Extraction, Purification, and Characterization of Catalase Enzyme From *Coriandrum Sativum* L. Leaves

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Abstract

Catalase enzyme isolated from *Coriandrum sativum* L. leaves was successively purified by 20-70 % ammonium sulphate precipitation method followed by dialysis and gel filtration chromatography on Sephadex G-100. Catalase activity in cell extracts was monitored through the decomposition of H_2O_2 by following the decrease in absorbance at 439 nm. Protein content was determined by the Biuret method using Bovine Serum Albumin (BSA) as standard at 550 nm. Specific activities, protein recovery, and degree of purification were evaluated in each purification step. Catalase has purified 3.7-fold over crude extracts and protein recovery was found to be 3.15 %. The pH optimum at 7 and the optimum temperature were observed at 30 ℃. The two different types of catalase present in *Coriandrum sativum* L. leaves have been found on non-reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of the protein subunit of the catalase enzyme was then investigated by reduced SDS-PAGE showing the isoforms of catalase at 60.26 kDa and 69.18 kDa, respectively.

***Keywords*:** specific activity of catalase, *Coriandrum sativum* L., molecular weight, SDS-PAGE

Introduction

Natural antioxidants are enzymatic and non-enzymatic antioxidants. Antioxidant enzymes include catalase, peroxidase, reductase, dehydrogenase, etc. while non-enzymatic antioxidants such as vitamins A, C, and E, altogether work in synergy to counterbalance oxidative stress. Catalase (EC 1.11.1.6, oxidoreductase) are powerful antioxidant enzyme with the ability to detoxification of highly reactive hydroxyl radicals by catalyzing the dismutation of H_2O_2 to water and dioxygen. It is widely distributed in a variety of life forms, including plants, animals, and microbes, and is usually absent from anaerobic organisms. They play crucial roles in promoting health by forming part of our bodies' primary system of defense against free radical damage (Dumen and Kaya, 2013). Catalase holds an important place in the enzymatic world because of its use in various industries and medicines. It also finds diverse industrial applications in textiles, waste treatment, cosmetics, and as a disinfectant agent. Three types of catalase enzymes are monofunctional catalases further subdivided into the small-and large-subunit categories, bifunctional catalases (catalase-peroxidases), and manganese catalases (Wang *et al*., 2017). Catalases possess a multiple diverse range of structures and molecular properties, with hundreds of gene and protein sequences being reported. Unlike animals, which involve a single catalase gene, in plants, this enzyme is encoded by a multigene family, which provides numerous isozymes whose number varies depending on the species (Rodriguez-Ruiz *et al*., 2019).

Since ancient times, there has been growing interest in plants. Plants can provide not only basic nutritional and energetic requirements but also additional physiological benefits. They possess a self-defense mechanism for protection from oxidative stress by the activation of many antioxidant defense enzymes. Some plants

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such as coriander have very high levels of catalase, far higher than they would likely ever need to prevent photorespiration damage. *Coriandrum sativium* L. has a very effective antioxidant profile showing radical scavenging activity, hydroxyl radical scavenging activity, superoxide dismutation, etc. (Demir *et al*., 2008). This study was designated to extract, purify, and characterize the molecular weight of catalase enzyme from *coriandrum sativium* L. leaves.

Materials and Methods

Sample Collection

The samples of coriander leaves were collected from Hledan market, Yangon Region. It was identified by an authorized botanist at the Department of Botany, University of Yangon.

Materials and Methods

Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich, England, and Sephadex G-100 was also purchased from Uppsala, Sweden. Hydrogen peroxide, catalase assay reagents, protein assay reagents, protein ladder, chemicals for electrophoresis, and all other chemicals used were all analytical reagent grade. In all investigations, the recommended standard methods and techniques involving both conventional and modern methods were provided.

Extraction and Purification of the Enzyme

A 20 g of fresh coriander leaves were washed with tap water, air dried, cut, and homogenized with 100 mL of pH 7 phosphate buffer solution. It was blended with an electrical blender and filtered. The suspensions were clarified by centrifugation at 5500 rpm for 30 min. Then the precipitate was discarded and the supernatant was collected. Solid ammonium sulphate was slowly added to this supernatant to obtain 20 % saturation. To homogenate for complete dissolution, it was stirred for 2 h in ice. After 2 h, the mixture was centrifuged again for about 30 min and discarded the precipitate. Solid ammonium sulphate was slowly added to this supernatant to obtain 20-70 % saturation. This supernatant was kept overnight in the refrigerator. The precipitated protein-containing catalase enzyme was isolated by centrifugation for about 30 min. The precipitate was collected with a small amount of pH 7 (phosphate buffer) and then dialyzed through a dialysis bag in the same buffer, for four and half hours with three changes of the buffer during dialysis. For further purification of catalase, the dialyzed enzyme sample was applied onto the (2 × 27) column filled with Sephadex G-100 equilibrated with the phosphate buffer at a flow rate of 1.5 mL/7 min. The catalase was eluted with the same buffer. Enzyme activity for each 1.5 mL fraction was estimated. Fractions with high catalase activity were combined and stored for further analysis at 4 ℃ (Dincler and Aydemir, 2001).

Characterization of Catalase Enzyme

The qualitative examination of the partially purified catalase of *Coriandrum sativium* L. leaves was carried out by a 6 % hydrogen peroxide test. The quantitative test was carried out using the method described by Hadwan. For enzyme assay, the catalase activity of coriander was determined with the carbonato-cobaltate (III) complex method in UV absorbance at 439 nm using H_2O_2 as a substrate. Although other methods of measuring catalase activity have been developed, this method is free from the interference that results from the presence of amino acids, proteins, sugars, and fats in the studied sample. Catalase was assayed by the depletion of hydrogen

peroxide. Protein concentrations in soluble cell extracts were determined by the Biuret method with Bovine Serum Albumin (BSA) as standard (Savory *et al*., 1968). Specific activities of different purification steps were calculated by using enzyme activity and protein content. Purification degrees were also determined by using specific activity in each purification step.

Effect of Reaction Time

In this study, different reaction times of 1, 2, 5, 8, 11, 14, 17 and 20 min were used for the investigation of the effective reaction time of the catalase enzyme.

Effect of pH

A study was made on the effect of pH on enzyme activity. Enzyme activity was determined in 10 mM phosphate buffer at different pH values, over the pH range of 5 to 8.

Effect of Temperature

For the determination of the optimum temperature for the purified enzyme using a constant temperature circulator, catalase activity was measured at different temperatures in the range from 10 to 50 ºC.

Molecular Weight Investigation

The molecular weight determination of native protein and protein's subunit of catalase enzyme were performed on the non-reduced and reduced (4-15) % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method along with a broad range protein ladder as standard. Just before loading, the prepared enzyme sample and protein ladder were treated with SDS. For reduced SDS-PAGE, 2-mercaptoethanol was also added and heated at 92 °C for 8 min on a dry block heater. Two gels were run under the same conditions. The electrophoresis was run until the dye had reached the bottom of the gel slab. After de-staining, the gel was stained with coomassie-brilliant blue R-250 until the background became clear at room temperature (Susmitha *et al*., 2016).

Results and Discussion

Simple Qualitative Examination of Catalase by Using Hydrogen Peroxide

The partially purified (20-70) % catalase was qualitatively examined by hydrogen peroxide test and catalase activity was confirmed by the liberation of oxygen gas bubbles to form a foam (Figure 1). The foam produced is a result of the conversion of hydrogen peroxide into water and oxygen by catalase, the bubbles are filled with this oxygen. The more bubbles produced the faster the reaction, or the more catalase present. However, without catalase, hydrogen peroxide did not liberate oxygen to form foam.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

hydrogen peroxide — water — oxygen

(a) (b)

Figure 1. 6 % H_2O_2 solution (a) with enzyme (b) without enzyme at room temperature

Catalase Activity, Protein Estimation, and Specific Activity in Purification Step

Hydrogen peroxide was used as a substrate for the determination of catalase activity. The absorbance at 439 nm has been used for the assessment of catalase activity. The cobalt-bicarbonate solution can act as a "stop bath" for reactions regulated by the catalase enzyme (Hadwan, 2018). Immediately after mixing the cobalt-bicarbonate reagent with the enzyme solution, cobalt (II) is oxidized to cobalt (III); any unreacted hydrogen peroxide resulting from the catalase activity has oxidized the cobalt (II) to cobalt (III) and then reacted with carbonate to produce a carbonato-cobaltate (III) complex ([Co $(CO_3)_3$] Co), which has an intense olive green colour. Catalase activity is always directly proportional to the rate of dissociation of hydrogen peroxide in the used samples. The decrease in colour intensity can be used as an index to represent the increased catalase activity. One unit of catalase activity is defined as the amount of enzyme that breaks down 1 µmol of H_2O_2 min^{-1} to water and oxygen under the defined assay conditions (Zelitch *et al*., 1991).

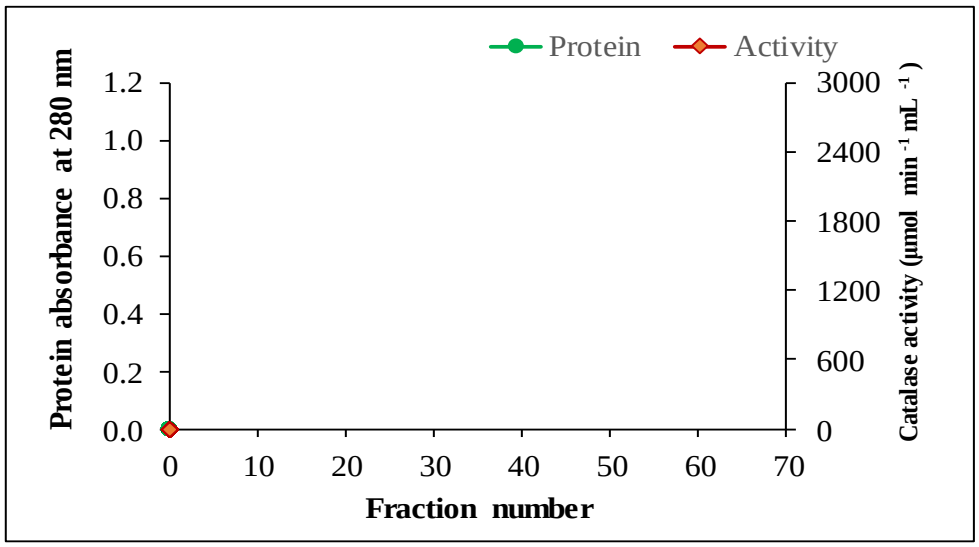

Figure 2. Elution pattern of catalase enzyme from gel filtration chromatography on Sephadex G-100

The protein content of elute was checked spectrophotometrically at 280 nm and 439 nm for enzyme assay. The enzyme eluted as a single peak (Figure 2). The fractions showing the highest catalase activity were pooled. Then purified enzyme solutions were stored at 4 ℃ and used for the characterization of the enzyme. The purification procedure for catalase from coriander leaves is summarized in (Table 1). The protein content of the purified fraction was determined by the Biuret method and it was observed to be 5.71 mg mL^{-1}. The specific activity was calculated to be 364.77 µmol min^{-1} mg^{-1}. After purification, catalase was purified to 3.7-fold over crude extract. The protein recovery percent was found to be 3.15 % (Table 1).

Table 1. Changes in Total Protein Content, Total Catalase Activity, Specific Activity, Protein Recovery, and Degree of Purity of Enzyme Solution during Different Purification Steps

Purification steps	Total protein content (mg)	Total catalase activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Protein recovery (%)	Degree of purity (fold))	Total volume (mL)
Crude extract	2721.79	270000	99.19	100.00	1.0	135.0
20 % $(NH_4)_2 SO_4$ saturation	2189.39	264550	120.83	78.49	1.2	130.0
70 % $(NH_4)_2 SO_4$ saturation	327.56	45185	137.95	12.04	1.3	3.7
Dialysis	197.95	62888	317.70	7.27	3.2	5.4
Gel filtration	85.59	31221	364.77	3.15	3.7	15.0

Effect of Reaction Time on Catalase-catalyzed Reaction

In this work, the effect of the reaction of the catalase on hydrogen peroxide was studied in a pH 7 phosphate buffer solution. The amount of carbonato-cobaltate (III) complex produced during the various reaction times of 1, 2, 5, 8, 11, 14, 17 and 20 min was determined by the spectrophotometric method. At the beginning of the reaction (for 2 min), the reaction is very fast. Then, velocity decreased rapidly (Table 2 and Figure 3). Therefore, in sequence studies, a reaction time of 2 min was chosen for the measurement of initial velocity in enzyme kinetic.

Table 2. Velocity of Catalase enzyme-catalyzed Reaction at Various Reaction Time

No.	Reaction time (min)	Velocity (M min^{-1})
1	1	0.1254
2	2	0.1134
3	5	0.0699
4	8	0.0518
5	11	0.0430
6	14	0.0384
7	17	0.0345
8	20	0.0296

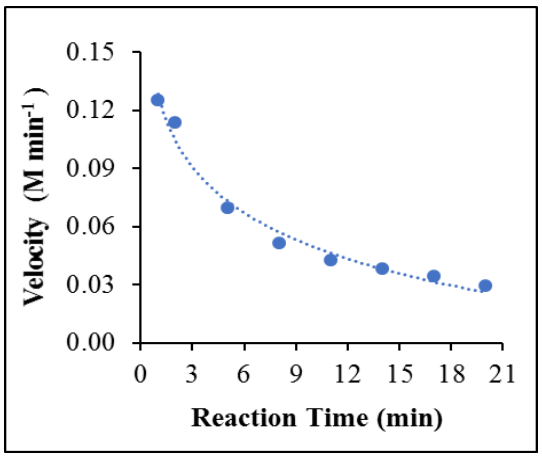

Figure 3. Plot of velocity of catalase-catalyzed reaction as a function of reaction time

Optimum pH of Catalase Activity

At an optimum pH, an enzyme's activity is the highest. At pH above and below optimum pH, the activity of the enzyme is reduced and reaction rates are slower (Henrickson *et al*., 2007). In this work, different buffers of pH values 5.5 to 8 (0.5 unit intervals) were used to determine the activity of the prepared catalase sample. The nature of the activity vs pH curve of the enzyme (Table 3 and Figure 4) was found to be unsymmetrical and the optimum pH was obtained at pH 7 with hydrogen peroxide as substrate.

Table 3. Effect of pH on the Catalase Enzyme-catalyzed Reaction

No.	pH	Catalase activity (µmol min^{-1} mL^{-1})
1	5.5	1430.09
2	6.0	1578.76
3	6.5	1628.32
4	7.0	1865.49
5	7.5	1748.67
6	8.0	1706.19

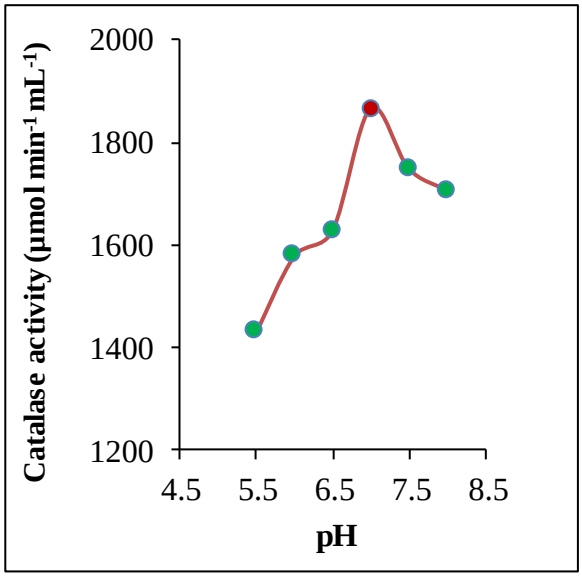

Figure 4. Plot of catalytic activity as a function of pH of solution

Optimum Temperature of Catalase Activity

Enzyme-catalyzed reactions also tend to go faster with increasing temperature until the maximum temperature is reached. Above this value, the enzyme molecule is denatured. In this study, the effect of the temperature on the catalase activity was investigated at the temperature between 10 to 50 ºC intervals. The optimum temperature for catalase was found to be 30 ºC in pH 7 phosphate buffer. (Table 4 and Figure 5).

Table 4. Effect of Temperature on the Catalase Enzyme-catalyzed Reaction

No.	Temperature (°C)	Catalase activity (µmol min^{-1} mL^{-1})
1	10	1315.89
2	20	1392.61
3	25	1599.14
4	30	1900.09
5	35	306.85
6	40	271.44
7	50	59.01

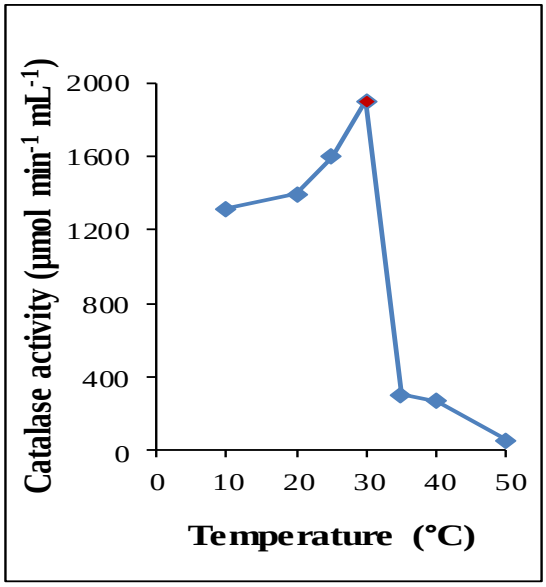

Figure 5. Plot of catalase activity as a function of temperature of solution at pH 7

Molecular Weight of Purified Catalase

The molecular weight of a protein under investigation was determined by comparing its electrophoretic mobility with that of protein standards of known molecular weight. The catalase enzyme revealed two bands with similar electrophoretic mobility on non-reduced SDS-PAGE due to the presence of two isoforms of catalase. They are designated as CAT 1 and CAT 2. Previous studies have shown catalase exists in multiple forms in several plants such as tobacco, cotton, maize, sunflower, etc. Multimeric isozymes may be the products of different genes or different gene-splicing events. Similar results were reported for catalase in safflower (Tayefi-Nasrabadi *et al*., 2016). Leaf extracts of coriander when examined by reduced SDS-PAGE showed similar protein profiles of non-reduced SDS-PAGE. Catalase was located between 50 kDa and 70 kDa of the standard protein (Figure 6,7). Two distinct catalase bands were detected throughout all stages of purification, these two were denoted as CAT 1 and CAT 2. For the standard protein and catalases, R_f values were calculated from the R_f vs Log MW graph and were obtained according to Laemmli's procedure showing a molecular weight of 60.26 kDa for CAT 1 and 69.18 kDa for CAT 2. Similar values were obtained for purified catalase from other sources (Dincler and Aydemir, 2001). Catalase from different sources mostly exists as a tetramer with molecular weight ranging from 220 to 270 kDa. According to the data, catalase from coriander mostly exists as can be formed from a monomer.

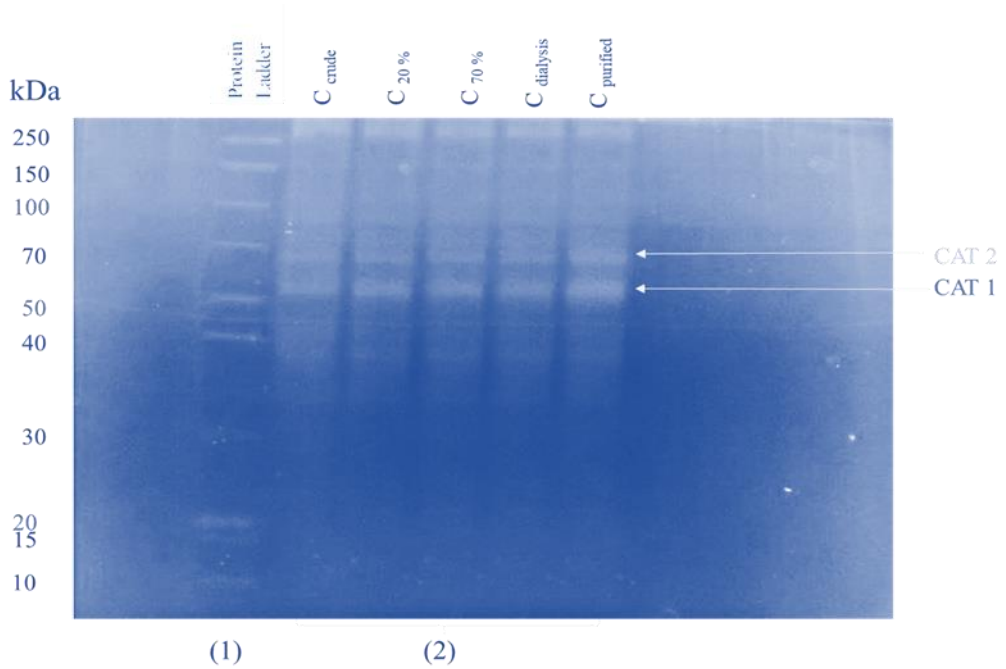

Figure 6. **Non-reduced and denaturing SDS-PAGE analysis of coriander catalase**
Lane 1: PageRuler Unstained Broad Range Protein Ladder
Lane 2: Enzyme catalase fractions obtained from each purification steps

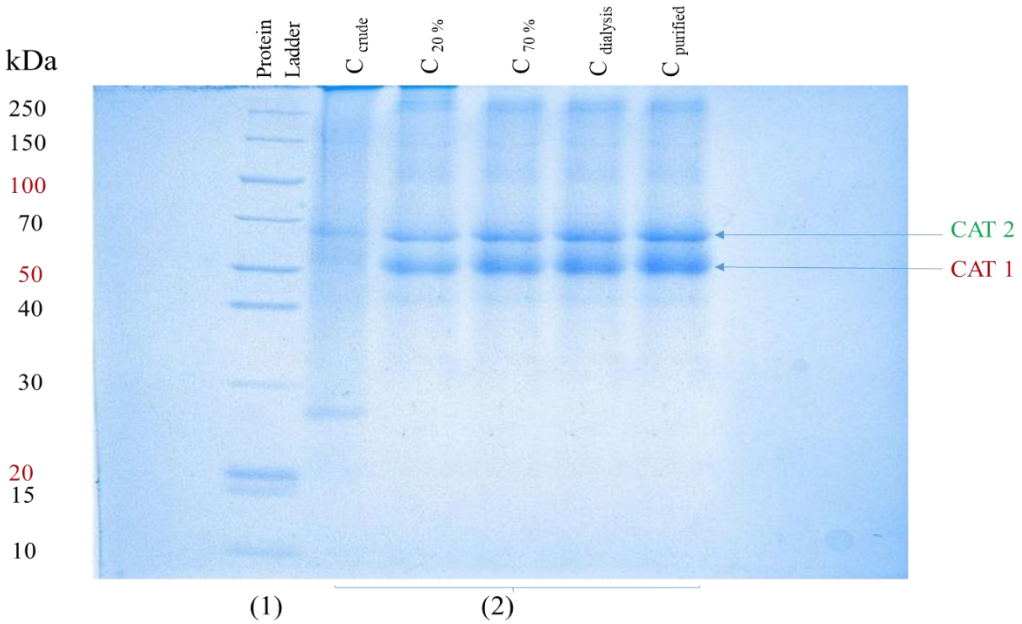

Figure 7. **Reduced and denaturing SDS-PAGE analysis of coriander catalase**
Lane 1: PageRuler Unstained Broad Range Protein Ladder
Lane 2: Enzyme catalase fractions obtained from each purification steps

An equal amount of enzyme of fraction number 16-23 with different catalase activity (Table 5) from the final purification steps was applied to the reduced SDS-PAGE gel, and the distribution of CAT 1 and CAT 2 among lanes 2 through 9 was the same (Figure 8).

Table 5. Catalase Activities of Fraction Numbers 16-23 on Sephadex G-100

Fraction numbers	Catalase activity (μmol min^{-1} mL^{-1})
16	49.56
17	750.44
18	2208.85
19	2860.18
20	2669.03
21	2817.69
22	2676.11
23	2654.87

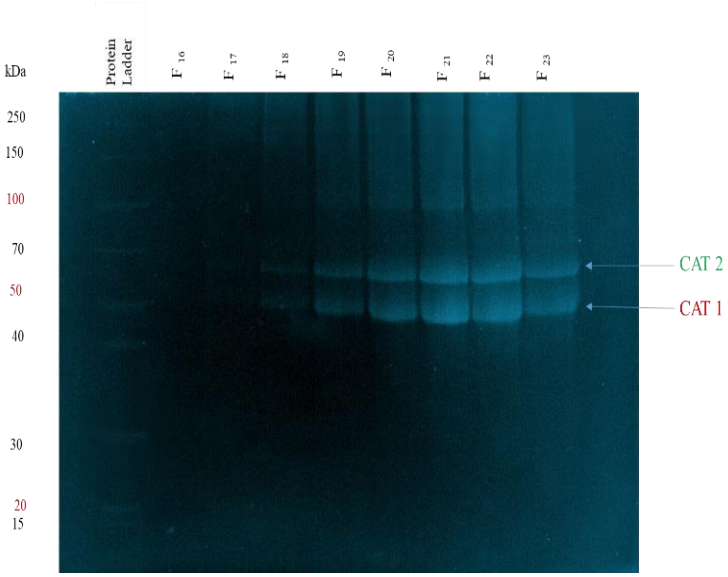

Figure 8. Distribution of catalase enzyme in purified fraction numbers of 16-23 on reduced SDS-PAGE

Thus, the increase in total catalase activity in the coriander cannot be explained by an increase in one form or isoenzyme, but rather two forms are increased proportionally.

Conclusion

In this research, purified catalase enzyme was isolated from matured green *Coriander sativum* L. leaves. The purified catalase was qualitatively examined by hydrogen peroxide test and catalase activity was demonstrated by the liberation of oxygen gas bubbles to form foam. Catalase isolated from coriander was purified by selective ammonium sulphate precipitations, dialysis, and gel filtration chromatography. Enzyme activity was increased when compared with its activity without purification. Catalase was purified 3.7-fold over crude extracts and the overall protein recovery after purification was found to be 3.15 %. The specific activity of purified catalase was 364.77 μmol min^{-1} mg^{-1} after gel filtration. The absorbance of the reaction mixture was read within only 2 min of reaction time at 439 nm against the reagent blank. The optimum pH of catalase was found to be 7 in phosphate buffer and the optimum temperature was found to be 30 °C. In the staining for catalase on non-reduced PAGE, two isoforms of catalases were detected in each purification step. CAT 1 was prominent while CAT 2 was faint. The protein subunit of catalase was investigated on reduced SDS-PAGE with the value of 60.26 kDa for CAT 1 and 69.18 kDa for CAT 2, respectively. Unlike most plant catalases, coriander enzyme acts as a monomer due to the data obtained using two different approaches, non-

reduced and reduced have similar protein profiles. According to the molecular weight of catalase observed from reduced SDS-PAGE, coriander catalase may be monofunctional catalase. Among the three types of catalase enzymes, monofunctional catalases constitute the largest and most extensively studied group of catalase enzymes.

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