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Development and Application of Organic Reagents for Analysis. IX.[1] A Sensitive Fluorometric Method for the Determination of Glutathione Peroxidase Activity

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A fluorometric method for the determination of rat liver glutathione peroxidase (GSH-Px) activity has been developed, based on the determination of the concentration of unreacted reduced glutathione in the assay mixture with *N*-{*p*-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide as a fluorogenic reagent. This method is very sensitive and can assay GSH-Px activity in 50 μg wet weight of rat liver. A linear relationship was obtained between GSH-Px activity and amount of sample over the range of 10—100 μg protein. GSH-Px activities obtained by this method correlated well with those obtained by the conventional colorimetric method with 5,5′-dithiobis(2-nitrobenzoic acid).

Keywords——glutathione peroxidase; reduced glutathione; fluorometric assay; *N*-{*p*-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide; rat liver; *tert*-butylhydroperoxide

Glutathione peroxidase (EC 1.11.1.9, GSH-Px), a selenium-containing enzyme, has been found in various mammalian tissues.[2–7] It has been demonstrated that GSH-Px can catalyze the reduction of many organic hydroperoxides as well as hydrogen peroxide, and that it may protect cell membranes against oxidative damage.[8–12]

Several assay methods for the determination of GSH-Px have been developed.[2b,13–17] The most commonly used method is the glutathione-reductase–NADPH coupled assay or some modification of this assay.[16,17] GSH-Px activity has also been assayed by measurement of reduced glutathione (GSH) with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[13,14] Except for a few instances,[15,17] these methods are usually tedious and insensitive.

Recently, we have reported a highly sensitive fluorometric method for the determination of GSH with *N*-{*p*-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide (DBPM) as a fluorogenic reagent.[1,18] The method permits the determination of as little as 2 pmol/ml of GSH. In this paper, a sensitive and simple method for the assay of GSH-Px activity based on the same fluorogenic reaction is described.

Experimental

Apparatus——Fluorescence intensities were measured with a Shimadzu RF-540 spectrofluorometer using quartz cells of 10 mm path length. A Toa HM-7B pH meter with a saturated calomel-glass electrode system was used for all pH measurements.

Materials——GSH, bovine erythrocyte GSH-Px, and *tert*-butylhydroperoxide (*tert*-BuOOH) were purchased from Nakarai Chemicals Co., Ltd., Kyoto (Japan). DTNB and Folin-Ciocalteu reagent were obtained from Wako Pure Chemical Ind., Ltd., Osaka (Japan). DBPM was prepared as described previously[19] and used as a 0.4 mM CH_3CN solution.[1] All other chemicals used were of analytical grade.

Animals——Male Wistar rats were used (8 weeks old). The animals were anesthetized with ether, then the livers

were removed and perfused with 0.9% (w/v) NaCl solution. The livers were homogenized in 20 mM ethylenediaminetetraacetic acid (EDTA) solution with a Teflon-glass Potter-Elvehjem homogenizer. The homogenate concentration was adjusted 1% (w/v) for the present method and 10% (w/v) for the DTNB method. Rat liver protein was determined by Lowry's method[20] using bovine serum albumin (fraction V, Sigma Chemical Co., Ltd., St. Louis, U.S.A.) as a standard.

Standard Procedure——The substrate mixture was prepared by adding 1 ml each of 0.8 mM GSH in 5 mM EDTA and 10 mM sodium azide to 20 ml of 0.1 M phosphate buffer (pH 8.5). The substrate mixture (2.2 ml) was added to 0.1 ml of rat liver homogenate ($\leqq 100\,\mu$g of protein) and the mixture was preincubated at 37 °C for 5 min. Then 0.2 ml of 0.4 mM *tert*-BuOOH was added and the mixture was incubated at 37 °C for 5 min. Immediately, CH_3CN (2 ml) and then 0.4 mM DBPM solution (0.5 ml) were added, and the mixture was heated at 60 °C for 30 min. The fluorescence intensity was measured at 457 nm with excitation at 355 nm to determine the concentration of GSH remaining in the assay mixture. For the blank, water (0.2 ml) was used instead of the indicated volume of *tert*-BuOOH solution.

A standard curve was obtained according to the standard procedure by using substrate mixture containing a known concentration of GSH (10—100 nmol), except that the liver homogenate was replaced by water and that the incubation was omitted.

GSH-Px activity was expressed as μmol of GSH destroyed per min at 37 °C and pH 8.5.

Results and Discussion

The reaction mixture of GSH and DBPM showed a fluorescence maximum at 457 nm with an excitation maximum at 355 nm. The standard curve against the reagent blank was linear up to at least 100 nmol of GSH and passed through the origin.

Mills[2b] reported that GSH-Px is most active at pH 8 or above with GSH as a substrate, and Flohé *et al.*[21] found the optimum pH to be 8.8. In the previous paper,[1] we showed that the optimum pH for the reaction of GSH with DBPM is 8.5. Thus, no further investigation of the pH dependency of the enzymatic reaction was made and 0.1 M phosphate buffer (pH 8.5) was used for the procedure. Sodium azide (1 mM) was included in the assay mixture to inhibit catalase activity.[16]

Substrate concentration effects on GSH-Px activity were examined. *tert*-BuOOH gave a maximum and constant activity in the concentration range of 25—40 μM with rat homogenate GSH-Px and 25—40 μM with bovine erythrocyte GSH-Px (Fig. 1), with apparent K_m values of 9.1 and 6.0 μM, respectively; 32 μM *tert*-BuOOH was used in the standard procedure.

The GSH-Px activity (ΔGSH) *vs.* GSH concentration plots gave a sigmoid curve for rat liver homogenate GSH-Px and a straight line for bovine erythrocyte GSH-Px (Fig. 2).

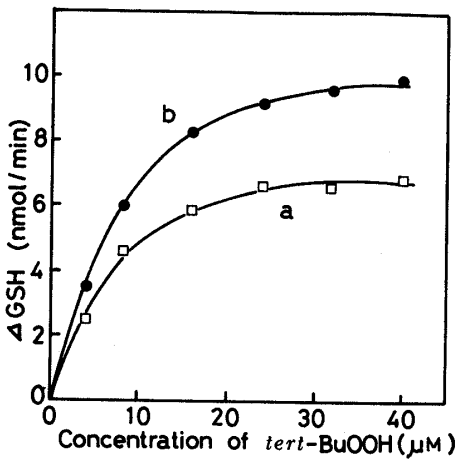

Fig. 1. Effect of *tert*-BuOOH Concentration on GSH-Px Activity

GSH, 32 μM; incubation time, 5 min at 37 °C. Mean values of triplicate determinations are shown. a) rat liver homogenate (0.5%), 100 μl; b) bovine erythrocyte GSH-Px (20 μg/ml), 100 μl.

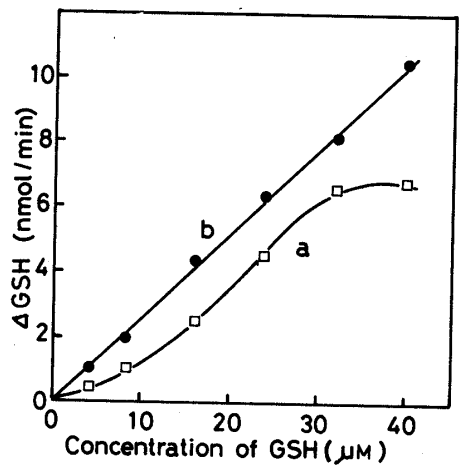

Fig. 2. Effect of GSH Concentration on GSH-Px Activity

tert-BuOOH, 32 μM; incubation time, 5 min at 37 °C. Mean values of triplicate determinations are shown. a) rat liver homogenate (0.5%), 100 μl; b) bovine erythrocyte GSH-Px (20 μg/ml), 100 μl.

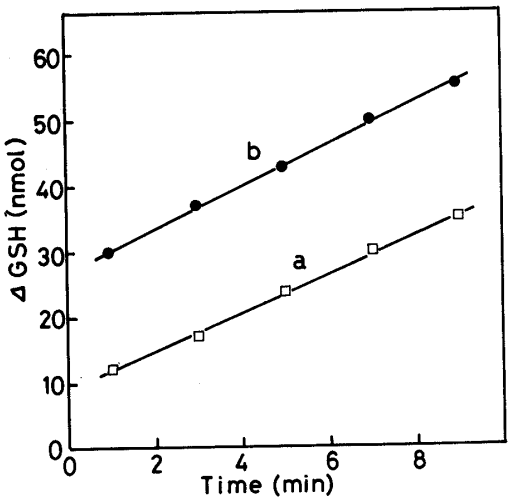

Fig. 3. Effect of Incubation Temperature and Time on GSH-Px Activity

GSH, 32 μM; *tert*-BuOOH, 32 μM. A 50 μl aliquot of rat liver homogenate (5%) was used. a) 37 °C; b) 25 °C. Mean values of triplicate determinations are shown.

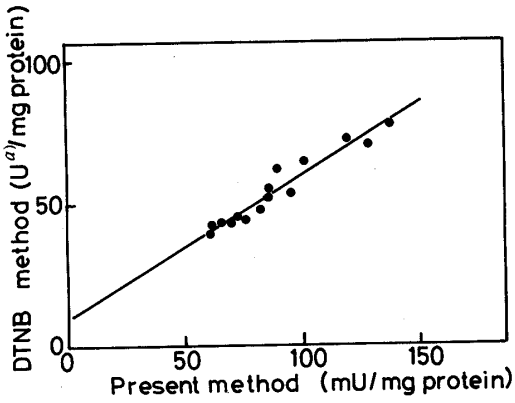

Fig. 4. Correlation between the Results of the Present and DTNB Methods

a) One unit of activity was defined as that amount causing a decrease in the log[GSH] of 0.001 per min after substraction of the decrease in log[GSH] per min due to nonenzymatic reaction.[13]

Therefore, K_m values for GSH could not be obtained by means of the Lineweaver-Burk plot. Though it was not always an optimum concentration, 32 μM GSH was used in the assay procedure.

The enzyme activity, expressed as ΔGSH, increased linearly with time for at least the initial 9 min of incubation with rat liver homogenate both at 25 °C and at 37 °C (Fig. 3). A similar result was obtained with bovine erythrocyte GSH-Px; an incubation time of 5 min at 37 °C was selected in the standard procedure.

The relationship between protein content of rat liver homogenate and GSH-Px activity was examined. A good linear relation in the range from 10 to 100 μg of protein (50 to 500 μg wet weight of rat liver) was obtained. The within-day precision of the present method was examined. The relative standard deviation was 3.9% ($n=10$) for rat liver homogenate with a mean activity of 130.7 mU/mg protein. In a comparison with the DTNB method,[13] using normal rat livers (Fig. 4), the correlation coefficient was 0.95 ($n=15$) and the regression equation for the present method (x) against the DTNB method (y) was $y=0.48x+11.4$.

The sensitivity of the proposed method is quite high and GSH-Px activity can be assayed in as little as 50 μg of tissue. This is comparable to the enzymatic method[17] based on fluorometric measurement of NADPH consumption. On the other hand, the DTNB method is insensitive and requires 100 times greater sample quantity than the present method. As the enzymatic and fluorogenic reactions can be done *in situ*, our method is simpler than the DTNB method. Further, the assay can be completed within an hour, so that many samples can be successively analyzed. The method should be useful for biological studies involving GSH-Px.

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