Glutathione Peroxidase from the Liver of Japanese Sea Bass *Lateolabrax japonicus*

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Glutathione Peroxidase, Japanese Sea Bass, Subunit Composition

Glutathione peroxidase (EC 1.11.1.9) present in the liver of Japanese sea bass (*Lateolabrax japonicus*) was extracted and purified by phenyl-toyopearl 650M, butyl-toyopearl 650M and DEAE-toyopearl 650M column chromatography. The molecular weight of the enzyme was estimated to be about 70 kDa by gel filtration by toyopearl HW-55F. On SDS-PAGE, this enzyme was composed of two identical subunits with 35 kDa and was a dimer. This enzyme was a typical SH-enzyme that was inhibited by iodoacetic acid, PCMB, DTNB, and Hg.

Introduction

This enzyme has been postulated to protect the erythrocytes from damage by H_2O_2 and will reduce lipid hydroperoxides led to the hypothesis that this enzyme may be protect tissue against oxidative damage due to lipid peroxidation. GSH-Px appears to be primary site of selenium action. The liver is a major site of detoxification and the first target of ingested oxidants and a very important tissue in the study of the role of GSH-Px in protection from lipid peroxidation. Until now, GSH-Px has been studies in many land animals (Cikryt *et al.*, 1982; Devore and Greene, 1982; Lee *et al.*, 1979; Richard and Raymond, 1978; Smith and Shrift, 1979; Tappel *et al.*, 1982). Although GSH-Px was purified from many mammals and characterized (Awasthi *et al.*, 1975; Chiu *et al.*, 1976; Lyons *et al.*, 1981; Nakamura *et al.*, 1974; Stults *et al.*, 1977; Yoshida *et al.*, 1982), there are many reports concerning the organ distribution of GSH-Px activity in some fish (Aksnes and Njaa, 1981; Braddon *et al.*, 1985; Diana and Thomas, 1996; Hai *et al.*, 1997; Kolayli *et al.*, 1997; Nagai *et al.*, 1999; Nakano *et al.*, 1992; Rana and Singh, 1996; Watanabe *et al.*, 1996; Wdzieczak *et al.*, 1981), few have been investigation that this enzyme was purified and characterized in an aquatic organism (Nakano *et al.*, 1992). From these reasons, we tried to purify and characterize this enzyme in the organs of fish. As a result, we have discovered a GSH-Px in the liver of Japanese sea bass. In this paper, we describe the characteristics of a GSH-Px in Japanese sea bass liver.

Materials and Methods

Fish

Japanese sea bass *Lateolabrax japonicus* (body weight, 1.0–1.3 kg) were obtained from a whole sale market, then cooled on ice and immediately transported to our laboratory. The liver was taken out and stored at −85 °C until use. All of the following procedures were carried out at 4 °C.

Assay of enzyme activity

The reaction mixture (0.5 ml) contained 32 mM sodium phosphate buffer (pH 7.0), 0.64 mM NaN_3, 1.28 mM ethylenediaminetetraacetic acid (EDTA), 0.13 unit glutathione reductase (EC 1. 6. 4. 2 from yeast), 0.65 mM glutathione [reduced form (GSH)], 0.097 mM NADPH, 0.16 mM *t*-butyl hydroperoxide (TBH) and an appropriate amount of enzyme. The reaction was started by the addition of TBH. The enzyme activity was measured by absorbance at 340 nm and was defined as nmol NADPH oxidized per min per mg protein. The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Molecular weight determination

The molecular weight of the purified enzyme was estimated using Toyopearl HW-55F (2.6 ×

90 cm) gel filtration and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Ferritin (MW: 450,000), catalase (240,000), aldolase (158,000), and albumin (68,000) were used as the standard markers for gel filtration. Native PAGE was also performed by the method of Davis (1964).

Results and Discussion

Purification of GSH-Px

The liver samples were weighed and homogenized with three volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol (2-ME), 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 30,000 × *g* for 30 min, and then the supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 1.3 M ammonium sulfate, 1 mM EDTA and 4 mM 2-ME. The dialysate was applied to a Phenyl-Toyopearl 650M column (1.0 × 7.0 cm), previously equilibrated with the same buffer. The enzyme was not absorved in this column. The active fractions were pooled and applied to a Butyl-Toyopearl 650M column (1.0 × 5.0 cm) previously equilibrated with the same buffer. Unfortunately, the enzyme was not also absorved in this column. After the enzyme solution was pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 4 mM 2-ME, it was applied to a DEAE-Toyopearl 650M column (1.0 × 4.0 cm) previously equilibrated with the same buffer. The enzyme was eluted without being absorved. The enzyme was pooled and stored at −85 °C. The purification of GSH-Px is summarized in Table I.

Estimation of molecular weight

The molecular weight of the purified GSH-Px was about 70 kDa, estimated by Toyopearl HW-55F (1.5 × 120 cm) gel filtration (data not shown). According to native PAGE, the enzyme produced a single protein band (Fig. 1-I). On SDS-PAGE, the purified enzyme appears as a single protein band of molecular weight about 35 kDa (Fig. 1-II). Japanese sea bass liver GSH-Px was composed of identical subunits with about 35 kDa and was a dimer. The molecular weight of the enzyme of some mammals and fish species have been reported as follows: rat liver, 75–76 kDa (Nakamura *et al.*, 1974), 80 kDa (Stults *et al.*, 1977; Yoshida

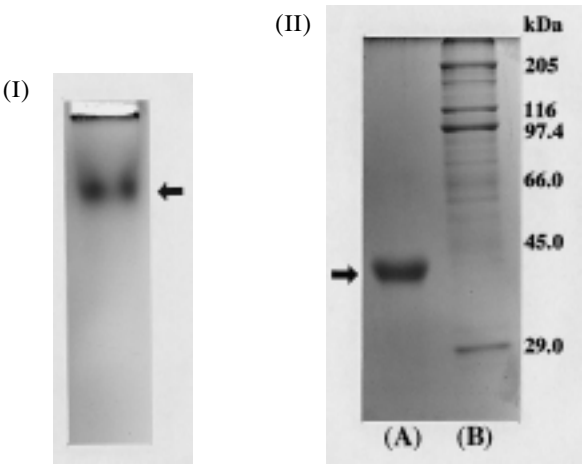

Fig. 1. Electrophoretic pattern of glutathione peroxidase using native PAGE (I) and SDS-PAGE (II). (A) Enzyme; (B) High molecular marker. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) were used as standards. After electrophoresis, the gel was stained using a Silver Stain Kit (Wako Pure Chemical Ltd. Osaka, Japan).

Table I. Summary of the purification of Japanese sea bass liver GSH-Px.

Step	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Purification fold
Crude	13492	5403	0.40	1
Phenyl-toyopearl 650 M	206.9	4117	19.9	49.8
Butyl-toyopearl 650 M	51.2	2579	50.4	126.0
DEAE-toyopearl 650 M	6.9	1887	273.5	683.8

Enzyme activity (U) was shown as 1 nmol/mg protein · min.

et al., 1982), and rat lung, 80 kDa (Chiu *et al.*, 1976). These values are similar to the molecular weight of the novel type GSH-Px. On the other hand, the molecular weight of the enzyme was estimated: human erythrocyte, 95 ± 3 kDa (Awasthi *et al.*, 1975), carp hepatopancreas, 100 kDa (Nakano *et al.*, 1992), and rainbow trout liver, 100 kDa (Bell *et al.*, 1984). These values are different from Japanese sea bass liver GSH-Px. Moreover, these organisms GSH-Px were composed of four identical subunits with 19–25 kDa and were a tetramer.

Effect of pH and temperature

The activity of purified enzyme was measured at different pHs at 25 °C for 5 min. The optimum pH of the enzyme was about 7.0. This value was different from those of rat lung (pH 8.8 and 9.0) (Chiu *et al.*, 1976), human erythrocyte (pH 8.5) (Awasthi *et al.*, 1975), and carp hepatopancreas D1 (pH 8.0) and D2 (pH 9.0) (Nakano *et al.*, 1992). The stability of the enzyme was examined at different pHs at 4 °C for 60 min. The enzyme retained more than 80% of the original activity at pH between 6.5 and 7.5, but became extremely unstable when the pH was higher than 8.0. Nakano *et al.* (1992) reported that these were stable at from pH 6.0 and 10.0 when the enzymes (D1 and D2) were stored at 4 °C for 12 h. Moreover, about 70% of the activity was retained after incubation of the enzyme for 36 h (Nakano *et al.*, 1992).

The effect of temperature on the activity of this enzyme was examined at 25 °C for 5 min in 10 mM sodium phosphate buffer (pH 7.0). The optimum temperature for the enzyme activity was around 40 °C (Fig. 2). On the other hand, the enzyme was incubated at different temperatures at pH 7.0 for 60 min. After cooling, the residual activity was measured. The enzyme was stable when incubated at 30 °C for 60 min, but only 18% of the original activity remained at 40 °C for 60 min (Fig. 2). Nakano *et al.* (1992) reported that both enzymes were stable at 40 °C for 10 min and D2 was stable at 50 °C for 10 min.

Effect of metal ions and SH-blocking reagents

The enzyme was strongly inhibited by Mg^{2+}, CH_2ICOOH, PCMB, and DTNB, and was fairly inhibited by Co^{2+}, Li^{+}, and Hg^{2+} (Table II). From the effects of the sulphydryl reagents such as iodoacetic acid, PCMB and DTNB, it can be suggested that this enzyme contains SH-groups in its active site. Moreover, because the addition of 2-ME throughout the purification was indispensable for enzyme stabilization, the instability of the enzyme seems to be due to the oxidation of SH-groups of the enzyme. While this enzyme was strongly activated by Mn^{2+}. Awasthi *et al.* (1975) reported that human erythrocyte GSH-Px was inhibited by PCMB, *N*-ethylmaleimide, iodoacetate, and Hg. This finding is the same as our report.

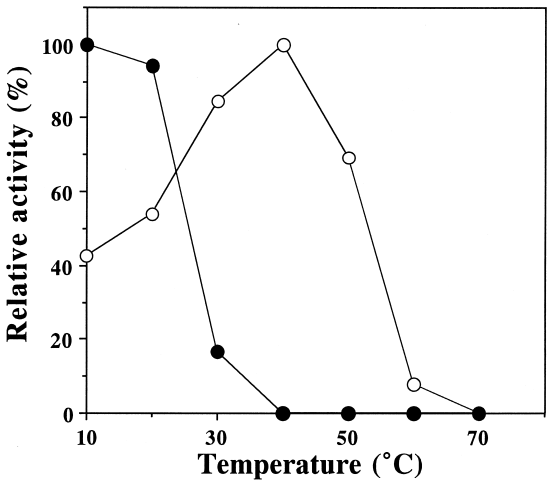

Fig. 2. Effect of temperature on activity and stability of glutathione peroxidase. Enzyme activity was assayed for each temperature after an incubation period of 5 min (○). The thermal stability experiments (●), the enzyme was prewarmed at the indicated temperature for 60 min, and then the remaining activity was determined.

Substrate specificity

The effects of various substrates (GSH, hydrogen peroxide (H_2O_2), TBH, and cumene hydroperoxide (CHP)) were examined and K_m values were calculated by Lineweaver-Burk plot analysis: 3.16×10^2 μM for GSH, 2.95 μM for H_2O_2, 6.0×10 μM for TBH, and 1.10×10^2 μM for CHP, respectively (Table III). H_2O_2 was the most suitable substrate for this GSH-Px. The other species enzymes showed the K_m values as follows: 4.1×10^3 μM (rat liver) (Nakamura *et al.*, 1974), 4.1×10^3 μM (human erythrocyte) (Awasthi *et al.*, 1975), $1.21–2.65 \times 10^3$ μM (carp hepatopancreas) (Nakano *et al.*, 1992), 3.0×10^3 μM (rainbow trout liver) (Bell

Table II. Effect of various ions and chemicals on the activity of Japanese sea bass liver glutathione peroxidase

Reagents [1 mM]	Relative activity [%]
None	100
KCl	94
NaCl	95
LiCl	29
$HgCl_2$	29
$MgCl_2$	0
$BaCl_2$	36
$ZnSO_4$	36
$MnCl_2$	221
$CuSO_4$	36
$CaCl_2$	93
$CoCl_2$	14
$FeCl_3$	36
CH_2ICOOH	0
PCMB	7
DTNB	0
Glutathione	0

PCMB: *p*-chloromercuribenzoic acid.
DTNB: 5,5′-dithiobis(2-nitrobenzoic acid).

Table III. K_m and V_{max} values for each substrate.

Substrate	K_m [μ_m]	V_{max} [nmol/mg protein · min]
GSH	316.2	0.52
H_2O_2	2.95	0.10
TBH	59.8	0.23
CHP	111.0	0.74

TBH: *t*-butyl hydroperoxide.
CHP: cumene hydroperoxide.

et al., 1984), for GSH, 4.8×10 μM (rat liver) (Nakamura *et al.*, 1974), 5.2×10 μM (human erythrocyte) (Awasthi *et al.*, 1975), $0.66–1.49 \times 10^2$ μM (carp hepatopancreas) (Nakano *et al.*, 1992), for TBH, $0.91–1.63 \times 10^2$ μM (carp hepatopancreas) (Nakano *et al.*, 1992), for CHP, and 10.0 μM (rainbow trout liver) (Bell *et al.*, 1984) for H_2O_2. In comparison with these results, Japanese sea bass liver GSH-Px had the same affinity for TBH and CHP, but had a high one for GSH and H_2O_2.

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