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Partial Purification of a Catalase from an Improved Nigerian Sorghum Grain Variety

C. I. Nnamchi[1*], B. C. Nwanguma[2] and O. C. Amadi[1]

[1]*Department of Microbiology, University of Nigeria, Nsukka, Nigeria.*
[2]*Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.*

Authors' contributions

This work was carried out in collaboration among all authors. Author CIN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BCN and OCA helped with analyses of the study and together with author CIN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Catalases are key components of cellular detoxification pathways that prevent the formation of highly reactive hydroxyl radicals through catalyzing the decomposition of hydrogen peroxide into water and molecular oxygen. Their presence in brewery grains prevent the inactivation of important brewery enzymes and also stop lipid peroxidation. To determine their occurrence and establish some of its properties in sorghum, which has become as an important brewery grain similar to barley, crude catalase was obtained from a sorghum grain variety. Preliminary purification of catalase from the sorghum grain variety used, NRL-3, showed that the enzyme was purified 3.2-fold from the crude protein to give a 49% yield of the partially purified enzyme, with a final specific activity of 32 Umg^{-1} proteins. There was also a positive indication of sorghum catalase presence on SDS PAGE with positive bands occurring between the range of 48-62 kDa. Therefore, the molecular weight of sorghum catalase most likely falls within the two bands. The enzyme showed a narrow pH range with optimum activity occurring at pH 7. Similarly, its optimum activity temperature occurred at 40°C. This work is the first reported attempt at purifying catalase from sorghum.

**Corresponding author: E-mail: chukwudi.nnamchi@unn.edu.ng;*

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1. INTRODUCTION

The many metabolic processes that cells undergo especially the aerobic ones, subject them to the production of reactive oxygen species (ROS) among which are the superoxide anion, hydroxyl radicals and hydrogen peroxide. These oxygen species are particularly transient because of their immensely high chemical reactivity. This causes them to react with cellular macromolecules such as DNA, proteins, carbohydrates and lipids in a destructive manner [1]. The major consequence of this development is that cells are equipped with an extensive antioxidant defense system with which to combat the excessive production of ROS and thus contain the destructive consequences due to oxidative stress that could result. The components of this defence system are the enzymes superoxide dismutase, peroxidases and catalase. Working singly or together, they form the most potent enzyme-based defence system of biological cells against reactive oxygen species and hydrogen peroxide [2] effectively degrading them before they damage cellular components [3]. Typically, the superoxide dismutase destroys the free radical superoxide by converting it to hydrogen peroxide which is the major ROS in the cell [1]. The primary defence mechanisms against hydrogen peroxide are mediated by the enzyme catalase [4]. Catalase is considered to be one of the most efficient enzymes known; it cannot be saturated by hydrogen peroxide at any concentration [5].

According to Hiraga et al. [6], catalases are heme containing enzymes that are typically classified as belonging to the second superfamily of peroxidases. The typical reaction of catalases is the reduction of hydrogen peroxide to form water and molecular oxygen [7]. Catalases occur in all aerobic organisms which of course includes plants and brewery cereals [8]. They also occur in many anaerobic organisms [3].

Reactive oxygen species (ROS) have also been observed to occur during the various processes associated with brewing [9]. Specifically, they are formed when partial reduction of oxygen causes them to acquire additional electrons in the p orbitals which results in the successive formation of superoxide, $O2^-$, hydrogen peroxide, H_2O_2 and hydroxyl radical, OH. These ROS then lead to toxic effects by inactivating important brewery enzymes and also cause lipid peroxidation [10]. The presence of ROS could lead to problems at various stages of the brewing process including the early part of malting such as during steeping and germination and also at the stage of mashing [9].

It is therefore not surprising that biological systems protect themselves against these toxic oxygen free-radicals using the above-mentioned scavenging enzymes viz: superoxide dismutase, peroxidase and catalase. The incidence of these important enzymes has been shown in many brewery and related industrial grains such as wheat, barley and also sorghum [11-15] Additionally, these enzymes are known to influence important brewing properties such as lipid peroxidation and flavor instability among others [12,16].

Sorghum has become recognized as an important alternative to many other brewing cereals in the brewing and malting industries. This is especially so in the tropics where it is extensively cultivated as a result of many advantages it possesses. These advantages among others include their physiological robustness which make them able to withstand extreme environmental circumstances like intense drought and heat as well as temporary water-logging [17-20]. Sorghum’s importance is also underlined by the fact that it is the fifth most important cereal in total world production, after wheat, rice, corn and barley [21-23].

So far, there has not been any reports of attempts to purify catalase from sorghum. Considering the above-mentioned importance of the enzyme and cereal, there is need to carry out such a study hence this work, which is a pioneering attempt aimed at partially purifying and characterizing catalase from some improved Nigerian sorghum grains.

2. MATERIALS AND METHODS

2.1 Enzyme Isolation and Assay

To isolate sorghum catalase, grains from sorghum variety NRL-3 was used. First, the grains were crushed to a fine powder using a blender and the protein extracted using a modified method of Nnamchi et al. [23]. Sorghum flour (0.2 g/mL) was incubated with 100 mM potassium phosphate buffer (pH 7.0) for 30 minutes at 4°C and then centrifuged at the same temperature for 30 minutes at 5000 rpm using a

SLC 6000 Sorvall Evolution centrifuge. The supernatant produced was used as the crude extract.

Thereafter, protein from the supernatant was precipitated using ammonium sulphate (Sigma), on ice and with continuous stirring. Ammonium sulphate (in the appropriate quantities) was added to the supernatant in a batch of four steps, first to 0-30%, then to 30-50%, 50-70% and finally 70-90% saturation points. Each of the four batches of saturation points was centrifuged (as described above) and then assayed for catalase activity as described (see below). The precipitates after centrifugation were redissolved in 50 mM phosphate buffer pH 7.0 and dialyzed against the same buffer in three 4-hour periods (3 x 4h i.e. 12 hours) and used for further purification.

Catalase activity was assayed using the method of Haywood and Large (1981). Exactly 0.1 ml of appropriately diluted enzyme preparation was added to 2.9 ml of a freshly prepared solution containing 0.1 ml of 30% (v/v) H_2O_2 in 50 ml of 50 mM phosphate buffer, pH 7.0. The decrease in absorbance at 240 nm was followed and the time taken for the A240 to fall by 0.05 absorbance units will be determined at room temperature (28$\pm$2°C). This corresponds to the decomposition of 3.45 micromole of H_2O_2 in the 3 ml solution. Catalase activity (expressed in sigma units) is equal to 3.45/ {time (min) required}.

2.2 Ion Exchange Chromatography

A 2.5 x 20 cm column was packed with DEAE cellulose (DE-52, Whatman International Ltd, Maidstone, England) and equilibrated with 50 mM phosphate buffer, pH 7.0. The dialyzed partially purified enzyme from the 30-50% ammonium sulphate cut which showed the highest catalase activity was loaded onto the column and washed with equilibration buffer at a flow rate of 150 ml/h. Protein bound after washing was then eluted from the column using a linear gradient of 0 – 0.6 M NaCl (1 L, in equilibration buffer) and 5 mL fractions collected. The absorbance of all collected fractions was monitored at 280 nm and assayed for catalase activity. Fractions showing catalase activity were pooled and concentrated by centrifugation at 4000 rpm using a 30 kDa molecular weight cut-off membrane in a SLC 6000 Sorvall Evolution Centrifuge. The concentrated protein was dialyzed against 50 mM phosphate buffer, pH 7.0 and monitored for catalase activity. These were then used for subsequent studies.

2.3 SDS-PAGE

The purity and molecular weight of the enzyme was assessed using SDS-PAGE under denaturing conditions. The experiment was conducted using a Mini-Protean system II (Biorad Laboratories) and 15% polyacrylamide gels were prepared according to the method described by Wenk and Fernandis [24]. Protein samples were prepared by mixing an equal volume of sample with sample buffer followed by incubation at 95°C for 5 minutes. Protein samples, including 1% and 0.1% bovine liver catalase (BLC) and broad range protein markers (New Englands Biolabs, UK) were loaded onto the gels and the gels run in SDS-containing running buffer at 150 V until the dye front reached the bottom of the gel. The protein bands were developed using standard staining and destaining protocols (BioRad Laboratories).

2.4 Kinetic Procedures

2.4.1 Temperature activity

The temperature activity profiles of the enzymes were determined using a modification of the method described by Okolo et al. [25]. The temperature activity profile was determined over a range of 30 – 70°C by incubating for a period of up to 30 minutes in a Grant water bath (Made in England), 0.1 ml of the enzyme preparations to 2.9 ml of freshly prepared solution of 0.1 ml of 30% (v/v) H_2O_2 in 50 ml of 50 mM phosphate buffer, pH 7.0. The decrease in absorbance at 240 nm was followed as described above.

2.4.2 pH activity

The effect of pH on the activity of the partially purified enzyme was studied over a pH range of 2 to 12.0. This will be done using relevant buffers, 0.1 M glycine-HCl buffer for pH 2, 0.1 M phosphate-citrate buffer for pH 3 to 5, 0.1 M Na-phosphate buffer for pH 6 to 8, 0.1 M glycine-NaOH for pH 9 and 10 and 100 mM Na-phosphate buffer for pH values 11 and 12. The enzyme and appropriate buffer for each pH were mixed and the absorbance change monitored as outlined above

3. RESULTS AND DISCUSSION

The preliminary catalase activity profile obtained from the crude NRL-3 sorghum variety and thereafter, from the different extract fractions obtained after they had been subjected to

sequential fractional precipitation with ammonium sulphate is presented in Fig. 1.

The result showed that the highest catalase activity from the ammonium sulphate precipitants was achieved with the 30-50% saturation cut, meaning that it contained the highest available amount of sorghum catalase. Therefore, that saturation point was used for further purification work. Of course, as expected, the highest overall catalase activity value here was achieved with the control, 0.1% bovine liver catalase. Table 1 shows a summary of the partial purification scheme of the sorghum catalase with the enzyme purified by 3.2-fold from the crude protein to give a 49% yield of the partially pure enzyme, and a final specific activity of 32 Umg^{-1} protein.

This was achieved by a combination of just the crude protein, 30-50% ammonium sulphate precipitation and anion exchange chromatography. Fig. 2 shows the SDS PAGE of the catalase fractions after the above partial purification process.

Shown in the figure are arrows that indicate similarity points and bands with those from the two bovine liver catalase lanes. These show that these points are most likely those containing catalase bands judging from similar bands from those of two bovine liver catalase bands. Since SDS denatures proteins from their conformational 3D shapes and separates them into their individual subunits by mass, it may seem that the proteins were separated into their different constituent polypeptides and subunits whose sizes likely lie between close to the 62 kDa band of the standard marker proteins. Catalases from different species have been found to be quite similar in properties [26]. The results of the SDS PAGE profile of our sorghum catalase shows the bands lie between 48 and 62 kDa band of the standard marker proteins (they are obviously closer to 62 kDa). The given value may therefore actually be 60 kDa which should be the molecular of each individual subunit, a further proof of the correct catalase identity of our enzyme. It is most probable that sorghum catalase comprises four individual subunits of equal weight bringing their overall molecular weight to 240 kDa. Catalases are typically said to be composed of four subunits with a combined molecular weight of about 240 kDa [27,28]. However, a greater proof of identity lie in the fact that a positive correlation occurred when a small nick from the SDS PAGE of sorghum catalase band was subjected to mass spectroscopy and the properties generated matched those of other catalases (personal communication between the first author and Andrew Bottrill of PNACL unit, University of Leicester, UK; data not shown). Switala and Loewen [3] had similarly found in their work on the diversity of properties among different catalases that their molecular weights lie mostly around 60 kDa.

Catalases have been found to have unique properties, among which is the fact that they not follow typical Michaelis Menten kinetics [29]. Therefore, we felt that it was not necessary to carry out such typical kinetic studies and concentrated on how different temperature and pH affected the activities of the partially purified enzyme. Fig. 3 shows how temperature affected the activity of the sorghum catalase.

From the result it was observed that the enzyme had its optimum activity at the temperature of 40°C. Shown in Fig. 4 is the effect of different pH on the activity of the sorghum catalase.

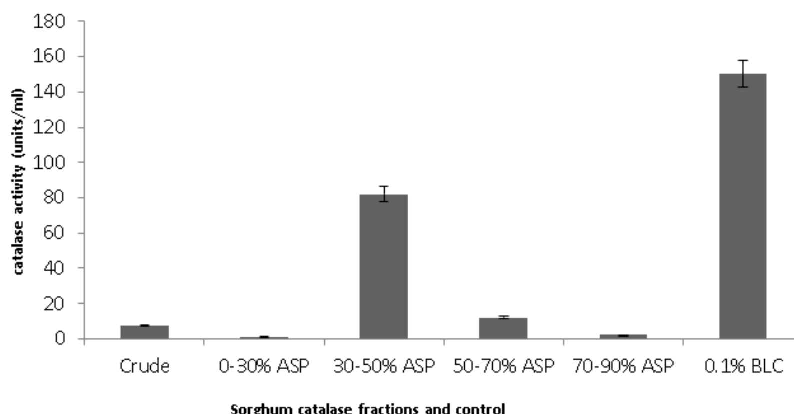

Fig. 1. Variations in catalase fractions after sequential $(NH_4)_2SO_4$ precipitation

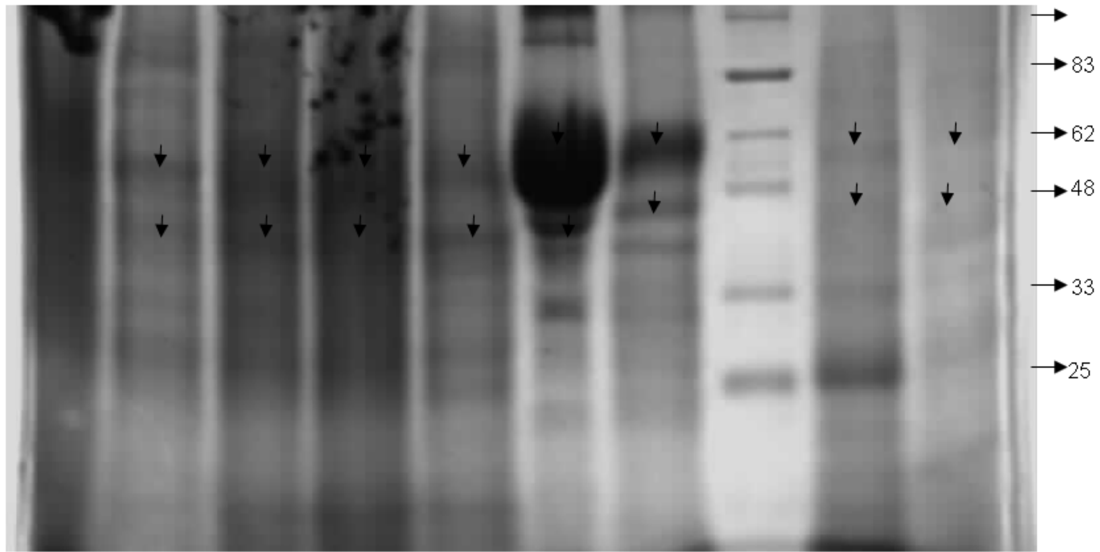

Fig. 2. SDS PAGE of NRL-3 sorghum catalase (From right: Lane 1 crude protein; lane 2 30-50% $(NH_4)_2SO_4$ precipitate; lane 3 marker proteins; lane 4 0.1% BLC; lane 5, 1% BLC; lane 6-10 anionic chromatography elution fractions). Arrows indicate similarity points that show catalase-containing bands judging from similar bands from the two bovine liver catalase-containing lanes. The figures on the left are molecular weights of the marker protein bands

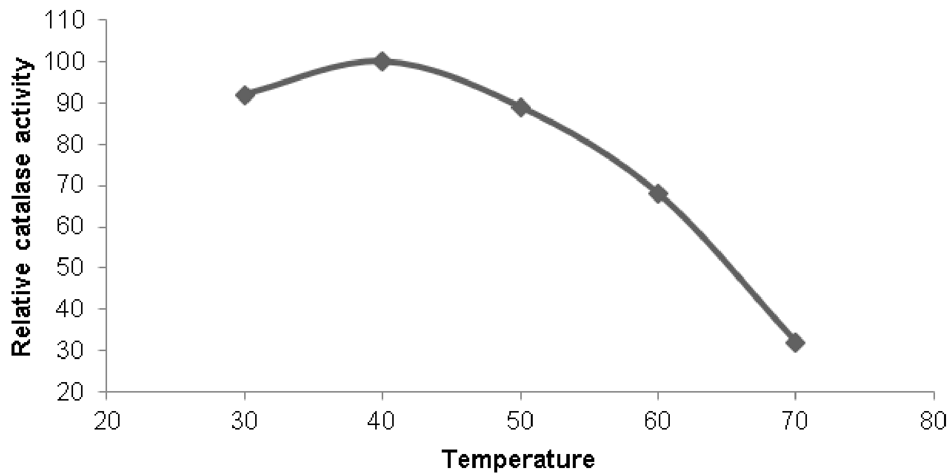

Fig. 3. Effect of different temperature on the activity of partially purified sorghum catalase

Table 1. Purification table of sorghum catalase

Purification Steps	Total Activity (U)	Total Protein (Mg)	Specific Activity ($U.mg^{-1}$)	Yield	Purification Fold
Crude enzyme extract	21550	2105	100	10	1
30 – 50% ammonium sulphate precipitate	17100	790	22	79	2.2
DEAE – 52 anion exchange Chromatography	10470	330	32	49	3.2

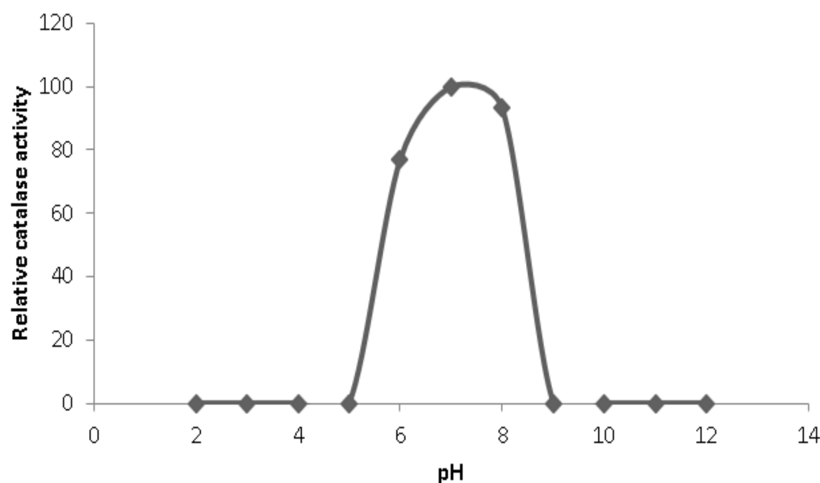

Fig. 4. Effect of different pH on the activity of partially purified sorghum catalase

The enzyme was found to have a very low pH range within which it was active. Thus, despite the broad span of the pH spectrum used in the assay, the enzyme showed activity only within the narrow pH range of 6 to 8, with the optimum occurring at pH 7. An even worse scenario in terms of range of pH effect was observed when attempts were made to purify the same enzyme from a malted version of the sorghum variety used for isolation, using the same methods (data not shown). In that case, activity was seen only at pH 7. However, the partially purified catalase obtained from that malted version of the NRL-3 sorghum variety showed even higher levels of activities at all the temperature range used for assay but with the optimum also occurring at 40°C. In their work with partially purified catalase from cabbage leaves, Gholamhoseinian et al. [26] had got a similar optimum pH of 7 but not such a narrow range as obtained in this work. Also Esaka and Asahi [28] in their work on a catalase obtained from sweet potato root microbodies obtained results that are in some ways similar, as their maximal pH of activity lay between the wide span of pHs 6.5 to 8.5. Catalases are considered unique and mysterious enzymes which have continued to show unpredictable and quite remarkable behaviours [30,31] but remain perplexing, enigmatic and utterly fascinating enzymes [7].

4. CONCLUSION

In this work we obtained crude catalase from a sorghum grain variety and partially purified it. Among other things, we found that its properties such as molecular weight, optimum activity pH and temperature are similar to those of other catalases. The present study evidently has some limitations and therefore will require more future works. However, it has in many ways furthered the search for more aspects of the interesting uniqueness and fascination associated with catalase research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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