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Direct assay of glutathione peroxidase activity using high-performance capillary electrophoresis

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ABSTRACT

A fast, sensitive and direct method has been developed for the determination of glutathione peroxidase activity (both selenium- and non-selenium-dependent) in cell-free preparations. The assay is based on the separation and quantitation of reduced and oxidized glutathione by capillary electrophoresis. The electrophoretic separation buffer was 100 m*M* sodium tetraborate (pH 8.2) containing 100 m*M* sodium dodecylsulphate. A micellar electrokinetic mechanism took place under these conditions, and a total mass recovery was observed for both peptides. The reproducibility of migration times was excellent (less than 3% variability). A linear detector response range was observed in the range 5–50 U/ml, and both the reproducibility and accuracy were satisfied. Samples out of this linear range could be analysed by either increasing the reaction time or diluting the enzyme preparation. The results obtained with the new direct capillary electrophoresis assay were compared with those derived from a reversed phase high-performance liquid chromatographic and spectrophotometric coupled assay. A very good agreement was found between the two direct assay methods in all samples. Capillary electrophoresis is a versatile technique that allows the automation of the glutathione peroxidase assay in a reproducible manner and within a relatively short time with sufficient accuracy and precision.

INTRODUCTION

Glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9, GSHPx) was first reported by Mills in 1957 [1], and catalyses the reduction of hydroperoxides by reduced glutathione. Based on their substrate specificity, two types of GSHPx are generally recognized: selenium-dependent [2] and selenium-independent [3] enzymes. The selenium-independent activity is attributed to an isoenzyme of glutathione-S-transferase acting on a variety of organic hydroperoxides but not on hydrogen peroxide [4], at difference with the selenium-dependent enzyme, active with both peroxides [5]. Both types are strictly specific for reduced glutathione (GSH) as the electron donor [6,7]. The enzyme plays an important role in the prevention of the deleterious effects of peroxides generated in the course of tissue metabolism [8].

The most commonly used assay for GSHPx is the photometric coupled method with glutathione reductase and NADPH, described by Paglia and Valentine [9]. Several modifications of this assay have been reported [10,11]. Other methods have been proposed measuring GSH consumption either directly [12] or indirectly with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [13]. None of these assays is useful for kinetic studies, since NADPH is an inhibitor of glutathione reductase [14] and the DTNB reaction is tedious

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[15]. A semi-automated method using DTNB to monitor GSH consumption [15] and a fully automated continuous-flow procedure using 2,6-dichloroindophenol (DCIP) to monitor the oxidation of GSH [16] have been reported to assay the GSHPx activity in a large number of samples. The use of high-performance liquid chromatography (HPLC) in enzymic analysis has been reviewed in detail [17]. This technique offers the potential to monitor several components simultaneously during the course of the reaction, making it a suitable technique for kinetic studies. A direct micro-assay for GSHPx based on the separation and quantitation of oxidized glutathione (GSSG) by reversed-phase HPLC was reported by Xia *et al.* [18].

Capillary electrophoresis (CE) [19,20] has become an important separation technique that combines the resolution power of conventional electrophoresis with the advances in instrument of modern HPLC. The achievements of CE make it "the most successful method of the last decade" [21]. The two most popular techniques in CE are capillary zone electrophoresis (CZE) or free solution electrophoresis (FSE) [22] and micellar electrokinetic capillary chromatography (MECC) [23]. In its different modalities, CE is proving to be of great utility in the separation of small biomolecules, such as peptides [24,25].

As far as we know, applications of CE to enzymic analysis have not been reported. In this paper we describe a fast and sensitive direct method for the determination of GSHPx activity in cell-free preparations, based on the separation and quantitation of reduced and oxidized glutathione.

EXPERIMENTAL

Chemicals

Glutathione peroxidase from ox erythrocytes (EC 1.11.1.9), disodium EDTA, reduced glutathione (GSH), glutathione disulphide (GSSG), sodium dodecylsulphate (SDS), phenylmethylsulphonyl fluoride (PMSF) and dithiothreitol (DTT) were from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and salts for the mobile phases were from E. Merck (Darmstadt, Germany). All other chemicals used were of analytical-reagent grade.

Equipment

HPLC was performed using a liquid chromatograph (Beckman, San Ramón, CA, USA) equipped with a programmable solvent module 126, a diode-array detector 168 and an Altex-Beckman 210A injection valve with a 20-ml injection loop. CE was carried out on the P/ACE System 2000 from Beckman, which includes a UV filter detector, an automatic injector and a capillary cartridge surrounded by coolant. Both systems were controlled by a Compaq Deskpro 386/20e computer (Houston TX, USA) fitted with Beckman's System Gold 6.0 software. Centrifugations were carried out on an L8-80M Beckman ultracentrifuge. High-purity water was obtained form a Milli-Q water purifier (Millipore, Milford, MA, USA). The solvents for HPLC and P/ACE were degassed under vacuum in an ultrasonic bath and filtered through 0.2-μm membrane filters. Spectrophotometric measurements and enzyme kinetics were carried out in a Beckman DU-62 spectrophotometer. An IKA Ultraturrax T-25 homogenizer (Janke & Kunkel, Staufen, Germany) was used for the preparation of fish-liver extracts.

Capillary electrophoresis conditions

The capillary cassettes used were fitted with either 57.5 mm × 75 μm I.D. or 27.5 cm × 50 μm I.D. uncoated fused-silica capillary columns from Beckman, 50 and 20 cm from the detector, respectively. Fresh capillaries were hydrated by flushing under pressure with 1.0 *M* NaOH for 15 min, followed by rinsing with water (5 min), 0.1 *M* HCl (5 min), and finally the running buffer (5 min). During electrophoresis the detection windows were located in the cathode side. The running buffer was 100 m*M* sodium tetraborate (pH 8.2)–100 m*M* SDS. Runs were performed at 16 kV (130 μA) and 8 kV (45 μA) for the 50-cm and 20-cm capillaries, respectively. All experiments were carried out at 30°C, and injections were performed in triplicate in the high-pressure mode for

1–3 s. To ensure reproducible migration times, the capillary was washed after three or four runs with water for 1 min followed by the running buffer for 2 min. Solutes were monitored at 214 nm. Buffer solutions were degassed and filtered before use to avoid capillary clogging.

HPLC conditions

A reversed-phase C_{18} Ultrasphere column (25 cm × 0.46 cm I.D.; particle size 5 μm) from Beckman was used to perform the GSHPx activity assay. The procedure was a modification of that previously described by Xia *et al.* [18]. Elution was carried out under isocratic conditions using methanol–water (15:85) as mobile phase. The observed elution times for GSH and GSSG were 4.9. and 5.6 min, respectively. UV detection was performed at 214 nm.

Enzymic assay procedures

Direct assays. All analyses were carried out at 25°C. The total GSHPx activity was determined with the following assay mixture: 50 m*M* sodium phosphate buffer (pH 7.2) containing 0.5 m*M* EDTA (assay buffer), 2 m*M* GSH, 0.5 m*M* organic hydroperoxide (cumene or *tert.*-butyl), 3.0 m*M* sodium cyanide, 5–50 μl of enzyme preparation, and water up to 500 μl final volume. The reaction was started by the addition of the hydroperoxide solution and stopped by the addition of 500 μl of 0.5% TFA solution. To assay the selenium-dependent activity, organic hydroperoxide was substituted by 0.150 m*M* hydrogen peroxide and 1 m*M* sodium azide. Unless otherwise stated, samples were incubated for 2 min before the reaction was stopped, and aliquots were taken for injection in the P/ACE and HPLC systems.

One glutathione peroxidase unit is defined as the enzyme amount that catalyses the formation of 1.0 μmol of glutathione disulphide per minute under the experimental conditions described above.

Coupled assays. The procedure followed was that previously described by Flohé *et al.* [7] using 1 m*M* GSH anmd 2 m*M* cumene hydroperoxide or 0.15 m*M* hydrogen peroxide. One unit of activity is defined as the amount of enzyme that causes the oxidation of 1 μmol of NADPH (equivalent to the formation of 1.0 μmol of oxidized glutathione) per minute.

Sample and standard preparations

Calf hemolysate. Cells from freshly drawn arterial blood were collected by centrifugation and lysed by osmotic shock with 5 m*M* sodium phosphate buffer (pH 7.0) containing 1 m*M* EDTA and 2 m*M* DTT (2 ml per ml of packed cells). Cellular debris were removed by centrifugation at 48 400 *g* for 10 min, and the supernatant was again centrifuged at 184 000 *g* for 1.5 h. The protein content was 14.93 mg/ml and the GSHPx specific activities found by the coupled assay were 0.79 and 0.63 U/mg with cumene and hydrogen peroxide, respectively.

Fish liver. Frozen *Mugil sp.* liver was ground in liquid nitrogen. The powder was washed twice with 0.96% sodium chloride solution to eliminate blood traces, and homogenized with 10 m*M* Tris–HCl buffer (pH 7.5) containing 1 m*M* EDTA, 5 m*M* GSH and 2 m*M* DTT (4 ml of buffer per gram of tissue). To avoid protease activity, 0.5 mg of PMSF per gram of tissue was added. The preparation was ultracentrifuged at 105 000 *g* for 1.8 h, the lipid layer was decanted, and the supernatant was used to assay enzyme activity. The extract contained 16.23 mg of protein per ml and 0.099 (cumene hydroperoxide) and 0.060 (hydrogen peroxide) U/mg of GSHPx, by the manual coupled assay.

Rat liver. Six livers obtained from freshly slaughtered male rats were removed and placed independently in a 0.96% sodium chloride solution to eliminate blood. Livers were then minced and homogenized in 10 m*M* Tris–HCl buffer (pH 7.5), containing 5 m*M* GSH, 2 m*M* DTT and 1 m*M* EDTA (4 ml/g of tissue). To inhibit protease activity, 0.5 mg of PMSF per gram of tissue was added. The cytosolic fraction was obtained by ultracentrifugation as described above for the fish liver extracts. The extract contained 18.76 mg/ml protein. The specific activities found by the coupled assay were 1.28 and 0.75 U/mg (cumene and hydrogen peroxide, respectively).

Preparation of standards. The standard stock

solutions, 20 m*M* GSH, 3 m*M* hydrogen peroxide and 10 m*M* *tert.*-butyl hydroperoxide, were prepared in cold distilled water. The standard 10 m*M* cumene hydroperoxide solution was prepared in 96% ethanol–water. All these solutions were prepared freshly each day and maintained in an ice-bath until used. The standard stock solution of commercial GSHPx from bovine erythrocytes was prepared at a nominal concentration of 0.1 mg/ml in assay buffer and contained 302.3 (with cumene hydroperoxide) and 520 (with hydrogen peroxide) U of GSHPx per mg of protein as determined by the conventional spectrophotometric coupled assay. All biological samples were stored in 1.5-ml Eppendorf tubes at −70°C and thawed at 4°C before the enzyme activity was assayed. No activity losses were detected in relation to the freshly prepared extracts. The assays were performed with dilutions (1:20 to 1:100) of enzyme preparations in assay buffer. Protein concentrations were estimated by the method described by Lowry *et al.* [26], using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The CE assay for GSHPx described here is based on the rapid determination of GSSG, produced by the reaction in the presence of other components of the assay mixture, particularly GSH, one of the substrates. In consequence, our first aim was to devise a protocol for the optimal separation of GSH and GSSG.

Adsorption of positively charged species onto the walls of fused-silica capillaries is a problem in CE. Resolution of basic peptides can be carried out at low pH values because silanol groups in capillary walls are protonated and thus unavailable for ionic interactions. However, separation of acidic peptides, such as GSH and GSSG, can be performed at any pH value above the isoelectric point because of the repulsion between the negatively charged peptide and the silanols of the capillary walls [27]. The influence of the pH and ionic strength on electroosmotic mobility in fused-silica capillaries has been reported [28–30]. It is generally accepted that the electroosmotic flow increases rapidly with pH up to pH 7–8. At higher pH values the mobility increases more slowly.

Other factors that affect the mobility, resolution and selectivity in CE are the applied voltage, the type of buffer, buffer additives and the electroosmotic flow [27–30]. We have studied the influence of these variables on the resolution of GSH and GSSG. Migration times observed using a 50 m*M* phosphoric acid solution (pH 2.5) as separation buffer and 20 kV (210 μA) and 30°C as running conditions for electrophoresis were longer than 20 min. Baseline instability and partial adsorption on the capillary walls were also observed under such conditions. In addition, it was necessary to regenerate the capillary by washing with 0.5 *M* NaOH (1 min), water (1 min), and separation buffer (2 min), after each run. At pH values above the isoelectric points, electroosmotic flow took place, and migration times observed using 50 m*M* sodium phosphate buffer (pH 7.5) as separation buffer were shorter (less than 12 min, using a 50-cm capillary). At both pH values, the mass recovery and reproducibility in migration times were unsatisfactory (results not shown).

Terabe *et al.* [23] have demonstrated that higher selectivity can be achieved using an ionic surfactant in the micellar electrokinetic capillary mode. Micelles can act as a pseudophase at or above the critical micelle concentration in the separation buffer. Although this mode of CE was developed as a means for separating electrically neutral solutes, it also provides enhanced selectivity in the separation of ionic species. The mechanism of the process implies charge and solute partitioning into detergent micelles. MECC also offers advantages over CZE for the separation of peptides in terms of improved selectivity and peak shape. The migration time is a function of three factors: (a) electrophoretic mobility; (b) distribution between the micellar phase and buffer; and (c) electroosmotic flow, which is clearly necessary to achieve shorter migration times [27].

The resolution of GSH and GSSG was improved using 100 m*M* sodium tetraborate buffer (pH 8.2) containing 100 m*M* SDS. Under these

conditions a micellar electrokinetic mechanism took place and a total mass recovery was observed for both peptides. Reproducibility of migration times was excellent, with less than 3% variability. Hence these conditions were adopted for the separation and quantitation of GSH and GSSG. The electropherogram shown in Fig. 1. corresponds to the direct assay of GSHPx, using hydrogen peroxide as the oxidant and calf hemolysate as the enzyme source. The migration times observed for GSH and GSSG were 6.0 and 6.5 min, respectively, using the 50-cm capillary column. For routine analysis a 20-cm capillary was used, providing excellent resolution with shorter migration times, 3.1 and 3.4 min for GSH and GSSG respectively, thus speeding up the process.

A standard curve for GSSG was obtained by plotting the peak areas expressed as arbitrary units *versus* the corresponding concentrations. Samples were prepared using the conditions described in Experimental for the direct assay of GSHPx activity. Each point plotted was the average of three injections of the same sample in the P/ACE apparatus. A linear regression analysis yielded a correlation coefficient of 0.999 for up to 2 m*M* GSSG in the assay mixture.

Peak areas of GSSG were used to calculate the GSHPx activity. Samples without enzyme were injected as a control to eliminate any interference from impurities and to follow the rate of the non-enzymic reaction. The activity was calculated from the following equation:

$$\text{activity (U/ml)} = \frac{(A - A') \cdot 10^3}{BCt}$$

where A is the peak area of the enzymic reaction, A' is the peak area of the control (reaction without enzyme), B is the area corresponding to 1.0 μmol of GSSG in the assay mixture, C is the volume (μl) of enzyme preparation added to the assay mixture, and t is the reaction time in minutes (usually 2).

The effect of the reaction time on the rate of GSSG formation is shown in Fig. 2. A good linearity was observed for times less than 4 min. Each point is the average of four determinations. Similar results were obtained with other enzyme sources tested. No significant progress of the non-enzymic reaction (chemical blank) was observed before 2 min, and therefore this was the reaction time selected for the standard direct assay procedure. Fig. 3 shows the relationship between GSHPx activity and the amount of calf hemolysate enzyme present in the assay mixture. A linear dependence was observed both with hydrogen peroxide and cumene hydroperoxide.

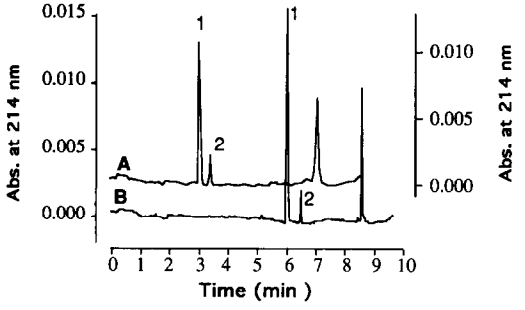

Fig. 1. Capillary electrophoresis migration pattern of GSH and GSSG. (A) Sample, 5 μl of rat liver extract in the assay mixture; oxidant, cumene hydroperoxide; reaction time, 3 min; capillary, 20 cm × 50 cm I.D.; voltage, 8 kV (45 μA). (B) Sample, an assay mixture containing 3 μl of calf hemolysate and hydrogen peroxide as oxidant; reaction time, 2 min; capillary, 50 cm × 75 μm I.D.; voltage, 16 kV (130 μA). For both cases: injection time, 3 s under the pressure mode; separation buffer, 100 m*M* sodium tetraborate (pH 8.2) and 100 m*M* SDS; temperature, 30°C. Peaks: 1 = GSH; 2 = GSSG.

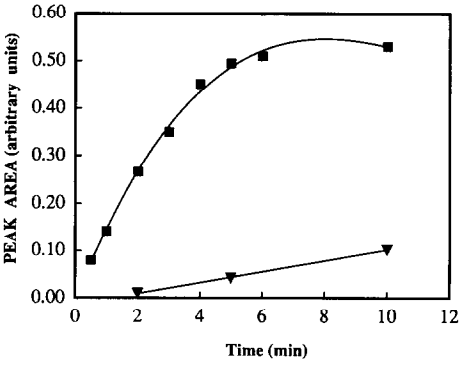

Fig. 2. Time-course of the GSHPx direct assay. Sample, 10 μl of calf hemolysate in 1.0 ml of assay mixture with 500 μ*M* cumene hydroperoxide as oxidant. Aliquots (50 μl) were drawn at the times indicated and mixed with 50 μl of 0.5% TFA solution. Other experimental conditions as in Fig. 1. (■) Enzymic reaction; (▼) non-enzymic reaction (chemical blank).

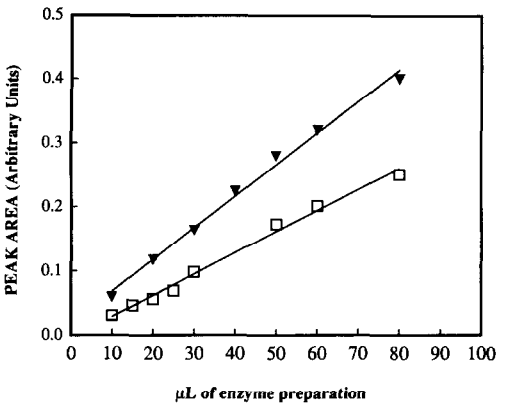

Fig. 3. Effect of enzyme amount on the reaction rate. Samples were prepared as described in Experimental for the direct assay, and contained different volumes of a 1:20 dilution of calf hemolysate in assay buffer. Experimental conditions as in Fig. 1. (▼) 500 μM cumene hydroperoxide; (□) 100 μM H_2O_2.

The conditions for the direct assay described in Experimental were selected by a trial-and-error analysis. The linearity of the detector response for a certain enzyme concentration had to be assured. Hence if the GSSG peak area was over 25% of the GSH initially present in the assay mixture, the reaction had gone too far and the linear range had been surpassed. In such cases it was necessary either to decrease the reaction time or to dilute the enzyme preparation. Using the standard conditions described for the direct assay, the linear detector response range was 5–50 U/ml and both the reproducibility and accuracy were satisfactory.

The results obtained with different enzyme sources are shown in Table I. The data are the average of four different samples of each enzyme source, injected three times in the CE and HPLC systems. Excellent agreement was found between the results obtained by the two direct assay methods (CE and HPLC) in all samples. The lowest R.S.D. values correspond in all cases to the CE assay. The photometric coupled assay yielded results numerically different, although related to the direct assays, ranging between 82 and 124% of the results obtained by CE and with the high-

TABLE I

RESULTS OBTAINED FOR GSHPx ACTIVITY IN CELL-FREE EXTRACTS

Enzyme source	GSHPx activity (U/ml)							
	CE			HPLC			Coupled assay	
	Se[a]		Total[b]	Se		Total	Se	Total
Ox erythrocytes[c]	32.85		22.06	33.80		21.98	40.83	23.73
R.S.D.[d] (%)	5.09		4.93	5.45		5.09	7.53	5.69
R[e]		0.67			0.65			0.58
Calf hemolysate[f]	10.35		13.05	10.58		12.93	9.50	11.89
R.S.D. (%)	3.30		4.11	3.20		5.01	6.60	5.52
R		1.26			1.22			1.25
Rat liver[f]	15.58		20.74	15.65		20.15	14.23	24.16
R.S.D.(%)	2.50		4.93	2.60		6.13	5.80	4.70
R		1.33			1.29			1.69
Fish liver[f]	1.18		1.66	1.12		1.53	0.98	1.61
R.S.D. (%)	2.49		2.15	4.10		3.12	7.70	5.60
R		1.41			1.34			1.64

[a] Selenium-dependent activity (with hydrogen peroxide).
[b] Activity with cumene hydroperoxide.
[c] Pure commercially available GSHPx.
[d] Relative standard deviation = (S.D./mean) × 100%.
[e] Ratio between the activities Total/Se.
[f] Cell-free extracts.

est R.S.D. of the assay methods compared. The GSHPx activity was determined both with hydrogen peroxide which is highly selective for the selenium-dependent enzyme, and with cumene hydroperoxide, which is active with both the selenium- and non-selenium-dependent enzymes and, thus, measuring "total" glutathione peroxidase activity. As expected, the proportion of selenium-dependent activity was higher in the commercial preparation from bovine erythrocyte, since this type of cell does not contain the selenium-independent GSHPx species [31]. However, the cell-free extracts analysed showed higher activities with cumene hydroperoxide as oxidant. The differences between the results obtained by the direct methods and the coupled assay could be due to the different experimental conditions used in this last case. In addition, the use of a coupling enzyme further complicates this assay. The situation is aggravated because GSHPx cannot be simultaneously saturated by GSH and peroxide [32], thus making this activity highly dependent on substrate concentrations and rendering interlaboratory comparisons almost impossible.

CONCLUSION

CE is a versatile technique that has the potential for automating the rapid assay of GSHPx activity in a reproducible manner and within a relatively short time. The direct CE assay described in this paper is cheaper and less labour-intensive than the HPLC and coupled assays, with improved accuracy and precision. Using the experimental conditions described for the 20-cm capillary column, it is possible to run up to twelve samples per hour in an automatic mode, as opposed to eight with HPLC and three to five with the photometric coupled assay.

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