Asian Journal of Chemistry Vol. 20, No. 3 (2008), 1927-1936

Purification and Characterization of Catalaze Enzymes from Coriander (*Coriandrum sativum*) Leaves

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Catalaze (H_2O_2:H_2O_2 oxidoreductase; E.C 1.11.1.6) was purified from Coriander (*Coriandrum sativum*) leaves. The kinetic behaviour and some properties of the enzyme were also investigated. The purification was done at 4 ºC in two steps: (a) ammonium sulfate fractionation and (b) DEAE-Sephadex A50 ion exchange chomatography. The enzyme was obtained with a yield of 10.67 % and had a specific activity of 89.68 EU/mg protein. Optimum pH, stable pH, optimum temperature, molecular weight, K_M and V_{max} values for H_2O_2 was also determined. The overall purification was about 64.06-fold. SDS-PAGE of the purified enzyme showed a single band. Enzymatic activity was spectrophotometrically measured at 240 nm. The molecular mass was estimated to be 60.95 kDa by SDS-PAGE and 58.12 kDa by Sephadex G-150 gel filtration column chromatography. The enzyme had optimum pH at 7.3 and was stable at pH 7.3 in 0.1 M *tris*-HCl buffer. The optimum temperature was at 30 ºC. The K_M value for H_2O_2 was 7.87 mM. The V_{max} value for this substrate was 42.19 EU/mL.

Key Words: Catalase, Purification, Characterization, Coriander (*Coriandrum sativum*).

INTRODUCTION

Coriander is an annual herb belonging to the carrot family and is grown primarily as a spice crop for use in different salads and sauces in various cuisines all over the world[1,2]. The green leaves and crushed seed provide the spicy flavour, while the seed oil is used in perfumes, cosmetics and soaps. Moreover, extracts obtained from the seeds of this plant have been shown to have a blood pressure lowering effect in anaesthetized rats[3].

Catalase (H_2O_2:H_2O_2 oxidoreductase, E.C. 1.11.1.6) is a common enzyme found in living organisms that changes hydrogen peroxide to water and oxygen. It is widely distributed in a variety of life forms, including plants, animals, microbes and usually absent from anaerobic organisms[4,5].

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Catalaze is located in a cell organelle called the peroxisome. Peroxisomes in plant cells are involved in photorespiration and symbiotic nitrogen fixation. Hydrogen peroxide is produced as an intermediate during these chemical processes and must be removed to prevent damage to cellular machinery[6].

Reactive oxygen species (ROS) denote a collection of oxygen radicals (O_2^-, $OH^•$) and some derivatives of oxygen like H_2O_2 and singlet oxygen. ROS are generated during the normal metabolism of eukaryotic cells, which involve mitochondrial electron transport and other systems. Controlled liberation of ROS has been advantageous to the systems like blastocyst implantation[7], iodination of tyrosine in the thyroxine biosynthesis[8] and mucous scretion in goblet cell[9]. Detrimental effects caused by ROS occur as a consequence of imbalance between the formation and inactivation of these species[10]. ROS mediated oxidative damage to macromolecules namely lipids, proteins and DNA have been implicated in the pathogenecity of major diseases such as cancer, rheunatoid arthritis and cardiovascular disease, *etc*.[11,12].

The antioksidant enzymes and free radical scavengers may provide a defensive mechanism against the deleterious actions of ROS. Some of the antioxidant enzymes that are found to provide a protection against the ROS are superoxide dismutase, catalase, peroxidase, glutathione peroxidase (GPx), glucose 6-phosphate dehydrogenase and ascorbate oxidase[13]. They are found to be wide spread in plant material[14]. Among the plant materials, fruits, vegetables and spices are reported to be rich in compounds with antioxidants[15,16]. Plants involved in antioxidative enzyme in which catalaze are the most efficient enzyme, influencing patterns of fruits[17]. Catalaze also removes electrons that can lead to the production of O_2^- free-radical[18]. Catalaze has been purified and characterized from various animal, fungal and bacterial sources[19] and from various plant sources, *e.g.*, spinach (*Spinacia oleracea*) leaves[20], lentil (*Lens culinaris*) leaves[21], cucumber (*Cucumis sativus*) cotyledons[22], sweet-potato (*Ipomoea batatas*) roots[23], maize (*Zea mays*) scutella[24], pumpkin (*Cucurbita* sp.) cotyledons[25,26] sunflower (*Helianthus annuus*) cotyledons[27] and *Zandedeschia aetgiopica* leaves[28]. The purification, characterization and some properties of the catalaze are also derived from apple[29]. The aim of the present study was to purify the catalaze from coriander leaves and some molecular properties and kinetic behaviour of the purified enzyme.

EXPERIMENTAL

DEAE-Sephadex A50, H_2O_2, protein assay reagent, chemicals for electrophoresis was purchased from Sigma Chem. Co. All other chemicals used were analytical grade and purchased from either Sigma or Merck.

Enzyme extraction and purification: Coriander (*Coriandrum sativum*) was grown in vermiculate for 2-3 weeks in a greenhouse. The leaves of plants were harvested and stored frozen in plastic bags until use. 30 g Plant leaves were pulverized in liquid nitrogen and homogenized with 125 mL of 0.125 M *tris*-HCl (pH = 7.3) containing 5 mM dithiothreitol. The suspension was centrifuged at 4 ºC for 0.5 h at 20,000 rpm and supernatant was collected[30]. The supernatant was brought to 20-50 % $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. Ammonium sulfate was slowly added to homogenate for completely dissolution. The mixture was centrifuged at 15,000 rpm for 0.5 h and precipitate was dissolved in 0.125 M *tris*-HCl (pH = 7.3) and than dialyzed at 4 ºC in the same buffer, 24 h with three changes of the buffer during dialysis[31].

For further purification of catalaze, the dialyzed enzyme extract was loaded onto a 30 × 3 cm column of DEAE-Sephadex (50 mL bed volume) equilibrated with the 0.125 M *tris*-HCl buffer (pH = 7.3) at a flow rate of 50 mL/h[31-33]. The elution process was performed in the presence of a decreasing linear gradient of NaCl (0-1 M). Eluates were collected in 3 mL tubes. Each 3 mL fractions were estimated for catalaze activity and UV absorbance was measured at 280 nm[31-33].

The fractions having catalaze activity were collected and purification degrees were determined by measuring specific activity before and after purification. For determining specific activity, catalaze activity and quantitative protein measurements were carried out. Protein contents were determined by the protein dye binding method[34].

Activity determination: The catalaze activity was determined by measuring the decrease in absorbance at 240 nm with a spectrophotometer. 10 μL of crude extract was added to 1 mL of substrate mixture containing 40 mM of 30 % H_2O_2 and 0.125 mM *tris*-HCl (pH = 7.3). One unit of activity was defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 per min calculated from the extinction coefficient[35] for H_2O_2 at 240 nm of 0.071 cm^2 μmol^{-1}.

Characterization of catalaze

Effect of ionic strength: Ionic strength effect on the enzyme was studied 40 mM substrate concentration using different concentration of the two buffers (0.0625-1.0 M potassium phosphate; 0.0625-1.0 M *tris*-HCl).

Effect of pH: The effect of pH on catalaze activity was different (0.125 M *tris*-HCl pH 7.0-9.0). After that, optimum pH was determined.

Stable pH determination: For this purpose, the enzyme activity was determined in 0.125 M *tris*-HCl buffer at pH of 7.0, 7.3, 7.5, 8.0, 8.5 and 9.0. In each experiment, the equal volumes of buffer and enzyme solutions

were mixed and kept refrigerated. Activity determinations were made with an interval of 3 h for 38 h.

Effect of temperature on catalazes activity: The effect of temperature on catalaze activity obtained at different temperature values (10-70 ºC, pH 7.3). After that optimum temperature was determined.

Molecular weight determination

Sephadex G-150 gel filtration chromatography: The molecular weight of enzyme was determined on the basis of Andrew's method[36]. The enzyme-containing tube was first determined. The void volume was observed with Blue Dextrane 2000. Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato β-amylase (200,000) were used as standards (Sigma: MW-GF-200).

SDS Polyacrylamide gel electrophoresis (SDS-PAGE): The subunit determination was made by SDS-PAGE[37]. Rabbit myosin (205,000), *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000) and chicken ovalbumin (45,000), bovine carbonic anhydrase (29,000) were used as standards (Sigma: MW-SDS-200).

Kinetic studies: For K_M and V_{max} evaluation, Lineweaver-Burk curves were used[38]. For catalase enzymes, 1/[S]-1/V graph was obtained at five different concentrations of H_2O_2 (0.075, 0.1, 0.15, 0.3, 0.6 mM). All kinetic studies were performed at 25 ºC and in optimal pH (0.125 M *tris*-HCl, pH 7.3).

RESULTS AND DISCUSSION

The purification process of coriander (*Coriandrum sativum*) leaves catalaze is summarized in Table-1. The first step used was ammonium sulfate fractionation. The catalaze activity in leaves was almost completely separated

TABLE-1
PURIFICATION SCHEME OF CATALASE FROM CORIANDER (*Coriandrum sativum*) LEAVES

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	53.52	70	38.190	2673.30	3746.4	1.40	100.00	1.00
Ammonium sulfate precipitation 20-50 %	341.12	10	31.960	319.60	3411.2	13.80	91.05	9.86
DEAE-Sephadex A50 ion exchange chromotography	40.00	10	0.446	4.46	400.0	89.68	10.67	64.06

(99 %) from catalase activity by subjecting the supernatant to 20-50 % saturated ammonium sulfate fractionation. The resultant pellet was dissolved with 0.125 M of *tris*-HCl (pH 7.3) buffer. After ammonium sulfate fractionation, DEAE-Sephadex A50 ion exchange chromatography was followed. The purification process showed a recovery of 10.67 % for the enzyme purification of 64.06-fold from coriander leaves and specific activity was calculated as 89.69 U/mg protein (Table-1).The purification of catalaze from coriander (*Coriandrum sativum*) leaves is rapid and can be performed in a day.

Fig. 1 shows the SDS-PAGE for the purity and subunit molecular weight of the enzyme. For the standard proteins and catalazes, R_f values were calculated and R_f-log MW graph (Fig. 2) was obtained according to Laemmli procedure[29] showing a molecular weight of 60.95 kDa for enzyme. The molecular weight of the enzyme was also determined by gel filtration chromatography. K_{av}-log MW graphs was obtained (Fig. 3), which showed a molecular weight of 58.12 kDa. The two methods showed similar molecular weights. Catalaze enzyme can be formed from monomer. Catalazes of different plant have similar molecular weight *i.e.*, potato[39], spinach[40] and cottonseed[41]. Similar values were obtained from purified catalaze of other sources[42,43].

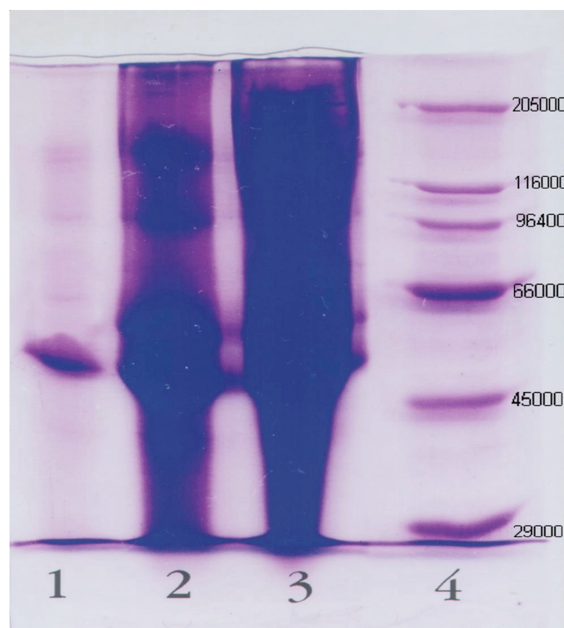

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified catalaze (Lane 1: catalaze; Lane 2: ammonium sulfate fractionation; Lane 3: homogenate; Lane 4: standard proteins)

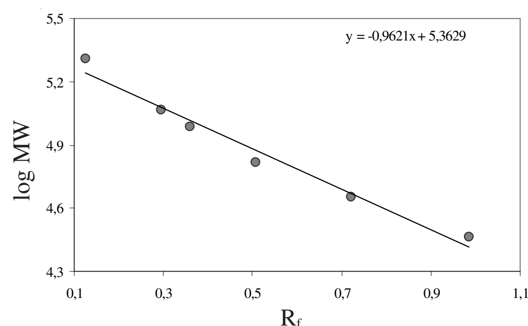

Fig. 2. Standard R_f-log MW graph of coriander (*Coriandrium sativum*) leaf catalaze using SDS-PAGE. (Standard: Rabbit myosin (205,000), *E. coli* β–galactosidase (116, 000), rabbit phosphorylase B (97, 400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000); R_f for enzyme: 0.60

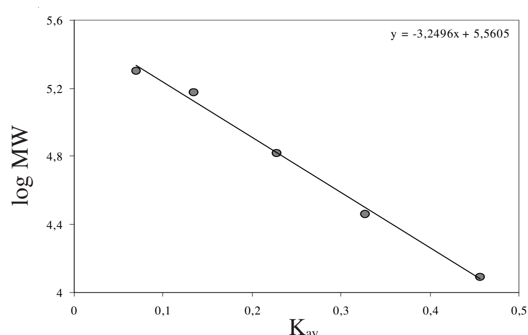

Fig. 3. Standards K_{av}-log MW GRAPH of coriander (*Coriandrium sativum*) catalaze using gel filtration. (Standards: Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydraze (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato β-amylase (200,000); K_{av} for coriander (*Coriandrium sativum*) leaf catalaze: 0.245.

The study of rate at which an enzyme works is called enzyme kinetics. Every enzyme has an optimal range. Activity decreases when an enzyme is exposed to conditions which are outside the optimal range.

The effects of ionic strength on the activity of catalaze is shown in Figs. 4 and 5. Appropriate ionic strength is 0.125 M KH_2PO_4 (pH = 7.0) and 0.125 M *tris*-HCl (pH = 7.5).

The optimum pH of the purified enzyme was *ca.* 7.3 using 0.125 M *Tris*-HCl (Fig. 6). The optimum pH was similar to that found in previous studies[33,44-46]. The stable pH profile of the enzyme was determined at six different pH (0.125 M *tris*-HCl) (Fig. 7). In general, most plants catalaze enzyme shows maximum activity between pH 6-8.

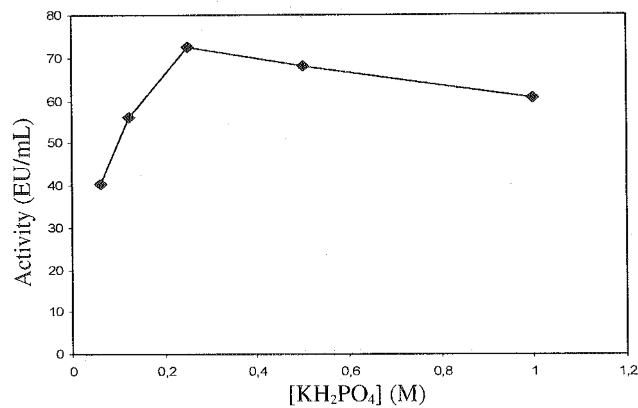

Fig 4. Activity-ionic strength graph of coriander (*Coriandrium sativum*) leaf catalaze in phosphate buffer

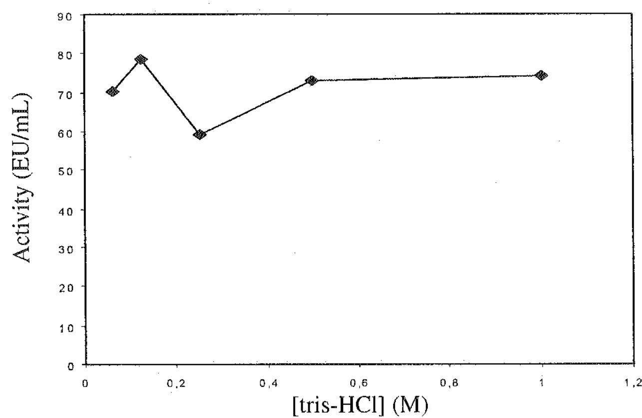

Fig 5. Activity-ionic strength graph of coriander (*Coriandrium sativum*) leaf catalaze in *tris*-HCl

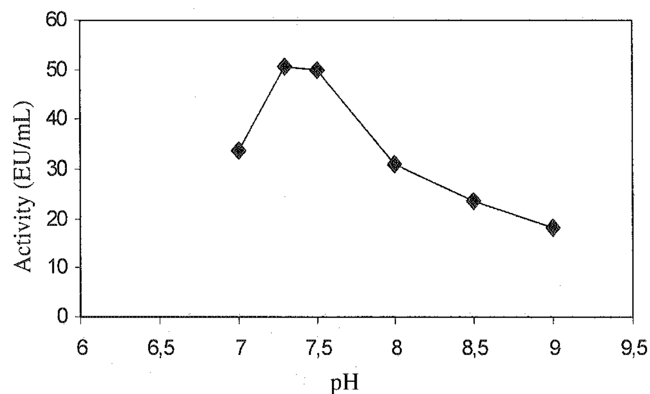

Fig. 6. Activity-pH graph of coriander (*Coriandrium sativum*) leaf catalaze

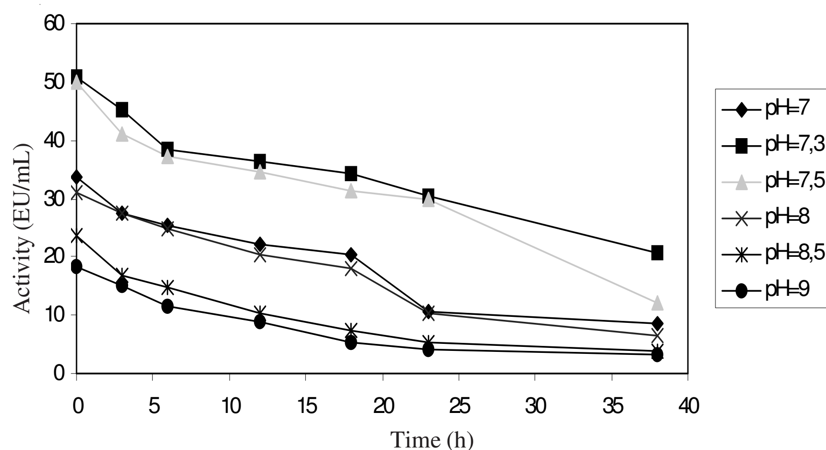

Fig 7. pH stability graphs of coriander (*Coriandrium sativum*) leaf catalaze in 0.125 M *tris*-HCl buffer

In general, chemical reactions speed up as temperature is increased. When the temperature increases, the reacting molecules have the kinetic energy required to undergo the reaction. Enzyme catalyzed reactions also tend to go faster with increasing temperature until optimum temperature is reached. Above this value the conformation of the enzyme molecule is disrupted. Changing the conformation of the enzyme results in less efficient binding of the substrate. Temperatures above 40-50 °C denature many enzymes[29]. The optimum temperature for catalaze activity was measured at different temperatures at 10-70 ° C. The highest activity was observed as 30 ºC (Fig. 8).

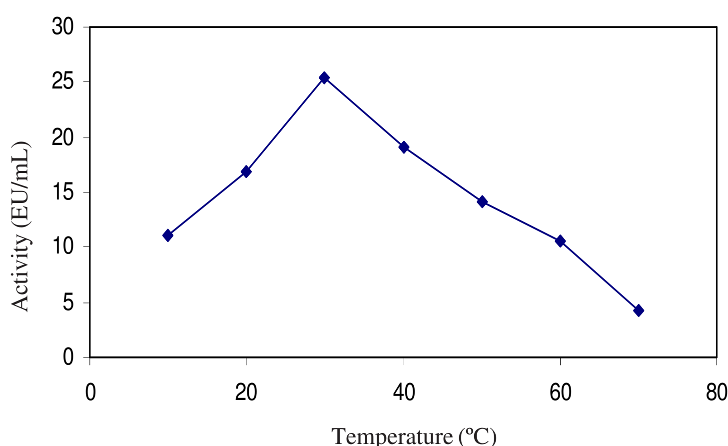

Fig. 8. Effect of temperature on coriander (*Coriandrium sativum*) leaf catalaze

K_M and V_{max} values for enzyme, at optimum pH and 25 ºC, were calculated from the Linewear-Burk graphs (Fig. 9). Getting K_M and V_{max} values are 7.87 mM and 42.19 U/mL for H_2O_2, respectively.

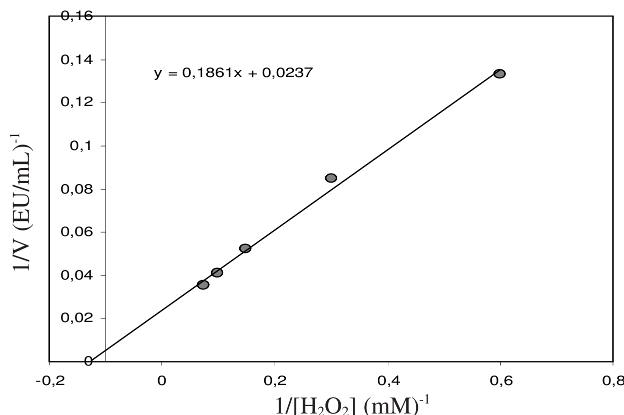

Fig. 9. Lineweaver-Burk graph in 5 different H_2O_2 concentrations

In conclusion, we described the purification of catalaze enzyme from coriander leaves by means of ammonium sulfate fractionation and DEAE-Sephadex A50 ion exchange chromatography and some molecular and kinetic properties of the enzyme.

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(*Received*: 10 March 2007; *Accepted*: 8 November 2007) AJC-6062