

## Purification and Characterization of Catalase Enzymes from Chicken Liver and Sheep Erythrocytes

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Catalase enzyme ( $H_2O_2$ :  $H_2O_2$  oxidoreductase; E.C. 1.11.1.6) was purified from chicken liver and sheep erythrocytes, using DEAE-Sephadex A50 ion exchange chromatography and some characteristics of the enzymes were investigated. The purification procedure was composed of 3 steps *i.e.*, homogenate/hemolysate preparation, ammonium sulfate precipitation and DEAE-Sephadex A50 ion exchange chromatography. Chicken liver and sheep erythrocytes enzymes, having the specific activity of 560.46 and 1017.5 EU/mg proteins were purified with a yield of 30.06 and 22.23 %; 190.63 and 643.9-fold, respectively. In order to control the purification of enzymes were done, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE showed a single band for each enzyme. Optimal pH, stable pH, optimal temperature,  $K_M$  and  $V_{max}$  values for  $H_2O_2$  were also determined for the each enzyme. In addition, molecular weight and subunit molecular weights were found by SDS-PAGE and gel filtration chromatography respectively.

**Key Words: Catalase, Purification, Characterization, Chicken, Liver, Sheep, Erythrocyte.**

### INTRODUCTION

Catalase is a very important enzyme for protection of cells from the toxic effects of  $H_2O_2$  and radical oxygen species. Such as superoxide, hydroxyl radical and hydrogen peroxide reactive oxygen species are generated in the metabolism and they attacks cell components such as DNA, protein and lipid membrane. Sometimes lethal damages may occur in the cells. These potentially injurious are neutralized by antioxidant enzymes such as catalases, superoxide dismutases and peroxidases<sup>1-6</sup>. Superoxide dismutase (which catalyses superoxide anion to  $H_2O_2$ ) and catalase are components of an antioxidant complex which used in the therapeutic such as oxidative injury and especially in myocardial ischaemia reperfusion oxidative injury<sup>7-9</sup>. Catalase catalyzes the decomposition of

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hydrogen peroxide to molecular oxygen and water without the production of free radicals<sup>1-3,8</sup>.

Most catalases are homotetramer with each possessing a ferric heme and tetramers have a relative molecular weight in the range from 225,000 to 270,000 Da<sup>6,7</sup>. Catalase is found in almost all aerobic organisms. Catalase enzyme of beef liver was one of the first intracellular enzymes crystallized<sup>10</sup>. Many catalases enzymes have been isolated from a number of animals, plants and microorganisms and their enzymatic properties have been studied<sup>11</sup>.

This paper describes the purification and characterization of chicken liver and sheep erythrocytes catalase. The aim of this study is to purify catalase enzyme in chicken liver and sheep erythrocytes by DEAE-Sephadex A50 ion exchange chromatography and characterization and comparison to purified enzyme from chicken liver and sheep erythrocytes with those the other living species.

## EXPERIMENTAL

DEAE-Sephadex A50, H<sub>2</sub>O<sub>2</sub>, protein assay reagents and chemicals for electrophoresis were purchased from Sigma Chem. Co. All other chemicals used for analytical grade were purchased from either Sigma or Merck.

**Preparation of the Homogenate:** 20 g of fresh chicken liver was cut with a knife. Excess blood, foreign tissues and membranes were removed from the samples. Tissue was suspended with 5 volumes (w/v) of 10 mM phosphate buffer (pH: 7.5) containing 5 mM CaCl<sub>2</sub> and was homogenized using a mixer at top speed for 3 min. The material was homogenized by ultrasonic homogenizer for 2 min. After that, the homogenate was centrifuged at 7,000xg for 20 min and the supernatant was used for ammonium sulfate precipitation. Temperature was maintained at 4°C during the homogenization process<sup>2</sup>.

**Preparation of the hemolysate:** 30 mL fresh sheep blood collected in tubes with EDTA (5 mM) was centrifuged at 2,500xg for 15 min and the plasma and leukocyte coat were removed by drip. The packed red cells were washed with KCl solution (0.16 M) 3 times. The samples were centrifuged at 2,500xg each time and supernatants were removed. One volume erythrocytes were haemolysed with 5 volumes of ice-cold water and centrifuged 10000xg for 0.5 h at 4°C, to remove the ghosts and intact cells<sup>12</sup>.

**Ammonium sulfate precipitation and dialysis:** Chicken liver homogenate and sheep red blood cell hemolysate were subjected to precipitation orderly with ammonium sulfate (10-20, 20-30, 30-40, 40-50, 50-60 and 60-70 %). For each respective precipitation, the enzyme activity was determined both in supernatant and in precipitate. Both enzymes were

observed to precipitate at 20-50 % precipitation. The precipitates were dissolved minimum volume of 10 mM phosphate buffer (pH: 7.5) and dialyzed in same buffer for 2 h with two changes of buffer<sup>2</sup>.

**Preparation of ion exchange chromatography material:** Ion exchange chromatography material was prepared from DEAE-Sephadex A50. 10 g dried DEAE-Sephadex A50 gel was used for 50 mL column volume. The gel was to blow up with distilled water at the 80-90°C and to remove foreign bodies and air of swollen gel was eliminated. The gel was suspended in 10 mM phosphate buffer (pH 7.5), then packed in a column (3 × 30 cm) and equilibrated and washed with same buffer. The flow rates for washing and equilibration were adjusted by peristaltic pump 50 mL/h<sup>1,2,13</sup>.

**Purification of catalase by ion exchange chromatography:** Dialyzed and filtered sample was loaded on DEAE-Sephadex A50 column and the gel was washed with 10 mM phosphate buffer (pH 7.5) until the absorbance of the column eluate at 280 nm is < 0.05. Bound proteins were eluted with a gradient of 0 to 400 mM sodium chloride in 10 mM phosphate buffer (pH 7.5) at flow rate of 20 mL/h. Eluates were collected in 2 mL tubes and each of their activity and absorbance were separately determined at 240 and 280 nm, respectively. Active fractions were collected. All of the procedures were performed at 4°C<sup>1,2,13</sup>.

**Protein determination:** Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin being used as a standard<sup>14</sup>.

**SDS Polyacrylamide gel electrophoresis (SDS-PAGE):** The control of enzyme purity, using Laemmli's procedure, was carried out in 3 and 8 % acrylamide concentrations for running and stacking gel, respectively. To the gel solution was added 10 % SDS. The gel was stabilized in the solution containing 50 % propanol + 10 % TCA + 40 % distilled water for 0.5 h. The staining was made for about 2 h in the solution of 0.1 % Coomassie Brilliant Blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in the solution of 50 % methanol + 10% acetic acid + 40 % distilled water until protein bands were cleared<sup>15</sup>.

**Optimal pH determination:** For the optimal pH determination, the enzyme activity was measured in 50 mM tris-HCl and phosphate buffers within the pH of 7.0 to 9.0 and of 5.0 to 8.0, respectively.

**Stable pH determination:** For this purpose, the enzyme activity was determined in 50 mM tris-HCl buffer at pH of 7.0, 7.5, 8.0, 8.5 and 8.9 and in 50 mM phosphate buffer at pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. In each experiment, the equal volumes of buffer and enzyme solutions were mixed and kept refrigerated (4°C). Activity determinations were made with an interval of 8 h for 24 h.

**Effect of temperature on CATs activity:** The enzymes activities were measured between 20 and 75°C at optimal pH for this purpose.

#### Molecular weight determination

**Sephadex G-200 gel filtration:** The molecular weight of the enzyme was determined on the basis of Andrew's method<sup>16</sup>. 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  $\beta$ -amylase (200,000) were used as standards (Sigma: MW-GF-200).

**SDS-PAGE:** The subunit determination was made by SDS-PAGE. *Rabbit myosin* (205,000), *E. coli*  $\beta$ -galactosidase (116,000), *Rabbit phosphorylase B* (97,400), *Bovine albumin* (66,000), *Chicken ovalbumin* (45,000) and 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<sup>17</sup>. For sheep and chicken catalase enzymes,  $1/[S]-1/V$  graph was obtained in 5 different concentrations of  $H_2O_2$  ( $5.44 \times 10^{-4}$ ,  $6.8 \times 10^{-4}$ ,  $1 \times 10^{-3}$ ,  $1.35 \times 10^{-3}$  and  $1.7 \times 10^{-3}$  mM). All kinetic studies were performed at 25°C and in optimal pH (50 M phosphate, pH 7.5).

## RESULTS AND DISCUSSION

Tables 1 and 2 shows purification characterized chicken liver and sheep erythrocytes catalase with a specific activity of 560.46 and 1017.5 EU/mg proteins, a yield of 30.06 % and 22.23 and a purification coefficient of 190.63 and 643.9, respectively. Elution profile of chicken liver and sheep erythrocytes CATs from DEAE-Sephadex A50 ion exchange column with a gradient of 0 to 400 mM sodium chloride in 10 mM phosphate buffer (pH 7.5) showed in Figs. 1a and 1b.

Fig. 2 exhibits the SDS-PAGE made for the purity and molecular weight of the chicken liver and sheep erythrocytes enzymes. For the standard proteins and CATs,  $R_f$  values were calculated and  $R_f$ -log MW graph (Fig. 3) was obtained according to Laemmli procedure, showing a molecular weight of 64,876 for both enzyme. The molecular weights of the enzymes were also determined by gel filtration chromatography.  $K_{av}$ -log MW graphs was obtained (Fig. 4), which showed a molecular weight of 245,595 (chicken liver CAT) and 242,128 Da (sheep erythrocytes CAT).

Optimal pHs of both enzymes have been determined as 7.5 using 50 mM phosphate buffer (Figs. 5a and 5b). The stable pH of the each enzyme was 7.5 in 50 mM phosphate buffer (Figs. 6a and 6b). Both enzymes were seen to show the highest activities at 30°C (Figs. 7a and 7b) between 20-90°C.

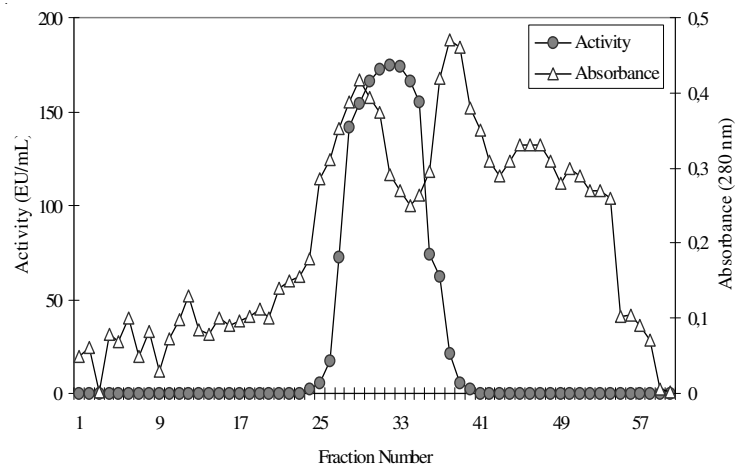


Fig. 1a. Elution profile of chicken liver CAT activity from DEAE-Sephadex A50 ion exchange column (3 × 30 cm) with a 0-400 mM sodium chloride in 10 mM phosphate buffer pH 7.5 at 4°C

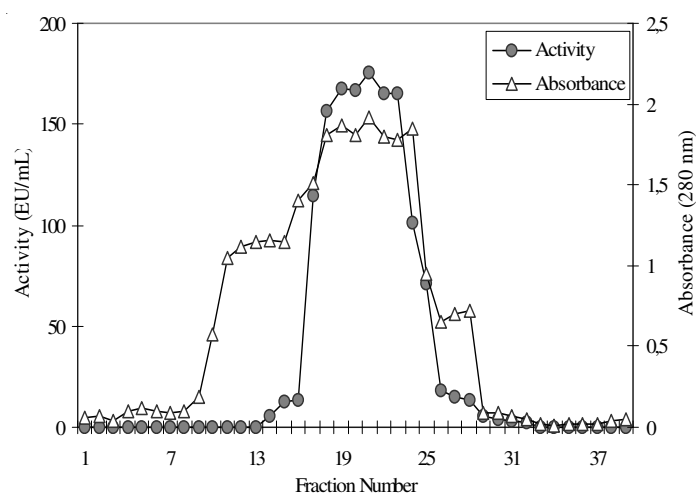


Fig. 1b. Elution profile of sheep erythrocytes CAT activity from DEAE-Sephadex A50 ion exchange column (3 × 30 cm) with a 0-400 mM sodium chloride in 10 mM phosphate buffer pH 7.5 at 4°C

The Lineweaver-Burk graphs were shown in Figs. 8a and 8b for chicken liver and sheep erythrocytes CATs. A  $K_M$  of 0.0119 mM and a  $V_{max}$  of 238 EU/mL were obtained for chicken liver and 0.015 mM and 303 EU/mL for sheep CAT.

In living cells, biological antioxidant systems are enzymes such as superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidases (APx), glutathione peroxidases (GPx) and glutathione reductase (GR) and non-

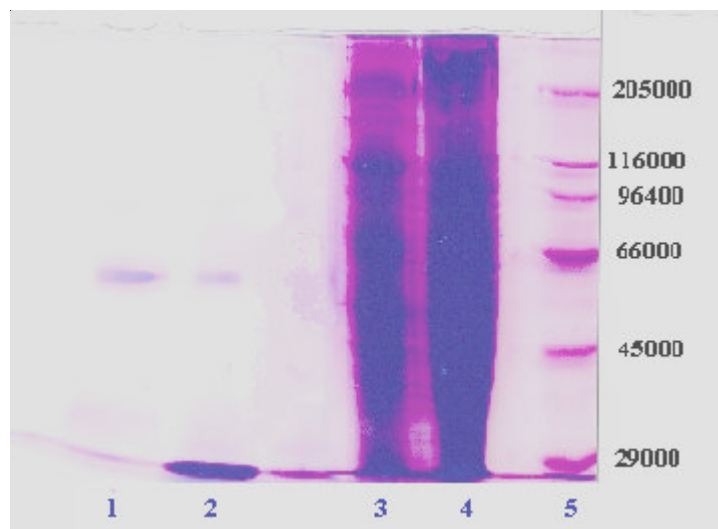


Fig. 2. SDS-PAGE bands of chicken liver CAT (Lane 1: Sheep erythrocytes CAT; Lane 2: Chicken liver CAT; Lane 3: Sheep erythrocytes ammonium sulfate precipitation sample; Lane 4: Chicken liver ammonium sulfate precipitation sample; Lane 5: Standard proteins)

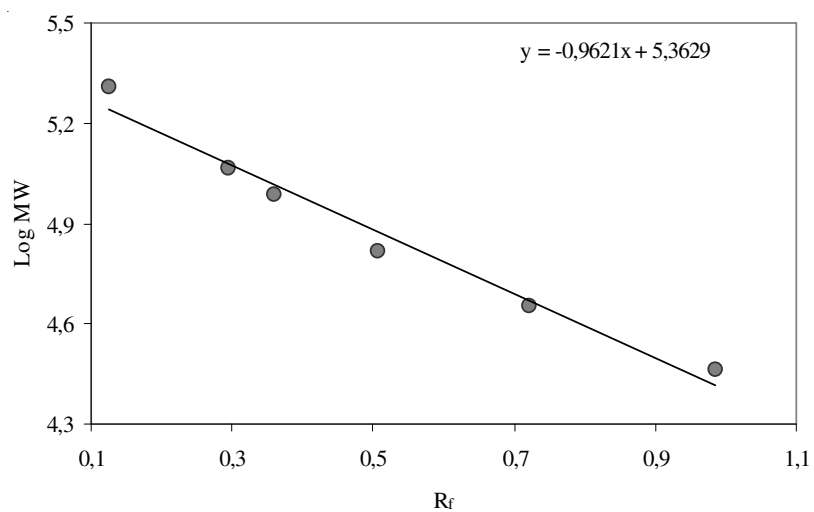


Fig. 3. Standard R<sub>f</sub> - log MW graph of chicken liver and sheep erythrocytes CATs using SDS-PAGE. (Standards: Rabbit myosin (205,000), *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000), R<sub>f</sub> for both enzymes: 0.5735)

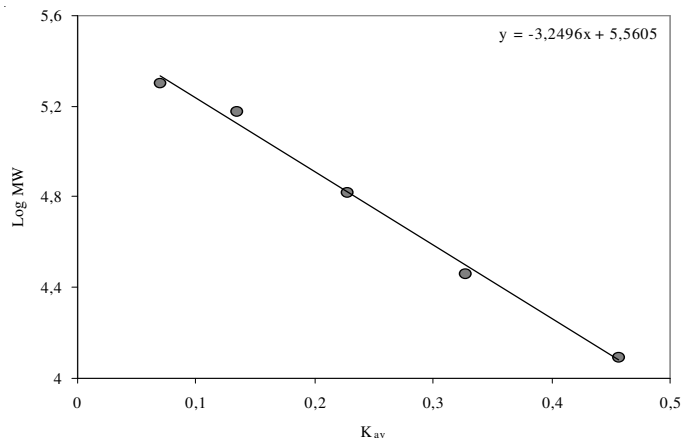


Fig. 4. Standard  $K_{av}$ -log MW graph of chicken liver and sheep erythrocytes CATs using gel filtration. (Standards: Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato  $\beta$ -amylase (200,000;  $K_{av}$  for chicken liver CAT: 0.0524;  $K_{av}$  for sheep erythrocytes CAT: 0.0543)

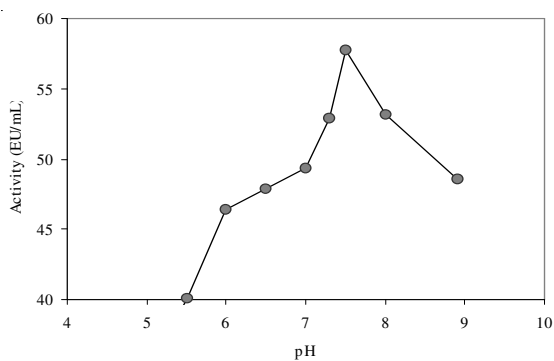


Fig. 5a. Activity-pH graph of chicken liver CAT

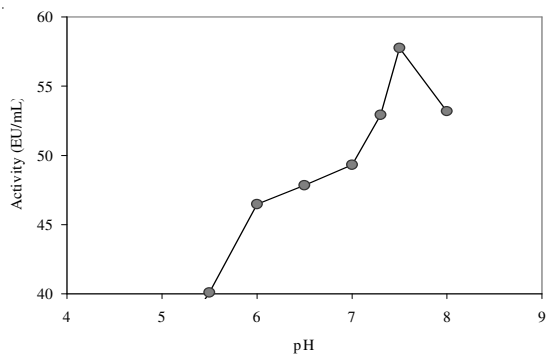


Fig. 5b. Activity-pH graph of sheep erythrocytes CAT

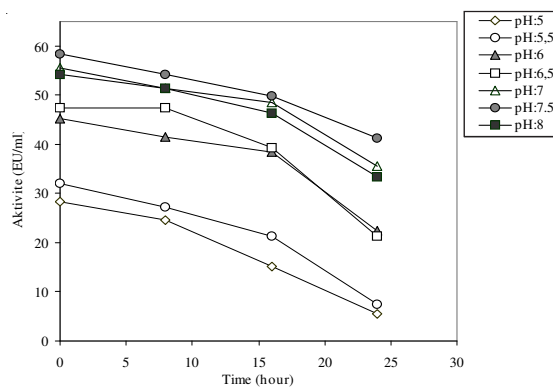


Fig. 6a. Stable pH graph of chicken liver CAT in 50 mM phosphate buffer

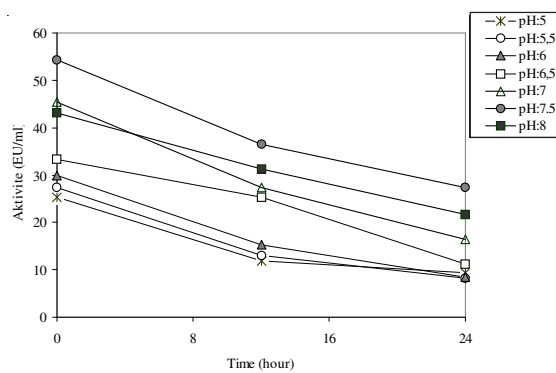


Fig. 6b. Stable pH graph of sheep erythrocytes CAT in 50 mM tris-HCl buffer

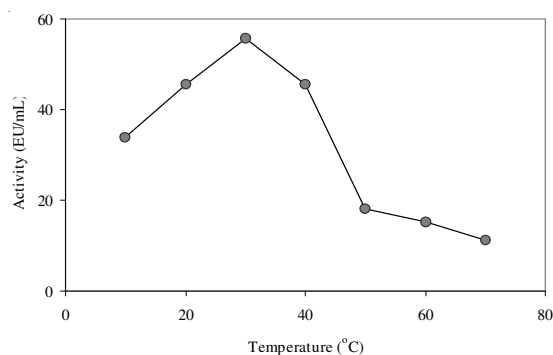


Fig. 7a. The effects of the temperature on chicken liver CAT



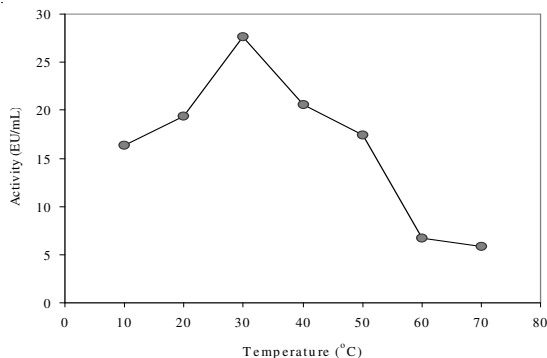


Fig. 7b. Effects of the temperature on sheep erythrocytes CAT

enzymic components *i.e.*, reduced glutathione (GSH), cysteine, hydroquinones, mannitol, vitamins C and E, flavonoids, some alkaloids and  $\beta$ -carotene. Biological antioxidants can prevent the uncontrolled formation of free radicals and activated oxygen species or inhibit their reactions with biological structures. The destruction of most free radicals and activated oxygen species relies on the oxidation of endogenous antioxidants, mainly scavenging and reducing molecules<sup>18-20</sup>.

In the present study, catalase enzymes were purified and characterized from chicken liver and sheep erythrocytes with a method which is consisted of 3 steps *i.e.*, homogenate/haemolysate, ammonium sulfate precipitation and DEAE-Sephadex A-50. Jang *et al.*<sup>2</sup> were made the purification of CAT from bullfrog liver with a method which is consisted of 6 steps (crude extract, ammonium sulfate, DEAE-Sephadex A-50, Sephadex G-150, DEAE-Sephacel, Sephacryl S-300). Goncalves *et al.*<sup>7</sup> purified CAT from human placenta with another method which is consisted of 6 steps. In another study Aydemir and Kuru<sup>1</sup> purified CAT from human erythrocytes with a method which is consisted of 4 steps<sup>1</sup>.

In present method, after ammonium sulfate precipitation (20-60 % fractionation) and dialyzed, the sample was loaded on DEAE-Sephadex A-50 ion exchange column and the enzyme was eluted with 0-400 mM NaCl / 10 mM phosphate buffer (pH 7.5) (Figs. 1a and 1b). By this way, CATs enzymes from chicken liver and sheep erythrocytes were purified and all purification steps were shown in Tables 1 and 2. We report here an application of this method to a rapid purification from 20 g of chicken liver and 30 mL sheep blood, which can be performed in a day. Both enzymes precipitated between 20-50 % in the ammonium sulfate saturation. In this way, a lot of unpurified proteins were removed from sample.

To control the purification of the each enzyme, SDS-PAGE was done for each purification step and the electrophoresis patterns were

TABLE-1  
PURIFICATION SCHEME OF CATALASE FROM CHICKEN LIVER

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	88.30	114	30.0	3420.0	10066.2	2.94	100.00	1.00
Ammonium sulfate precipitation (20-50)%	147.59	43	47.0	2021.0	6346.3	3.14	63.04	1.06
DEAE-Sephadex A50 ion exchange chromatography	168.14	18	0.3	5.4	3026.5	560.46	30.06	190.63

TABLE-2  
PURIFICATION SCHEME OF CATALASE FROM SHEEP ERYTHROCYTES

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	73.2	140	46.30	6482.00	10252.2	1.58	100.00	1.00
Ammonium sulfate precipitation (20-50)%	123.5	34	36.60	1244.40	4199.0	3.37	40.95	2.13
DEAE-Sephadex A50 ion exchange chromatography	162.8	14	0.16	2.24	2279.2	1017.50	22.23	643.90

photographed (Fig. 2). Both enzymes have a single band after DEAE-Sephadex A-50 ion exchange gel chromatography step. By using the  $K_{av}$  from the Sephadex G-150 gel filtration chromatography and  $R_f$  values from SDS-PAGE, the molecular weight of chicken liver and sheep erythrocytes CAT were calculated as 64,876 - 245,595 and 64,87 - 242,128 Da, respectively (Figs. 3 and 4). Catalase enzyme generally in the different sources can form homotetramers of the identical subunits<sup>1,2,7,21-24</sup>. Since the molecular weights determined by the two methods were different to each other for both enzymes, suggesting the enzymes to be a homotetramer in the active state.

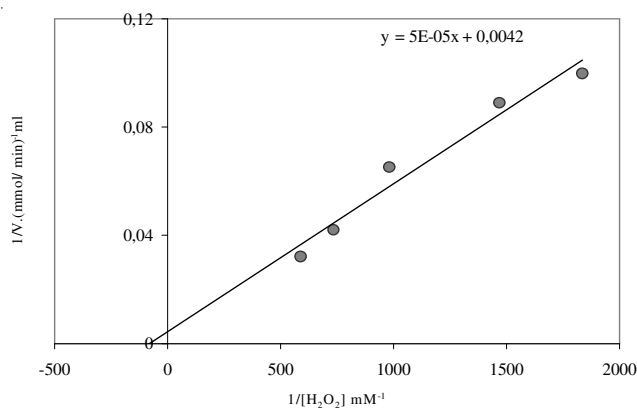


Fig. 8a. Lineweaver-Burk graph in 5 different H<sub>2</sub>O<sub>2</sub> concentrations

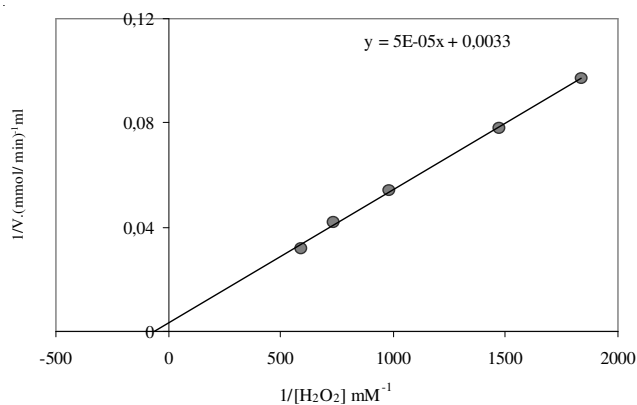


Fig. 8b. Lineweaver-Burk graph in 5 different H<sub>2</sub>O<sub>2</sub> concentrations

The stable pHs profile of enzymes were determined in 50 mM phosphate (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and 50 mM tris-HCl buffers (pH 7.0, 7.5, 8.0, 8.5 and 8.9). The stable pHs were found as pH 7.5 in 50

mM phosphate for both enzymes (Figs. 6a and 6b). Similar result had been found for chicken erythrocytes<sup>1</sup>.

In order to determine optimum pH, CATs activities were calculated with 50 mM phosphate and 50 mM tris-HCl buffers and the optimum pHs were found as pH 7.5 in 50 mM phosphate for both enzymes (Figs. 5a and 5b). Similar results had been found for chicken, human, pig, horse erythrocytes and bullfrog liver<sup>1,7,21-24</sup>.

The effects of temperatures between 10 and 70°C on CAT activity were assayed and were shown in Figs. 12 and 13. As seen in Figs. 7a and 7b, maximum activities were found at 30°C for both enzymes. Similar result had been found for chicken erythrocytes<sup>1</sup>.

$K_M$  and  $V_{max}$  values for enzyme, at optimum pH and 25°C, were calculated from the Lineweaver-Burk graphs (Figs. 8a and 8b).  $K_M$  values were 0.0119 mM for chicken liver, 0.015 mM for sheep erythrocytes and  $V_{max}$  values were 238 EU/mL for chicken liver and 303 EU/mL for sheep erythrocytes. According to these  $K_M$  values,  $H_2O_2$  has higher affinity to chicken liver CAT than sheep erythrocytes CAT. The  $K_M$  values determined in our study for sheep erythrocytes and chicken liver were similar to those obtained in bullfrog liver, chicken erythrocytes, goat liver and bovine liver<sup>2,25,26</sup>.

Herein we described the purification of catalase enzyme from chicken liver and sheep erythrocytes and investigation of their kinetic properties such as, optimum pH, optimum temperature, stable pH, molecular weight,  $K_M$  and  $V_{max}$ .

## REFERENCES

1. T. Aydemir and K. Kuru, *Turk. J. Chem.*, **27**, 85 (2003).
2. M.J. Jang, P.J. Park, W.K. Jung and S.K. Kim, *J. Food Biochem.*, **28**, 435 (2004).
3. R. Lemberg and J.W. Legge, *Hematin Compounds and Bile Pigments*, New York, Interscience, pp. 415-424 (1949).
4. N.J. Brown-Peterson and M.L. Salin, *J. Bacteriol.*, **175**, 4197 (1993).
5. I. Fridovich, *Arch. Biochem. Biophys.*, **274**, 1 (1986).
6. C. DeDuve and P. Baudhum, *Physiol. Rev.*, **46**, 323 (1966).
7. V.M. Goncalves, L.C.C. Leite, I. Raw and J. Cabrera-Crespo, *Biotechnol. Appl. Biochem.*, **29**, 73 (1999).
8. R.A. Greenwald, *Free Radicals Biol. Med.*, **8**, 201 (1990).
9. M.E. Zughuib, X.L. Tang, J.Z. Sun and R. Bolli, *Ann. N.Y. Acad. Sci.*, **723**, 218 (1994).
10. J.B. Sumner and A.L. Dounce, *J. Biol. Chem.*, **121**, 417 (1937).
11. I. Yamazaki, *Prot. Nucl. Acid Enzyme (Tokyo)*, **33**, 282 (1988).
12. S. Beydemir, M. Çiftçi and Ö.I. Küfrevioğlu, *J. Enzyme Inhib. Med. Chem.*, **17**, 271 (2002).
13. A. Çoban, M. Çiftçi and Ö.I. Küfrevioğlu, *Preparative Biochem. Biotechnol.*, **32**, 173 (2002).
14. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
15. U.K. Laemmli, *Nature*, **227**, 680 (1970).

16. P. Andrews, *Biochem. J.*, **96**, 595 (1965).
17. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **57**, 685 (1934).
18. J.G. Scandalios, *Plant Physiol.*, **101**, 7 (1993).
19. R.D. Allen, *Plant Physiol.*, **107**, 1049 (1995).
20. J. Chaudiere and R. Ferrai-Iliou, *Food Chem. Toxicol.*, **37**, 949 (1999).
21. A. Hochman and I. Goldberg, *Biochim. Biophys. Acta*, **1077**, 299 (1991).
22. A. Saha, D.H. Campbell and W.A. Schroeder, *Biochim. Biophys. Acta*, **85**, 38 (1964).
23. K. Nakamura, M. Watanabe, S. Sawai Tanimato and T. Ikeda, *J. Biochem. Cell Biol.*, **30**, 823 (1998).
24. A. Takeda, K. Hirano, Y. Shiroya and T. Samejima, *J. Biochem.*, **93**, 967 (1983).
25. U. Chatterjee, A. Kumar and G.G. Sanwall, *Indian J. Biochem. Biophys.*, **26**, 140 (1989).
26. A.I. Fernandes and G. Gregoriadis, *Biochim. Biophys. Acta*, **1293**, 90 (1996).

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