Purification and Properties of Glutathione Peroxidase from *Mucor hiemalis*

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Selenium-independent glutathione peroxidase was purified from a cell-free extract of *Mucor hiemalis* by ammonium sulfate fractionation, column chromatographies on DEAE-Sephadex and hydroxylapatite, and gel filtration on Bio-Gel P-100. The purified enzyme was homogeneous on ultracentrifugation. The enzyme had a molecular weight of 45,000 and an isoelectric point of 5.2. The enzyme could reduce cumene hydroperoxide and *t*-butyl hydroperoxide, but could not reduce hydrogen peroxide. The enzyme was highly specific for glutathione as a hydrogen donor. *Mucor* glutathione peroxidase was proved to be different from mammalian selenium-dependent glutathione peroxidase I and selenium-independent glutathione peroxidase II in some physicochemical and enzymatic properties.

In the previous studies,^{1,2)} *Mucor hiemalis* was found to form a large quantity of selenium-independent glutathione peroxidase (EC 1.11.1.9) in the mycelium, and the cultural conditions for the production of glutathione peroxidase were described. The present paper describes the purification and characterization of glutathione peroxidase from *M. hiemalis*, and its comparison with mammalian selenium-dependent glutathione peroxidase I and selenium-independent glutathione peroxidase II.

MATERIALS AND METHODS

Microorganism and cultural conditions. The culture of *Mucor hiemalis* was conducted in a 300-liter fermentor. The medium composition and cultural conditions were as described previously.²⁾

Enzyme assay. Glutathione peroxidase activity was assayed by the following two methods.

(I) **Coupled enzymatic method.** The enzyme activity was assayed by determining the oxidized glutathione (GSSG) formed by the peroxidase with glutathione reductase (EC 1.6.4.2), as described previously.²⁾ This method was used throughout the purification procedures and for most investigations of the enzymatic properties. One enzyme unit was defined as the amount of enzyme which oxidized 1 μ mol of glutathione per min, and specific activity was

expressed as the enzyme units per mg of protein.

(II) **DTNB method.** For the sulfhydryl substrate specificity and some inhibition experiments, the enzyme activity was assayed by determining the decrease of sulfhydryl compounds with Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), according to the method of Sedlak and Lindsay.³⁾ The reaction mixture contained 3 μ mol of cumene hydroperoxide, 3 μ mol of glutathione or an other sulfhydryl compound, 150 μ mol of potassium phosphate buffer, pH 7.0, 3 μ mol of sodium azide, 3 μ mol of EDTA and a suitable amount of enzyme in a total volume of 3.0 ml.

Protein determination. Protein concentration was determined by the method of Lowry *et al.*⁴⁾ with bovine serum albumin as standard.

Ultracentrifugal analysis. A Hitachi UCA-1 analytical ultracentrifuge with double-sector cells and an RA-60 rotor were used for the sedimentation analysis. The temperature was maintained at 20°C throughout.

Amino acid analysis. The amino acid composition was analyzed with a Nihon Denshi Model 5AH automatic amino acid analyzer. The sample was hydrolyzed in 6 N HCl in a sealed evacuated tube at 110°C for 20 hr.

Molecular weight determination. Gel filtration was performed according to the method of Andrews,⁵⁾ with a column (2.5 $\times$ 50 cm) of Sephadex G-150 equilibrated with 0.01 M potassium phosphate buffer, pH 6.0, containing 0.1 M KCl. Elution was performed with the same buffer. Myoglobin (mol. wt. 17,800), chymotrypsinogen (mol. wt.

25,000), ovalbumin (mol. wt. 45,000), bovine serum albumin (mol. wt. 67,000) and alcohol dehydrogenase (mol. wt. 150,000) were used as molecular-weight markers. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn,⁶ with columns of 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA per column. Cytochrome *c* (mol. wt. 13,000), myoglobin, chymotrypsinogen, ovalbumin and bovine serum albumin were used as molecular-weight markers.

Isoelectric focusing. Isoelectric focusing was performed according to the method of Vesterberg and Svensson,⁷ with carrier ampholytes (pH 3.5~10, LKB-Produkter AB). After focusing at a constant potential of 900 V for 48 hr at 3°C, the column was carefully drained through the bottom tubing, and 2.0 ml fractions were collected. Each fraction was tested for pH, protein concentration and enzyme activity.

Chemicals. DEAE-Sephadex A-50 and Sephadex G-150 were purchased from Pharmacia Fine Chemicals, Sweden. Bio-Gel P-100 was purchased from Bio-Rad Laboratories, USA. Hydroxylapatite was prepared as described by Tiselius *et al.*⁸ Other chemicals were the best commercial products available.

RESULTS

Purification of glutathione peroxidase

All operations were carried out at 0~5°C unless otherwise specified, and all buffers used were potassium phosphate buffer, pH 6.0.

1. *Preparation of cell-free extract.* Washed mycelia (ca. 400 g, wet weight) of *M. hiemalis* were suspended in 4,000 ml of 0.01 M buffer, and then disrupted with a Dyno Mill KDL (W. A. Bachofen, Switzerland). The cell debris was removed by centrifugation at $20,000 \times g$ for 20 min.

2. *Ammonium sulfate fractionation.* The cell-free extract was brought to 70% saturation with ammonium sulfate, and the precipitate formed was removed by centrifugation at $20,000 \times g$ for 20 min. The resultant supernatant solution was made to 90% saturation with ammonium sulfate, and the precipitate formed was collected by filtration using celite as a filter aid. The enzyme precipitate contained in the filter aid was extracted three times with 200 ml of 0.01 M buffer, and the extracts were dialyzed overnight against the same buf-

fer. The dialyzed enzyme preparation was concentrated by ultrafiltration with a YM 10 membrane (Amicon Corp., USA).

3. *DEAE-Sephadex column chromatography.* The concentrate (100 ml) was applied onto a DEAE-Sephadex A-50 column (5.5×50 cm), previously equilibrated with 0.01 M buffer. The column was washed first with the same buffer, and then the enzyme was eluted with a linear gradient of NaCl, from 0 to 0.4 M. The active fractions were combined and concentrated by ultrafiltration. The concentrate was dialyzed overnight against 0.01 M buffer.

4. *Bio-Gel P-100 gel filtration.* The dialyzed enzyme solution (20 ml) was applied onto a Bio-Gel P-100 column (5.5×48 cm), previously equilibrated with 0.01 M buffer. Elution of the enzyme was performed with the same buffer. The active fractions were combined and concentrated by ultrafiltration and dialyzed overnight against 0.01 M buffer.

5. *Hydroxylapatite column chromatography.* The dialyzed enzyme solution (40 ml) was applied onto a hydroxylapatite column (2.5×11 cm), previously equilibrated with 0.01 M buffer. The column was washed first with the same buffer and then with 0.05 M buffer. The enzyme was eluted with 0.10 M buffer. The active fractions were combined and dialyzed overnight against 0.01 M buffer. The dialyzed enzyme was concentrated by ultrafiltration and stored at -20°C.

The results of purification are summarized in Table I. Glutathione peroxidase was purified about 2000-fold from the cell-free extract of *M. hiemalis* with a recovery of about 10% of the initial activity.

Properties of glutathione peroxidase

Homogeneity. The purified enzyme preparation showed a single and symmetrical moving peak on ultracentrifuge analysis (Fig. 1). The apparent sedimentation coefficient, $S_{20,w}$, was calculated to be 2.24. The enzyme also gave a single protein band on polyacrylamide gel electrophoresis carried out at pH 8.3, as described previously.¹

Amino acid composition. The amino acid

TABLE I. PURIFICATION OF GLUTATHIONE PEROXIDASE FROM *M. hiemalis*

Step	Total protein (mg)	Total activity (units)	Specific activity (u/mg)	Recovery of activity (%)
Cell-free extract	62,700	6,350	0.10	100
(NH ₄) ₂ SO ₄ (70~90%)	1,680	1,470	0.88	23.1
DEAE-Sephadex	108	1,270	11.8	20.0
Bio-Gel P-100	16.3	1,100	67.5	17.3
Hydroxylapatite	3.16	657	208	10.3

FIG. 1. Sedimentation Patterns of Glutathione Peroxidase from *Mucor hiemalis*.

The purified enzyme was used at a concentration of 4.1 mg protein/ml in 0.01 M phosphate buffer, pH 6.0, containing 0.1 M KCl. Photographs were taken, A to D, at 6, 24, 48 and 72 min after reaching 55,430 rpm.

TABLE II. AMINO ACID COMPOSITION OF GLUTATHIONE PEROXIDASE FROM *M. hiemalis*

Amino acid	Analyzed value ^a (μmol/mg)	Nearest integer (residues/mol)
Lysine	0.684	31
Histidine	0.180	8
Arginine	0.138	6
Aspartic acid	1.05	47
Threonine	0.470	21
Serine	0.495	22
Glutamic acid	0.843	38
Proline	0.521	23
Glycine	0.479	22
Alanine	0.584	26
Half-cystine ^b	N.D.	N.D.
Valine	0.512	23
Methionine	0	0
Isoleucine	0.305	14
Leucine	0.793	36
Tyrosine	0.088	4
Phenylalanine	0.412	19
Tryptophan ^b	N.D.	N.D.
Total		340

^a Analytical methods are described in METHODS.

^b Not determined.

composition of glutathione peroxidase is shown in Table II. The results show that the enzyme has about 340 amino acid residues per mol and that it is rich in aspartic acid, glutamic acid and leucine. From the amino acid composition, the partial specific volume of the enzyme was calculated to be about 0.74 cm³ g⁻¹ according to the method of Cohn and Edsall.⁹⁾

Molecular weight. The molecular weight of glutathione peroxidase was determined to be 45,000 by gel filtration on Sephadex G-150 (Fig. 2). SDS-polyacrylamide gel electrophoresis gave a single protein band with a mobility corresponding to a molecular weight

of 27,000 (Fig. 3). These results suggest that the enzyme consists of two apparently identical subunits.

Isoelectric points. The isoelectric point of glutathione peroxidase was estimated to be 5.2 by isoelectric focusing (Fig. 4).

Effect of pH. The effect of pH on the activity and stability of glutathione peroxidase was examined. The enzyme activity was maximum at around pH 6.0 as shown in Fig. 5. The enzyme was stable between pH 5.0 and 7.0 on treatment at 4°C for 24 hr.

Thermostability. The effect of temperature

FIG. 2. Determination of Molecular Weight of Glutathione Peroxidase by Sephadex G-150 Gel Filtration.

Experimental conditions are described in METHODS. A, myoglobin; B, chymotrypsinogen; C, glutathione peroxidase; D, ovalbumin; E, bovine serum albumin; F, alcohol dehydrogenase. V_o and V_e are the void volume of the column and the elution volume of protein, respectively.

FIG. 3. Determination of Molecular Weight of Glutathione Peroxidase by SDS-Polyacrylamide Gel Electrophoresis.

Experimental conditions are described in METHODS. A, cytochrome c; B, myoglobin; C, chymotrypsinogen; D, glutathione peroxidase; E, ovalbumin; F, bovine serum albumin.

on the stability of glutathione peroxidase was tested. The enzyme was stable up to 50°C when treated at pH 6.0 for 15 min, and lost about 55% of the original activity at 60°C.

Substrate specificity. The substrate specificity of glutathione peroxidase was determined for several hydroperoxide and sulfhydryl substrates. Cumene hydroperoxide was

FIG. 4. Isoelectric Focusing of Glutathione Peroxidase. Purified protein (6 mg) was applied to an isoelectric focusing column of 110 ml. The conditions for electrophoresis are described in METHODS. ●, glutathione peroxidase activity; ○, absorbance at 280 nm; — — —, pH.

FIG. 5. Effect of pH on the Activity of Glutathione Peroxidase.

The enzyme activity was determined by two assay methods, namely, the coupled enzymatic method (●) and the DTNB method (○), under the respective standard conditions with the 0.05 M buffer indicated. Acetate buffer was used from pH 4.0 to 5.0, phosphate buffer from pH 6.0 to 8.0, and borate buffer from pH 9.0 to 10.

most effectively reduced, and *t*-butyl hydroperoxide showed about 90% of the enzymatic reaction rate with cumene hydroperoxide (Table III). On the other hand, the enzyme did not act on hydrogen peroxide or dialkyl peroxides (R-OO-R) such as dicumene peroxide and di-*t*-butyl peroxide. The enzyme was highly specific for glutathione as a hydrogen donor, and other sulfhydryl compounds tested were not effective at all (Table IV). The apparent K_m values for cumene hydroperoxide, *t*-butyl

TABLE III. SUBSTRATE SPECIFICITY OF GLUTATHIONE PEROXIDASE IN THE REDUCTION OF VARIOUS HYDROPEROXIDES

The rates of reduction were determined by the coupled enzymatic method (I) and were expressed as a percentage of that of cumene hydroperoxide.

Substrate	Relative activity (%)
Cumene hydroperoxide	100
<i>t</i> -Butyl hydroperoxide	90.1
Hydrogen peroxide	0
Dicumene peroxide	0
Di- <i>t</i> -butyl peroxide	0

TABLE IV. SUBSTRATE SPECIFICITY OF GLUTATHIONE PEROXIDASE IN THE HYDROGEN DONOR

The enzyme activity was assayed by the DTNB method (II), and was expressed in μmol of sulfhydryl residue oxidized per min.

Thiol compound	Activity
Glutathione	36
L-Cysteine	0
Dithiothreitol	0
2-Mercaptoethanol	0
Cysteamine	0
Thioglycolic acid	0

hydroperoxide and glutathione were calculated to be 0.13 mM, 0.13 mM and 0.67 mM, respectively. *Mucor* glutathione peroxidase did not show glutathione *S*-transferase activity, as described previously.¹⁾

Effect of various chemicals. The effect of various chemicals on the activity of glutathione peroxidase was examined (Table V). Various kinds of chelating agents did not affect the enzyme activity. This suggests that metal ions are not concerned in the enzyme activity directly. Anionic detergents such as SDS and bile acids inhibited the enzyme activity strongly, whereas non-ionic detergents did not affect the enzyme activity. Of divalent cations tested, only the cupric ion was inhibitory. ATP, which is an allosteric inhibitor for mammalian selenium-dependent glutathione peroxidase I,¹⁰⁾ and bilirubin, which is a potent inhibitor for glutathione *S*-transferase,¹¹⁾ did

TABLE V. EFFECT OF VARIOUS CHEMICALS ON THE ACTIVITY OF GLUTATHIONE PEROXIDASE

The enzyme was incubated in 50 mM phosphate buffer, pH 7.0, containing various chemicals for 15 min at 25°C. Then, the reaction was initiated by addition of cumene hydroperoxide. Activities were determined by the coupled enzymatic method (I) or the DTNB method (II) (*), and were expressed as a percentage of the case of no addition, respectively. In this experiment, sodium azide and EDTA were omitted from both standard reaction mixtures.

Chemical	Concentration	Relative activity
No addition	—	100
No addition	—	100*
EDTA	1 mM	106
<i>o</i> -Phenanthroline	1 mM	110
α, α' -Dipyridyl	1 mM	102
Diethyldithiocarbamic acid	1 mM	114
<i>o</i> -Phenylenediamine	1 mM	104
KCN	1 mM	105
NaN ₃	1 mM	112
Hydroxylamine	1 mM	117
Iodoacetic acid	0.1 mM	95.9
	1 mM	64.8
Iodoacetamide	0.1 mM	98.9
	1 mM	84.3
Chlortetracycline	1 mM	102*
Quinacrine	1 mM	82.4*
Sodium dodecyl sulfate	0.1%	1.0*
Cholic acid	0.1%	37.4
Deoxycholic acid	0.1%	18.3
Triton X-100	0.1%	84.6
Tween 40	0.1%	100
Span 40	0.1%	99.0
CuSO ₄	1 mM	33.0
FeSO ₄	1 mM	87.5*
ATP	1 mM	104
Bilirubin	0.02 mM	90.5

not inhibit this microbial glutathione peroxidase.

DISCUSSION

Glutathione peroxidase from *Mucor hiemalis* was purified to homogeneity. The specific activity of the final preparation was 208 units/mg protein. The specific activities of purified selenium-dependent glutathione peroxidases I were reported to be 41.8, 103, 123, 70.4, 1036 and 1852 units/mg for bovine lens

TABLE VI. PROPERTIES OF GLUTATHIONE PEROXIDASE OF VARIOUS ORIGINS

Physicochemical and enzymatic properties of three kinds of glutathione peroxidases were compared.

	<i>Mucor</i> GSH-Px ^a	GSH-Px I ^b	GSH-Px II ^b
Se content (atoms/mole)	0	4	0
Molecular weight	45,000	75,000~96,600	39,000~45,000
Subunit number	2	4	2
Isoelectric point	5.2	4.9~7.0	8.5~9.5
Substrate specificity			
Reduction of H ₂ O ₂	No	Yes	No
Transferase activity	No	No	Yes
Michaelis constant			
Cumene hydroperoxide	0.13 mM	0.027~0.08 mM	0.18~0.57 mM
Glutathione	0.67 mM	0.017~0.13 mM	0.2 mM
pH Optimum	6.0	8.5~9.1	N.D. ^c

^a Glutathione peroxidase from *M. hiemalis*.^b Glutathione peroxidases I and II of various mammalian origins, and their properties are cited from many reports described in the text.^c Not determined.

enzyme,¹²⁾ human erythrocyte enzyme,¹³⁾ rat liver mitochondrial enzyme¹⁴⁾ and rat liver cytosolic enzymes,^{15~17)} respectively. The specific activities of purified selenium-independent glutathione peroxidases II were also reported to be 5.07, 8.0 and 11 units/mg for human liver enzyme,¹⁸⁾ guinea pig liver enzyme¹⁹⁾ and rat liver enzyme,²⁰⁾ respectively. The specific activity of *Mucor* glutathione peroxidase was close to those of selenium-dependent glutathione peroxidases I.

When the properties of *Mucor* glutathione peroxidase were compared with those of mammalian glutathione peroxidases I and II,^{21~25)} this microbial enzyme was found to be different from the two mammalian enzymes in some physicochemical and enzymatic properties, as summarized in Table VI. The important characteristics of *Mucor* glutathione peroxidase were that it is selenium-independent and does not show glutathione *S*-transferase activity. From these results, we concluded that the *Mucor* glutathione peroxidase is a novel type of enzyme.

The following peroxidases are known at present: protoheme peroxidases and flavo-protein peroxidases.²⁶⁾ *Mucor* glutathione peroxidase was suggested to contain no heme because it had no Soret band at 400~420 nm (data not shown) and was insensitive to azide

and cyanide inhibition, and X-ray fluorescence analysis failed to detect a significant amount of iron as described previously.¹⁾ No inhibition by quinacrine or chlortetracycline, both inhibitors of flavoenzymes,²⁷⁾ suggest that a flavin nucleotide is not involved in the *Mucor* enzyme activity. It remains unknown what moiety in the *Mucor* enzyme is concerned in the oxidation-reduction reaction. Further characterization of the *Mucor* glutathione peroxidase is now underway.

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