione as a substrate. An extinction coefficient E^{mM}(340 nm)=6.22 was used for calculations. The activity was expressed in nmol NADPH oxidized per minute per milligram protein (Mohandas et al., 1984).

Glutathione *S*-transferase (GST) activity measurement was based on the spectrophotometric determination of 1-chloro-2,4-dinitrobenzene (CDNB) conjugate formed in a GSH coupled reaction. A molar extinction coefficient E^{mM}(230 nm)=9.6 was used to calculate activity which was expressed as nanomoles of CDNB conjugated with glutathione per minute per milligram protein. (Mohandas et al., 1984).

2.3. Statistical analysis

Multiple comparisons against a single control group were made by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test using the GraphPad Prism Software (Version 4.02, San Diego, CA, USA), $P<0.05$ was considered to be a statistically significant difference.

3. Results

The most resistant enzyme against freeze–thawing stress was catalase; even 15 freeze–thaw cycles did not change its activity (Fig. 1A).

Glutathione peroxidase was resistant against 4 freeze–thaw cycles. After 10 cycles, 51% of initial activity was lost, after 15 cycles only 5% of initial activity was observed (Fig. 1B).

As few as 3 freeze–thaw cycles decreased the activity of GST up to 81% of the initial value and the subsequent cycles caused a further decrease in its activity (Fig. 1C).