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Purification and Characterization of Catalase from Chard (*Beta vulgaris var. cicla*)

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Catalase is a major primary antioxidant defence component that primarily catalyses the decomposition of H_2O_2 to H_2O. Here we report the purification and characterization of catalase from chard (*Beta vulgaris var. cicla*). Following a procedure that involved chloroform treatment, ammonium sulfate precipitation and three chromatographic steps (CM-cellulose, Sephadex G-25, and Sephadex G-200), catalase was purified about 250-fold to a final specific activity of 56947 U/mg of protein. The molecular weight of the purified catalase and its subunit were determined to be 235 000 and 58 500 daltons, indicating that the chard catalase is a tetramer. The absorption spectra showed a soret peak at 406 nm, and there was slightly reduction by dithionite. The ratio of absorption at 406 and 275 nanometers was 1.5, the value being similar to that obtained for catalase from other plant sources. In the catalytic reaction, the apparent Km value for chard catalase was 50 mM. The purified protein has a broad pH optimum for catalase activity between 6.0 and 8.0. The enzyme had an optimum reaction temperature at 30 °C. Heme catalase inhibitors, such as azide and cyanide, inhibited the enzyme activity markedly and the enzyme was also inactivated by β-mercaptoethanol, dithiothreitol and iodoacetamide.

Keywords: Catalase, Purification, Characterization, Chard, *Beta vulgaris var. cicla*

INTRODUCTION

Highly reactive oxygen forms such as superoxide radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH^-$) and hydrogen peroxide, can biologically cause a significant form of cellular stress. These oxygen species can initiate peroxidation of membrane fatty acids, mark proteins for proteolysis, cause DNA damage, inhibit photosynthesis and destroy chlorophyll.[1,2] Organisms have evolved specific enzyme systems to neutralize potentially lethal reactive oxygen species. Among these systems is the group of proteins designated hydroperoxidases. One of these hydroperoxidase is catalase and it protects cells from oxidative damage by converting these oxygen species into oxygen and water.

Catalase (H_2O_2:H_2O_2 oxidoreductase EC 1.11.1.6) is a tetrameric, heme-containing enzyme found in all aerobic organisms. Catalase is located in peroxisomes where it is responsible for scavenging H_2O_2 produced during the oxidative reactions that occur there. In plants high

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levels of H_2O_2 are also increased during photorespiration as a product of glycolate oxidase activity.[1,2]

Catalase has been purified and characterized from various animal, bacterial, fungal and a number of plant sources such as spinach, tobacco and lentil leaves, cucumber, pumpkin and cotton cotyledons, maize scuttella, castor bean endosperm, and sweet potato roots.[3–10] These typical catalases are active in the pH range 5–10.[11]

Here, we described the purification and characterization of catalase from chard leaves (*Beta vulgaris var. cicla*).

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), hydrogen peroxide, L-arginine, L-aspartic acid, L-alanine, L-glycine, L-glutamic acid, sodium azide, chloroform, ammonium sulfate, potassium cyanide, sodium flouride were purchased from Merck, Germany. CM-cellulose, Sephadex G-25, Sephadex G-200, β-mercaptoethanol, dithiothreitol, urea, sodium dodecyl sulfate (SDS), iodoacetamide, molecular weight standards for gel exclusion and SDS/PAGE were obtained from Sigma Chemical Company, USA. Other reagents were analytical grade.

Enzyme Assay

The catalase activity in crude extracts and in purified catalase fractions was determined spectrophotometrically by direct measurement of the decrease in light absorption at 240 nm by the decomposition of hydrogen peroxide by catalase.[12] The reaction mixture contained, in a total volume of 1 ml, 10 mM H_2O_2 and 0.05 M potassium phosphate buffer (pH 7.0).

Protein Determination

Protein content was determined according to the dye binding method of Bradford[13] using BSA as a standard.

Purification

All purification steps were carried out at 4 °C. 40 g of chard leaves (*Beta vulgaris var. cicla*) was homogenized in 0.05 M phosphate buffer (pH 7.0) using a Waring blender for 30 s at low speed followed by 60 s at high speed. The resulting homogenate was filtered through two layers of muslin and the filtrate centrifuged at 14000 rpm for 15 min. The supernatant was extracted with chilled chloroform (1:1), to liberate the catalase from the suspended chloroplasts and bring it into aqueous solution and the aqueous phase was separated by centrifugation at 14000 rpm for 5 min. The enzyme was precipitated by addition of ammonium sulfate to give a concentration of 45% and the precipitate was collected by the centrifugation and dissolved in 0.05 M phosphate buffer (pH 7.0).

CM-Cellulose and Sephadex G-25 Column Chromatography

The ammonium sulfate fraction was applied onto a CM-cellulose column (1.5 × 60 cm) previously equilibrated with 0.05 M phosphate buffer (pH 7.0). Elution was done by using a linear gradient of 0–1.0 M NaCl in 0.05 M potassium phosphate buffer (pH 7.0). Fractions of 5 ml were collected at a flow rate of 1.5 ml/min. The fractions containing catalase activity was applied to a column of Sephadex G-25 (1.5 × 60 cm) which had been equilibrated with 0.05 M phosphate buffer (pH 7.0) and the column was eluted with 0.1 M phosphate buffer (pH 7.0). Fractions of 2 ml were collected at a flow rate of 1.5 ml/min. The active fractions were pooled and concentrated using a 30 000 NMWC ultrafilter.

Sephadex G-200 Gel Filtration

The concentrated catalase solution was adsorbed onto a Sephadex G-200 column (1 × 90 cm) previously equilibrated with 0.05 M phosphate buffer (pH 7.0) and the enzyme was eluted with the 0.1 M phosphate buffer (pH 7.0) at a flow rate of 0.25 ml/min.

Determination of Molecular Weight

Chromatography was performed through a Sephadex G-200 column (1 × 90 cm) which was equilibrated with 0.05 M phosphate buffer (pH 7.0) using bovine serum albumin (M_r 66 000), alcohol dehydrogenase (M_r 150 000), α-amylase (M_r 200 000) and apoferritine (M_r 443 000), as calibration markers.

Electrophoresis

For determination of the molecular weight of denaturated subunits, SDS/PAGE in tricine buffer system was used to enhance resolution of low molecular weight polypeptide as described by Laemmli.[14] The following standards were used to calibrate the gel: carbonic anhydrase (M_r 29 000), ovalalbumin (M_r 45 000), bovine plasma albumin (M_r 66 000), phosphorylase b (M_r 97 400), β-galactosidase (116 000), myosin (205 000). Gels were stained with coomassie blue for protein detection.

Characterization of the Catalase Enzyme

Effect of pH

A study was made of the effect of pH on enzyme activity. Enzyme activity was determined in 0.05 M phosphate buffer at different pH values, over the pH range 4.0–10.0.

Effect of Temperature

For determining 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 80 °C. The heat stability of the purified enzyme was determined by measuring catalase activity at different temperatures in the range 40°–80 °C for 60 min duration.

Storage Stability

A series of studies were undertaken to determine the effects of storage temperature and storage time on activity. The catalase activity was measured every day for the first week and then at weekly intervals.

Enzyme Kinetics

Determination of the Michaelis constant (Km) value for catalase activity was determined using H_2O_2 at varying concentrations (1–20 mM) in phosphate buffer pH 7.0. The Km value of catalase were calculated from a plot of 1/V *vs* 1/[S] by the method of Lineweaver-Burk (not shown).

Effect of Some Iinhibitors on Enzyme Activity

Purified enzyme was incubated in 0.05 M phosphate buffer (pH 7.0, 25 °C) containing different concentrations of inhibitors and the changes in activity were observed relative to a control experiment in the absence of inhibitor.

The inhibitor concentration that reduced the enzyme activity by 50% (I_{50}) was determined by regression analysis graphs of percentage inhibition *vs* [I].

RESULTS

Purification Steps

The purification procedure for catalase from chard leaves (*Beta vulgaris var. cicla*) is summarized in Table I. The first purification step of catalase from the crude extract was achieved by

TABLE I Purification steps for chard (*Beta vulgaris var. cicla*) catalase

Purification Step	Total Units	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (Fold)
Crude Extract	167360	736	227.39	100	
Chloroform Treatment	161160	616.2	261.23	96.3	1.1
Ammonium Sulfate Fractionation	64584	58.5	1 104	38.58	5.1
CM-cellulose Chromotography	15855	0.69	22 978	9.47	101
Sephadex G-25 Chromotography	12920	0.38	34 000	7.72	149.5
Sephadex G-200 Chromotography	5410	0.095	56 947	3.23	250

fractional separation of chlorophylls using chloroform, *n*-butanol, petroleum ether and *n*-hexane. Chard catalase in the crude extract was not resistant to *n*-butanol, petroleum ether and *n*-hexane and the activity yield was decreased dramatically by using these organic solvents. The highest recovered activity obtained was 261.23 U/mg after chloroform treatment. Chloroform fractionation of the initial extract followed by ammonium sulfate precipitation steps resulted in 5.1 fold purification of catalase with 38.58% recovery. Several precipitations with solid ammonium sulfate at concentrations between 20–90% were tested to find the best saturation point. Catalase activity of the precipitate obtained with 45% $(NH_4)_2SO_4$ gave the highest activity and this saturation point was used all the extraction processes. As can be seen from Figures 1 and 2 the degrees of purification of chard catalase was 101-fold after CM-cellulose chromatography and 250-fold after Sephadex G-200 gel filtration.

Molecular Weight

The molecular weight of the native purified protein was estimated to be approximately 235 000 by gel filtration through a Sephadex G-200 column. Upon SDS/PAGE, the enzyme yielded a single band with a subunit molecular weight of

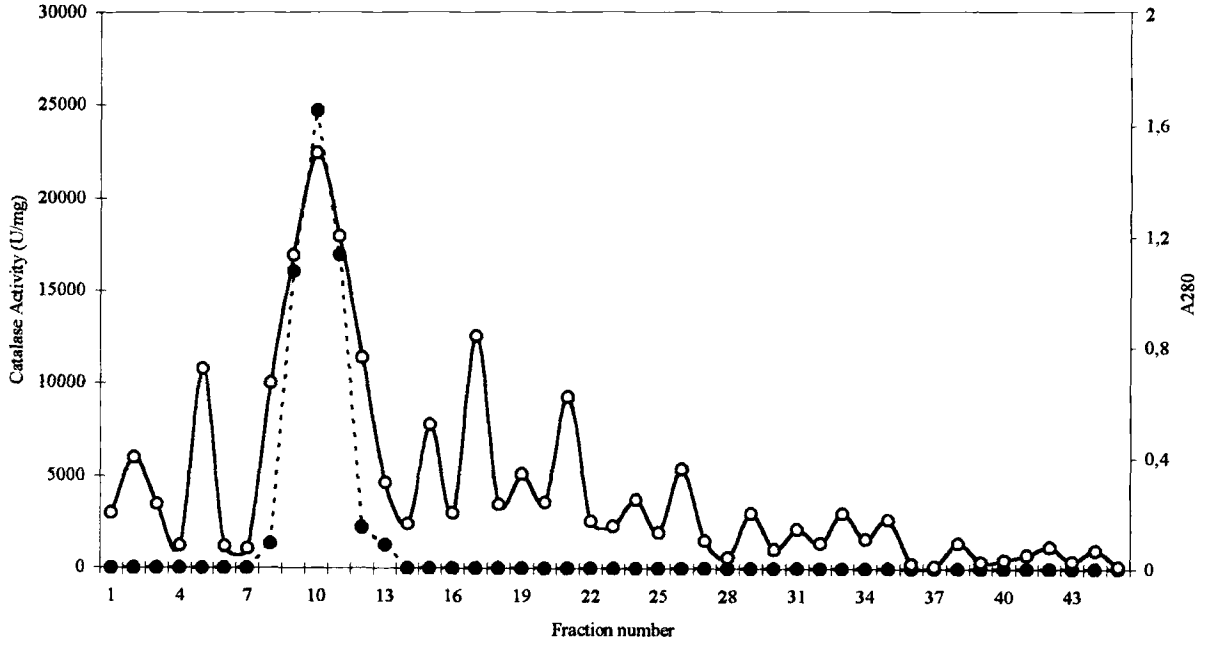

FIGURE 1 Elution profile of catalase from CM-cellulose column chromatography. Absorbance at 280 nm (—○—); catalase activity (---●---).

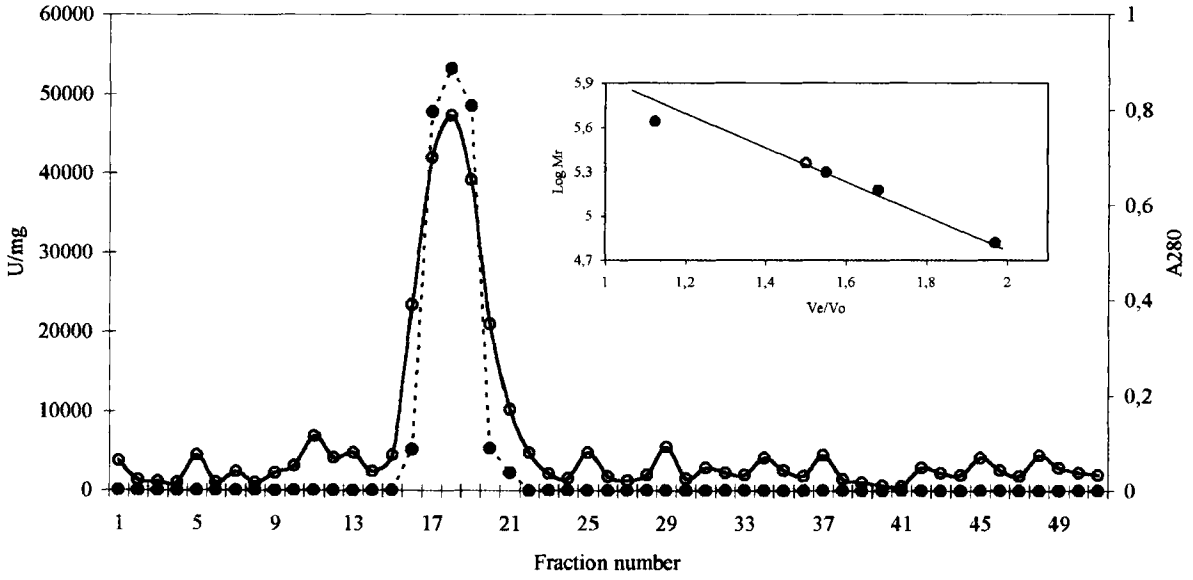

FIGURE 2 Elution pattern of chard catalase from gel filtration chromatography on Sephadex G-200. Absorbance at 280 nm (—○—), catalase activity (- - -●- - -) Inset: molecular weight calibration curve. Protein standards (- - -●- - -); purified chard catalase (—○—).

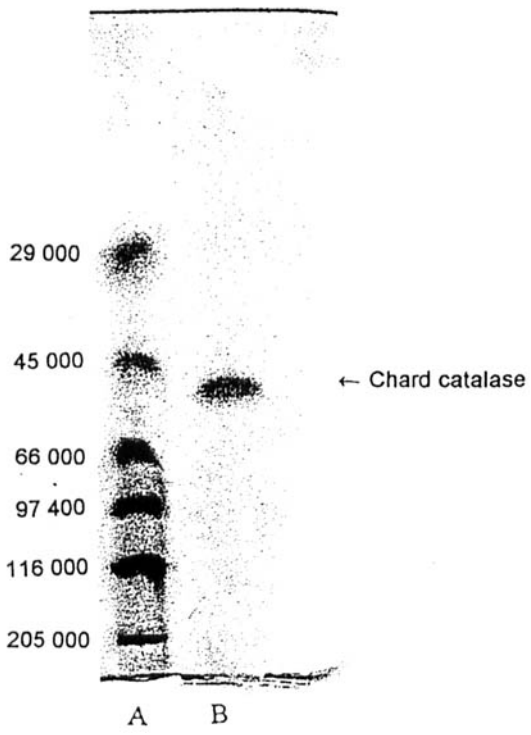

FIGURE 3 SDS/PAGE results for purified chard catalase. Lane A, protein standards, Lane B, purified catalase.

58 500 Da (Figure 3). Similar values were obtained for purified catalase from other sources.[9,15–17] From these data, it is highly likely that the chard catalase has a tetrameric structural organization.

Effect of pH

The pH optimum for chard catalase activity was found to have a very broad optimum between pH 6.0–8.0 with more than 95% of the maximum activity occuring between these limits.

As can be seen from Figure 4, catalase was stable for 60 min in the range pH 6.0–8.0 at 25 °C. Incubation of the enzyme for 60 min at pH 4 and 10 caused 40% and 60% loss of activity respectively. The stability of the purified enzyme in basic media was lower than that in acidic media.

Effect of Temperature

The effects of temperatures between 10°–80 °C on catalase activity showed that the optimum temperature for the purified enzyme was 30 °C.

Heat stability of the catalase was investigated between 40 °C and 80 °C over a 1 h period (Figure 4). A decrease in catalase activity of 42% and 62% was found at 40 °C and 50 °C respectively. The time required to inactivate 50% of the enzyme was estimated to be 30 min at 50 °C, 10 min at 60 °C and 7 min at 70 °C. Catalase was completely inactivated after 50 min at 60 °C, 30 min at 70 °C and 2 min at 80 °C.

Stability on Storage

The crude enzyme preparation was unstable. On storage at 25 °C and 4 °C, the initial extract lost its activity after 24 h and 72 h respectively. The purified enzyme lost its activity after 72 h at 25 °C, however it was observed to be much more stable at 4 °C and only lost 6% activity on storage for 3 months. This results are consistent with results reported in the literature by others.[3,18–20]

Kinetic Properties

The enzyme had a Km value of 50 mM with H_2O_2 substrate as determined by a Lineweaver-Burk plot.

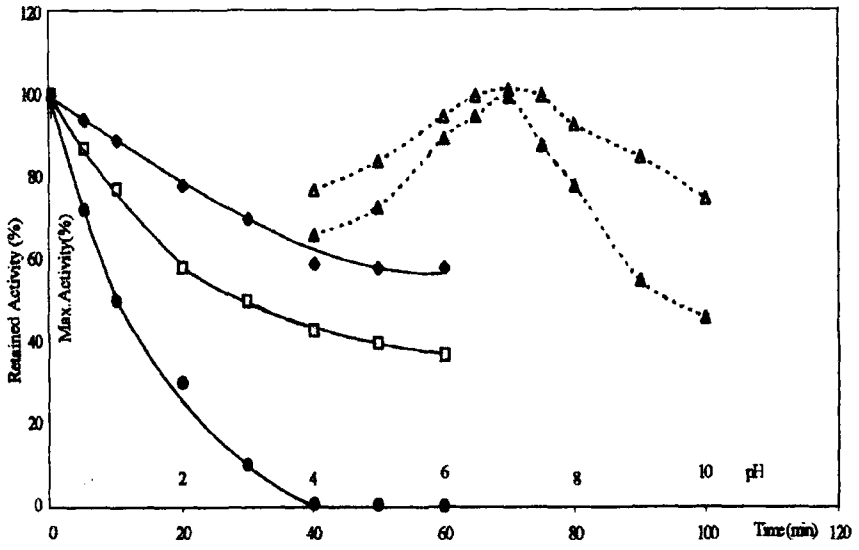

FIGURE 4 Effect of pH and temperature on chard catalase. Effect of pH on chard catalase (--Δ--); pH stability of chard catalase after incubation for 60 min at 25 °C (--▲--); heat stability of chard catalase at 40 °C (–♦–), 50 °C (–□–), 60 °C (–●–) on incubation for 60 min at pH 7.0.

Absorption Spectra

The absorption spectrum of the purified enzyme was typical of a heme protein, having two major peaks at 275 and 406 nm (Soret band) and three minor peaks 540, 575 and 635 nm respectively (Figure 5). The ratio of the absorption peaks at 275 to 406 nm was 1.5. The peak at 406 nm disappeared on addition of alkali to pH 11.5 with appearence of a shoulder at 406 nm and the peak at 275 nm was absent.

Addition of cyanide to the purified enzyme resulted in a shift of the Soret band to 416 nm and the peak at 635 nm was abolished.

Chard catalase was only slightly reduced by dithionite.

Treatment of catalase with thiol compounds (3 mM) such as β-mercaptoethanol and dithiothreitol (DTT), caused a reduction in the Soret band with a shift of 1–3 nm as shown in Figure 5. Decrease of the Soret band of the β-mercaptoethanol-treated enzyme was greater than that for the DTT-treated enzyme. These results may indicate that the two thiol reagents not only reacted with the enzyme molecule as simple reducing agents but also caused some conformational change in the vicinity of the heme group.

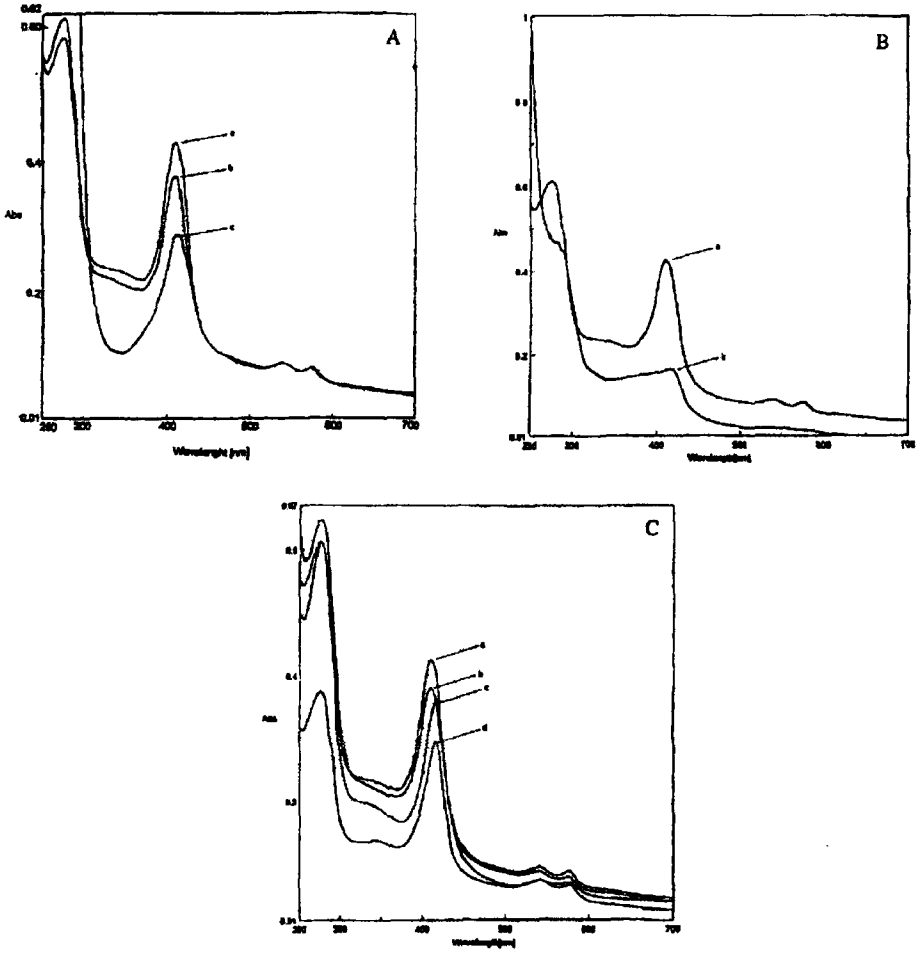

FIGURE 5 (A) The changes in the soret absorption of purified catalase with thiol compounds. (a) control; (b) 3 mM DTT; (c) 3 mM β-mercaptoethanol. (B). Absorption spectrum of chard catalase in alkali. (a) control; (b) alkali denaturated (pH 11.5). (C) Absorption spectra of catalase with some inhibitors. (a) control; (b) 2 mM sodium dithionite; (c) 10 mM urea; (d) 10 mM KCN.

The Soret band of catalase treated with 10 mM urea decreased with a shift of the peak to 416 nm and resulted in a ratio A_{275}/A_{416} of 1.277.

Effect of Ions

Anions such as SO_4^{2-}, Cl^-, and F^- at 1 mM concentrations did not effect the enzyme activity. As can be seen from Table II metal ions Cu^{2+} and Mn^{2+} inhibited enzyme activity but Fe^{2+} ions produced weak inhibition.

Effect of NaN_3 and KCN

Catalase was inhibited by the heme-protein ligands, cyanide and azide. The purified enzyme was incubated with different NaN_3 concentrations in the range of $0.5–5 \cdot 10^{-9}$ M for 2 minutes. 50% inhibition was observed at $1.8 \cdot 10^{-9}$ M NaN_3. The purified enzyme was also incubated with different concentrations of KCN in the range of $0.5–5 \cdot 10^{-3}$ M for 2 minutes when 50% inhibition was observed at $6.5 \cdot 10^{-4}$ M KCN.

Effect of Thiols

The treatment of catalase with β-mercaptoethanol and DTT caused a gradual reduction in activity as shown in Table III and 50% inhibition was observed at 3 and 10 mM concentrations of the respective thiols after 60 minutes incubation. When the purified enzyme was preincubated with 20 mM β-mercaptoethanol, the enzyme initially lost activity quickly, losing 50% activity after a 15 min period. Complete inhibition was obtained after about 2 hours incubation. Complete inhibition by the same concentration of DTT was observed after 4 hours incubation.

When the purified catalase was treated with 1 mM β-mercaptoethanol only 3% inhibition occured. The decrease in catalase activity is dependent on the incubation time and the nature of the thiol compounds; β-mercaptoethanol was the most potent reagent for reduction of enzyme activity. Miyahara has reported similar results.[21]

TABLE II Effect of β-mercaptoethanol and Dithiothreitol on chard catalase after 2 hours incubation

	Remaining Activity(%)	
Concentration (mM)	β-mercaptoethanol	Dithiothreitol
0.5	63	68
1	45	62
3	32	50
10	24	33
20	0	20
30	0	0

TABLE III Effect of Cu^{2+}, Fe^{2+}, and Mn^{2+} ions on the purified enzyme activity

	Relative Activity(%)		
Concentration (mM)	$CuSO_4$	$MnCl_2$	$FeSO_4$
0.2	53	98	99
0.4	21	87	93
1	8	44	89
1.5	2	18	86

Effect of Glutaraldehyde

The enzyme was incubated in the presence of various concentration of glutaraldehyde for 30 min in 0.05 M phosphate buffer (25 °C, pH 7.0). It was stable in the presence of 2% glutaraldehyde but at 8% concentration lost 24% of its activity.

Effect of Iodoacetamide

Purified catalase was incubated with different concentration of iodoacetamide for 60 minutes in phosphate buffer (25 °C, pH 7.0). 50% inhibition was observed at 2 mM iodoacetamide.

Effect of SDS

The time-dependence of changes in the activity of purified enzyme was investigated in various concentrations of SDS. When incubated for 60 minutes in the presence of 1%, 0.1% and 0.01% SDS, the enzyme lost 86%, 58% and 45% of its activity respectively.

Although the activity of the catalase was completely lost in 1% SDS solution after 2 hours, the enzyme showed only a slight loss of activity in 0.01% SDS.

Effect of Urea

Incubation of the enzyme in the presence of 4 M and 8 M urea for 3.5 minutes and 1 minute respectively caused 50% inhibition. Purified enzyme was completely inhibited after 12 minutes in the presence of 8 M urea but was still 17% active in the presence of 4 M urea. However 30 minutes incubation with 4 M urea resulted in complete inactivation of the enzyme.

Effect of Amino Acids

L-Glycine, L-arginine and L-alanine (5–30 mM) in phosphate buffer pH 7.0 at 25 °C after 2 minutes incubation with the enzyme did not show any effect on enzyme activity. L-glutamic acid (5, 15, 30 mM) caused 16%, 41% and 84% inhibition respectively. L-aspartic acid (5, 15, 30 mM) inhibited the enzyme by 7%, 38% and 92% respectively.

Effect of Hydrogen Peroxide

Hydrogen peroxide, in addition to being a substrate of catalases, causes time-dependent inhibition of the enzyme. Incubation of purified catalase for 60 minutes with 2 mM H_2O_2 in phosphate buffer pH 7.0 at 25 °C did not affect enzyme activity. When incubated for 60 minutes in the presence of 10 mM and 30 mM H_2O_2 the purified catalase lost 55% and 78% of its activity respectively.

DISCUSSION

Purification of the chard catalase preparation used in this research led to a 250-fold increase in specific activity. This specific activity of 56 947 U/mg is similar to the values reported for catalase from leaves of *Nicotiana sylvestris*, sunflower and cotton seed cotyledons 54 000, 66 720 and 47 760 U/mg respectively but is higher than that for the purified enzyme from spinach and lentil green leaf 28 000 and 30 000 U/mg respectively.

The molecular weight of the chard catalase was found to be 235 000. Catalases isolated from higher organisms have molecular weights in the range 220 000 to 270 000 and contain four equally sized subunits each with a ferric heme prosthetic group.[11]

The chard catalase has a Km of 50 mM and with H_20_2 as a substrate it showed an apparent maximal velocity and a decrease in activity at higher substrate concentrations. Havir *et al.* reported a Km of 57 mM for the enzyme from the leaves of *Nicotiana sylvestris* and Kendall *et al.* found half maximal activity at 18 mM for the enzyme from the barley leaves.[9,22] Our result is similar to that for the *Nicotiana sylvestris* enzyme.

The purified enzyme preparation showed a broad optimum pH in the range 6.0–8.0. This characteristic is shared by typical catalases. Kendall *et al.* reported that the barley leaf enzyme has a broad optimum pH between 7.0 and 9.0.[22] pH stability tests performed at 25 °C showed that the chard catalase is most stable at pH 7. The enzyme was inactivated quickly at more alkaline and more acidic values. However, Jones and Suggett have shown a pH-independence of the reaction in the absence of buffers from 4.7 to 10.5.[23] Galston *et al.* found that purified spinach catalase was stable in the cold at a pH between 5.3 and 8.9, and was destroyed rapidly at lower pH values.[3]

As shown in Figure 4, temperature had a very important effect on the stability of chard catalase. The enzyme was inactivated very rapidly initially but the rate progressively decreased as the period of heating was increased. It also appears that the catalase in chard has a greater thermal stability than that in spinach.[24]

A value of 1.52 for the ratio A_{275} and A_{406} compares favorably with those reported for other plant catalases. The absorption spectrum of chard leaves catalase showed a soret peak at 406 nm suggesting the heme nature of the enzyme similar to that of other catalases.[25,26] The ratio of absorption A_{280}/A_{405} for several catalases of plant origin[3,27,28] is about 1.5, indicating[3] that the number of heme groups per enzyme molecule is approximately 2. In contrast[25,26,29] the ratio for some mammalian catalases as well as the bacterial enzyme containing 4 heme groups per molecule is close to 1.

The chard catalase was inhibited by the heme protein ligands cyanide and azide. Spinach catalase showed similar inhibition effects with cyanide and azide the former being a more effective inhibitor. Galston reported that 50% inhibition by KCN and NaN_3 occured at concentrations of $5 \cdot 10^{-6}$ and $2 \cdot 10^{-5}$ M respectively.[3] Its ferric heme can be reduced by dithionite.

Inhibition of chard catalase activity by thiol compounds increased gradually with time. Thiol inhibition of catalase has been established by early workers. Miyahara *et al.* studied the effect of thiol on goat liver catalase and concluded on the basis of absorption and circular dichroism spectral studies that there was interaction between thiol reagents and heme groups in catalase directly and/or with some amino acid residues in the proximity of the heme group causing conformational alteration near the heme group which reduces enzyme activity.[21]

Previous work has reported that bovine liver catalase was insensitive to glutaraldehyde.[30] Similar results were also found for chard catalase at low concentrations of glutaraldehyde.

SDS is a denaturant for proteins as well as a separating reagent for proteins having subunits. The inhibition effect of SDS on purified chard catalase depended on incubation time and SDS concentration. This is understandable since the rate of SDS binding to proteins depends upon the quaternary structure. SDS binds rapidly to proteins composed of a single polypeptide chain, whereas it binds slowly to proteins consisting of subunits causing irreversible dissociation of proteins into subunits.[31]

Treatment of chard catalase with urea led to a decrease in absorbance at 406 nm with an associated loss of catalase activity. In the presence of 8 M urea, similar enzyme activity losses were reported for the human erythrocyte catalase.[32] According to some authors, when beef catalase was treated with 8 M urea, a complete loss of the helical content with time was observed.[33] Takeda *et al.* revealed that the porcine erythrocyte catalase molecule dissociated into presumably identical one quarter sized subunits on treatment with urea, although human and beef erythrocyte catalase dissociated into dimer subunits with 8 M urea, as reported by Scherz *et al.* and Samejima.[32,33]

Therefore it would seen that urea treatment causes a dissociation of the tetrameric native enzyme into components half or one quarter of its size, accompanied by the loss of the catalytic function.

SUMMARY

Catalase has been purified from chard leaves. The specific activity of the purified chard catalase was higher than that reported for the purified catalase from spinach and lentil leaves. Chard leaves catalase has a similar molecular weight to that from other plants but has a lower molecular weight than the liver and erythrocyte catalase. Purified enzyme showed a broad pH optimum in the range 6.0–8.0. The absorption spectrum of catalase showed a soret peak at 406 nm suggesting the heme nature of the enzyme similar to other catalases. A lower value for the Michaelis constant of 50 mM for chard catalase compared to 110 mM and 70 mM for liver and lung catalase, shows greater affinity of purified catalase was towards hydrogen peroxide. The chard catalase was inhibited by the protein heme ligands cyanide and azide and its

ferric heme slightly reduced by dithionite. These characteristics are shared by typical catalases.

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