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Purification and characterization of catalase from sprouted black gram (*Vigna mungo*) seeds

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ABSTRACT

Black gram (*Vigna mungo*) is a legume which belongs to *Fabaceae* family. It is a rich source of protein. It has been known to have interesting small molecule antioxidant activity. However, its enzymatic antioxidant properties have not been explored much. In the present work we studied catalase, a principal antioxidant enzyme from black gram seeds. Day four sprouted black gram seeds were found to have a significant catalase content approximately of 15,240 U/g seeds. IMAC (Seph 4B-IDA-Zn(II)) was used for purifying this catalase, a purification fold of 106 and a high specific activity of 25,704 U/mg was obtained. The K_m and V_{max} of the purified catalase were found to be 16.2 mM and 2.5 μmol/min. The effect of inhibitors like Sodium azide (NaN_3) and EDTA and different metal ions on catalase activity were studied. NaN_3, Fe^{3+} and Cu^{2+} were found to have profound inhibitory effects on the enzyme activity. Other metal ions like Ni^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} had both enhancing and inhibitory effects. The enzyme showed optimal activity at a temperature of 40 °C and pH 7.0. It was stable over a broad range of pH 6.0–10.0 and had a half life of 7 h 30 min at 50 °C.

1. Introduction

All aerobic organisms during the course of metabolism form reactive oxygen species (ROS) as by products [1]. Superoxide ($^{\bullet}O_2^-$), nitric oxide ($^{\bullet}NO$), hydroxyl ion radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are the common ROS [1]. Mitochondria are the major intracellular source of ROS because of its respiratory chain [2]. The ROS when accumulated causes oxidative stress resulting in various pathological conditions like neurodegenerative diseases, cardiovascular diseases, cancer, ophthalmic disorders, etc. [3]. ROS are maintained under certain levels by a battery of enzymatic and non-enzymatic molecules with antioxidant capacity [4]. The enzymatic defense against oxidative stress primarily comprises of superoxide dismutase, catalase and glutathione peroxidases [4]. Catalase (CAT, H_2O_2: H_2O_2 oxidoreductase; EC 1.11.1.6) is one of the principle antioxidant enzymes. It is a heme-containing homotetramer of approximately 250 kDa found ubiquitously in both eukaryotes, prokaryotes [6] and scavenges $2H_2O_2$ to $2H_2O$ and O_2 [5]. Catalase has very high industrial significance with its applications varying from removing residual H_2O_2 from milk used in the process of cold pasteurization during cheese production [9,10] to fabrication of porous materials in textile industry.

Legumes are a rich source of protein [7,8]. Black gram (*Vigna mungo*) belonging to *Fabaceae* family serves as the major source of proteins among the pulse crops of India. It was chosen as the source material in this study for extraction and purification of catalase. The existing literature reports [11–13] on purification of catalase from plant sources demonstrated the use of two to four chromatographic steps with a purification fold of 6.25 [11], 138.4 [12] and 302.5 [13] respectively. Purification of catalase from plant sources using IMAC system has not been explored earlier. Although there are a few reports which discuss about the purification of catalase using IMAC from animal sources [16–18]. The present study deals with an efficient single step purification process for purifying catalase and characterization of the same from sprouted black gram seeds.

2. Materials and methods

2.1. Collection of plant material and growth conditions

Black gram (*Vigna mungo*) seeds identified as LBG-17 (unprocessed variety) [8] were purchased from the local market (Guntur, Andhra Pradesh, India) which was used as starting material for this study. The seeds were surface sterilized with 10% sodium hypochlorite and were washed thoroughly with tap water to remove traces of sodium hypochlorite. The washed seeds were soaked in sufficient

Abbreviations: CAT, H_2O_2, H_2O_2 oxidoreductase and EC 1.11.1.6: catalase; ROS, reactive oxygen species; BLC, bovine liver catalase; Seph, sepharose.

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volume of tap water and incubated in a dark place overnight at room temperature. After the overnight incubation, seeds were transferred to a moist layer of cotton and whatman filter paper and then were allowed to sprout until day 4. On day 4 the sprouted seeds were transferred to green house and were allowed to grow in to seedlings until day 15.

2.2. Enzyme extraction from sprouted black gram seeds

To 1 g of plant material (sprouted seeds from day 1 to 4 or seedlings from day5 to 9 or leafs from day10 to 15) 8% PVP, 10 mM PMSF, 4 mL of 50 mM phosphate buffer, pH 7.0 with 0.1 mM EDTA and 30 mM KCl was added and homogenized into a fine homogenous paste in a pre chilled pestle and mortar [11,12]. The extract was centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatant collected was quantified for its protein content and catalase activity.

2.3. Quantification of total protein

Protein estimation was determined using the method described by Bradford [19] using bovine serum albumin as the standard.

2.4. Spectrophotometric analysis of catalase activity

One unit of Catalase is defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 in a minute [20]. The substrate used for analysis was 10 mM H_2O_2 solution which was made in 50 mM phosphate buffer, pH 7.0 [21]. The reaction was started by adding 475 μL of substrate to 25 μL of diluted enzyme and the rate of decomposition of the H_2O_2 was determined spectrophotometrically at $\lambda_{240\,nm}$ per min. Catalase activity was calculated as described by Beers and Sizer [22].

2.5. Purification of catalase by IMAC

A 0.8 mm in diameter column was packed with 2 mL of sepharose 4B gel coupled with IDA and was washed thoroughly with 15 column volumes of ultra pure water (milli q). After the water wash sorbent was charged with 15 column volumes of 50 mM $ZnSO_4 \cdot 7H_2O$ solution. Excess metal from the column was removed by washing the column with ultra pure water and was later equilibrated with 15 column volumes of 50 mM phosphate buffer, pH 7.0 containing 0.5 M NaCl and 5 mM imidazole (equilibration buffer). After the equilibration 1.0 mL of 20% to 60% ammonium sulphate precipitate with a total protein concentration of 8 mg was loaded on to the column. After loading the sample, it was washed with 39 column volumes of equilibration buffer to remove the unbound proteins followed by elution with 66 column volumes of 10 mM imidazole and 15 column volumes of 25 mM imidazole in equilibration buffer. 3 mL fractions were collected at a flow rate of 1 mL/min. The eluates (10 mM imidazole and 25 mM imidazole) were pooled and concentrated to 1 mL each respectively with 50 kDa centricon filters (Millipore, India) and was stored at 4 °C until further analysis.

2.6. Determination of the purity of black gram catalase

2.6.1. SDS–PAGE

SDS–PAGE was performed according to the method described by laemmli, U.K. [38] using a 10% polyacrylamide gel. The 20–60% ammonium sulfate precipitate, non retained protein fraction, concentrated eluates of 10 mM and 25 mM elutions were treated with 10% SDS and heat denatured at 100 °C before loading on to the gel, the prepared samples were loaded on to the gel and electrophoresis was carried out at 100 V for 120 min. After the electrophoresis the gel was subjected to silver staining and the molecular weight was estimated with the help of a medium range (97.4–14.3 kDa) protein ladder (Genei).

2.6.2. Zymogram (Native-PAGE)

The 20–60% ammonium sulfate precipitate, non retained protein fraction and concentrated eluates from 10 mM, 25 mM imidazole elutions were loaded on to an 8% polyacrylamide gel in their native state. The electrophoresis was carried out at 50 V for 300 min at 4 °C. Catalase was qualitatively assayed as described by Woodbury et al. [23]. After the electrophoresis the 8% native PAGE gel was incubated with 50 ml of 10 mM H_2O_2 made in 50 mM phosphate buffer, pH 7.0 for 10 min followed by a quick rinsing with distilled water and staining with 1% potassium ferricyanide and ferric chloride solution.

2.7. Characterization studies

2.7.1. Determination of K_m and V_{max} of catalase

Different concentrations of H_2O_2 (5 mM to 25 mM) were prepared in 50 mM phosphate buffer, pH 7.0. Maintaining the Units of purified enzyme (10 mM eluate) constant, the rate of H_2O_2 decomposition was estimated spectrophotometrically at λ_{240nm}. Lineweaver–Burk plot was plotted with reciprocal values of rate (1/V) against the reciprocal values of substrate concentration (1/[S]) from which K_m and V_{max} of the enzyme were calculated [29].

2.7.2. Effect of inhibitors and metal ions on catalase activity

The effect of varying concentrations (0.5 mM to 2.0 mM) of inhibitors EDTA and sodium azide (NaN_3) and metal ions Fe^{3+}, Cu^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+} on catalase activity was determined. The concentrated eluate obtained from 10 mM imidazole elution was incubated for 15 min at room temperature (25 °C) with inhibitors made in 50 mM phosphate buffer, pH 7.0. Similarly with metal salt solutions made in 50 mM HEPES buffer, pH7.0. The percentage activity retained was calculated. For all the different inhibitor and metal salt concentrations the enzyme (U/mL) used for incubation was kept constant.

2.7.3. Determination of thermal stability of catalase

The concentrated eluate from 10 mM imidazole elution was incubated at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 15 min and the percentage activity retained was calculated. Enzyme half life was determined by incubating the enzyme for 24 h at 50 °C and by determining the catalase activity for every 30 min. The enzyme (U/mL) used for incubation at various temperatures was kept constant.

2.7.4. Effect of pH on catalase activity

The concentrated eluate obtained from 10 mM imidazole elution was analyzed for its pH stability. The enzyme activity was determined at different pH, pH 3.0, 4.0 and 5.0 (50 mM sodium acetate buffer), pH 6.0 and 7.0 (50 mM sodium phosphate buffer), pH 8.0 and 9.0 (50 mM tris–HCl buffer) and pH 10.0 (50 mM carbonate buffer). Incubation was done for a period of 15 min at room temperature (25 °C) and the percentage activity retained was calculated. The enzyme (U/mL) used for incubation at different pH was kept constant.

3. Results and discussion

3.1. Catalase activity during the germination of black gram seeds

In order to find out the best growth stage to obtain maximum catalase activity, specific activity of the catalase in black gram seeds was measured as a function of sprouting, seedling formation and up to level of fully grown plant (Fig. 1). Maximum specific activity was

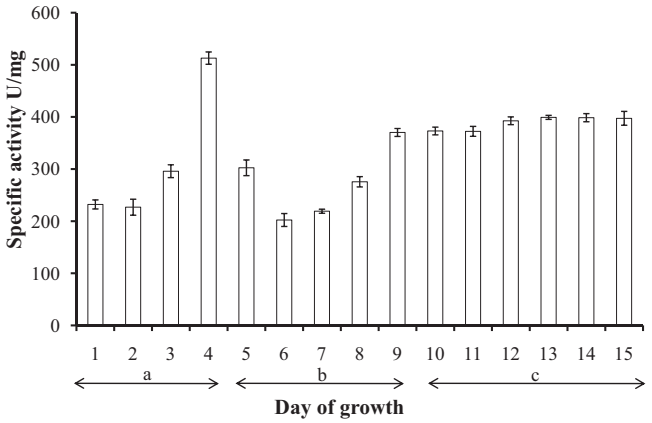

Fig. 1. Specific activity of catalase (U/mg). (a) sprouts (day1–4), (b) seedlings (day5–9) and (c) leafs of seedlings (day10–15) (mean ± SE, $n = 3$).

observed in sprouted seeds of day four. During the period of germination it is known that the stress levels are high, the high catalase activity found on day four might be attributed to the seeds reaction to combat stress [11]. During the evolution a clear decrease in the activity which shoots off slightly on day nine due to the secondary leaf formation was observed. In order to understand catalase from this widely used plant source and to compare catalase from other well known sources studied, we chose day four sprouted black gram seeds as a model for purifying and characterizing catalase.

3.2. Purification of catalase by IMAC

The turbid supernatant obtained after centrifugation of homogenized day four sprouted black gram seeds was subjected to 20–60% ammonium sulphate ($(NH_4)_2SO_4$) precipitation. The precipitate was dissolved in 50 mM phosphate buffer, pH 7.0 containing 30 mM KCl and dialyzed against the same for a period of 16 h with 4 buffer exchanges. The 20–60% $(NH_4)_2SO_4$ fraction having the maximum specific activity of 486 ± 25.8 U/mg and a total protein concentration of 8 mg/mL was purified by IMAC.

IMAC is an effective tool for purification of proteins, it works on the basis of affinity between surface accessible histidine residues of a protein molecule and the transition metal ion [14,15]. The four commonly used metal ions are Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} with each metal ion having unique demand of surface accessible histidine residues for interaction. For example Zn^{2+} and Co^{2+} are the metal ions which require clusters of histidine residues or more than two histidine residues located at specific intervals (His-x-x-x-His) [14] for them to interact with a protein molecule.

The 20–60% $(NH_4)_2SO_4$ fraction was loaded onto two individual columns (0.8 mm × 4 cm) of sepharose-4B-IDA-Cu(II) and Ni(II) which yielded 53% and 46% of catalase from 50 mM imidazole elution with a purification fold of 10.5 and 15.8, respectively (data not shown). In order to achieve a better purity of catalase next metal ion in the series Zn^{2+} was chosen, which has a different selectivity of histidine residues [14]. The sample was loaded on to sepharose-4B-IDA-Zn(II) column which was pre equilibrated with 50 mM phosphate buffer, pH 7.0 containing 0.5 M NaCl and 5 mM imidazole (equilibration buffer). The non retained protein fractions obtained by washing the column with equilibration buffer did not show any catalase activity (Fig. 2a). Elution was performed with increasing concentrations of imidazole in the form of step gradient. We obtained a percentage yield of 39.7 ± 5.7 with a purification fold of 106.8 ± 10 in 10 mM imidazole elution and a percentage yield of 10 ± 1 with 23 ± 1.9 fold purity in 25 mM imidazole elution (Table 1). The distribution of catalase in two different elutions depicting differential strength of binding towards the same metal

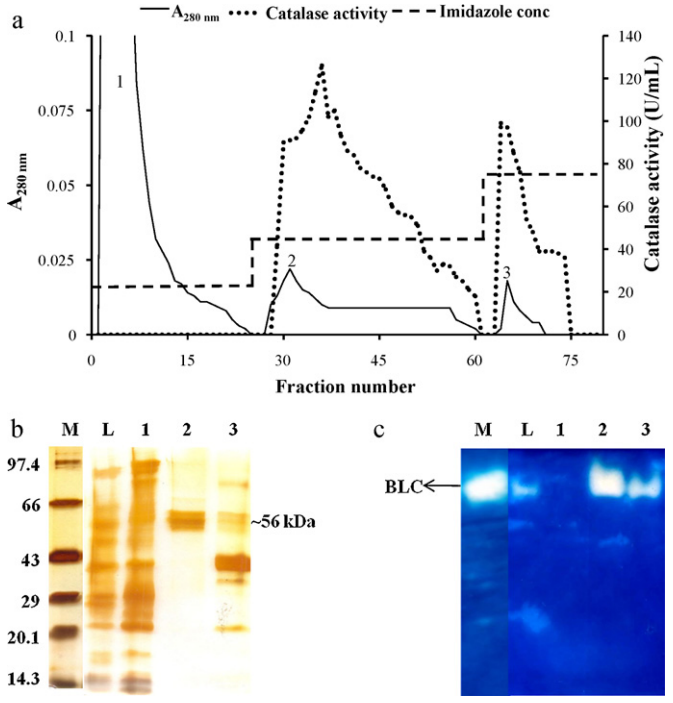

Fig. 2. (a) The chromatographic profile of catalase from black gram sprouted seeds using Seph 4B-IDA-Zn(II), Protein concentration in the load: 8 mg/mL, column was equilibrated with 50 mM phosphate buffer (pH-7.0) + 0.5 M NaCl + 5 mM imidazole, elution was performed with 10 mM and 25 mM imidazole in the equilibration buffer. (b) SDS–PAGE (10%), 2 μg protein concentration, lane M: low molecular weight protein marker, lane L: 20–60% $(NH_4)_2SO_4$ precipitate, lane1: non-retained fraction of Seph 4B IDA-Zn(II), lane2: concentrated eluate of 10 mM imidazole elution and lane3: concentrated eluate of 25 mM imidazole elution. (c) Zymogram (Native-PAGE) (8%) -H_2O_2 (substrate), ferricyanide-ferric chloride stained gel, 2 μg protein concentration, lane M: Bovine liver catalase (BLC), lane L: 20–60% $(NH_4)_2SO_4$ precipitate, lane1: non-retained fraction of Seph 4B IDA-Zn(II), lane 2: concentrated eluate of 10 mM imidazole elution and lane3: concentrated eluate of 25 mM imidazole elution.

might be due to isoform of enzyme with differential histidine exposure. However, further studies are required for the confirmation of this possibility. The high purity obtained using Zn^{2+} makes it the metal of choice for purification of black gram catalase. This corroborates with the work of Yang and DePierre [17] reporting the purification of catalase form mouse liver using Zn^{2+} metal ion. The selectivity of Zn^{2+} towards catalase compared to Cu^{2+} and Ni^{2+} can be attributed to the number of accessible histidine residues. As explained earlier Zn^{2+} metal ion requires clusters of accessible histidine residues in order to interact with a protein molecule. Approximately 10–19 exposed histidine residues are present on a native bovine liver catalase and it has been reported that catalase has a highly conserved protein sequence and structure [25,35,36]. This could be one of the reasons that explain the high purity of catalase achieved with Zn^{2+} as the metal ion.

The concentrated eluate fractions of 10 mM and 25 mM imidazole elutions were analyzed by SDS–PAGE and Zymogram (Native-PAGE). The 10 mM eluate (Lane 2, Fig. 2b) shows a single band corresponding to approximately 56 kDa, which could be the monomeric weight of catalase. It has been reported that monomers of catalase are not held together via disulphide bridges [36]. SDS treatment and heat denaturation were possibly able to disassociate the homotetramer in to monomers. Similarly, 25 mM eluate (lane 3, Fig. 2b) shows a less intense band at 56 kDa with other protein bands. The difference in the intensities of the bands observed between lane 2 and 3 of SDS–PAGE and Zymogram (Native-PAGE) (Fig. 2b and IIc) highlights the specific activity of catalase in respective elutions (Table 1).

Table 1
Purification table of catalase purified from black gram sprouted seeds using Seph 4B-IDA-Zn(II) sorbent, (mean ± SE, $n = 5$).

Fractions	Protein concentration (mg/ml)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	9.1 ± 0.3	2260 ± 326.5	274,320 ± 37,980	251.31 ± 29.8	1	100
$(NH_4)_2SO_4$ precipitate (20–60%)	8.1 ± 0.06	3960 ± 213.5	158,400 ± 8541.6	486.3 ± 25.8	2.04 ± 0.27	61.8 ± 8.8
Seph 4B IDA-Zn(II)Non retained fraction	2.9 ± 0.1	–	–	–	–	–
Elution 10 mM	0.09 ± 0.01	2548.8 ± 163.2	101,952 ± 6527.7	25,704 ± 697.2	106.8 ± 10	39.7 ± 5.7
Elution 25 mM	0.1 ± 0.01	650 ± 20	26,008 ± 805.4	5649 ± 263.5	23.3 ± 1.9	10.1 ± 1.3

3.3. Determination of K_m and V_{max} of catalase

In order to determine K_m and V_{max} of the enzyme we used different concentrations of substrate and measured its rate of decomposition. Calculating different velocities at which substrate was scavenged by catalase a Lineweaver–Burk plot was constructed. From the plot (Fig. 3) K_m and V_{max} of the enzyme were found to be 16.2 mM ± 0.64 mM and 2.5 μM/min ± 0.088 μM/min. The values of K_m and V_{max} of black gram catalase corroborate with the reported K_m and V_{max} values of catalase from different sources [37].

3.4. Effect of inhibitors and metal ions on catalase activity

The percentage of the catalase activity retained at different concentrations of enzyme inhibitors and metals were determined (Fig. 4). Inhibitors EDTA and sodium azide (NaN_3) and the metals ferric chloride ($FeCl_3$), Copper Sulphate ($CuSO_4{\cdot}5H_2O$), Nickel Chloride ($NiCl_2{\cdot}6H_2O$), Calcium Chloride ($CaCl_2{\cdot}5H_2O$), Magnesium Chloride ($MgCl_2{\cdot}6H_2O$), and Manganese Chloride ($MnCl_2{\cdot}4H_2O$) were used for the study. Only 4.6% of catalase activity was retained in the presence of 0.5 mM sodium azide which was also observed by Blaschko [34]. In presence of 2 mM EDTA 65% activity was retained. EDTA known to be a metal chelator would have chelated the Fe^{3+} metal ion from the active site of catalase there by inhibiting the activity [37]. Metals act as cofactors for various enzymes. Some metal ions contribute to the structural stability of enzymes and some participate in the reaction mechanism [29]. Catalase has a metal ion in the active site. Addition of other metal ions to the enzyme might affect the stability of the structure or affect the redox state of metal in active site leading to a change in activity [33]. The Fe^{3+} and Cu^{2+} metal ions inhibited the activity of catalase progressively with the increase in the concentration of respective metal ions. This observation corroborates with reported inhibition pattern of catalase from *Beta vulgaris* [37]. Increase in the catalase activity was observed with the increase in metal ion concentration of Ni^{2+}, Ca^{2+} till 1 mM (132.6%, 130%) and then there was a decrease in the activity at 1.5 mM (103%, 125%) and 2.0 mM (90%, 89%) metal ion concentration. Increase in the catalase activity was also observed in presence of Mg^{2+}, Mn^{2+} till a concentration of 1.5 mM (137%, 115%) and declined at 2.0 mM (114%, 74%) metal ion concentration. The increase in catalase activity in the presence Ni^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} may be due to the stability attained in the enzyme structure at that particular concentration of metal ion, at which they might serve as cofactors [33].

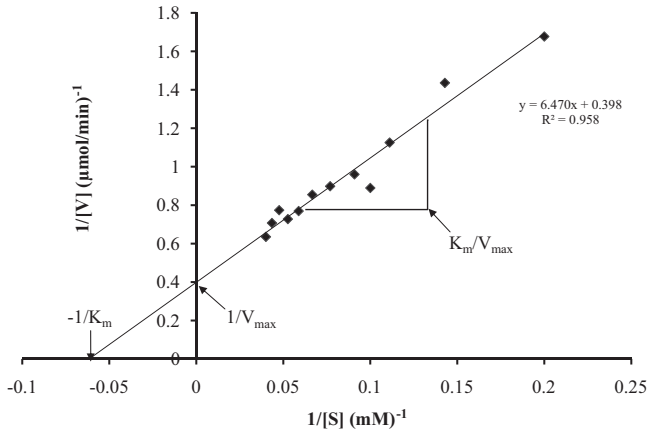

Fig. 3. Lineweaver–Burk plot of catalase using H_2O_2 as substrate (mean ± SE $n = 3$).

3.5. Determination of thermal stability of catalase

In order to determine the thermal stability of catalase aliquots of the enzyme were incubated at different temperatures varying from 30 °C to 90 °C for a period of 15 min. Thermal stability study indicated that the optimal activity was at 40 °C and then declined beyond 50 °C (Fig. 5a). The half life of catalase was determined by incubating the enzyme at 50 °C for 24 h and estimating the activity every 30 min (Fig. 5b). The half life of the enzyme at 50 °C was found to be 7 h and 30 min. Similar activity pattern of catalase was also observed in *Triganopsis variabilis* [26], *Porphyra yezoensis* [27] and dog liver [28]. The effect on the activity can be attributed to the changes occurring in the structure of the enzyme at a given temperature [24,29]. As the catalase monomers are held together via ionic interactions [36] at higher temperatures disassociation of the tetramer can occur leading to the loss in activity.

3.6. Effect of pH on catalase activity

The pH optimum of catalase was found to be 7.0 [37]. The effect of pH on the activity of catalase was analyzed under different buffer conditions. Catalase was found to be stable in a broad pH range of

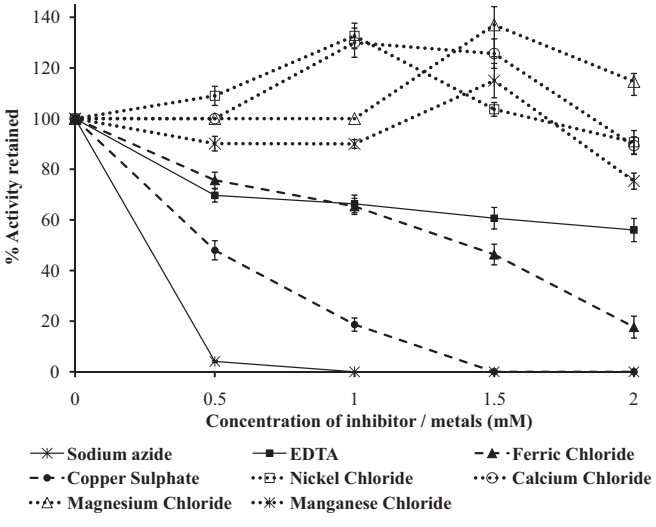

Fig. 4. Percentage catalase activity retained at different concentrations of enzyme inhibitors and metals (mean ± SE, $n = 3$).

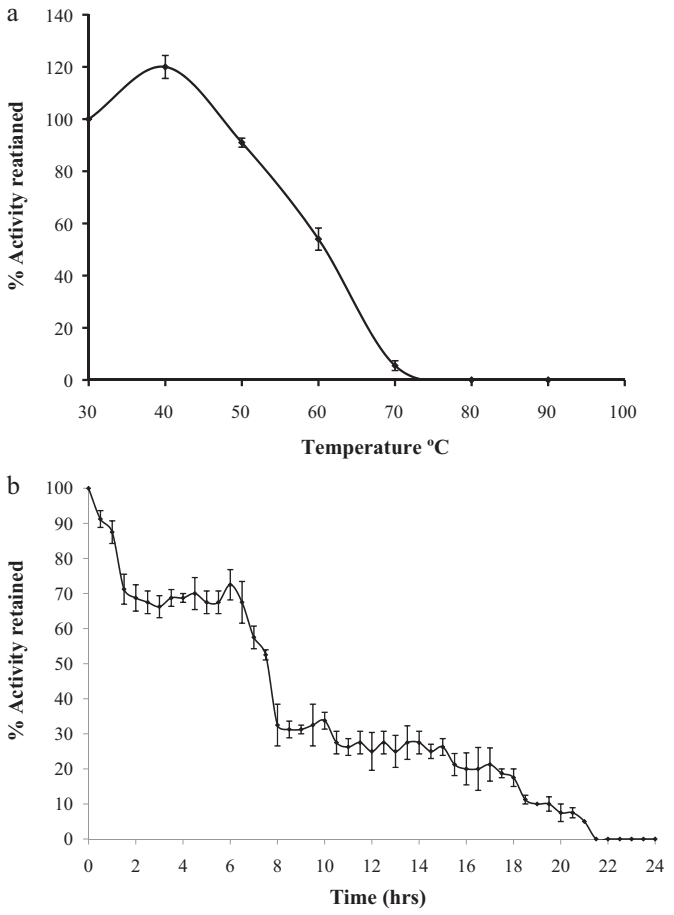

Fig. 5. (a) Thermal stability of catalase at different temperatures (mean ± SE, $n = 3$). (b) Thermal stability of catalase at 50 °C for 24 h at different time intervals (mean ± SE, $n = 3$).

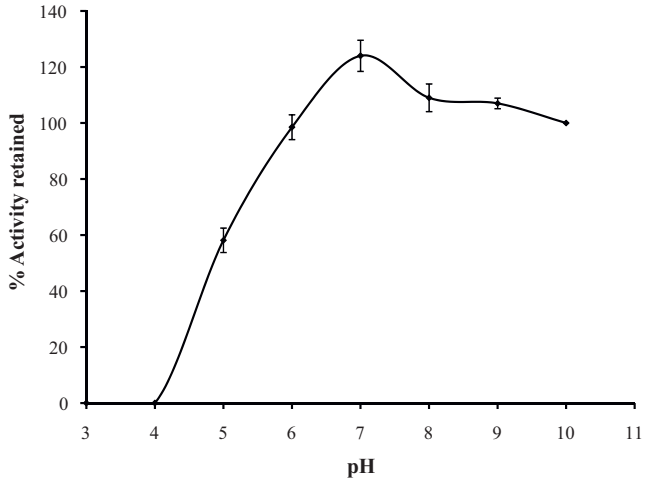

Fig. 6. Stability of catalase at different pH conditions (mean ± SE, $n = 3$).

6.0 to 10.0 (Fig. 6). The enzyme showed maximum activity at pH 7.0, retained 50% activity at pH 5.0 and it was completely inactive at acidic pH 3.0, 4.0 (Fig. 6). This observation is in correlation with the pH sensitivity of catalase purified from *Thermoascus aurantiacus* and bovine liver [30,31]. The loss in the activity of catalase at pH 3.0, 4.0, 5.0 and retention in the activity from pH 6.0 to 10.0 may be attributed to the changes occurring in the ionization states of amino acid chains [29,32] hence effecting the enzyme conformation.

4. Conclusion

The experimental results lead to the conclusion that black gram had a high amount of catalase. The Seph 4B-IDA-Zn(II) (IMAC) column employed for the purification of catalase from sprouted black gram seeds yielded 39% of catalase with a purification fold of 106. Both the SDS–PAGE and Zymogram analysis resulted that the recovered protein was catalase. Further the kinetic studies based on determining K_m and V_{max} values as well as the inhibition studies were in corroboration with the catalase purified from other sources. The physical studies demonstrated the optimum temperature and pH as 40 °C and 7.0, respectively. There by it can be concluded that the purification method used proves to be a potential approach for purifying catalase from black gram seeds in a single step.

Acknowledgements

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References

[1] D. Harman, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 7124–7128.
[2] Y.H. Wei, H.C. Lee, Exp. Biol. Med. (Maywood) 227 (2002) 671–682.
[3] D. Harman, J. Gerontol. 11 (1956) 298–300.
[4] J.M. Palma, A. Jimenez, L.M. Sandalio, F.J. Corpas, M. Lundqvist, M. Gomez, F. Sevilla, L.A. del Rio, J. Exp. Bot. 57 (2006) 1747–1758.
[5] H.N. Kirkman, G.F. Gaetani, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 4343–4347.
[6] M.J. Mate, M. Zamocky, L.M. Nykyri, C. Herzog, P.M. Alzari, C. Betzel, F. Koller, I. Fita, J. Mol. Biol. 286 (1999) 135–149.
[7] N.A. Tejera Garcia, C. Iribarne, F. Palma, C. Lluch, Plant Physiol. Biochem. 45 (2007) 535–541.
[8] M.P. Parashar, B.D. Sherkar, Post Harvest Profile of Black Gram, Department of Agriculture and Cooperation India, 2006.
[9] L.J. Teply, P.H. Derse, W.V. Price, J. Dairy Sci. 41 (1958) 593–605.
[10] J.S. White, D.C. White, Sourcebook of Enzymes, CRC, 1997.
[11] E.A. Havir, N.A. McHale, Plant Physiol. 84 (1987) 450–455.
[12] R.T. Mullen, D.J. Gifford, Plant Physiol. 103 (1993) 477–483.
[13] J. Yamaguchi, M. Nishimura, Plant Physiol. 74 (1984) 261–267.
[14] S. Eugene, Trends Biotechnol. 3 (1985) 1–7.
[15] I.T. Bresolin, M. Borsoi-Ribeiro, W.M. Tamashiro, E.F. Augusto, M.A. Vijayalakshmi, S.M. Bueno, Appl. Biochem. Biotechnol. 160 (2010) 2148–2165.
[16] L. Yang, L. Jia, H. Zou, Y. Zhang, Biomed. Chromatogr. 13 (1999) 229–234.
[17] Q. Yang, J.W. DePierre, Protein Expr. Purif. 12 (1998) 277–283.
[18] T. Aydemir, K. Kuru, Turk. J. Chem. 27 (2003) 85–98.
[19] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[20] H. Bisswanger, Practical Enzymology, Wiley Online Library, 2004.
[21] Y. Li, H.E. Schellhorn, J. Biomol. Tech. 18 (2007) 185–187.
[22] R.F. Beers, I.W. Sizer Jr., J. Biol. Chem. 195 (1952) 133–140.
[23] W. Woodbury, A.K. Spencer, M.A. Stahman, Anal. Biochem. 44 (1971) 301–305.
[24] R.K. Scopes, Protein Purification: Principles and Practice, Springer, 1994.
[25] M.G. Klotz, G.R. Klassen, P.C. Loewen, Mol. Biol. Evol. 14 (1997) 951–958.
[26] D. Monti, E. Baldaro, S. Riva, Enzyme Microb. Technol. 32 (2003) 596–605.
[27] T. Nakano, M. Watanabe, M. Sato, M. Takeuchi, Plant Sci. 104 (1995) 127–133.
[28] K. Nakamura, M. Watanabe, Y. Sasaki, T. Ikeda, Int. J. Biochem. Cell Biol. 32 (2000) 89–98.
[29] A.L. Lehninger, D.L. Nelson, M.M. Cox, Lehninger Principles of Biochemistry, WH. Freeman & Co, 2005.
[30] H. Wang, Y. Tokusige, H. Shinoyama, T. Fujii, T. Urakami, J. Ferment. Bioeng. 85 (1998) 169–173.
[31] S. Prajapati, V. Bhakuni, K.R. Babu, S.K. Jain, Eur. J. Biochem. 255 (1998) 178–184.
[32] A.M. Vetrano, D.E. Heck, T.M. Mariano, V. Mishin, D.L. Laskin, J.D. Laskin, J. Biol. Chem. 280 (2005) 35372–35381.
[33] E.P. Medyantseva, M.G. Vertlib, G.K. Budnikov, Russ. Chem. Rev. 67 (1998) 225–232.
[34] H. Blaschko, Biochem. J. 29 (1935) 2303–2312.
[35] A. Deisseroth, A.L. Dounce, Physiol. Rev. 50 (1970) 319–375.
[36] C.D. Putnam, A.S. Arvai, Y. Bourne, J.A. Tainer, J. Mol. Biol. 296 (2000) 295–309.
[37] A. Chang, Springer handbook of enzymes: Class 1, Oxidoreductases X EC 1. 9-1.13, Springer Verlag, 2005.
[38] U.K. Laemmli, Nature 227 (1970) 680–685.