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GLUTATHIONE PEROXIDASE ACTIVITY OF D,L-SELENOCYSTINE AND SELENOCYSTAMINE

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SUMMARY: The activity of glutathione peroxidase (EC 1.11.1.9) was found for D,L-selenocystine and selenocystamine at the concentration of 10^{-5}M Se. The reaction depends upon the pH and the optimum pH's were 8.0 for D,L-selenocystine and 9.0 for selenocystamine. Based upon the chromatographic separation of the intermediate seleno-sulfur (-S-Se-) compound, the mechanism was discussed in relation to the selenoenzyme, glutathione peroxidase.

Glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) catalyzes the reaction between hydroperoxides and reduced glutathione (GSH) in mammalian tissues (1,2). This enzyme plays an important role in preventing the lipid layer of the cell membrane from peroxidation (3). Mammalian glutathione peroxidases were found to contain selenium (4), and the chemical form of selenium at the catalytic site in the reduced rat liver glutathione peroxidase was identified as selenocysteine (5,6). The selenium in the enzyme is reported to be the only atom that undergoes oxidation-reduction (6).

It is well known that some selenium compounds catalytically decompose hydroperoxides and oxidize thiols to disulfides (7,8). In relation to the reaction of selenium in glutathione peroxidase (GSH-Px), this paper examines the glutathione peroxidase activity of D,L-selenocystine and selenocystamine.

MATERIALS AND METHODS

Materials: D,L-Selenocystine, selenocystamine, reduced and oxidized glutathione were purchased from Sigma Chemical Co. Glutathione reductase (Sigma G 4751) was dialyzed against sodium phosphate buffer (0.01M, pH7.0) prior to use. Sodium selenite was from Wako Pure Chemical Co. An amino acids' mixture was from Ajinomoto Co.Ltd.

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Apparatus: A Shimadzu MPS-5000 UV-VIS spectrophotometer was used for the enzyme assay. A Thomas Scientific TRL-111 SP thermostatic circulator was used to control the assay temperature.
Ion-exchange chromatography: An automatic amino acid analyzer (Sibata AA600) with a 0.25 cm X 100 cm Aminex A-4 (Bio-Rad Lab.) column at 58 ± 0.5 °C was used. For elution, two 0.1 M citrate buffers of pH 3.25 and 4.25 were pumped successively at the rate of 0.3 ml/min. The buffers were changed at 100 min. The effluent from the column was mixed with ninhydrin, and absorbances at 570 nm and 440 nm were recorded.
Thin layer chromatography: Precoated cellulose thin layer plates (E.Merck, Art 5577) were used. The solvent system consisted of acetic acid, n-butanol, water, phenol and acetone (V:V:V:W:V=9.2:16.8:26.0:4.0:2.5). Spots were visualized with a ninhydrin reagent (Tokyo Kasei Co.).
Preparation of samples for chromatography: Reduced glutathione (GSH) was dissolved in 0.75 mM D,L-selenocystine or selenocystamine at the concentration of 1.5 mM. Then, after 5-6 hrs the pH of the mixture was adjusted to about 9.0 with dilute NaOH.
Enzyme assay: The GSH-Px activity was measured by a coupled assay originally developed by Paglia and Valentine (9) with some modifications. In the standard assay, the final concentrations of the reagents were: 0.1 M Tris-HCl (pH 8.0), 2.0 mM GSH, 0.2 mM NADPH, 0.5 mM EDTA and glutathione reductase 1 U/ml. The final volume was 3.0 ml. The assay temperature was 37 °C. The reaction was initiated by adding hydroperoxides, and the activity was measured by the decrease of the absorbance at 340 nm. The molar absorption coefficient of NADPH, 6,220 $M^{-1}cm^{-1}$, was adopted.
Reduction of diselenides to selenol: The diselenide bond (-Se-Se-) was cleaved by the addition of $NaBH_4$ under alkaline conditions. Since selenol (-SeH) is very labile to oxidation, nitrogen gas (oxygen-free) was continuously passed through the solution in order to maintain the reduced state.

RESULTS AND DISCUSSION

The GSH-Px activity of the selenium compounds was observed in the magnitude of 10^{-5}M Se (Fig.1). The activity increased in proportion to the selenium concentration. This indicates that the activity was clearly associated with the presence of selenium. The GSH-Px activity at the same selenium concentration was found to decrease in the order of selenite, selenocystamine and D,L-selenocystine. The reduced forms of the latter two, D,L-selenocystine and selenocystamine, showed the same activity as the oxidized forms of each compound, probably because selenol was oxidized in the assay system and the diselenide compound produced exhibited the activity.

The GSH-Px activity of diselenides varied markedly with pH as presented in Fig.2 . The net activity was very low under acidic conditions (negligible at pH's lower than 6.0) and the apparent optimum pH of the activity of D,L-selenocystine and selenocystamine was at pH 8.0 and 9.0, respectively. The blank reaction, which is the direct oxidation of GSH with hydroperoxides, in-

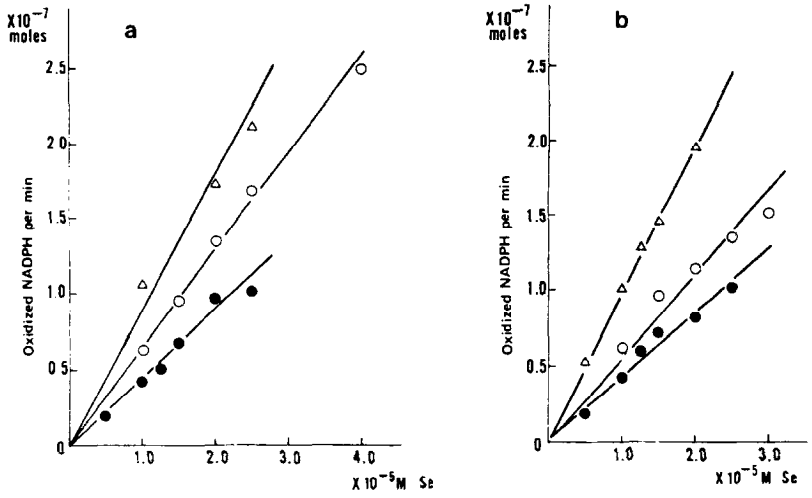

Fig.1 Glutathione peroxidase activity vs. selenium concentration. a:0.7 mM t-butyl hydroperoxide as a substrate, b:0.17 mM H_2O_2 as a substrate. Experimental conditions are: GSH;2.0 mM, NADPH;0.2mM, pH;8.0, temperature;37°C. Symbols are: ● - ● ; D,L-Se-cystine, ○ - ○ ; Se-cystamine, △ - △ ; sodium selenite.

creased as the pH increased, however, no optimum was observed over the pH range investigated. The increased net activity over the pH range 6.5-7.5 may be due to the thiol-diselenide interchange reaction as discussed later. The reason for the decrease of the net activity above pH 9.0 is uncertain, but glutathione

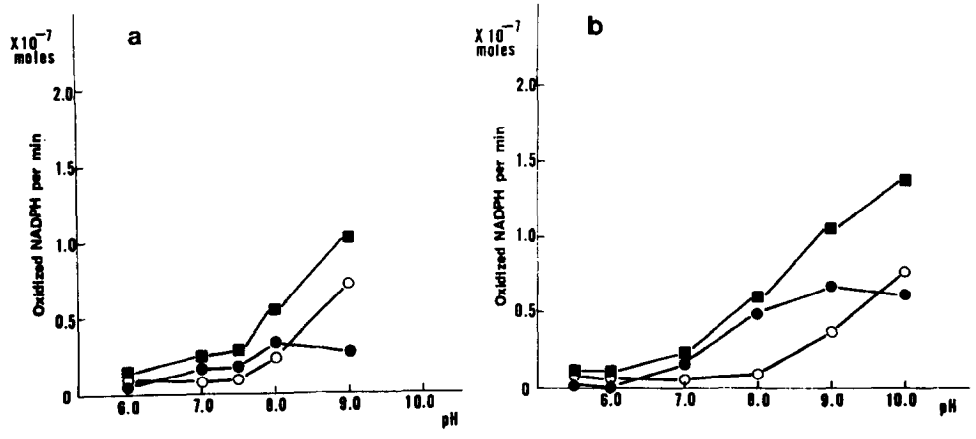

Fig.2 pH dependence of glutathione peroxidase activity of diselenide compounds.
a:D,L-Se-cystine;1.0 x 10^{-5}M, b:Se-cystamine;1.0 x 10^{-5}M. Experimental conditions are the same as in Fig.1 except hydroperoxide;t-butyl hydroperoxide,0.7 mM. Symbols are: ■ - ■ ;total activity, ● - ● ;net activity, ○ - ○ ;blank.

Table 1 Apparent K_m and V_{max} of GSH-Px of diselenide compounds

K_m and V_{max} for ROOH (GSH:1.0 mM)

Chemical form of Se	ROOH	app K_m(M)	app V_{max}($M \cdot min^{-1}$)
D,L-Se-cystine	H_2O_2	$8.51X10^{-5}$	$3.57X10^{-8}$
D,L-Se-cystine	t-butyl	$1.87X10^{-4}$	$2.55X10^{-8}$
D,L-Se-cystine	cumene	$2.06X10^{-4}$	$4.59X10^{-8}$
Se-cystamine	t-butyl	$2.08X10^{-4}$	$2.68X10^{-8}$

K_m and V_{max} for GSH(ROOH:0.7 mM)

Chemical form of Se	ROOH	app K_m(M)	app V_{max}($M \cdot min^{-1}$)
D,L-Se-cystine	t-butyl	$5.60X10^{-4}$	$2.94X10^{-8}$
D,L-Se-cystine	cumene	$2.06X10^{-4}$	$2.54X10^{-8}$

reductase, the coupled enzyme, would not be the limiting factor for the entire reaction rate, because this enzyme was added in excess. The pH dependency of the GSH-Px activity of diselenides was quite similar to that of the true enzyme which has the optimum pH at 8.8 (10).

Based upon the Lineweaver-Burk plot of the activity, the apparent K_m and V_{max} for various peroxides were measured under the standard assay conditions, and are shown in Table 1. The apparent K_m of diselenides was about one hundred times higher than that of the true GSH-Px reported by Flohe et al (10).

In order to realize the mechanism of the pseudo-selenoenzyme, we supposed that the thiol-diselenide interchange reaction occurred first and an intermediate might be present. The interchange between S- and Se-compounds has been reported for some cases (11-13). The reaction consists of two sequential steps as follows:

$$XSH + RSeSeR \underset{}{\overset{K_1}{\rightleftharpoons}} XSSeR + RSeH \qquad (1)$$

$$XSSeR + XSH \underset{}{\overset{K_2}{\rightleftharpoons}} XSSX + RSeH \qquad (2)$$

where XSH means thiol, and K_1 and K_2 are the equilibrium constants for the equations. As to GSH and diselenides, reliable evidence about the reaction process has so far not been reported.

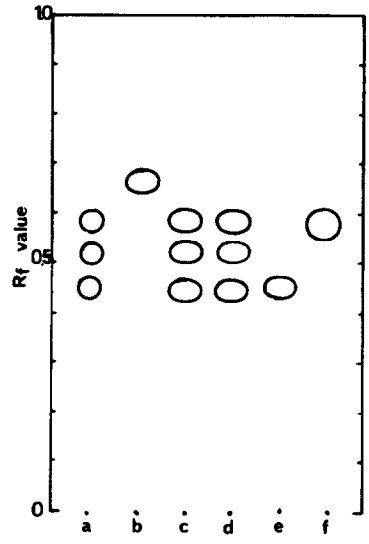

Fig.3 Thin layer chromatogram of the mixture of GSH and D,L-Se-cystine. GSH;1.5 mM, D,L-Se-cystine;0.75 mM. Solvent system: acetic acid/n-butanol/water/phenol/acetone = 9.2/16.8/26.0/4.0/2.5 (V/V/V/W/V).

The mixture of D,L-selenocystine and GSH was developed on TLC plates and an ion-exchange column. As shown in Figs 3 and 4, both chromatograms revealed an unknown compound which appeared at a midposition between GSSG and selenocystine. Dickson and Tappel (13) reported the equilibrium constant of equation 1 to be in the magnitude of 10^{-2} and that the complete reduction of selenocys-

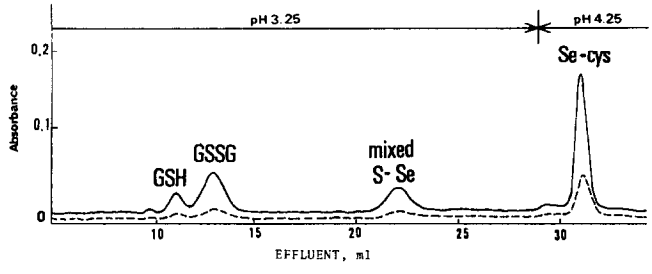

Fig.4 Ion-exchange chromatogram of the mixture of GSH and D,L-Se-cystine, using an amino acid analyzer. GSH;1.5 mM, D,L-Se-cystine;0.75 mM. Two sodium citrate buffers of pH 3.25 and 4.25 were used. The solid line shows the absorbance at 570 nm, and the broken line at 440 nm.

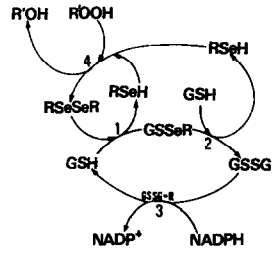

Fig.5 Schematic mechanism for glutathione peroxidase activity of diselenide compounds. Reactions 1 and 2 correspond with the thiol-diselenide interchange reaction. Reaction 4 is the oxidation of RSeH to RSeSeR with hydroperoxide.

tine (-Se-Se-) or -S-Se- compound to selenocysteine (-SeH) might occur in the case where the ratio of [GSH]/[selenocystine] is over 1,000. As the ratio was 2 in the present case, an intermediate GSSeR could be detected in chromatograms as an unknown spot or peak.

When a hydroperoxide such as H_2O_2, t-butyl hydroperoxides or qumene hydroperoxide was added to the mixture of D,L-selenocystine and GSH, the TLC spots corresponding to GSSG and GSSeR increased in density as compared with the case for no hydroperoxide addition. Hydroperoxides should accelerate the oxidation of RSeH to RSeSeR, as shown in equations 1 and 2. As to selenocystamine, taking a similar pH dependency of the activity of selenocystamine into consideration, the same thiol-diselenide interchange reaction might take place in the course of the assay.

In summary, we depict the following scheme for the GSH-Px activity of diselenides in Fig.5. The GSH-Px activity was measured as the production of GSSG and where the diselenides were at first reduced to selenol, then oxidized back to diselenides. In this scheme, the decomposition of peroxides is shown to couple with the oxidation of selenol. In the true enzyme GSH-Px, the presence of a diselenide bond has not been recognized. Though the mechanisms for the

pseudo-enzyme and the true one are quite different from each other in this respect, the occurrence of the intermediate GSSeR enables us to suppose the common characteristics of the selenocysteine moiety in both enzymes.

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REFERENCES

1. Mills, G.C. (1957) J. Biol. Chem. 229, 189-197.
2. Mills, G.C. (1959) J. Biol. Chem. 234, 502-506.
3. Sies, H and Summer, K.H. (1975) Eur. J. Biochem. 57, 503-512.
4. Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D. and Hoekstra, W.G. (1973) Science 179, 588-590.
5. Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) Biochemistry 17, 2639-2644.
6. Forstrom, J.W. and Tappel, A.L. (1979) J. Biol. Chem. 254, 2888-2891.
7. Caldwell, K.A. and Tappel, A.L. (1964) Biochemistry 3, 1643-1647.
8. Caldwell, K.A. and Tappel, A.L. (1965) Arch. Biochim. Biophys. 112, 196-200.
9. Paglia, D.E. and Valentine, W.N. (1967) J. Lab. Clin. Med. 70, 158-169.
10. Flohe, L., Loschen, G., Gunzler, W.A. and Eichele, E. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 987-999.
11. Walter, R., Schlesinger, D.H. and Schwartz, I.L. (1969) Anal. Biochem. 27, 231-243.
12. Olson, O.E. and Palmer, I.S. (1976) Metabolism 25, 299-306.
13. Dickson, R.C. and Tappel, A.L. (1969) Arch. Biochim. Biophys. 130, 547-550.
14. Ladenstein, R., Epp, O., Bartels, K., Jones, A., Huber, R. and Wendel, A. (1979) J. Mol. Biol. 134, 199-218.