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Physiochemical properties of purified Catalase enzyme from *Azolla*

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A B S T R A C T

Enzymes are the reaction catalysts of biological systems which accelerate and direct specific biochemical reactions. Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. Catalase catalyzes the reduction of hydroperoxides, thereby protecting mammalian cells against oxidative damage. The aim of the project is to analyze the presence of antioxidant enzyme and to purify the enzyme from the aquatic fern *Azolla.* The catalase enzyme presence was confirmed by standard assay procedure and purified through DEAE cellulose and Sephadox G-75 Column chromatography. The purified catalase enzyme was subjected for Molecular weight determination by SDS-PAGE analysis. Since the separated enzyme appeared as a single band, it was concluded that catalase enzyme as tetrameric. The purified catalase was found about 55,000 Da molecular weight. Maximum enzyme activity observed at pH 7 which was the optimum pH level of the catalase enzyme purified from *Azolla* and the optimum temperature level was 10°C.

Introduction

Enzymes are the reaction catalysts of biological systems which accelerate and direct specific biochemical reactions. Great specificity of enzymes is a very important biological phenomenon which assures high coordination to yield a harmonious interplay among many different metabolic activities necessary to sustain life. It is well known that activities of intracellular and extracellular enzymes depend on numerous constituents of medium or circumstances.

The most important factors which influence enzyme activity are presented by enzyme concentration, the amount of specific enzyme substrate, electrochemical reaction of medium for enzyme activity (pH), the presence of activators (specific or nonspecific) as well as the presence of inhibitors.

Antioxidant enzymes are capable of stabilizing, or deactivating free radicals

before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals. Catalase (E.C. 1.11.1.6; H_2O_2: H_2O_2-oxidoreductase) belonging to the oxidoreductase family are a group of metallo enzymes with the ability to catalyze the decomposition of hydrogen peroxide into water and dioxygen. Four classes of catalases have been defined, including mono functional hem-containing catalases, catalase peroxidases, manganese catalases, and minor catalases.

CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity:

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

Catalase protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. Catalase is a tetrameric enzyme consisting of four identical tetra hedrally arranged subunits of 60 kDa that contains a single ferri protoporphyrin group per subunit, and has a molecular mass of about 240 kDa (Aebi, 1980). CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity. Catalase catalyzes the reduction of hydroperoxides, thereby protecting mammalian cells against oxidative damage. In addition, catalase is active in neutralizing reactive oxygen species and so removes cellular superoxide and peroxides before they react with metal catalysts to form more reactive species. Catalase protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. So our approach was to purify the catalase enzyme from *Azolla* and its characterization.

Materials and Methods

Azolla cultivation and maintenance

Azolla sample was collected from Tamilnadu Agricultural University, Coimbatore. About 0.5-1kg of pure mother *azolla* culture seed material was spread uniformly over the water, after mild stirring of soil and water in the *azolla* bed. Fresh water was sprinkled over the azolla immediately after inoculation to make the *azolla* plants upright. A mixture of 20 g of Super Phosphate and about 1 kg of cow dung was added once in 5 days in order to maintain rapid multiplication of the *azolla* and to maintain the daily yield of 500g. A micronutrient mix containing magnesium, iron, copper, sulphur etc., was added at weekly intervals to enhance the mineral content of *azolla.* About 5 kg of bed soil was replaced with fresh soil, once in 30 days, to avoid nitrogen build up and prevent micro-nutrient deficiency. 25 to 30 percent of the water also needed to be replaced with fresh water, once every 10 days, to prevent nitrogen build up in the bed.

Conditions maintained for *Azolla* growth

Temperature 20°C - 28°C
Light 50% full sunlight

Relative Humidity 65 - 80%
Water (standing in the tank) 5 - 12 cm
pH 4-7.5

Extraction preparation

The *Azolla* fern was collected from the upper position of bed and used for further enzyme purification. 10g of *Azolla* fern was crushed with 50 ml of cold 0.067M Phosphate buffer (pH 7) using homogenizer.

Phytochemical screening

Phytochemical analyses were carried out according to the methods described by Trease and Evans (1989) of the crude extract of *Azolla* for the identification of phytochemicals like, alkaloids, saponin, steroids, flavonoids, cardiac glycosides, cynogenic glycosides and phlobatannins.

Antioxidant, Protein and Phenolic Determination

Protein determination

Protein content in the supernatant was estimated by the method described by Lowry et al. (1951). 0.2 ml and 0.4 ml of *Azolla* extract was used to determine the protein content.

Total phenolic content

The concentration of phenolic compounds in the extract was determined as described by Jayaprakasha et al. (2001) and results were expressed as tannic acids equivalents. The extracts were dissolved in a mixture of methanol and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of tenfold diluted Folin–Ciocalteu reagents and 0.8 ml of 7.5% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm. The estimation of phenolic compounds in the fractions was carried out in triplicate and the results were averaged.

Total antioxidant content (Prieto et al. 1999)

An aliquot of each extracts (0.05ml) were mixed with 0.5ml of reagent (0.6M H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate) in 1.5ml eppendorff tube. The tubes were capped and boiled in a boiling water bath at 95ºC for 90 min and cooled. The absorbance of each sample was measured at 695nm against blank in a spectrophotometer. A typical blank contained 0.5ml of reagent solution and 0.05ml of buffer and treated in the same manner as test. The antioxidant capacity was expressed as micromoles of ascorbic acid equivalents of antioxidant capacity.

Catalase enzyme assay (Luck, 1974)

Catalase activity was measured spectrophotometrically by monitoring the decrease in A_{240} resulting from the elimination of H_2O_2, using a Hitachi U-3210 spectrophotometer. The standard reaction mixture for the assay contained 0.067M potassium phosphate buffer (pH 7.0), 30mM H_2O_2, and 30µl of catalase-containing solution for a total volume of 3.0 ml.

On decomposition of hydrogen peroxide by catalase, the absorption decreased with time. Noted the time required for a decrease in absorbance from 0.45-0.40. The enzyme activity arrived at from this point. The extinction coefficient of hydrogen peroxide at 240 nm was assumed to be 0.036 µm/ml and one unit (U) of catalase activity was defined as the amount of enzyme required to degrade this 1µm/ml of hydrogen peroxide.

Purification of catalase enzyme (Tony ching and Gordin, 1973)

The supernatant brought to 45-90% saturation and kept at over night to precipitate the enzyme. The supernatant was centrifuged at 10,000rpm for 30mins to purify the enzyme. The purified enzyme was subjected to dialysis against 50mM Pottasium phosphate buffer (pH 7) for changing the buffer thrice at cold condition. The dialyzed sample considered as partially purified and subjected for further purification. A column (1 by 3cm) of DEAE-cellulose equilibrated with buffer A. Thirty milliliters of the dialyzed enzyme solution was applied to the column and washed with 4 ml of buffer A. The column was eluted with distilled water. Active fraction detected by the standard assay method were collected and pooled for subsequent Sephadex G-75 column.

A column of (0.5 X 5 cm) of sephadex G-75 that had been equilibrated with buffer A. The active fractions (8 ml) were diluted to 32 ml with the buffer A in order to reduce the salt concentration.

The fraction was then applied to the column and washed with the 3 ml of buffer A. Elution was performed with a continuous linear gradient of 0 to 0.5M NaCl in buffer A (total volume 15 ml) and then with 3 ml of 0.5M NaCl in buffer A. The volume of 1 fraction was 1 ml. the active fractions (2 ml) were further purified by rechromatography on the same column and under elution conditions. Then the enzyme were stored at 4°C and used for the characterization of the enzyme.

Molecular weight determination

Purified catalase enzyme was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel as per method by Sambrook and Russel (2001) along with standard molecular weight protein markers. The sample and marker proteins were treated with 2% SDS and 5% 2- mercaptoethanol at 100°C for 5 min just before loading. The gel was stained with Coomassie-brilliant blue R-250.

Physio-chemical properties of catalase

Analysis of pH profile of catalase

The pH profiles for the activity of catalase was obtained using 50mM Pottasium phosphate buffer (pH 3 to pH 8). In each pH buffer (1 ml), 30µl of enzyme solution was mixed and kept for overnight at 4°C. After this period, enzyme activity of sample was analyzed under the standard assay condition.

Analysis of thermostability of catalase

Thirty micro liters of catalase was placed in water bath at a temperature of 10, 20, 30, and 40°C for periods of 60 min. Enzyme activity after treatment was analyzed under the standard assay condition.

Storage stability

Thirty microliters catalase was mixed with 1ml of 50mM Pottasium phosphate buffer (pH 7) buffer and stored at the 4°C. Enzyme activity was analyzed every day up to 1 week in order to find out the storage stability of enzymes.

Results and Discussion

Phytochemical analysis

The extract of *Azolla* contains alkaloids, saponin, steroids, flavonoids, cardiac glycosides and cynogenic glycoside. Phlobatannins was absent. The observed results were tabulated (Table 1).

Protein estimation

Standard graph was plotted for bovine serum albumin using spectrophotometric reading at 660 nm. Total protein obtained for *Azolla* was 20.5 mg/g (Table 2).

Total antioxidant and phenolic content

The absorbance of the extract of each tube was measured for total anti oxidant content at 695 nm against blank it was observed that leaves contain 13.71 mg/g.

The absorbance was measured at 765 nm for Phenolic content. The estimation of phenolic compound in the fractions was carried out in triplicate and the results were averaged. *Azolla* extract contained 11.56 mg/g. The results were tabulated in table 2.

Catalase assay

Catalase assay was performed for the mushroom extracts supernatant, enzyme activity were measured at 240nm wavelength. Time taken for the reduction of A_{240} values from 0.45-0.4 was noticed. The enzyme activity for *Azolla* was 121 U/ml.

Purification and molecular weight of catalase from *Azolla*

The enzyme eluted as a single species in the initial DEAE-cellulose chromatography step. Additional purification of the enzyme was achieved by gel filtration on Sephadex G-75. Table 3 summarizes the results of each step of the catalase purification.

The enzyme was purified about 51.07-fold, with a final specific activity of 14.3U/mg. The overall recovery of the purification was 14%. The molecular mass of purified catalase was 55 kDa (Figure 1).

Effect of pH on catalase activity

The pH profile on the activity of catalase enzymes from *Azolla* was shown in figure 2. The optimum pH for the strains of *Azolla* was pH 7. All the catalase enzymes had a minimum activity at pH 3.5.

Effect of temperature and storage stability of catalase activity

Purified catalase from *Azolla* was stable in 50mM Pottasium phosphate buffer (pH 7) for more than 4 days at 4°C, as a decrease in activity observed after 5 days (Figure 3). After being heated at 10-30°C for 60 minutes, catalase from *Azolla* retained 26.98 units/ml of the enzyme activity measured at 30°C for 60 minutes but became completely inactive after treatment between 40-60 °C for 60 minutes.

Storage stability of purified catalase from *Azolla*

Purified catalase from *Azolla* was stable in Potassium phosphate buffer (pH 7) for more than 4 days at 4°C, as a decrease in activity observed after 5 days (Figure 4). *Azolla* catalase was also completely inactive under heat treatment between 40-60°C for 30 minutes. This catalase enzyme has 28.08 units/ml of activity after being heated at 10-30°C for 60 minutes.

The main aim of analyzing the phytochemical properties of *Azolla* is to determine its biochemical composition. The phytochemical analysis of *Azolla* showed that the presence of metabolic compounds. Protein content of *Azolla* taken for analysis revealed that the presence of total protein.

Table.1 Phytochemical analysis of *Azolla*

Phytochemical constituents	Observation
Alkaloids	+
Steriods	+
Saponin	+
Cynogenic glycosides	+
Cardiac glycosides	+
Flavonoids	+
Phlobatannins	-
Phenols	+

'+' – Present '-'–Absent

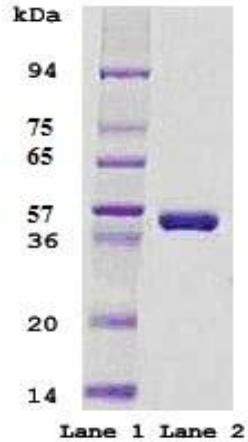
Table.2 Total anti oxidant and phenol content of *Azolla*

Sample extraction	Protein Content OD at 660nm (mg/g)	Anti oxidant content OD at 695 nm (mg/g)	Phenolic content OD at 765 nm (mg/g)
Azolla extract	20.5	13.71	11.56

Table.3 Purification of catalase from *Azolla*

S.No	Purification step	Total amount of protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
1	Supernatant	430	121	0.28	100	1
2	Dialyzsate	92	82	0.89	67.7	3.1
3	DEAE- cellulose	87	52	1.67	42.9	5.9
4	Sephadox G-75 fraction	1.2	17.2	14.3	14	51.07

Fig.1 Molecular mass of purified catalase:



Lane 1 Marker
Lane 2 *Azolla* catalase

Figure 2. Effect of pH on Catalase activity

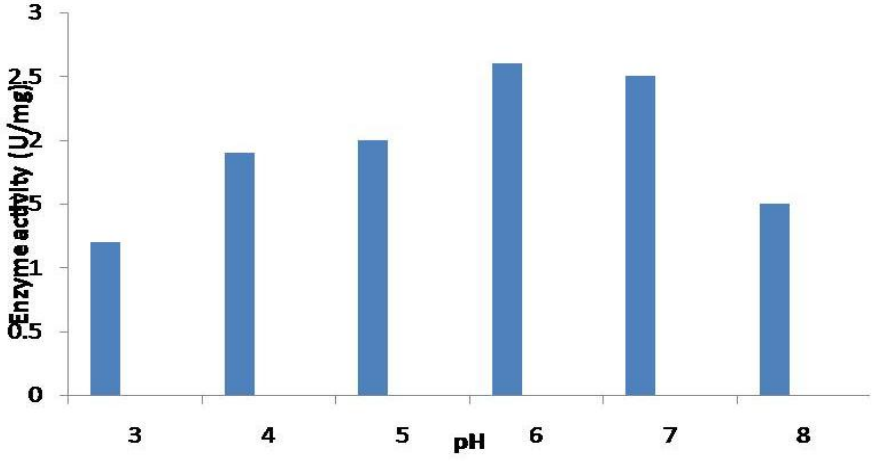


Figure 3. Temperature stability of Catalase

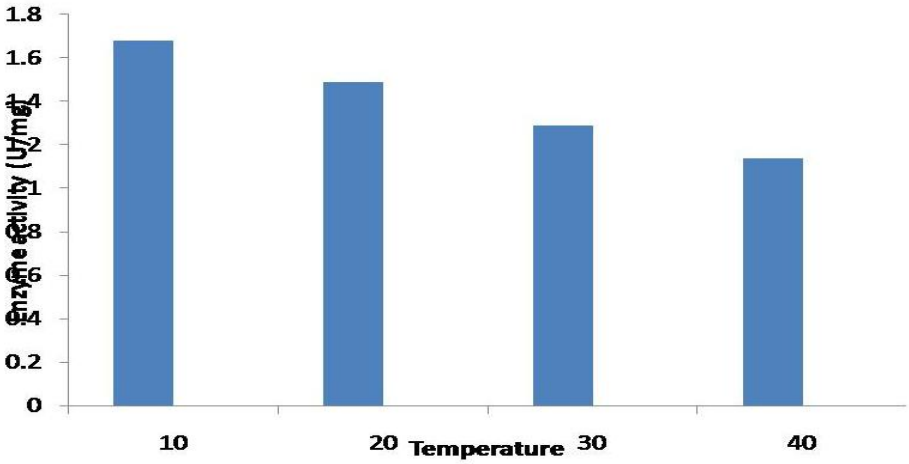
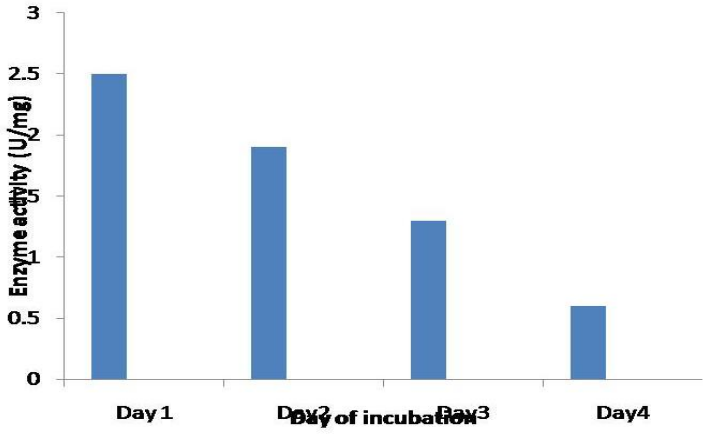


Figure 4. Storage stability of Catalase



Catalase is wide spread in nature having been found in all aerobic organisms studied to date. Most of the work has been performed on the enzyme obtained from mammalian and bacterial sources where enzyme activity was present in high concentration. Previous studies had shown catalase exist in multiple forms in several plants such as tobacco, saffron, cotton, mustard, maize, wheat, sunflower, castor bean, spinach, pepper, loblolly pine and kohlrabi (Havir and McHale, 1987). Beevers, (1979) and Huang et al. (1983) reported that catalase catalyzes the decomposition of H_2O_2 produced from the β-oxidation of fatty acids. In the present study, catalase enzyme from *Azolla* was 12.05 U/ml.

Most catalases described until now contain four equally sized subunits with each possessing a ferric heme (protoporphyrin IX), and are tetramers with a relative molecular weight in the range from 225,000 to 270,000 Da (Dunford and Stillman 1976). Although a large amount of information about the physiological function of catalase is now available (Yang and DePierre 1998), the information on the studies of catalase for amphibia has been scarce until now.

Catalase activity of *Azolla* was determined in the presence of hydrogen peroxide. The peak specific activity of catalase was 14.3 U/mg observed. There was rise in specific activity in each purification step. Loewen et al. (1987) were observed the rise of specific enzyme activity in every purification step. The native PAGE method developed by Hedrick and Smith (1968) provides site estimation for proteins analyzed on gels of successive high acrylamide concentration. As it has been mentioned above, the molecular weight of catalase T is between 225 - 250 kD, and for catalase A – considerably lower 170 - 190 kD. Ultracentrifugation studies of purified mouse hepatic catalase revealed that 5-7% of the total material consists of a form with a higher molecular weight than the bulk of the catalase. The two components were separated by sucrose-gradient centrifugation. Polyacrylamide-gel electrophoresis (in borate buffer) demonstrated that high molecular weight catalase is enriched in a more slowly migrating component, and sodium dodecyl sulphate polyacrylamide gel-electrophoresis demonstrated that the molecular weight of the subunits of the high-molecular-weight material is identical with that of the subunits of the major form. These results suggest that high-molecular-weight catalase consists of subunits that are not markedly distinct from those present in the normal catalase tetramer.

Noted that the most interesting characteristics of catalase from *Azolla* was the optimum pH and temperature. The optimum temperature and optimum pH for purified catalase from *Azolla* on enzymatic reaction were 30°C and pH 7 respectively. Singh et al. (2007) studied on *Archaeoglobus fulgidus,* the purification, and characterization of catalase at various pH and found the similar pH characterization. Similar temperature and pH has also been reported by Aydemir and Kuru (2003) on catalase enzyme of blood erythrocytes.

Azolla is a small aquatic fern which floats on the water surface. It contains within its leaf cavities a symbiotic cyanobacterium– Anabaena azolae. The N-fixing capacity of Anabaena azolae enables *Azolla* to thrive on nitrogen-free waters. The importance of *Azolla* for lowland rice production has been evaluated in numerous investigations. After an investigation for several years, we found that *Azolla* is also a promising plant to be applied in controlled ecological life support system.

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