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Novel assay for glutathione reductase activity by high-performance liquid chromatography with electrochemical detection

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Abstract

A new method was devised for the direct measurement of glutathione reductase activity in crude tissue samples using high-performance liquid chromatography and electrochemical detection with a mercury/gold electrode. This assay depends on the high sensitivity of the electrode to detect SH groups produced from a substrate, oxidized glutathione, the interference of which can be excluded by setting the oxidation voltage as low as 100 mV. The sensitivity was high enough to detect the activity in crude samples containing less than 1 μg of protein. This method proved to be sensitive and simple for the measurement of this biologically important reductant activity without the need for a further purification procedure. © 1998 Elsevier Science B.V.

Keywords: Glutathione reductase; Enzymes

1. Introduction

Normally, the production of reactive oxygen species (ROS) is balanced by the antioxidant defense system. If the production of ROS increases or the antioxidant capacity falls, ''oxidative stress'' is induced. Under oxidative stress, many cellular components, such as proteins, nucleic acids and membrane lipids, are damaged, which further accounts for various pathological processes. Major free radical-scavenging enzymes are superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1), glutathione peroxidase (glutathione: hydrogen-peroxide oxidoreductase, EC 1.11.1.9, GSH-Px) and catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC 1.11.1.6). Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide, which is further converted into water by GSH-Px and catalase. GSH-Px removes peroxides via oxidation of reduced glutathione (GSH) into glutathione disulfide (GSSG) [1], indicating that the level of GSH essentially affects the scavenging capacity of ROS. The GSH level is regulated mainly by the activity of NADPH-dependent glutathione reductase [glutathione disulfide reductase (NAD(P)H), NAD(P)H: glutathione-disulfide oxidoreductase, EC 1.6.4.2, GSH-R] [2]. Especially in the brain, where catalase activity is low and superoxide dismutase activity is only moderately expressed, hydrogen peroxide is detoxicated by the glutathione system [3–5].

The quantitative determination of GSH-R activity in crude tissue samples has been difficult. Usually, GSH-R activity has been assayed spectrometrically

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by measurement of NADPH consumption during the reduction of GSSG to GSH [6,7]. To increase the sensitivity, $NADP^+$, the oxidation product of NADPH, was measured fluorometrically [8]. More recently, GSH-R activity was determined in plant extracts utilizing high-performance liquid chromatography (HPLC) by quantitation of $NADP^+$ after induction of fluorescence under alkaline conditions [9]. This method can be applied to the purified enzyme, but it is insensitive and not reliable for the measurement of GSH-R activity in crude tissue samples, because of the high background of NADPH absorbance at 340 nm and the presence of other NADPH-dependent enzyme systems. Another assay method is based on the measurement of GSH generated from GSSG, or of GSSG consumed by GSH-R [6,7]. The thiol group of GSH was derivatized with 5,5′-dithio-bis(2-nitrobenzoic acid) [10] or *N*-(9-acridinyl)maleimide [11] and the products were measured spectrometrically or fluorometrically. However, it is not known if these methods can be applied to crude tissue homogenates containing various thiol compounds. For the assay, the crude sample should be pre-washed to remove interfering compounds of small molecular mass.

Another method for measuring GSH and GSSG directly, electrochemical detection (ECD) with HPLC, has been used. The detection of dithiols with a carbon graphite electrode in an amperometric or coulometric method requires a very high oxidation voltage; 730 and 860 mV for GSH and GSSG, respectively [12]. On the other hand, using a dual gold–mercury cell in series, GSH and GSSG separated by HPLC are reduced to GSH at the first electrode, and detected by the catalytic oxidation of mercury at the second electrode [13]. This dual electrode system can detect both GSH and GSSG with relatively low oxidation voltages.

Using this highly sensitive detection method for GSH, catalytic oxidation with a mercury/gold electrode, a new, sensitive and simple assay of GSH-R activity was devised and applied to crude tissue homogenates. When the electrode was set at a low oxidation voltage, the presence of a high dose of a substrate, GSSG, did not interfere with the detection of the GSH produced. This method may be the first direct detection method for GSH-R activity in crude samples and may be applicable to various biological samples without the need for a purification or derivatization procedure.

2. Experimental

2.1. Reagents

All chemicals were of analytical grade. Perchloric acid (PCA) and other chemicals were purchased from Katayama (Osaka, Japan); GSH, GSSG and β-NADPH were from Sigma (St. Louis, MO, USA). Monochloroacetic acid was from Nacalai tesque (Kyoto, Japan).

2.2. GSH assay

For the detection of GSH, an HPLC system comprising a Shimadzu LC-9A pump (Kyoto, Japan), an LC-4C amperometric detector (Bioanalytical System, West Lafayette, IN, USA) and an autosampler AS-8020 (Tosoh, Tokyo, Japan) was used. A biophase ODS-IV analytical column (110×4 mm, 5 μm, Bioanalytical System) was used. The mobile phase was 0.1 *M* monochloroacetic acid, the pH of which was adjusted to 3.0 with 1 *M* sodium hydroxide, and it was perfused with nitrogen gas. The flow-rate of the mobile phase was 0.8 ml/min. GSH was quantitatively determined using a mercury/gold electrode (Dual gold working electrode, MF 1002, Bioanalytical System). The first electrode was set at an oxidation voltage of 100 mV and the output was monitored.

2.3. Sample preparation

Male Wistar rats (eight weeks old, 220–240 g body weight) were used as the source of the enzyme samples. The experimental protocols were approved by the Ethical Committee of the Department of Biosciences, Nagoya Institute of Technology. Heart, liver and the whole brain were rapidly dissected, washed with chilled physiological saline and frozen in liquid nitrogen. Tissue samples were weighed and sonicated with five or ten volumes of distilled water in a Branson sonicator at 40 W with a 50% duty cycle for 1 min. The sonicated sample was diluted

20–100 times with 0.2 *M* sodium phosphate buffer, pH 7.0, just before analysis.

2.4. Assay for GSH-R activity

The enzyme sample (about 1–5 μg of protein) was incubated in a reaction mixture (total volume, 100 μl) of 0.1 *M* sodium phosphate buffer, pH 7.0, containing 1 m*M* GSSG and 100 μ*M* NADPH. The mixture was incubated at 37°C with gentle shaking for 20 min. The reaction was terminated by the addition of 100 μl of 0.1 *M* perchloric acid containing 0.1 m*M* EDTA and 0.1 m*M* sodium metabisulfite. After mixing, the solution was allowed to stand for 10 min in an ice-bath, then centrifuged at 22 000 *g* for 10 min at 4°C, the supernatant was filtered through a Millipore filter (0.45 μm pore size), and GSH was quantitatively assayed by HPLC–ECD.

2.5. Kinetics of GSH-R

The enzyme sample prepared from rat liver (protein, 1.66 μg) was incubated at 37°C for 20 min with eight different concentrations of GSSG, ranging from 1 m*M* to 58.5 μ*M*, and 100 μ*M* NADPH in a total volume of 100 μl. The effects of NADPH concentration were also examined from 100 to 5.85 n*M* with 1 m*M* GSSG. The reciprocal of the reaction velocity (nmol GSH produced/min/mg protein) was plotted against that of the concentration of either GSSG (m*M*) or NADPH (n*M*) (Lineweaver–Burk plot).

2.6. Protein assay

The protein concentration of tissues samples was determined using the method of Bradford [14], with bovine γ-globulin as a standard.

3. Results and discussion

The voltammograms of GSH and GSSG are shown in Fig. 1. GSH was detected by oxidation from 0 to 250 mV, and with an oxidation voltage ranging from 200 to 250 mV, the electrochemical response reached a plateau, whereas GSSG was not oxidized at voltages up to 250 mV. The oxidation voltage needed to detect GSH or GSSG was so markedly different that GSH was measured without interference from large excess of GSSG using an oxidation voltage lower than 250 mV. To reduce interferences by the contaminants in a crude sample, 100 mV was chosen to monitor GSH produced from GSSG by GSH-R.

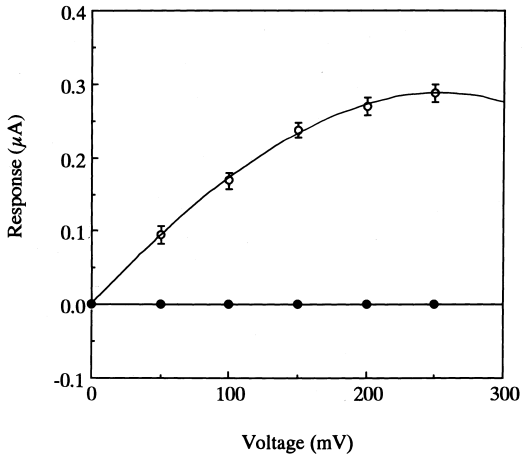

Fig. 1. Voltammograms for GSH (open circle) and GSSG (filled circle). The circle and bar represent the mean and the standard deviation of triplicate measurements at each potential. The HPLC conditions are described in the text.

Fig. 2 shows typical chromatograms of authentic GSH standard (Fig. 2A) and GSH produced after incubation with a tissue sample (Fig. 2B). Using the HPLC–ECD conditions described in Section 2, GSH was clearly detected after incubation of GSSG and NADPH with an enzyme sample prepared from rat heart (Fig. 2B).

Table 1 summarizes the results on the reproducibility of the GSH assay by this HPLC–ECD system. The inter- and intra-day precision of the GSH assay was sufficiently high; the coefficients of variation (C.V.) was 3.0 and 2.8%, respectively. In the reaction mixture without the substrate, GSSG, no detectable amount of GSH was produced after the incubation, indicating that the amount of GSH in the enzyme sample did not interfere with the assay (Fig. 2C). In the reaction mixture containing the heart sample (0.73 μg protein), GSH (185.9 pmol) was produced after an incubation time of 20 min. GSH standard (250 pmol) was spiked into the reaction

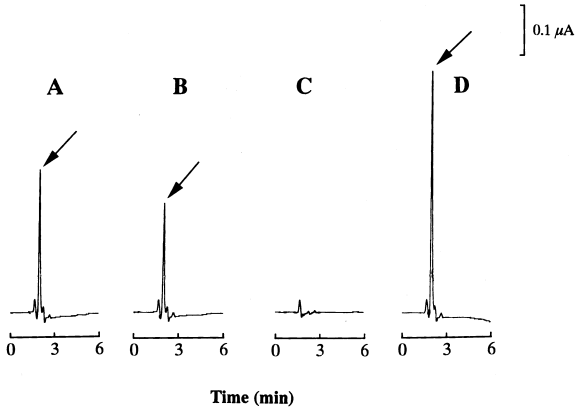

Fig. 2. HPLC chromatograms of GSH standard and the reaction mixture containing rat heart homogenates. The enzyme sample prepared from rat heart was incubated with GSSG and NADPH, and treated as described in the text, at 37°C for 20 min. (A) Standard GSH (250 pmol injected), (B) the complete reaction mixture; 185.9 pmol of GSH were produced after incubation with the enzyme sample (0.73 μg protein), (C) the reaction mixture incubated without GSSG, (D) reaction mixture (as in B) that was spiked with GSH standard (250 pmol). The amount of GSH detected was 421.1 pmol.

mixture and incubated in a similar way. The GSH recovered was estimated to be 421.1±7.2 pmol, suggesting that, under the assay conditions, quantitative recovery of GSH could be expected. The producibility for analysis of GSH spiked into heart samples was found to satisfy recommended guidelines (15%) for assay precision and accuracy [15].

The effect of the amount of protein on GSH synthesis is shown in Fig. 3. After a 20-min incubation at 37°C with the enzyme sample prepared from rat liver, GSH production was linear. The correlation coefficient obtained was 0.999.

Fig. 4 represents the relationship between GSH production and incubation time at 37°C. It shows that GSH synthesis was linear with incubation time up to

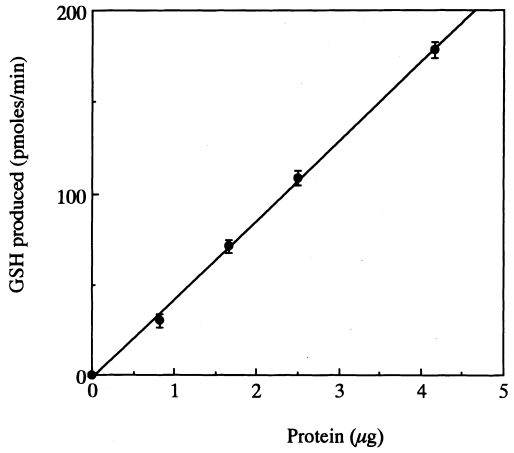

Fig. 3. Effect of the amount of protein on GSH synthesis. GSSG (1 m*M*) and NADPH (100 μ*M*) were incubated with rat liver homogenates. The point and bar represent the mean and the standard deviation of triplicate determinations.

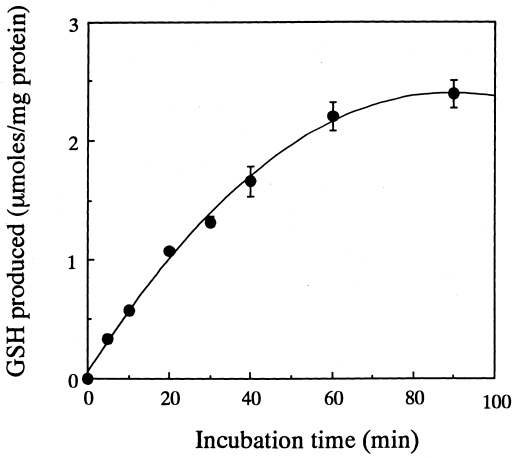

Fig. 4. Effect of incubation time on GSH production after incubation with liver homogenate. GSSG (1 m*M*) and NADPH (100 μ*M*) were incubated with liver homogenates (3.8 μg of protein) at 37°C. Each point and bar represent the mean and the standard deviation of triplicate determinations.

Table 1
Intra-day and inter-day precision of this assay for GSH

	Intra-day		Inter-day (two days)	
	Amount (pmol)	C.V.[a] (%)	Amount (pmol)	C.V.[a]
GSH standard	242.70±7.33	3.0	240.74±6.81	2.8

Values are the mean±SD of six experiments.
[a] Coefficients of variation.
The reaction mixture was spiked with standard GSH (250 pmol), mixed with perchloric acid and analyzed as described in the text.

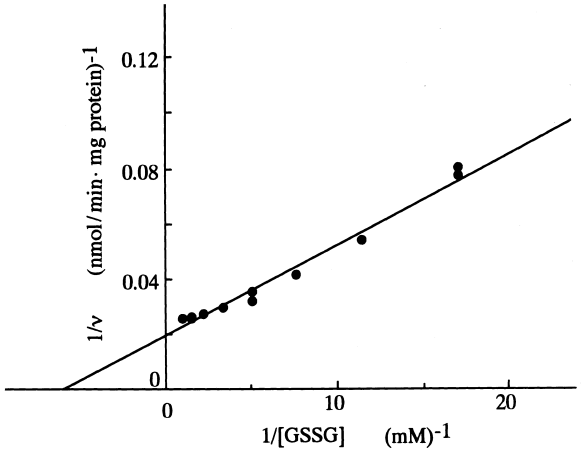

Fig. 5. Effect of the concentration of GSSG on the velocity of GSH production. The sample prepared from liver was incubated with eight different concentrations of GSSG in the presence of 100 μ*M* NADPH at 37°C for 20 min, and then treated as described in the text. The reciprocal of the reaction velocity (μmol GSH produced/min/mg protein) was plotted against that of the substrate concentration (m*M*) (Lineweaver–Burk plot). Each point represents the reaction velocity (measured in duplicate) at a definite concentration of GSSG.

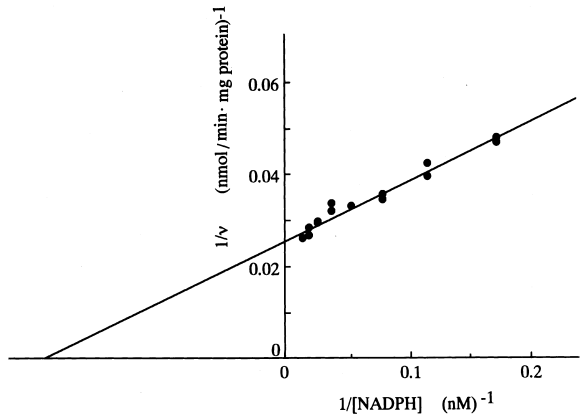

Fig. 6. Effect of the concentration of NADPH on the velocity of GSH synthesis. The sample prepared from liver was incubated with eight different concentrations of NADPH in the presence of 1 m*M* GSSG. The reciprocal of the reaction velocity (μmol GSH produced/min/mg protein) was plotted against that of the substrate concentration (n*M*) (Lineweaver–Burk plot). Each point represents the reaction velocity (measured in duplicate) at a definite concentration of NADPH.

40 min. The correlation coefficient was estimated to be 0.978.

Kinetic properties of GSH-R were studied using the liver sample. As shown in Figs. 5 and 6, the reciprocal of the reaction velocity was plotted against that of the concentration of GSSG (Fig. 5) or NADPH (Fig. 6), respectively, using a Lineweaver–Burk plot.

Table 2 summarizes the values of the Michaelis constant, K_m, and the maximal velocity, V_{max}. The K_m value of NADPH was much lower than that of GSSG, 4.73 n*M* and 164.6 μ*M*, respectively. Table 3 summarizes the specific activity of GSH-R in rat liver, heart and brain, using GSSG and NADPH at concentrations of 1 m*M* and 100 μ*M*, respectively. Activity was highest in liver, followed by the brain.

Table 3
Activity of glutathione reductase in rat tissues

Tissue	Specific activity (nmol/min/mg protein)
Liver	56.0±1.9
Heart	10.8±0.6
Brain	29.4±4.4

The specific activity was measured with 1 m*M* GSSG and 100 μ*M* NADPH.
The values represent the mean±SD of triplicate measurements in three separate experiments.

A sensitive, simple and rapid assay for determin-

Table 2
Kinetics of glutathione reductase in rat liver homogenate

	K_m value	V_{max} value (nmol/min/mg protein)
In terms of NADPH[a]	4.71±0.33 (n*M*)	37.54±7.61
In terms of GSSG[b]	165.20±13.87 (μ*M*)	50.69±2.59

[a]In the presence of 1 m*M* GSSG.
[b]In the presence of 100 μ*M* NADPH.
The values of K_m and V_{max} were obtained from a Lineweaver–Burk plot.
The values represent the mean±SD of duplicate values determined in two separate experiments.

ing the activity of GSH-R, a key enzyme in the defense system against ROS, has been attained. In contrast to previous methods, our novel method is based on the selective measurement of GSH generated from GSSG using a mercury/gold electrode. The mercury detector is very sensitive, but it is very fragile in the presence of electrochemically active contaminants in crude samples or excess amounts of the substrate (GSSG) in the assay system. The stability and reproducibility of the method were improved by using a gold electrode, model MF 1002, from Bioanalytical System. This method can reduce the oxidation voltage to 100 mV, which is much lower than the oxidation voltage used in the amperometric [16] or coulometric [12,17,18] detection systems. This low oxidation voltage increases the stability of the electrode and reduces the interference by other electrochemical contaminants.

The reaction conditions for the assay of GSH-R activity were examined and the conditions were deemed to be adequate for crude enzyme samples. The concentrations of GSSG and NADPH required for the assay were estimated to be 1 m*M* and 100 μ*M*, respectively. The amount of protein used is about 1 to 5 μg per test, and the reaction time is within 30 min at 37°C and pH 7.0. The reaction conditions are almost the same as reported previously [7]. This method was successfully applied to crude enzyme samples prepared from rat tissues and is sensitive enough to measure the activity in samples containing 1 μg of protein, whereas previous methods (measuring $NADP^+$) required more than 15 μg [8] or even 100 μg of protein per assay. By reducing the sample size, the blank value of GSH in the sample can be reduced to less than the detection limit (0.1 fmol/injection).

The K_m value for GSSG using the rat liver sample was found to be approximately 160 μ*M*, which is much lower than the GSSG level in rat liver or heart; 330±30 or 300±20 μ*M*, respectively [19]. This suggests that, in liver and heart, the GSH-R activity may regulate the intracellular level of GSH.

As a key enzyme in the regeneration of GSH as a potent antioxidant, GSH-R is now gaining increasing attention in relation to oxidative stress induced by endogenous or environmental factors. The changes in the activity of GSH-R and its possible involvement in diabetes or in aging have been reported [20,21]. On the other hand, the induction of GSH-R was reported by exposure to air pollutants [22]. Our new HPLC–ECD method will be applicable to the study of glutathione metabolism in tissues under physiological and pathological conditions.

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