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Purification and Partial Characterization of Catalase from Chicken Erythrocytes and the Effect of Various Inhibitors on Enzyme Activity

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Catalase plays a major role in the protection of tissues from the toxic effects of H_2O_2 and partially reduced oxygen species. A nearly 136-fold enzyme purification was obtained from chicken erythrocyte by acetone precipitation, ethanol-chloroform treatment, CM-cellulose and Sephadex G-200 chromatography. The specific activity of purified enzyme was 42,556 U/mg. The molecular weight of the native chicken erythrocyte catalase was estimated at 240 kDa by gel filtration. SDS-gel electrophoresis results indicated that chicken erythrocyte catalase consists of four apparently identical subunits, with a molecular weight of around 57.5 kDa. The optical spectrum of the purified enzyme shows a Soret band at 406 nm, which is the characteristic for the heme group. Dithionite treatment of the enzyme resulted in the reduction of enzyme. The Km of chicken erythrocyte catalase was 33 mM H_2O_2. The maximal activity of catalase was observed between pH 6.0 and 8.0. Enzyme activity was stable at temperatures between 10 and 30°C. The activity of purified catalase was inhibited by azide, cyanide, β-mercaptoethanol, dithiotreitol (DTT) and iodoacetamide.

Key Words: Chicken erythrocyte, antioxidant enzyme, purification, characterization, isolation, inhibitors.

Introduction

Metabolism in an environment containing oxygen results in the generation of reactive oxygen species such as superoxide, hydroxyl radical, and hydrogen peroxide. These reactive oxygens attack almost all cell components, DNA, protein and lipid membrane, and sometimes cause lethal damage to the cells. Organisms have evolved sophisticated and efficient enzyme systems to neutralize these potentially injurious reactive oxygen species, including catalases, superoxide dismutases, and peroxidases. The iron-containing enzyme catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1,16,1,6) catalyzes the breakdown of H_2O_2, which is a potentially destructive agent in cells. It is primarily an intracellular enzyme, as confirmed by Agner in the case of blood catalase[1−5].

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Catalases isolated from higher organisms resemble each other in that they have molecular weights in the range of 225,000 to 270,000, contain four equally sized subunits each containing one ferric heme prosthetic group (protoporphrin IX) and show activity at a broad pH range of 5.0 to 10.5[6–8].

The erythrocyte catalase has been purified from man, the horse, pig, rabbit, rat, dog and cattle[7–14]. However, little to no detailed investigation of chicken erythrocyte catalase has appeared in the literature.

In this work, we studied the purification and characterization of catalase from chicken erythrocyte. The inhibitory effects of various chemicals on erythrocyte catalase activity were also examined.

Materials and Methods

Chemicals

Hydrogen peroxide, bovine serum albumin, ammonium sulfate, and chloroform were purchased from Merck AG, Germany. Sephadex G-200, CM-cellulose, β-mercaptoethanol, dithiothreitol, sodium azide, urea, glysin, arginine, alanine, glutamic acid, aspartic acid, molecular weight standards for size exclusion and SDS/PAGE were purchased from Sigma Chemical Company, USA. Other reagents were all analytical grade and supplied by various companies.

Enzyme assay

The catalase activity was measured by the Aebi method[15]. Then 20 μl enzyme solution was added to the 1 ml 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 25 °C for 1 min. Initial reaction rate was measured from the decrease in absorbance at 240 nm.

Protein determination

Protein was determined by the Bradford dye-binding method using bovine serum albumin (BSA) as the standard[16].

Purification of catalase from chicken erythrocytes

Unless otherwise stated, all purification steps were carried out at 4°C. Fresh venous chicken blood with 15% (v/v) citric acid solution as an anticoagulant was used throughout the study. Venous blood plasma was removed by centrifugation at 4000 g for 15 min. Erythrocytes were washed three times with cold 0.9% saline and hemolyzed by adding two volumes of cold distilled water, stirring for 10 min and then freeze-thawing.

Fractional precipitation of proteins in hemolysate was achieved by chilled acetone in the range of 20-75%. Precipitated proteins were separated by centrifugation at 14,000 g for 30 min. The precipitate was suspended in 20 mM potassium phosphate buffer, pH 7.0. The samples were stirred for 15 min and the insoluble material removed by centrifugation at 14,000 g for 15 min.

Then 0.5 ml of ethanol-chloroform mixture (3 parts of chloroform to 1 part of ethanol) was added to each ml of the supernatant and mixed vigorously for one minute. The denaturated hemoglobin is removed by centrifugation (14,000 g, 30 min). The ethanol–chloroform mixture is then evaporated in vacuo.

The supernatant of the ethanol-chloroform treatment was applied to a CM-cellulose column (1.5 x 60 cm) previously equilibrated with 20 mM phosphate buffer pH 7.0. The column was washed with 2-bed volumes of the equilibration buffer and the enzyme was eluted with a linear gradient of phosphate buffer

(50-100 mM, pH 7.0). Fractions of 5 ml were collected at a flow rate of 1 ml/min. The active fractions were pooled and concentrated by 30,000 NMWC ultrafilter.

The concentrate obtained was loaded to the Sephadex G-200 gel filtration column (1.0 x 90 cm) equilibrated with 0.05 M phosphate buffer pH 7.0. Enzyme elution was carried out with the 100 mM phosphate buffer pH 7.0 at a flow rate of 0.2 ml/min. Fractions of 3 ml were collected and catalase activity assayed.

Molecular weight determination of purified catalase

The molecular weight of the chicken erythrocyte catalase was determined by size exclusion on a Sephadex G-200 column previously equilibrated in 50 mM phosphate buffer, pH 7.0[17].

Bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-Amilase (200 kDa), and apoferritin (443 kDa) were used as molecular weight calibration markers.

Sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis of purified catalase

Subunit molecular weight of chicken erythrocyte catalase was estimated electrophoretically with SDS-PAGE by the Laemmli method[18] using 7% stacking gel and 12% running gel. Electrophoresis was performed at a constant 125V (8.0 mA) for 4 h in Tris-HCl buffer pH 8.3. After electrophoresis, proteins in the separating gel were made visible by staining with Coomassie Brilliant Blue. Plasma albumin (66 kDa), phosphorilase (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa) were used as molecular weight markers.

Effect of pH

The effect of pH on catalase activity was investigated in the range of 4.0 to 10.0 of 0.05 M phosphate buffer. Then 10 μl enzyme solution (specific activity: 42,500 U/mg, protein concentration: 9.5 μg/ml) was mixed with 0.990 ml phosphate buffer at various pH values and incubated at 25°C for 1 h. Thereafter retained enzyme activity was measured as indicated in the "Enzyme Assay" section.

Effect of temperature

The effect of temperature on enzyme activity was investigated by incubating the enzyme in 50 mM phosphate buffer, pH 7.0 for 60 min in the range of 10 to 80°C. Then 10 μl enzyme solution (specific activity: 42500 U/mg, protein concentration: 0.0095 mg/ml) was mixed with 0.990 ml phosphate buffer and incubated. Thereafter retained enzyme activity was measured as indicated in the "Enzyme Assay" section.

Storage stability

The storage stability of catalase was determined by 16 weeks of incubation of the enzyme at three different temperatures (-15, 4 and 25°C). Then 100 μl enzyme solution with 42,000 U/mg specific activity and 9.5 μg/ml protein in 50 mM phosphate buffer pH 7.0 was stored at the temperatures indicated above. Then the retained activity of the enzyme was measured at weekly intervals. Catalase samples stored at –15 °C were kept in separate vials for each observation to avoid repeated freeze-thaw effect on the enzyme.

Enzyme kinetics

All kinetic studies were performed with the enzyme having specific activity: 39,400 U/mg and 9.5 μg/ml protein concentration. Reactions for activity measurements were carried out for 1 min at 25°C in 0.05 phosphate buffer pH 7.0 using increasing H_2O_2concentrations in a range of 5 to 25 mM. Initial reaction rates were determined spectrophotometrically by measuring the decrease of absorbance at 240 nm. The apparent Km H_2O_2 value was estimated by the Lineweaver-Burk plot.

Effect of some compounds on enzyme activity

The effect of inhibitors (β-mercaptoethanol, DTT, urea, SDS, glutaraldehyde, iodoacetamide, L-Gly, L-Ala, L-Glu, L-Asp) on catalase activity was determined by measuring the enzyme activity in various concentrations of inhibitors in 50 mM phosphate buffer pH 7.0.

To determine the inhibitor concentration that reduced the enzyme activity by 50% (I_{50}) regression analysis graphs were drawn by using percent inhibition values. I_{50} values were determined from these graphs.

Results

Purification of catalase

The purification profile of the catalase from chicken erythrocytes is given in Table 1. The elution profiles of enzyme from CM-Cellulose and Sephadex G-200 columns are shown in Figures 1 and 2. The highest specific activity (42,557 U/mg) was obtained in the pooled fractions from the Sephadex G-200 column. The overall yield of highest purification was 1.68 and purification was nearly 136-fold.

Table 1. Purification of chicken erythrocyte catalase.

Purification Steps	Volume (ml)	Total Protein (mg)	Volum Activity (U/ml)	Specific Activity (U/mg)	Yield %	Purification fold
Initial extract	40	436	3421.1	313.86	100.00	1.00
Acetone fractionation (20-75%)	20	82	3552.3	866.41	59.33	2.70
Ethanol-chloroform treatment	8 2.64	1091.9	3308.78	7.29	1.00	
CM-Cellulose	12	0.372	697.57	22504.25	6.99	71.70
Sephadex G-200	5	0.0475	404.29	42556.84	1.68	135.59

The molecular weight of the purified enzyme was determined by gel filtration to be approximately 240 kDa as shown in Figure 2. The molecular weight of the subunits of chicken erythrocyte catalase was estimated by SDS gel electrophoresis (Figure 3). The resulting gel reveated only a wide single band with a molecular weight of around 57.5 kDa. This indicates that the chicken erythrocyte catalase molecule consists of four identical subunits.

Changes in the absorption spectra of catalase in various inhibitors

The absorption spectrum of purified enzyme showed two major peaks, at 270 and 406 nm. Besides the Soret band at 406 nm, several other very small bands were observed in the visible part of spectrum at 539, 577 and 635 nm (Figure 4). A_{406}/A_{270} ratio was 0.57.

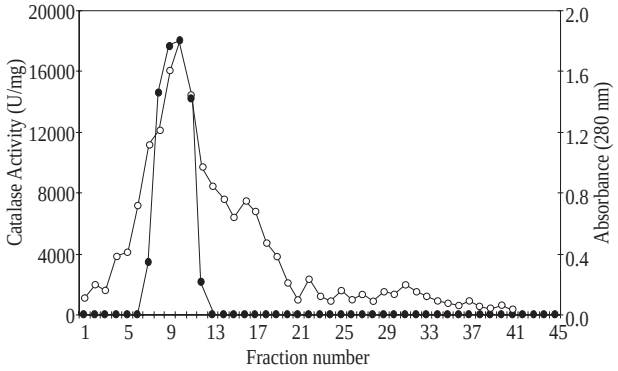

Figure 1. Chromatographic separation of chicken erythrocyte catalase on CM-cellulose. Catalase activity (U/mg) (–●–), Absorbance at 280 nm (–○–).

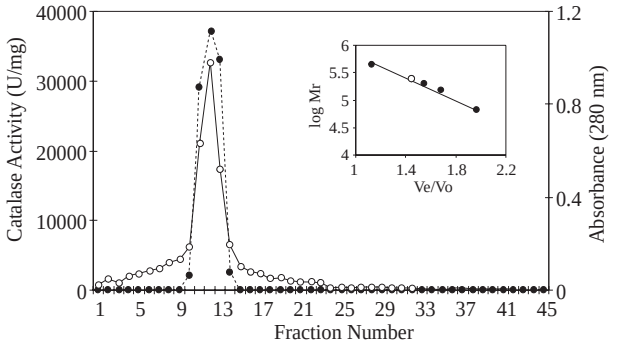

Figure 2. Separation of chicken erythrocyte catalase by gel filtration chromatography on Sephadex G-200. Catalase activity (U/mg)(–●–), Absorbance at 280 nm (–○–). Inset: Molecular weight calibration curve. Protein markers (–●–), Purified chicken erythrocyte catalase (–○–).

The peak at 270 nm vanished in alkaline conditions (pH 11.5). The maxima at 406 nm decreased immediately with the shift of the peak to 415 nm (Figure 4A).

The Soret band of the catalase treated with β-mercaptoethanol decreased with the shift of the peak to 411 nm, and the peaks at 270 and 635 nm vanished. The decrease in the Soret band of β-Mercaptoethanol-treated catalase was greater than that in the DTT-treated enzyme (Figure 4B). These observations indicate that these two thiol reagents reduce the disulfide bonds of enzyme molecules and cause conformational changes in the vicinity of heme groups.

The treatment of enzyme solution with cyanide, dithionite and urea resulted in a shift of the Soret band from 406 nm to 417, 414 and 412 nm respectively. The peak at 635nm vanished after cyanide and dithionite treatments. Urea treatment also reduced the A_{406}/A_{270} ratio from 0.57 to 0.42 (Figure 4C). Similar decreases in the A_{405}/A_{280} ratio were also reported by Scherz et al.[19,20].

The time dependence of changes in the Soret absorption spectra of catalase in 1% SDS is shown in Figure 4D. The absorption maximum at 406 nm of purified catalase decreased gradually within 1 h with an accompanying shift toward 400 nm.

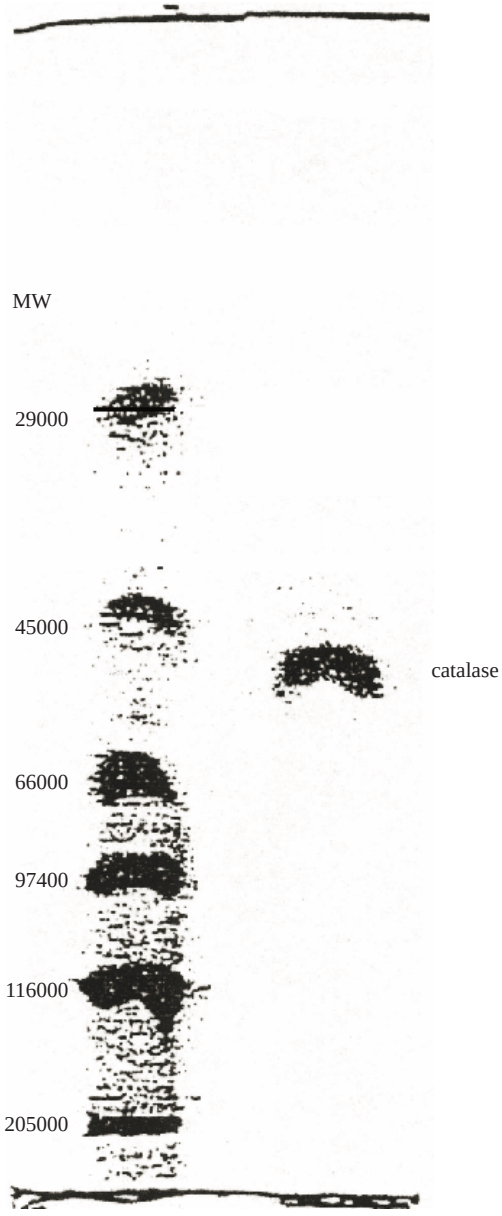

Figure 3. Polyacrilamide gel electrophoresis of chicken erythrocyte catalase. Lane 1, molecular weight markers; lane 2, chicken erythrocyte catalase.

Optimal pH and temperature

Maximal enzyme activity was observed at pH values between 6.0 and 8.0. The activities at pH values other than pH 7.0 were expressed as relative activities, taking the activity at pH 7.0 as 100% (Figure 5A).

The highest enzyme activity was at 25°C. Relative activities at other temperatures were estimated by taking the activity at 25°C as 100% (Figure 5B).

Enzyme activation energy was estimated from the slope of the Arrhenius plot obtained by plotting ln v values versus 1/T (K^{-1}) values and calculated at 2.583 kcal/mol (10.8082 kJ/mol) at a temperature interval of 10 to 30°C (Figure 6).

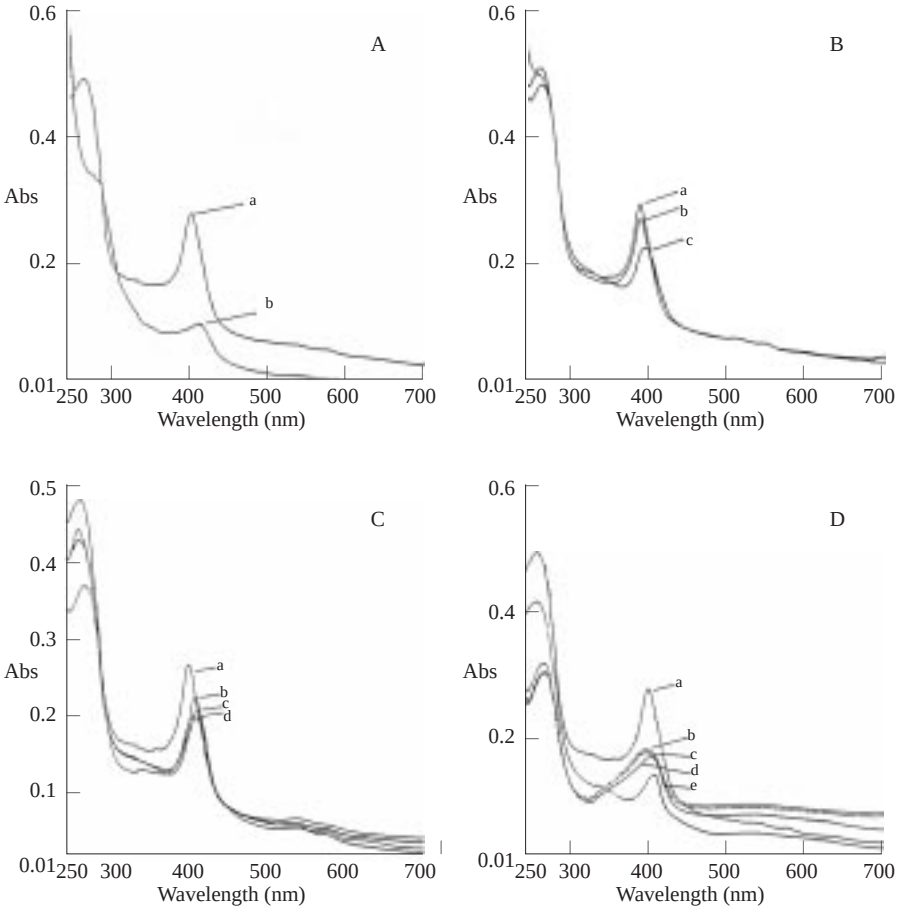

Figure 4. A) The changes in the Soret absorption of purified catalase in alkali pH. a, Native; b, alkali denaturated (pH 11.5). B) The changes in the absorption spectra in the Soret and visible region of chicken erythrocyte catalase with thiol compounds. a, Native; b, DTT; c, β-mercaptoethanol. C) The changes in the Soret absorption of purified catalase in various compounds. a, Native; b, 3 mM dithionite; c, 10 mM urea; d, 5 mM KCN. D) The changes in the Soret absorption of purified catalase over time in 1% SDS solution. a, Native (without SDS); b, 10 min; c, 30 min; d, 60 min; e, 24 h.

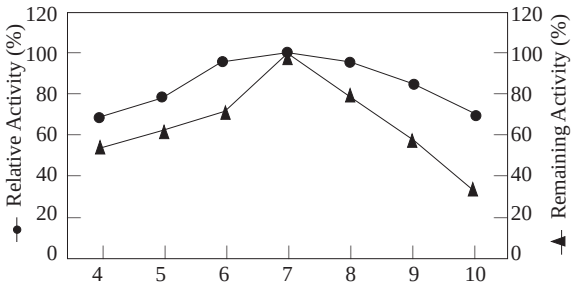

Figure 5. A) pH and the activity and stability profiles of chicken erythrocyte catalase (–●–) Relative activity, (%); (–▲–) Remaining activity (%).

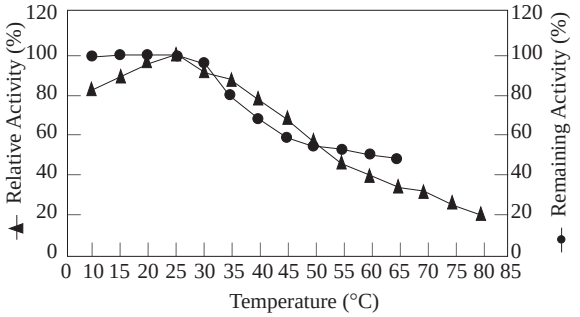

Figure 5. B) Temperature and the activity and stability profiles of chicken erythrocytes catalase: (–■–) Relative activity, % (–●–) Remaining activity, %.

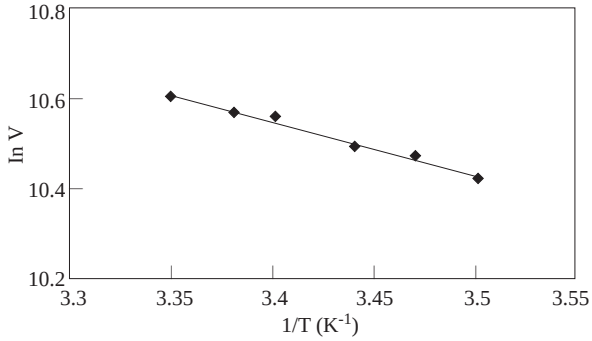

Figure 6. Arrhenius plot for estimation of the activation energy of chicken erythrocytes catalase.

Enzyme stability

Enzyme stability was expressed as remaining activity, taking the activity before incubation at different pH and temperature values as 100%. The stability at different pH values is shown in Figure 5A. When catalase was stored at 25°C for 1 h, it was stable at pH 7.0, 45 and 64% inhibitions were observed when the enzyme was incubated at pH 4.0 and 10.0.

The thermal stability of the enzyme was determined (Figure 5B) by incubation at various temperatures for 60 min. Activity was stable at temperatures between 10 and 30°C, but decreased with increasing temperatures and almost completely disappeared at 80°C.

The storage stability of crude enzyme preparation was poor. A complete loss of crude enzyme activity was observed when stored at 4°C for 96 h. A loss of 52% was observed at the same temperature after 7-day incubation of partially purified enzyme (acetone-fractionated enzyme); however, only 10% activity loss was observed for highly purified enzyme after 4-month storage at 4°C. 62% of initial activity was retained after 5 weeks of storage at -15°C.

Kinetic parameters

Km and Vmax values of enzyme were found to be 33 mM H_2O_2 and 128,000 U/mg from the Lineweaver-Burk plot (Figure 7). The K_{cat} value of the enzyme was calculated as $6.6.10^8$ min^{-1}.

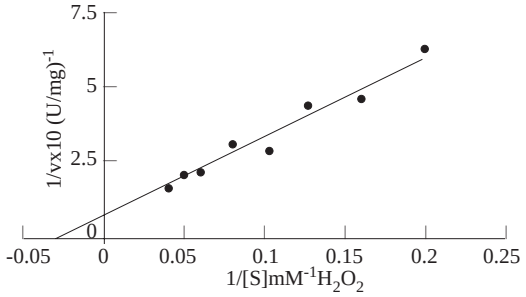

Figure 7. Lineweaver-Burk plot of chicken erythrocytes catalase for estimation of kinetic parameters of enzyme with a r^2 value of 0.9766.

Effect of various compounds on chicken erythrocyte catalase activity

Twelve, 36 and 89% enzyme inhibition was observed in the 5, 15 and 30 mM concentrations of L-Glu respectively after 2 min incubation. L-Asp caused 5, 21 and 73% inhibition at the same concentrations.

The enzyme was stable in 2 mM H_2O_2 pH 7.0 at 25°C when incubated for 60 min. However, 10 and 30 mM H_2O_2 concentrations caused a 46 and 60% enzyme activity loss respectively within the same incubation time. Substrate inhibition is characteristic of the other catalase-peroxidase proteins[2,21,22].

SO_4^-, Cl^- and F^- anions at 1 mM did not display any inhibitory effect on enzyme activity. The effect of various ions on the purified enzyme activity after 2 min incubation is shown in Table 2. Fifty percent inhibition was observed at 0.5 mM for Cu^{2+} ions, but Fe^{2+} and Mn^{2+} ions produced weak inhibition.

Table 2. Effect of some metal ions on enzyme activity after 2 min incubation.

	Relative Activity (%)		
Concentration (mM)	$CuSO_4$	$MnCl_2$	$FeSO_4$
0.5	49	87	94
0.6	43	82	91
1.0	31	76	81
1.5	26	70	74

The purified enzyme was incubated with different concentrations of NaN_3 and KCN, for 2 min. Fifty percent inhibition was observed at $5.2.10^{-4}$ mM NaN_3 and $8.0.10^{-1}$ mM KCN. Catalase contains heme IX as a prosthetic group, exhibiting a typical ferric-heme high-spin spectrum that is converted to low-spin following cyanide and azide binding. The enzyme consequently undergoes inhibition. The magnetic susceptibility measurements of beef liver catalase demonstrated that the helix content of protein decreases by complex formation between the cyanide and azide ligands and heme group[10].

Thiols such as β-mercaptoethanol and DTT inhibited enzyme activity. The incubation of catalase with different concentrations of thiol compounds for 3 h caused a gradual reduction in the activity as shown in Table 3. Fifty percent inhibition for β-mercaptoethanol and DTT was observed at 20 and 30 mM concentrations respectively. β-mercaptoethanol was the more potent reagent in reducing enzyme activity. Complete inhibition was obtained by 20 mM β-mercaptoethanol in 5 h. The remaining enzyme activity after treatment was obtained by 20 mM DTT for 5 h and established at 44%.

Twenty, 30 and 60 min incubation of enzyme in 4 M urea resulted in 48%, 64% and 88% enzyme inhibition respectively. When the enzyme was incubated in the presence of 8 M urea a 79% activity loss was

observed in 20 min, and the enzyme was completely inactivated after 30 min incubation. Inactivation of the enzyme coincided with the decrease of absorption maximum of the enzyme at 406 nm in the Soret region. This finding indicates that the native catalase requires some specific conformation in the vicinity of heme groups necessary for the catalytic action of the enzyme[10].

The effect of SDS on the denaturation of the enzyme was investigated by incubating the enzyme in different SDS concentrations at different time intervals, and the results are shown in Figure 8. Fifty percent enzyme inhibition was observed in 15, 30 and 60 min with 1, 0.1 and 0.01% SDS concentrations respectively. These results are in agreement with other studies[23].

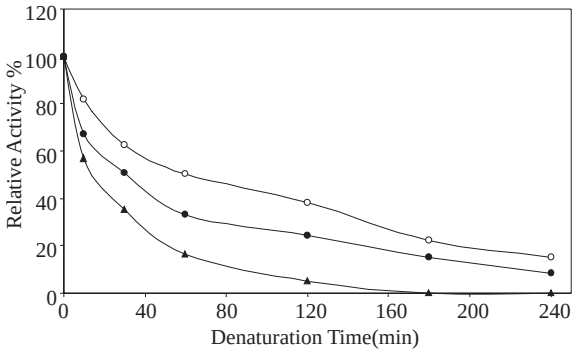

Figure 8. Effect of SDS concentration and time on the denaturation of chicken erythrocytes catalase. 0.01% SDS (–○–), 0.1% SDS (–●–), 1% SDS (–▲–) concentration.

Purified catalase was incubated in the presence of various concentrations of glutaraldehyde for 30 min at 25 °C in 50 mM phosphate buffer pH 7.0. Fifty percent inhibition was determined at 1.68 M concentration.

The chicken erythrocyte catalase was incubated with different concentrations of iodoacetamide for 60 min at 25°C in phosphate buffer pH 7.0. Fifty percent and complete inhibition were observed at 0.9 and 7 mM concentrations respectively. Enzyme solutions containing iodoacetamide at different concentrations were incubated at different time intervals. The initial rates were measured. ln ($E_i/E_\circ$) values were plotted against time at each concentration. Irreversible inhibition constants were calculated from the slopes of linearly regressed lines (Figure 9). These were 0.0116, 0.0259, 0.0756 and 0.1302 min^{-1} for 1.0, 3.0, 10 and 15 mM iodoacetamide concentrations respectively.

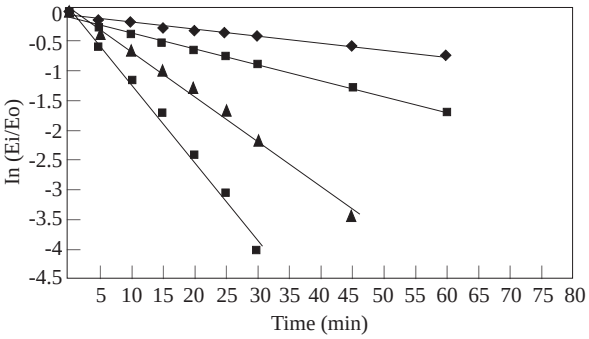

Figure 9. Inactivation kinetics of chicken erythrocytes catalase at different iodoacetamide concentrations function of time. Ei: The initial rate of the enzyme catalyzed reaction after a certain time incubation in the presence of inhibitor. Eo: The initial rate of the enzyme catalyzed reaction in inhibitor-free media. (–●–) 1.0 mM, $r^2 = 0.9646$; (–■–) 3 mM, $r^2 = 0.9862$; (–▲–) 10 mM, $r^2 = 0.9925$; (–♦–) 15 mM, $r^2 = 0.9944$ iodoacetamide.

Discussion

In this work, the purification of catalase from chicken erythrocytes was carried out by acetone fractionation, ethanol-chloroform treatment, CM-cellulose and Sephadex G-200 chromatography steps. The stable behavior of chicken erythrocyte catalase was observed against ethanol-chloroform and acetone treatment like the other types of catalase[6,10]. However, the denaturation of beef erythrocyte catalase was reported by Bonnichen[24] under the same treatment.

The specific activity of the purified chicken erythrocyte catalase was lower than that for purified catalase from other erythrocyte sources. In addition 86,000 U/mg specific activity of porcine erythrocyte catalase has been reported[25]. Varied specific activities between 85,000-110,000 and 60,000-78,000 U/mg have been reported for human and horse erythrocyte catalases respectively[7,9,14,26,27]. However, 144,000 U/mg specific activity was reported by Deutsch for horse erythrocyte catalase[28]. These variations in specific activity values can be explained by the different protein composition of various types of catalase. The rigorous conditions applied in purification prodecures and the variation in heme content of erythrocytes may also cause alteration of specific activity values.

The molecular weight of the chicken erythrocyte catalase was approximately 240 kDa and according to the SDS-PAGE results it contains four identical subunits at 57.5 kDa MW. The molecular weights of dog, human, pig and horse erythrocyte catalases were 240, 269, 230 and 243 kDa respectively[6,7,13,29].

The absorption spectrum of chicken erythrocyte catalase showed a Soret peak at 406 nm suggesting that the enzyme contains heme group like the other catalases[27,30–32]. The absorption peak at 270 nm disappeared with alkaline denaturation, and the Soret band at 406 nm vanished and a new, small peak appeared at 415 nm. The ferric heme can be reduced by dithionite. Miyahara et al. observed inactivation of goat liver catalase in the presence of β-mercaptoethanol or DTT and reported the changes in the absorption and circular dicroism spectra of goat liver catalase after thiol treatment. The thiol reagents interact with the heme groups in catalase directly or with amino acid residues in the proximity of the heme groups. This results in alteration of the conformational structure of protein and consequently in critical alteration of enzyme activity[30].

Chicken erythrocyte catalase showed a broad optimum pH value between 6.0 and 8.0. Catalase is generally regarded as an enzyme without a pH optimum[33]. In the case of other catalases, the enzyme has been shown to exhibit sharp optimum pH[34,35] or a broad pH optima[36]. The stability of the purified enzyme in a basic region was lower than that in an acidic region. Thermal stability of the purified enzyme is better than that obtained from bovine liver catalase[37].

The chicken erythrocyte catalase was inhibited by Asp and Glu. The enzyme was resistant to Gly, Arg and Ala.

The lower K_m value of 33 mM for chicken erythrocyte catalase compared with 110 mM for goat liver catalase and 70 mM for bovine liver shows a greater affinity of catalase towards hydrogen peroxide[38,39]. Our results show that the inhibition of catalase activity by thiol compounds increased gradually over time. Like other catalases, heme protein ligands such as KCN and NaN_3 are powerful inhibitors of chicken erythrocyte catalase. Takeda et al. reported that β-mercaptoethanol was the most potent reagent for the reduction of porcine erythrocyte catalase activity[25]. Wasserman and Hultin indicated that bovine liver catalase was insensitive to glutaraldehyde[37].

Scherz et al. reported that human erythrocyte catalase loses 97% of its activity if treated with 8 M urea for 3 min at 20°C[20]. Samejima and Kita reported a complete loss of helical content over time when beef

liver catalase was treated with 8 M urea[40]. It is therefore evident that urea treatment causes a dissociation of the tetrameric native enzyme by the loss of catalytic function.

Many proteins combine with SDS to form protein-SDS complexes with the unfolding of the rigid conformation of proteins[23]. Our results indicate that the inhibition effect of SDS on chicken erythrocyte catalase depends on denaturation time and SDS concentration. Interaction with SDS disrupts all non-covalent protein bands and causes the dissociation of subunits of catalase.

The inhibition of enzyme with iodoacetamide indicates the existence of cystein residues at the active site or at a site near to the active site of the enzyme. It has been reported that human and horse erythrocytes catalases contain 16 cystein residues per molecule[10].

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