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Effect of Desiccation on the Isozyme Profile of Antioxidative Enzyme Catalase in Cyanobacterium Lyngbya Arboricola

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Abstract:
Lyngbya arboricola inhabiting the bark of Mangifera indica is known to survive desiccation for a long period. The possible role of the antioxidative enzyme catalase in providing tolerance to the cyanobacterium was studied in desiccated mats both in dark and light conditions. An increase in catalase activity was observed in growing and desiccated (dark, light) conditions. But the increase was upto160% in dark desiccated mats whereas it was only 50% in growing mats. The catalase isoforms were studied with the help of the antibody developed against catalase (anticatalase). The partial purification of the enzyme was carried out on FPLC, a gel filtration column chromatography after sequential precipitation with acetone. The isozyme profile of catalase showed that there was induction of new isoform in mats desiccated in dark in addition to the three isoforms present in the growing mats. These three isoforms were in their native state which was revealed by the immunodiffusion experiments. Residual enzyme activity followed by Quantitative precipitin assay also suggested that there was formation of a new isozyme in addition to those present in the freshly collected mats. There was inactivation of the two isoforms in mats desiccated in light condition which can be inferred from the thickness of the CBB R250 stained precipitin lines.

Keywords: *Antioxidant enzyme, catalase, desiccation, column chromatography, Quantitative Precipitation, immunodiffusion,*

1. Introduction

Cyanobacteria (blue green algae) are thought to have originated during the Precambrian era and are the only group of organisms next to bacteria that have sustained large variations in habitats. Cyanobacteria of extreme habitats are known to withstand various environmental stresses such as water, temperature, light, pH etc. Thus, they are considered as model organism to understand the basis of tolerance and survival in a wide range of stress, especially water stress. It has been seen that on rewetting the cyanobacterial mats they have regained their normal metabolic function, even after 30 years (Potts 1994). The response of prokaryotic cells to desiccation at molecular level has been implemented to air dry human (eukaryotic) cells (Potts et al. 2005). An understanding of the tolerance of cells to almost complete removal of intracellular water (anhyhydrobiosis) may find its application in genetic engineering, seed storage, gene banks and the preservation of dry foods and pharmaceutical products.

A common feature of plants exposed to water stress is their potential to increase the production of reactive oxygen species (ROS) in their tissues (Menconi et al. 1995, Price et al. 1991). Although ROS is produced during normal metabolism in electron transport chains of respiration and photosynthesis (Halliwell 1984) but desiccation enhances their production (Smirnoff 1993). The ROS are highly reactive and can damage proteins, chlorophylls, lipids and are linked to various cellular injuries, including membrane lipid peroxidation, protein denaturation and enzyme inactivation etc.

Cyanobacteria being the first oxygenic phototrophs on earth must have been the first organism to elaborate mechanism for the detoxification of partially reduced ROS originating from one (i.e. O^{-}_{2} or HO^{-}_{2}) and two electron reduction of O_2 (H_2O_2) (Regelsberger et al. 2002). But surprisingly there is a lack of knowledge on the type, role and the mechanism(s) of peroxide degrading enzymes in these cyanobacteria (Bernroitner et al. 2009). Desiccation tolerant organisms seem to apply two strategy to cope with the danger of O_2 toxicity (1) increased efficiency of antioxidant defense mechanism and (2) metabolic control of both energy producing and energy consuming processes (Oliver et al. 2001). The desiccation tolerant plants are shown to be associated with up regulation of antioxidant genes (McKersie et al. 1999, Hsieh et al. 2002). In Baker's yeast over expression of superoxide dismutase exhibited increased tolerance to dehydration (Pereira et al. 2003). In higher plants like sunflower and bean seeds as well as in maize leaves catalase activity is shown to be increased during dehydration (Bailly et al. 2001, 2004; Jiang and Zhang 2002). *Tolypothrix scytonemoides,* a cyanobacterium is also reported an increase in enzyme activity and band intensity on gel following desiccation (Rajendran et al. 2007).

Cyanobacteria inhabiting the surfaces of bark of trees in tropical countries like India faces frequent drying and wetting during rainy season and are found invariably exposed to extremes of desiccation and irradiation of solar light (Tripathi et al. 1991). *Lyngbya arboricola* inhabiting the bark surface of Mangifera indica is one such cyanobacterium which survives desiccation. The present study has been carried out to understand the role of antioxidative enzyme catalase in desiccation tolerance of the cyanobacterium with the help of anticatalase produced in the rabbit to understand the survival strategy of the cyanobacterium at its natural habitat.

2. Materials and Methods

The desiccation tolerant cyanobacterium *L. arboricola* was isolated from the bark of *Mangifera indica* from the campus of Banaras Hindu University, Varanasi (India).

Desiccation treatment: The mats were desiccated over silica gel in dark at 25°C±1°C and in light intensity of 600 µmol m^{-2} s^{-1} for 12 weeks.

Incubation for growth: The cyanobacterial samples collected were placed over filter paper soaked in double distilled water and incubated in light intensity of 35µmol$m^{-2}s^{-1}$ at 25°C±1°C.

Preparation of cell free extract: The cyanobacterial material (20gm) crushed in liquid nitrogen was homogenized in 200 ml extraction buffer (100 mM potassium phosphate buffer, pH 7.5) at 4°C. The homogenate was disintegrated at 4°C by using ultrasonicator (Sonics & Materials,USA) at 130W, 20 kHz for 5 min at 50% amplitude and centrifuged at 25,000 g for 1 h at 4°C. The supernatant was filtered with filter (Millipore 45µm) and was used as cell free extract for enzyme assays and further purification of catalase. Protein estimation was performed by using the method described by Lowry et al. (1951) by using lysozyme as standard.

Purification of CAT: Acetone precipitation The enrichment of the enzyme in the cell --free extract was done by 40% (v/v) acetone precipitation method for 12h at 4°C. The extract was then centrifuged for 15 min at 17,000g. The pellet obtained was lyophilized to remove acetone.

Chloroform and ethanol treatment. The catalase enriched pellet was dissolved in extraction buffer and further treated with ethanol and chloroform in the ratio of 10:5:3 (sample: ethanol: chloroform) by vortexing for 5 min at 4°C . The uppermost fraction obtained after centrifugation at 5000rpm for 30min at 4°C was lyophilized and dissolved in KPB (extraction buffer). The sample was filtered with 22µm filter (Millipore). The absorbance was measured at 240 and 280nm and the ratio was calculated.

Gel filtration chromatography: Gel filtration of the above sample was carried out by using Sephacryl S 300HR matrix packed in a FPLC column. (GE Healthcare, USA). The column was loaded with 0.5 ml sample (2 mg protein ml^{-1}) in 500 µl loop and gel filtration was carried out at a flow rate of 0.5 ml min^{-1}. The fraction showing enzyme activity was pooled and concentrated in a dialysis sac. The absorbance was measured at 240 and 280 nm and the ratio was calculated.

Enzyme assay: Activity of CAT was determined by monitoring the consumption of H_2O_2 (extinction coefficient 39.4Mm cm^{-1}) at 240 nm for 1 min at 15 sec intervals in 3 ml reaction mixture containing potassium phosphate (100mM pH 7.0) with 0.2 ml enzyme extract (1mg protein ml^{-1}). The reaction was initiated by adding 10ul of H_2O_2 (30% v/v) (Rao et al. 1996).

Native PAGE Cell free extracts with equal amount of protein were subjected to discontinuous PAGE at 8% under non denaturing non reducing conditions as described by Laemmli (1970), supported by 10% glycerol (Mittler and Zilinskas 1993). Electrophoresis separation was performed at 4°C for 4 h with a constant current of 30mA per gel and after completion the was stained for activities of CAT. The staining was done by incubating the gel in a solution of H_2O_2 (.003% v/v) for 10 min. After washing the gel with double distilled water a solution of freshly prepared 1% ferric chloride and 1% potassium ferricyanide was poured over it and shaken till white regions showing the CAT isoforms appeared against dark blue background.

Electroelution Technique: Electroelution of individual isoform was carried out on electroeluter (Bio-Rad Model 422). Native-PAGE of catalase was performed as above. One of the lanes in each 10 gel was stained for detecting the enzyme activity and gel-slices corresponding to the individual isoforms were cut from the rest of the lanes. The running buffer was same as used for PAGE. The collection buffer used was Tris HCl 20mM (pH 7.5), DTT (5mM). The Electroelution was carried out at 10 mA per tube for 4 h at 4˚C. The eluate was diluted with fresh collection buffer and concentrated with the help of Ultracel YM-100 (10 kDa cut-off, Millipore USA) to the protein concentration of 1mg ml^{-1}.

Anticatalase production: The partial purified catalase after gel filtration chromatography was used for the production of anticatalase. The enzyme fraction was brought into PBS (phosphate buffer saline, pH 7.4). The antigen (catalase) solution (0.5ml) containing 1mg protein ml^{-1} was mixed with an equal volume of Freund's complete adjuvant (Sigma, USA). The mixture was injected intramuscularly into thigh muscle of a rabbit having 3kg weight. The consecutive three injections were given once a week with the same concentration of antigen but with incomplete Freund's Adjuvant. The booster dose was given with 1ml antigen (1mg ml^{-1}) mixed with an equal amount of incomplete adjuvant.

Blood collection and serum preparation: Blood sample (5 ml) was collected after 7 days of the booster dose by using syringe and needle which was washed with anticoagulant (Acid Citrate Dextrose-ACD) from marginal veins of ear of the rabbit under aseptic conditions. The collected blood sample was allowed to stand in glass tubes for 2 h at room temperature then it was kept at 4°C for overnight. The sample was then centrifuged at 5000rpm for 15 min and the supernatant was collected. The antiserum was precipitated with 33% (v/v) saturated $(NH_4)_2SO_4$ solution for 12 h at 4° C . The precipitate so obtained was dissolved in PBS buffer (pH 7.4) and was dialysed against the same buffer at 4°C for 12 h. The anticatalase (IgG) thus obtained was used for the immunodiffusion experiments and quantitative immunoassay.

Immunodiffusion Experiment: Ouchterlony's double diffusion precipitin technique was used for detection of the antibody in the antiserum (Ouchterlony 1949). The diffusion plates were prepared in a Petri-dish by using 1% (w/v) agarose in PBS containing NaN_3

(0.5% w/v) at pH 7.4. The wells were prepared in the gel by cutting with stainless steel cork borer (6mm diameter) at a distance of 1.5 cm between two wells.

Plate 1: The central well was filled with the 40µl antiserum (4mg protein ml^{-1}). The peripheral wells were filled with 40µl catalase isoforms (1mg protein ml^{-1}) obtained after electro elution. The plate was allowed to stand for 10 min at room temperature then it was kept in dark and moist chamber at 4^oC for 12 h.

Plate 2: The central well (1) was filled with the 40µl antiserum (4mg protein ml-1). The peripheral wells were filled with ethanol fraction of catalase (40µg each) from desiccated mats in dark (2) and light (3). The plate was allowed to stand for 10 min at room temperature then it was kept in dark and moist chamber at 4^oC for 12 h. The plate was thoroughly washed with 0.5M NaCl solution and stained with CBBR

Quantitative precipitin assay: The catalase fraction obtained after acetone precipitation followed by ethanol, chloroform treatment was used in this experiment. Varying amount of catalase (50 to 350µg) was added to 50µl of anticatalase and the volume of each solution was maintained to 1ml with PBS buffer. Then it was allowed to stand overnight at 4^o C . The solution was centrifuged in eppendorf microfuge for 5 min and the precipitate was collected. The protein content of the precipitate was determined and a graph was plotted between catalase and the precipitate (protein). The point of equivalence was calculated from this graph. This amount of calculated catalase from the growing mats as well as two months desiccated mats in dark and light were then used to precipitate same amount of IgG as above. The precipitates were removed by centrifugation and the enzyme activity was measured in the supernatants separately.

3. Results

Catalase activity and Native PAGE: The catalase activity in cell free extract of growing and one and two months desiccated mats in dark and light were compared. It was seen that the enzyme activity was increased with the increase in incubation period in case of mats desiccated in dark but the mats desiccated in light shows initial increase then decrease in catalase activity (Figure 1). The activity staining of catalase of desiccated mats in dark shows increase in band intensities of two lower isoforms in addition to some faint bands between the first and second band. The over expression of lower two bands was also seen in case of light desiccated mats but the upper portion of the gel show smear formation which suggests inactivation of the isoform(s) (Figure 2).

Purification of catalase: The catalase isoforms have been purified from two months desiccated mats of *L.arboricola*. The cell free extract of the cyanobacterium was precipitated with acetone which results in enrichment of the enzyme in the pellet. The treatment of the sample with ethanol and chloroform effectively removes phycobiliproteins and mycosporin like amino acids (MAAs) from the enzyme fraction. The ethanol fraction showing enzyme activity was further subjected to gel filtration chromatography on FPLC. The elution profile of FPLC shows two major peaks one from fraction number 12 to 20 and other from 21 to 27(Figure 3) . The peaks were separately collected and tested for enzyme activity. The activity was seen in both of these peaks so these fractions were pooled together. Further purification of the gel filtered enzyme fractions on ion exchange chromatography (DEAE Sephadex A-50) results in loss of some isozymes so this purification step was avoided. The specific activity was calculated at each step of purification and it shows an increase from 2.2 U/mg in crude extract to 9.46 U/mg in FPLC eluted enzyme fraction.

Anticatalase production and detection: Antibody against purified catalase enzyme was produced in the rabbit. The three isoforms electroeluted separately from the growing mats of *L. arboricola* were tested for their presence in desiccated mats in dark.The appearance of precipitin lines against all the three isozymes suggest that these isoforms are present in the desiccated mats in their native state (Figure 4). The immune diffusion experiment was also performed with the partially purified catalase (ethanol fraction) from mats desiccated in light and dark. The precipitin lines obtained show the difference in thickness of the two lines. The precipitin line obtained in case of dark desiccated mats was thicker than the line obtained from desiccated mats in light and seems that the two precipitin lines have been fused in case of mats desiccated in dark(Figure 5) .

Quantitative precipitin assay (QPA) and residual enzyme activity: The point of equivalent was calculated with catalase from growing mats from the graph of QPA**.** It was seen that 50µl of IgG was completely precipitated by 135.59 µg of catalase. This amount of catalase was then used from desiccated mats in dark and light to precipitate 50µl antiserum. After removal of the antigen antibody complex (precipitate), the supernatant was tested for the enzyme activity. The supernatant of two months desiccated mats in dark shows maximum enzyme activity followed by desiccated mats in light and the growing mats shows negligible activity (Figure 7).

4. Discussion

Catalase is an important antioxidative enzyme which plays a key role in surviving acute water deficit. Cytoplasmic catalase maintains the intracellular redox balance and also prevents oxidative damage to membranes during dehydration (Franca et al.2005;Bailly et al.2004). The increase in catalase activity in *L. arboricola* during desiccation has been reported (Tripathi et al. 2001) and the study undertaken here investigates its possible role in desiccation tolerance.

The results of present study show that the activity of catalase increases on increasing the desiccation period in dark and light for 2 months but on further increasing the incubation period for 3 months, the enzyme activity decreases in case of light desiccated mats. The activity has increased more than two fold in desiccated cells in dark for 3 months when compared with the growing mats. This is in accordance with the results found in sunflower and bean seeds, as well as in maize leaves where catalase activity increases during dehydration (Bailly et al. 2001.2004; Jiang and Zhang 2002). Some desiccation tolerant cyanobacteria like *Tolypothrix scytonemoides* and *L. arboricola* have also been reported to increase the catalase activity upon desiccation.

The activity staining on native PAGE shows that the two lower isoforms have increased in intensity whereas a faint band appears inbetween the first and second band which suggests that there is induction of new isoform as a result of desiccation. Srivalli et al.

(2003) also established an induction of new catalase isoform in severely water stressed rice seedlings. So this is very interesting to see that the filamentous cyanobacterium is adopting the survival strategy that is adopted by the higher plants. The native PAGE of catalase in case of desiccated mats in light shows inactivation of the upper isoform whereas the lower two isoforms shows an increase in intensity. The inactivation of the upper isoform can be explained by the phenomena of photo inactivation and the lower two bands shows no effect of radiation and are over expressed in desiccated mats in dark as well as in light. The role of first isoform in desiccation tolerance was confirmed by the double immunodiffusion experiment. All the three isoforms present in the growing mats show the formation of precipitin line with the anticatalase suggesting that all of these are present in their native state and play an important role in providing tolerance against desiccation. The response of different isoforms of catalase to different stress factors have been observed here in case of *L.arboricola*. This is in agreement with the results observed by many authors (Scandalios 1984; Tsang et al. 1991; Wang et al. 2004).

In the present study anticatalase has been used to probe the catalase which was produced in the rabbit. For the production of anticatalase the enzyme was to be purified from the crude extract of *L. arboricola* . The purification procedures adopted for the purification of catalase can be considered comparatively a suitable procedure for enzyme purification as compared to the purification of catalase-peroxidase from Synechococcus PCC 7942 (Mutsuda et al. 1996) and from *Anacystis nidulans* (Obinger et al.1997) and was more similar to the purification procedure adopted for higher plants (Shivali 2008) .The purification of the catalase was analysed at each step of purification by calculating the specific activity which shows an increases from crude extract to gel filtration. There are three types of catalases classified in the literature. The typical or monofunctional catalases, the catalase peroxidases that have both peroxidative and catalytic activity and the Mn-catalase or pseudocatalase (Zamocky and Koller 1999). It is known since 1923 (Tsuchihashi 1923, c.f. Goldberg and Hochman 1989) that though other proteins are denatured, the typical or monofunctional catalases arte stable against ethanol-chloroform and acetone treatment, but the denaturation of beef erythrocyte catalase is reported by Bonnichen (1955) under the same treatment. Typical catalases, the largest group of H_2O_2 dismutating enzymes that segregate early in the evolution and even occurring in anaerobic bacteria are unusual in cyanobacteria and till date is reported only in *Nostoc punctiforme* PCC73102 (Zamocky et al. 2008). However, acetone precipitation and ethanol-chloroform treatment adopted in the present case makes it quite apparent that the cyanobacterial catalases possesses hydrophobic properties and are likely to be typical catalases (Shivali *et al.* . 2013).

QPA was performed with partially purified catalase (ethanol fraction) from both dark and light desiccated cells and the enzyme activity was seen in the supernatants.The enzyme activity was maximum in dark desiccated mats followed by desiccated mats in light whereas no activity was observed in the case of growing mats. The presence of catalase activity in desiccated cells in dark and light can be explained by the over expression of lower isoforms in both cases and the formation of new isoform in case of dark desiccated mats. This result was further analysed by cross reacting the catalase with the anticatalase. The immunodiffusion plate stained with CBB R250 shows the difference in thickness of the two precipitin lines. The precipitin line formed with the catalase of desiccated mats in dark was thicker than the desiccated mats in light. The close view of the thick line reveals that two precipitin lines have been fused together, which suggests hat there is some structural differences between the new isoform formed and the isoforms already present in the non desiccated cells. Catalase isoforms under the control of different genes have been detected in higher plants (Ota et at. 1992). Chandlee et al. (1983) and Scandalios (1987) have also shown that the maize has three genetically different catalase molecules that are tissue specific and age dependent.

5. Acknowledgment

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6. References

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Annexure

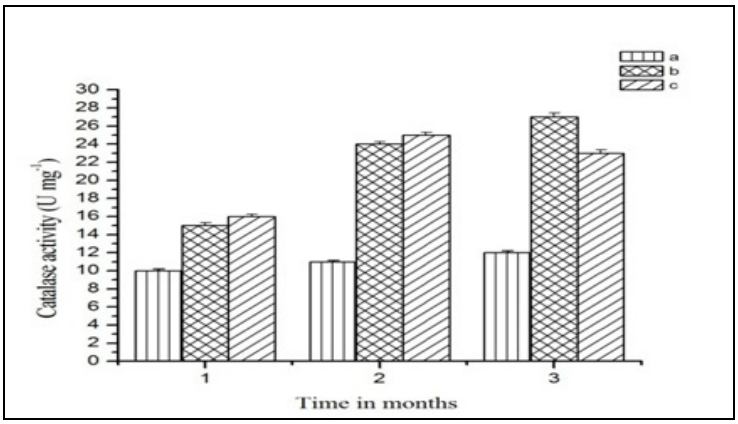

Figure 1: Catalase activity in L. arboricola (a-growing mats, b-desiccated mats in dark, c- desiccated mats in light).

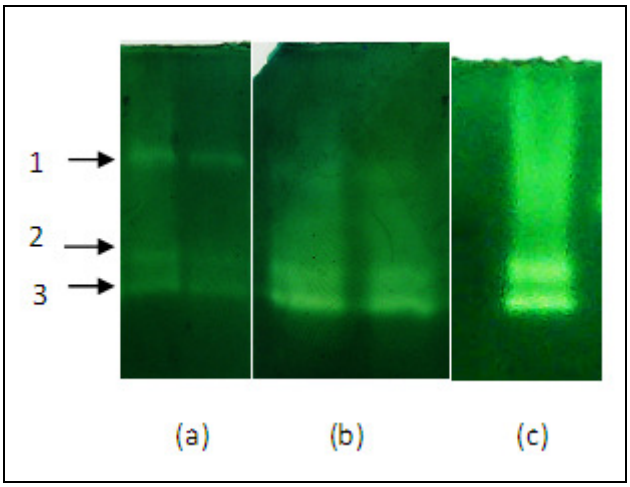

Figure 2: Catalase activity on Native PAGE of
(a) growing mats (b) desiccated mats in dark and (c) desiccated mats in light of L. arboricola after 3 months.

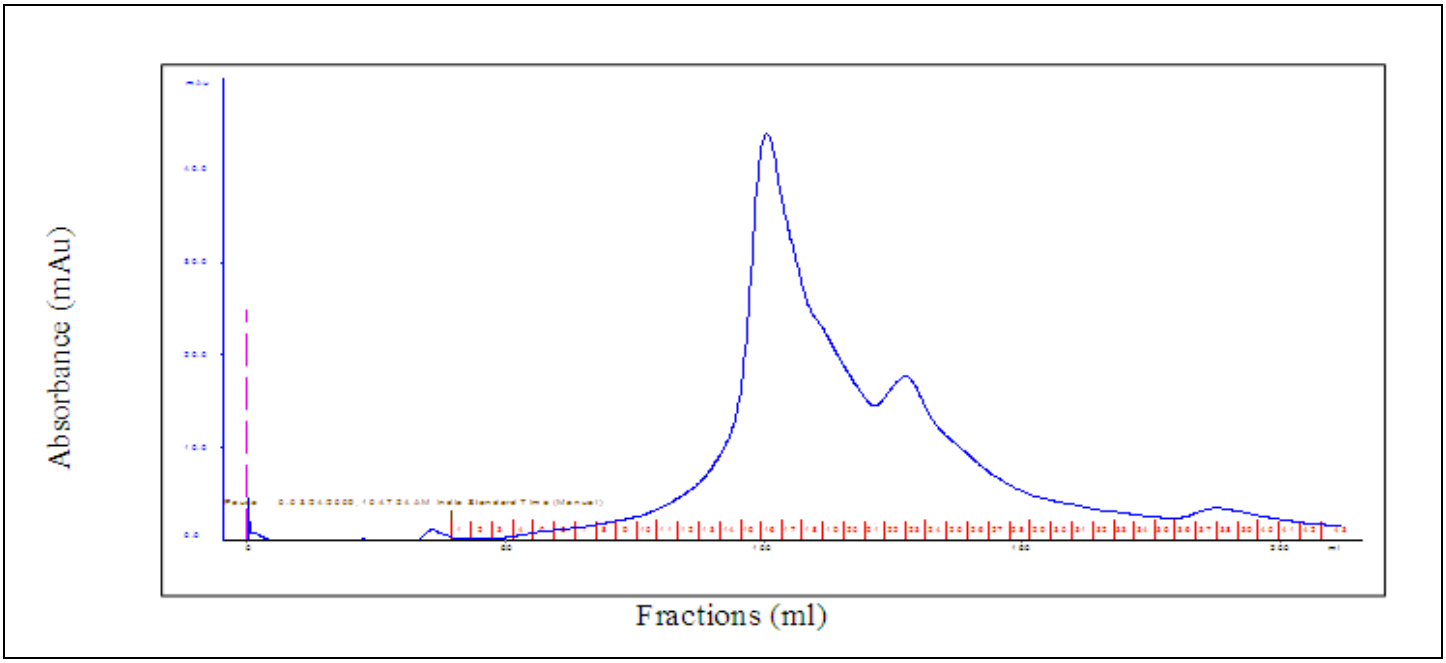

Figure 3: Elution curve of fractions obtained after gel filtration chromatography on Sephacryl S300 HR performed on FPLC.

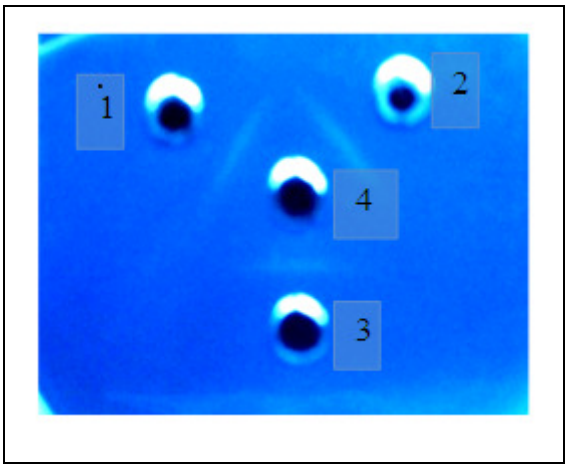

Figure 4: Double immunodiffusion plate showing precipitin lines. The three electroeluted isoforms of catalase (1, 2 and 3) from fresh mats shows the precipitin line formation against the anticatalase (4) developed from desiccated mats in dark.

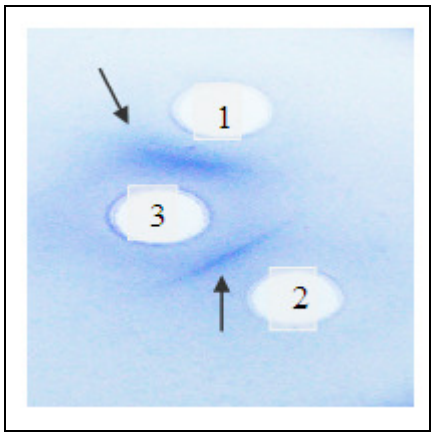

Figure 5: CBB R250 stained precipitin lines. The catalase from dark desiccated mats (1) shows thicker precipitin line formation than the catalase from light desiccated mats (2).

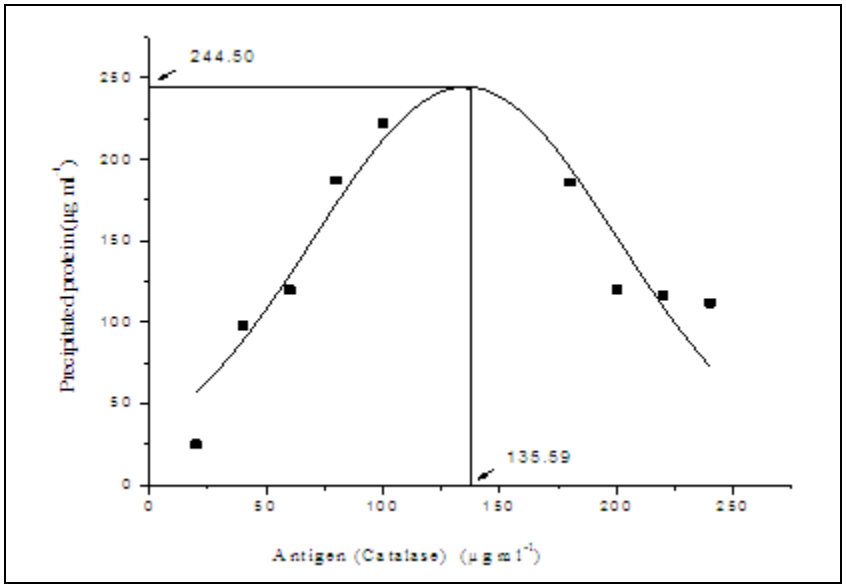

Figure 6: Precipitin curve of catalase with anticatalase on increasing antigen (catalase) concentration.

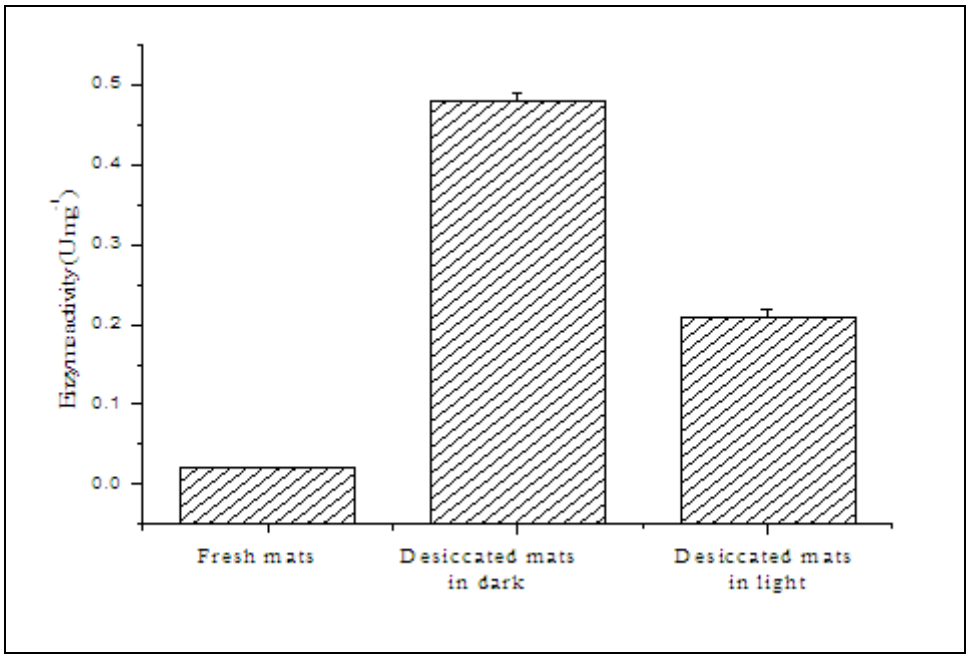

Figure 7: Residual catalase activity in the supernatant after removal of catalase-anticatalase (Ag:Ab) complex.