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Purification and properties of a glutathione peroxidase from Southern bluefin tuna (*Thunnus maccoyii*) liver

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Abstract

A glutathione peroxidase (GPX) protein was purified approximately 1000-fold from Southern bluefin tuna (*Thunnus maccoyii*) liver to a final specific activity of 256 μmol NADPH oxidised min^{-1} mg^{-1} protein. Gel filtration chromatography and denaturing protein gel electrophoresis of the purified preparation indicated that the protein has a native molecular mass of 85 kDa and is most likely a homotetramer with subunits of approximately 24 kDa. The K_m values of the purified enzyme for hydrogen peroxide, cumene hydroperoxide, *t*-butyl hydroperoxide and glutathione were 12, 90, 90 and 5900 μM, respectively. The K_m values for cumene hydroperoxide and *t*-butyl hydroperoxide were approximately 8-fold greater than the K_m value for hydrogen peroxide. Thus, the SBT liver GPX has a considerably greater affinity for hydrogen peroxide than for the other two substrates. The pH optimum of the purified enzyme was pH 8.0. Immunoblotting experiments with polyclonal antibodies, raised against a recombinant human GPX, provided further evidence that the purified SBT enzyme is a genuine GPX.

Keywords: Southern bluefin tuna; Glutathione peroxidase; Liver; Purification; K_m; V_{max}; Molecular mass; pH optimum

1. Introduction

Glutathione peroxidase (GPX) is one of the key enzymes in the antioxidant defence system of living cells (Halliwell and Gutteridge, 2003). The antioxidant defence system protects cells against the damaging effects of reactive oxygen species (ROS). ROS include the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). ROS are generated as byproducts of normal aerobic metabolism and this is why cells have evolved antioxidant defence systems to protect themselves. The major components of the cellular antioxidant defence system are dietary antioxidants (e.g. vitamin C, vitamin E), cellular antioxidants (e.g. glutathione) and antioxidant enzymes (e.g. GPX, catalase and superoxide dismutase). GPX detoxifies hydrogen peroxide and fatty acid hydroperoxides formed when ROS attack cellular lipids. Fish lipids are especially susceptible to attack by ROS because of the high proportions of polyunsaturated fatty acids (PUFAs) they contain (Bell and Cowey, 1985; Sargent et al., 1999). Lipid peroxidation, initiated by the attack of ROS on cellular lipids, is a key factor affecting the product quality and shelf life of fish.

The GPX enzyme family consists of four major selenium containing isozymes which have been extensively studied in mammals (Arthur, 2000). The four major mammalian isozymes are classical GPX (GPX-1), gastrointestinal GPX (GPX-2), plasma GPX (GPX-3) and phospholipid hydroperoxide GPX (GPX-4). GPX-1 is abundant in the liver but also found in other tissues. GPX-2 is found mainly in the gastrointestinal tract but the mRNA has also been found in the liver of humans (Chu et al., 1993). GPX-3 is a glycoprotein which was originally purified from human plasma (Takahashi et al., 1987) and GPX-4 is believed to be membrane associated in vivo (Urisini et al., 1985). GPX-4 differs from GPX-1, GPX-2 and GPX-3 in that it is monomeric whereas the others are tetrameric. Currently little is known about the localization or the number of the different isozymes of GPX in finfish.

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Many researchers have assayed GPX enzyme activity in various finfish species and various finfish tissues (Bagnyukova et al., 2005; Braddon et al., 1985; Kai et al., 1995; Kolayi et al., 1997; Nagai et al., 2002; Nakano et al., 1992b; Perez-Campo et al., 1993; Watanabe et al., 1996). However, there has never been a thorough investigation to identify the various isozymes of GPX in fish. The best that has been done is to differentiate between selenium-dependent and selenium-independent isoforms of GPX using alternate substrates in the enzyme assay that allow differentiation between these two classes (Carmagnol et al., 1983). To increase our understanding of GPX in finfish, it is therefore important to isolate and biochemically characterise the different isoforms of this enzyme from a range of species.

We are interested in extending the shelf life of South Australian farmed Southern bluefin tuna (SBT) by reducing the rate of lipid peroxidation post-harvest. South Australian farmed SBT is exported either fresh or frozen mostly to Japan (Glencross et al., 2002). The best prices are obtained for sashimi grade fresh SBT. Thus, we are interested in extending the shelf life of the fresh product and thereby extending the window of opportunity for sale at premium prices. A major aim of our investigations is to develop an understanding of the antioxidant system in SBT and how its various components interact to delay lipid peroxidation post-harvest. Our preliminary work has shown that GPX activity in muscle samples taken from SBT fed diets supplemented with selenium and high concentrations of vitamin C and vitamin E remains stable for longer post-harvest than GPX activity in muscle samples taken from control SBT fed the normal diet (Philip Thomas, Jeff Buchanan, Janene Thompson, Kathryn Schuller, unpublished). These results suggest there is a positive synergistic interaction between elevated dietary vitamin intake in live fish and GPX stability post-harvest. With this in mind, we decided to undertake a detailed investigation of the biochemical properties of SBT GPX.

Very little is known about the individual isozymes of GPX in finfish except for the work of Bell et al. (1984) with rainbow trout liver GPX, Nakano et al. (1992a) with carp hepatopancreas GPX and Nagai et al. (2002) with Japanese sea bass liver GPX. The trout liver GPX is a homotetramer with a subunit molecular mass of approximately 25 kDa and one atom of selenium per subunit whereas the Japanese sea bass liver GPX is a homodimer with a subunit molecular mass of 35 kDa. Two different forms of GPX have been isolated from carp hepatopancreas. The two forms differed in their pH optima and their affinity for various fatty acid hydroperoxide substrates, but no information was available with respect to the molecular mass of either the subunit(s) or the native enzyme.

Here we report on the purification and biochemical characterization of a GPX enzyme from SBT liver. This is the first time that any enzyme has been investigated in detail in this large and economically highly valuable fish. Our results form the basis of a better understanding of the antioxidant defence system in SBT.

2. Materials and methods

2.1. Fish

Southern bluefin tuna (*Thunnus maccoyii*) muscle, liver and plasma samples were obtained from a commercial fish farm located in Boston Bay near Port Lincoln in South Australia. The fish were harvested as described by Thomas et al. (2003). The samples were transported from Port Lincoln to Adelaide on dry ice. Upon arrival in Adelaide they were stored at −65 °C.

2.2. Reagents

All reagents were purchased from Sigma-Aldrich except for dithiothreitol (DTT) which was purchased from Astral Scientific and the denaturing protein gel electrophoresis molecular mass standards which were obtained from Bio-Rad.

2.3. Glutathione peroxidase enzyme assay

Glutathione peroxidase (GPX) activity was assayed using a modification of the method described by Nakano et al. (1992a). The standard reaction mixture contained 50 mM K-phosphate buffer (pH 8), 2.7 mM ethylenediaminetetraacetic acid (EDTA), 1.8 mM NaN_3, 5.4 mM reduced glutathione (GSH), 0.12 mM NADPH, 1 U of glutathione reductase (E.C. 1.6.4.2., from yeast), 0.24 mM cumene hydroperoxide and 5–40 μL of enzyme extract in a final volume of 1.13 mL. The reaction was initiated with the addition of cumene hydroperoxide and the reaction rate was determined by monitoring the change in absorbance at 340 nm due to the oxidation of NADPH. Control assays were performed without the addition of the enzyme extract and the rates obtained for these assays were subtracted from the rates obtained for the assays with the enzyme extract. All assays were performed at 25 °C in a temperature-controlled cuvette holder in a Beckman DU650 spectrophotometer. One unit of GPX enzyme activity is defined as the amount required to oxidize 1 μmol NADPH min^{-1}. For some assays, cumene hydroperoxide was replaced by either hydrogen peroxide or *t*-butyl hydroperoxide as the peroxide substrate (see legends to tables and figures). The K_m and V_{max} values for the various peroxide substrates were determined by varying the concentration from 0 to 0.24 mM and then analysing the reaction rates using Michaelis–Menten plots generated by GraphPad Prism software version 4.02. For the determination of the pH optimum for GPX activity, the potassium phosphate buffer used in the standard reaction mixture was replaced by a mixed buffer. The final concentrations of the mixed buffer components in the assay were 22 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 22 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 22 mM [(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]-1-propanesulfonic acid (TAPS), 22 mM sodium acetate and 0.44 mM ethylene glycol-bis (β-aminoethylethere) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The pH of the mixed buffer was adjusted using either glacial acetic acid or NaOH. The mixed buffer had a buffering range of around pH 4–9.