Purification and Characterization of Monofunctional Catalase in Post-mitochondrial Fractions from *Chironomid* Larvae (Bloodworms)

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A Thesis Submitted in Partial Fulfilment

of the Requirements for the Degree of

Master of Philosophy

in

Biochemistry

August 2001


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Acknowledgements

I am particularly grateful to my supervisor, Prof. W.S. Ho, for his patience, guidance, encouragement and critical discussions throughout the entire period of this study. I would like to thank Prof. L.K. Leung and Prof. T.B. Ng for acting as internal examiners. Thanks are also due to Prof. S. Manahan for acting as external examiner.

Special thanks to Prof. H.K. Lee for letting me use the FPLC system and Mr. W.K. Yuen for his advice in the project.

Last but not the least, I would like to thank my beloved family for their love and support.

Abstract

Monofunctional catalases (HPII) are heme-containing proteins and exist as homotetramer proteins that appear to play an important role in defense against oxidative stress. They catalyze the basic reaction, the dismutation of hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$) without consuming cellular reducing equivalents. The enzyme can protect the cell by removing peroxide before causing cell damage by peroxide radicals that are generated in the reaction.

Chironomidae are believed to be the most widely distributed and frequently the most abundant insects in freshwater. Earlier studies of *Chironomidae* have shown that they can survive in different contaminated environments of extreme pH, temperature and salinity and can undergo strong oxidation stresses of rapid tissue differentiation and metamorphosis. The ability of *Chironomidae* to survive in such adverse conditions prompts our study of catalase. The common *chironomid* larvae (Order Diptera, Class Insecta) that can be cultured in chicken manure will provide a model for our studies.

Catalase from *Chironomidae* was isolated by different chromatographic methods. The post-mitochondrial fractions were obtained by differential centrifugation. The supernatants were mixed with ethanol and chloroform in a ratio of 10:4:3. The monofunctional catalase was purified using Blue Sepharose and Mono S column.

The purified catalase showed an optimal activity between pH 7 and 10 at 40^0C. It showed one band on SDS gel electrophoresis. Its subunit and native molecular mass was found to be about 53.2kDa and 205kDa, respectively. The purified enzyme was quite stable at temperature up to 40^0C and could maintain 70% activity up to 60^0C. In spectroscopic analysis, the A_{406}/A_{280} ratio was determined to be about 0.97 and the presence of protoheme IX was recorded. A 10-minutes incubation of the enzyme at 25^0C with 33μM KCN, 83μM NaN_3 and 0.34mM 3-amino-1,2,4-triazole resulted in 50% inhibition of the catalase activity. The apparent K_m and V_{max} of the enzyme were 25mM and 125,000U(mg of protein)$^{-1}$, respectively. The N-terminal amino acid sequence (GRKFAVFFYT) identity and positive of the purified catalase towards the other catalases was greater than 66% and showed 77% homology. These results indicate that the purified catalase belongs to the class of monofunctional catalases (HPII).

摘要

單一功能過氧化氫酶是一種含有血紅素的蛋白質，通常以同一四聚物形態出現，並且在對氧化壓力防衛的過程中扮演着十分重要的角色。單一功能過氧化氫酶能歧化過氧化氫爲水和氧($2H_2O_2 \rightarrow 2H_2O + O_2$)，從而刪除過氧化物，防止過氧化物引致細胞損壞和死亡，但它們在催化過程中卻沒有利用細胞中的還原劑。

搖蚊科是一種廣泛分佈在世界各地，而品種和數量相信是昆蟲類之冠的昆蟲群體。它們無論在高温、低温、高鹽度和極端的酸鹼度等不同惡劣環境下都可生存。大家對於它們這種强頑的生命力產生了濃厚興趣，希望從過氧化氫酶的研究中得知它們如何面對和適應不同的生活環境，而我們利用了搖蚊科的幼蟲(順序: 雙翅目,類: 昆蟲)作爲這研究計劃的生物模型。在香港，搖蚊科的幼蟲(俗稱：紅蟲)主要是用作魚類的飼料，是在朽糞或棄耕的禾田中培養出來。

要從搖蚊科的幼蟲中淨化出過氧化氫酶，可使用一系列不同色譜法，而這個研究曾使用乙醇三氯甲烷沉淀法、親合力及正離子移變色譜法來淨化，當中只有一個過氧化氫酶能從搖蚊科的幼蟲淨化出來。這個過氧化氫酶的最佳活動性是在攝氏四十度及在氫離子指數七至八點五之間，而它在熱力穩定測試中表現相當穩定，在攝氏六十度時仍能維持百分之七十的活動性。此外，依據十二烷基苯磺酸鈉膠化電泳的結果，這種過氧化氫酶的重量大約是五十三點五千道爾頓的業分子量。紅蟲的過氧化氫酶的最佳濃度和速度的數值大約是二十五毫模和十二萬五千個單位，經分光鏡的分析,A_{406}/A_{280} 比率決定是大約零點九五，也記錄到正鐵血紅素(九)的存在。在比較過氧化氫酶的氨基酸末端序列的過程後，我們發現搖蚊科幼蟲的過氧化氫酶跟其他動物的

過氧化氫酶有着相當密切的關係。這些實驗結果顯示搖蚊科幼蟲的過氧化氫酶屬於單一功能過氧化氫酶。

Abbreviation

Basic Local Alignment Search Tool	BLAST
Bovine Serum Albumin	BSA
N-Cyclohexyl-3-aminopropanesulfonic Acid	CAPS
Dithiothreitol	DTT
Ethylenediaminetetraacetic	EDTA
Isoelectric Focusing	IEF
Nitrogen Free Extract	NFE
Nicotinamide Adenine Dinucleotide	NAD
Nicotinamide Adenine Dinucleotide Phosphate	NADP
Polyvinylidene Difluoride	PVDF
Reduced Nicotinamide Adenine Dinucleotide	NADH
Reduced Nicotinamide Adenine Dinucleotide Phosphate	NADPH
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis	SDS-PAGE
N,N,N',N'-tetramethylethylenediamine	TEMED
Trifluoroacetic Acid	TFA
Tris(hydroxymethyl)aminomethane	TRIS
3-amino-1,2,4-triazole	3AT

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CHAPTER 1
INTRODUCTION

1.1 Catalases

Catalases (EC 1.11.1.6) are found in virtually all aerobic organisms. They appear to be an important part of the system that virtually all cells growing under aerobic conditions posses for their defense against oxidative stress. Catalases are unique among hydrogen peroxide-degrading enzymes in that it can degrade hydrogen peroxide without consuming cellular reducing equivalents. Hence, when cells are under stress and in need of energy, they are rapidly generating hydrogen peroxide through "emergency" catabolic processes. Hydrogen peroxide is degraded by catalase in an energy-efficient manner. This should result in a net gain of reducing equivalents and, therefore, cellular energy. While not essential for growth it plays a significant role in the survival of cells subjected to oxidative stress by hydrogen peroxide.

Catalases are normally located in peroxisomes where many hydrogen peroxide producing enzymes are present (Van Den et al., 1992; Del Rio et al., 1992). However, insect catalases are unusual in that the activities are not restricted to the microsomal fraction that contains the peroxisomes, but are also present in the cytosol and mitochondria (Ahmad et al., 1988b). The tissue distribution and characterization of catalases have been examined in only a few insect species. The ability of catalases to be induced or not is still unclear. For example, catalase is induced in

larvae of the geometrid moth (*Anaitis plagiatatum)* (Lee et al., 1989) but it is not induced in malpighian tubules (*Helicoverpa zea).*

1.2 Classification of catalases

Catalases are divided into two main groups amid the considerable heterogeneity, based on a survey of the properties and sequences of the enzymes. They are heme-containing catalases and Mn-catalases. Heme-containing catalases can be subdivided into HPI and HPII. HPI and HPII are also called catalase peroxidase and monofunctional catalase, respectively.

1.2.1 Catalase peroxidase (HPI)

Catalase peroxidases are found in bacteria (Yumoto et al., 1990; Hicks, 1995), yeasts (Fraaije et al., 1996), fungi (Levy et al., 1992), mammals and plants (Redinbaugh et al., 1988). They usually exist as homotetramers. The sizes of subunits of catalase peroxidase are about 80kDa, but variants with both smaller and larger subunits and with homodimeric structures have also been found. The subunit structure of catalase peroxidase was found to be similar to cytochrome c peroxidase (Hillar et al., 1995) and the conserved regions of these two enzymes were shown in figure 1.1. For the catalytic properties, catalase peroxidases are inhibited by common catalase inhibitors such as cyanide, azide and ascorbate but are not inhibited by 3-amino-1,2,4-triazole (Loewen, 1995). The catalase peroxidase activity

is also found to be sharp pH-dependent (Loewen, 1995).

Catalase peroxidases can either catalyze the direct dismutation of hydrogen peroxide into water and oxygen (catalatic mode) or use hydrogen peroxide to oxidize substrates such as methanol, ethanol, formaldehyde, formate, or nitrite (peroxidatic mode). At low concentration ($<10^{-6}$M) of hydrogen peroxide, they act "peroxidatically", where a variety of hydrogen peroxide donors can be oxidized in the following manner (equation 1 & 2). However, the peroxidatic reaction mechanism of catalase peroxidase is still unclear. At high concentration of substrate, catalase peroxidases can decompose toxic hydrogen peroxide at an extremely rapid rate using the catalatic reaction in which hydrogen peroxide acts as both acceptor and donor of hydrogen molecules (equation 1 & 3).

$$\text{CAT-Fe-OH} + H_2O_2 \rightarrow \text{CAT-Fe-OOH} + H_2O_2 \quad (1)$$

$$\text{CAT-Fe-OOH} + C_2H_5OH \rightarrow \text{CAT-Fe-OH} + H_2O + CH_3CHO \quad (2)$$

$$\text{CAT-Fe-OOH} + H_2O_2 \rightarrow \text{CAT-Fe-OH} + H_2O + O_2 \quad (3)$$

Keys: CAT-Fe-OOH can also be called compound 1.

Figure 1.1 Conserved regions of cytochrome c peroxidase and HPI subunit (Hillar et al., 1995).

1.2.2 Monofunctional catalases (HPII)

Monofunctional catalases are heme-binding enzymes and usually exist as homotetramers of 50 to 65kDa. They are associated with one heme b (protoheme IX) per subunit, but a number of variants are evident. Dimeric (Youn et al., 1995) and hexameric (Bol et al., 1991) structures are also found in some species. They are active in a broad range of pH and usually found to be stable up to 40^{0}C. They can be inhibited by cyanide and azide. Besides, monofunctional catalases is specifically inhibited by 3-amino-1,2,4-triazole (Margoliash et al., 1960).

Monofunctional catalases can only catalyze the direct dismutation of hydrogen peroxide into water and oxygen (catalatic mode) but they do not use hydrogen peroxide to oxidize substrates such as methanol, ethanol, formaldehyde, formate, or nitrite (peroxidatic mode). The enzymatic mechanism of monofunctional catalases was shown using equations 1 & 3 in the section 1.2.1.

For the structure of HPII, the four heme groups are deeply buried inside the molecule due to complex intersubunit interactions that appear strongly preserved in all heme-containing enzymes, and protein proximal iron ligand is invariably a deprotonated tyrosine residue (Bravo et al., 1997). There are three essential amino acids directly involved in the catalytic activity (Schonbaum et al., 1976): A histine and an asparagine in the distal side and a tyrosine in the proximal side (His-74, Asn-147,

and Tyr-357. The structure of a HPII subunit of *Escherichia coli* (Loewen et al., 1993) is shown in the figure 1.2.

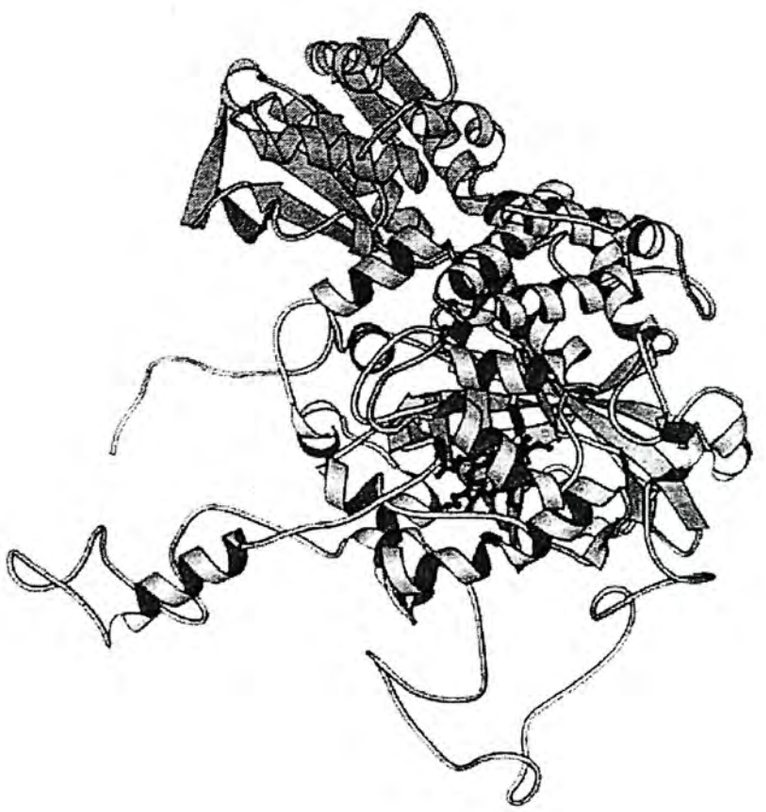

Figure 1.2 Subunit structure of catalase of HPII of *Escherichia coli* (Loewen et al., 1993).

1.2.2.1 NADPH in catalases

NADPH binding to catalases was totally unexpected when it was found to occur in some mammalian catalases (Kirkman et al., 1987) and bacterial catalases (Jouve et al., 1989). Catalases can also bind NADPH-like cofactors, the order of affinities being: NADPH > NADH >> $NADP^+$ > NAD^+ (Kirkman et al., 1984). In all cases, one NADPH molecule is bound per catalase monomer. The reaction of NADPH with catalase was described earlier (Hillar et al., 1994). The reaction mechanism of NADPH with catalase is shown in the figure 1.3.

The biochemical function of NADPH is not fully understood, but it seems clear (Kirkman et al., 1987; Jouve et al., 1989; Hillar et al., 1994) that NADPH is not essential for dismutation of hydrogen peroxide; prevents the formation of compound II; speeds up the decomposition of compound II to the ground state Fe(III) if compound II has been previously formed.

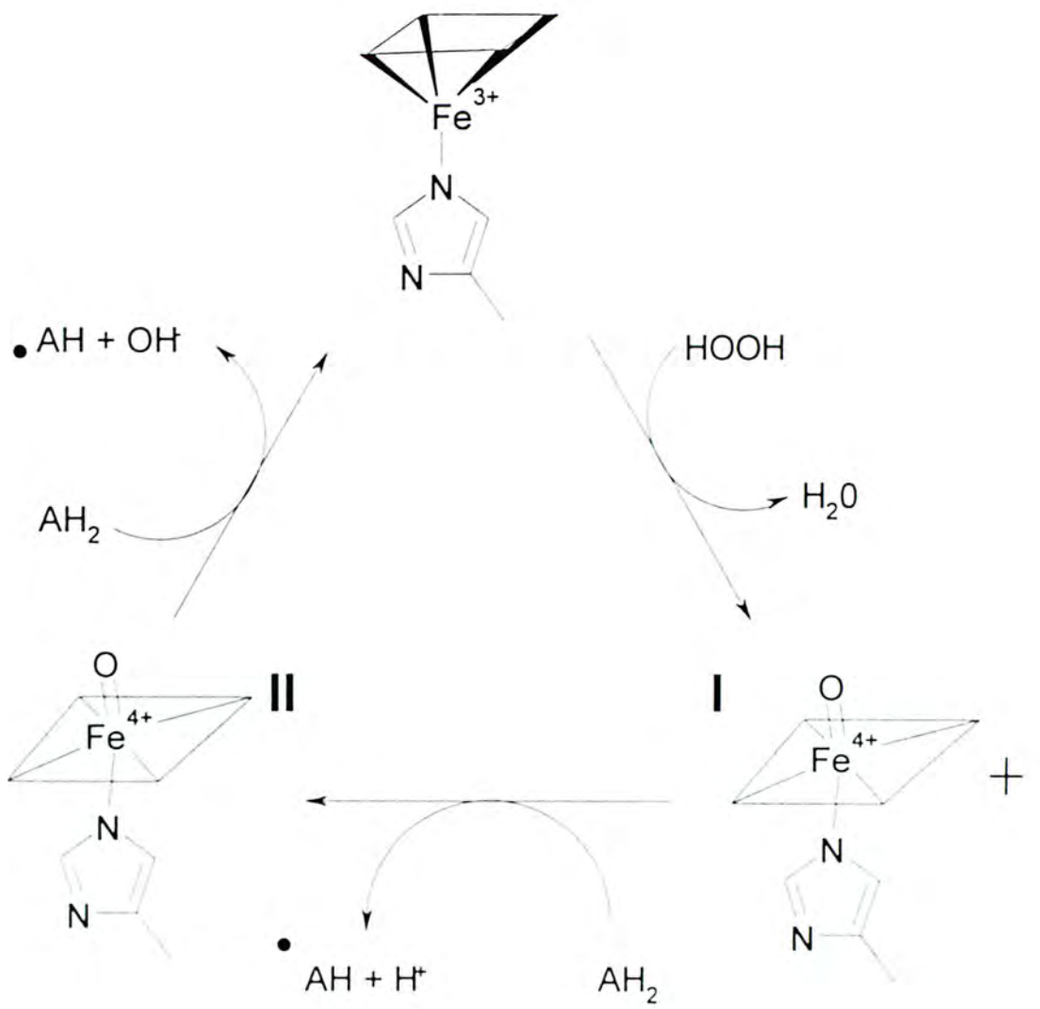

Figure 1.3 The reaction mechanism of NADPH with catalase

1.2.3 Mn-catalases

Most catalase enzymes have a heme group at their active sites. However, a number of bacteria (Kono et al., 1983; Allgood et al., 1986) is known to utilize a manganese dimer in order to function. Both tetrameric (Allgood et al., 1986) and hexameric (Baryin et al., 1986) structures are found in Mn-catalases. Mn-catalases are insensitive to the common catalase inhibitors such as cyanide and azide. Subunit structure sizes of Mn-catalases are smaller than that of the heme-containing catalases, and some of the Mn-catalases are found to be stable up to 80^0C. The enzymatic mechanism of Mn-catalases involves the changes of oxidation states of Mn. The interactions may be with one of three oxidation states: Mn(II)/Mn(II), Mn(III)/Mn(III), and Mn(III)/Mn(IV). Only the two lower oxidation states are catalytically active. A working model of Mn-catalases that recently proposed by Yoder (Yoder DW et al., 2000) is shown in the figure 1.4.

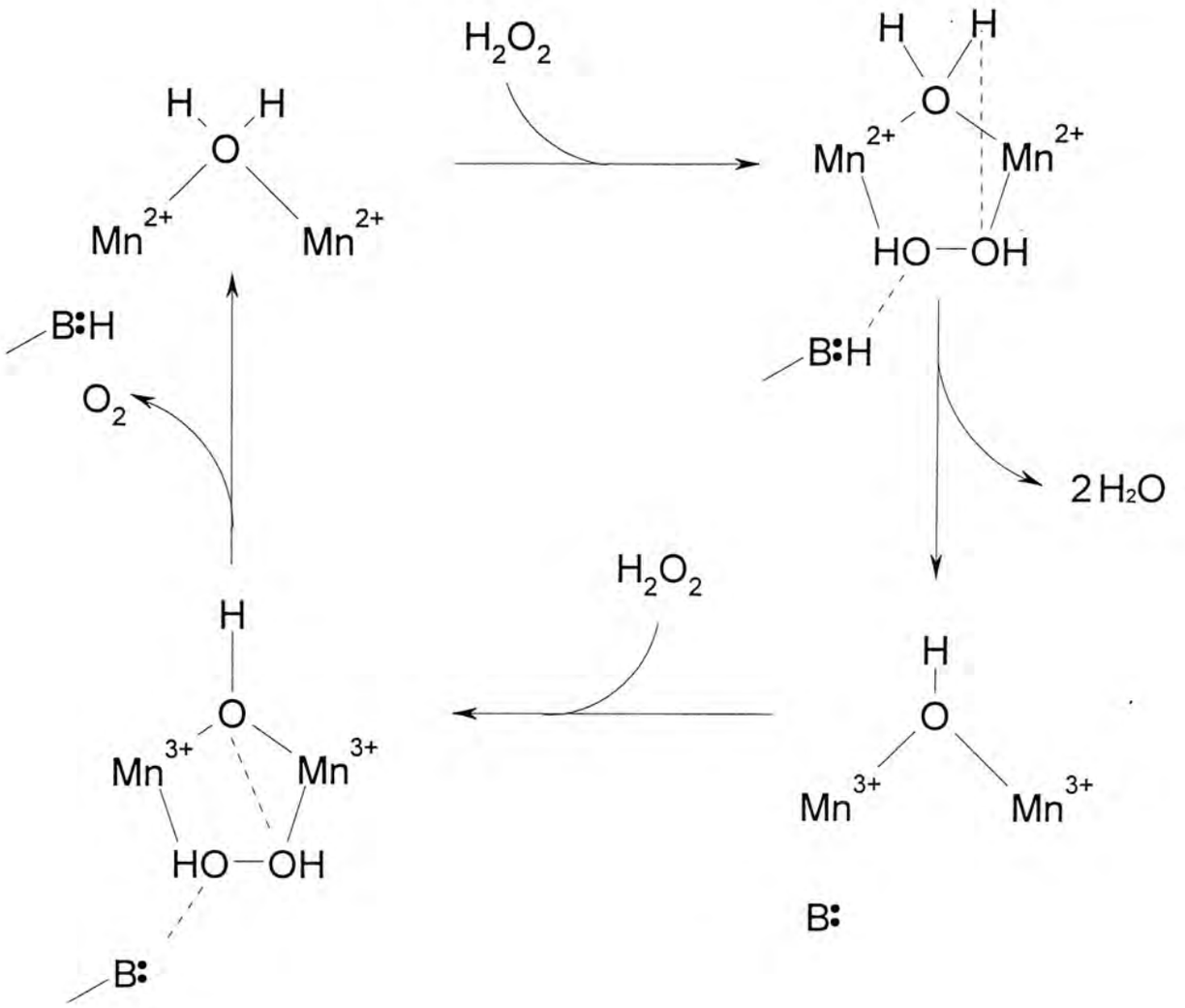

Figure 1.4 Proposed reaction mechanism of Mn-catalases (Yoder DW et al., 2000).

1.3 Sources and cytotoxic effects of hydrogen peroxide

Hydrogen peroxide is a pale-blue covalent liquid, freely miscible with water and apparently able to cross cell membranes readily (Halliwell et al., 2000). The cellular sources of hydrogen peroxide are many, and this species stems from non-enzymatic or enzymatic reactions. The former are usually encompassed by the spontaneous disproportionation of the superoxide radical to hydrogen peroxide. The latter are usually describing the enzymatic reaction of NADPH oxidases, xanthine oxidases, xanthine dehydrogenase and enzyme involved in microsomal electron-transfer chain (Hauptmann et al., 2001).

In chemical terms, hydrogen peroxide is poorly reactive. It can act as a mild oxidizing or as a mild reducing agent, but it does not oxidize most biological molecules readily, including lipids, DNA and proteins (Spector et al., 1996; Levine et al., 1999). The harmful effects of hydrogen peroxide largely comes from its ready conversion to the indiscriminately reactive hydroxyl radical, either by exposure to ultraviolet light (Ueda et al., 1996) or by interaction with a range of transition metal ions, of which the most important *in vivo* is probably iron (Halliwell et al., 1990). The formation of hydroxyl radical is shown in the following equations.

$$H_2O_2 \rightarrow 2OH\bullet \text{ (exposed to UV)} \qquad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe^{3+} + OH\bullet + OH^- \quad (5)$$

Hydroxyl radical has high electrophilicity and high thermochemical reactivity. It can attack virtually all macromolecules, leading to serious damage in cellular components, DNA lesions, and mutations, and often resulting in irreparable metabolic dysfunction and cell death (Hauptmann et al., 2001).

1.4 The *Chironomidae*

Chironomidae are the most widely distributed and frequently the most abundant insects in fresh water. There are estimated to be as many as 15,000 species of chironomids worldwide (Cranston, 1995). *Chironomidae* are found to be living in extremes of temperature, pH, salinity, depth and current velocity. They can live in the glaciated areas of the highest mountains, including at elevation of up to 5600m in the Himalaya and are active at temperatures of -16^0C. Larvae of *Sergentia* live at over 1000m in depth in the abyssal of the world's deepest body of freshwater, lake Baikal.

1.4.1 Life cycle of *Chironomidae*

The life cycle of *Chironomidae* is divided into four distinct stages, i.e. egg, larva, pupa and adult. Notwithstanding the large number of species within the family, chironomids share one conspicuous life history characteristic in that the last two stages are generally very short in duration, while the egg and larval stages vary

substantially between and within species. The short durations of last two stages are probably used to minimize the energy output of the body. As a result, the highest reproductive output will be achieved (Tokeshi, 1995). The detailed description of the life cycle is as follows.

1. Eggs

The eggs are laid immediately following copulation after which the female soon dies. The whole process may be completed in 10 minutes or an hour at the most. Most chironomids deposit their eggs on firm substrata such as macrophytes, stones or leaf litter, close to the water's edge (Nolte, 1993). Typical egg-masses of freshwater chironomids contain from 20 to 30 eggs, in the case of some smaller species, to 2000 or more for the larger species. The highest recorded number of eggs in a single mass is 3300, for *Chironomus (Camptochironomus) tentans* (Fabricius) (Nolte, 1993). The time taken for eggs to develop to a point of hatching into next stage is usually within a few days to one month.

2. Larva and pupa

The newly hatched larvae are usually not more than 1 mm long but they measure up to 10-15 mm when they reach the last stage of the larva period. Each larva moults four times before it reaches the pupal stage. The growth rate of larva can be affected by some environmental factors, such as temperature (Ward et al., 1979;

Hauer et al., 1991), food (Storey, 1987) and biotic interaction. Oxygen content of water, pH and toxic substances can affect the growth of larva but only a few studies of the larvae have been investigated. Besides, larva of smaller sizes usually grows faster than that of the larger one. For the length of pupal stage, it varies among different species (2 days to several weeks). The pupal stage can provide a suitable condition for *chironomids* to develop into adults within a short period of time.

3. Adult

Adult chironomids are short-lived and their behavior is concerned largely with reproduction. The adult life is defined by the emergence of the free adult midge from the pupal skin. Emergence is rapid and the adult is able to fly almost immediately. They only live 3 to 5 days and mating and oviposition take place during this period. The adults occur in great numbers in the vicinity of ponds, lakes and streams because eggs are laid in water and the young stages are aquatic.

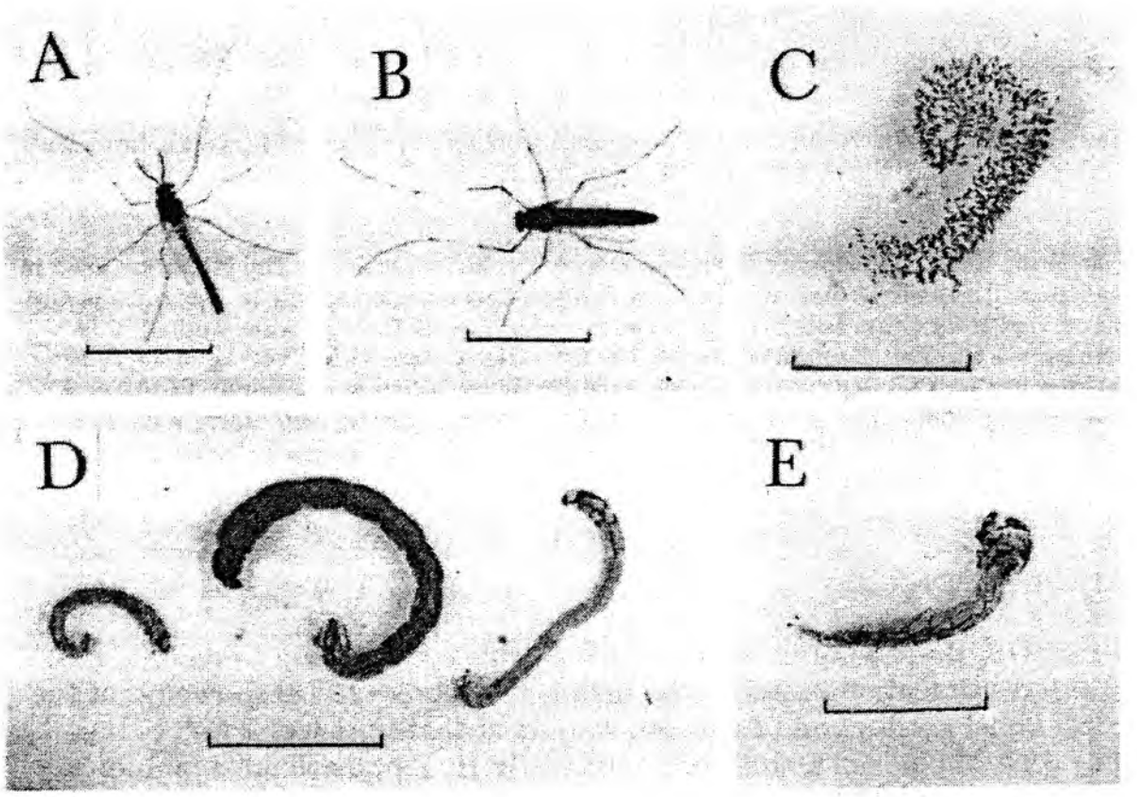

Figure 1.5 Life history of *Chironomus.*

Legend: A, Male adult; B, female adult; C, eggs; D, larvae; E, pupa. The bars indicate 0.5 cm (Shaw et al., 1980).

1.4.2 Bloodworms

Bloodworms are larvae of the non-biting midges of the Family *Chironomidae* (Order Diptera, Class Insecta). In Singapore over fifty species have been recorded (Letha, 1969). Not all the *chironomid* larvae are red in color. Some of them are greenish or whitish but only those that contain haemoglobin are red and hence their name bloodworms. The *chironomid* larvae and pupae are highly nutritious and nourishing and constitute one of the staple food items in the ratio of many fishes in the natural environment. The importance of *chironomid* larvae as live food for tropical fish culture is well known in Asian countries. All carnivorous fishes, such as oscar, discus, Siamese fighting fish and cichlids will greedily devour them when they are offered, and the fish grow faster and spawn earlier. They have been reported in literature to be very adequate for growth in fishes (Yashouv, 1970). It has been found that if carps are provided with bloodworms as supplementary food they gain better weight and the growth rate is more uniform (Yashouv, 1956). The nutritional value of bloodworm is considered to be very good. Chemical analysis shows that bloodworms contain 9.3% dry matter and of this there are 62.5% crude protein, 10.4% crude fat and 11.6% ash with 15.4% nitrogen free extract (NFE). They are also a good source of iron for the fish since they contain haemoglobins in their blood as the vertebrates.

Bloodworms are benthic organisms. They live in contaminated sediments and often feed on detritus and algae associated with the sediments (Larsson, 1984) which are often reservoirs for pollutants (Pesch et al., 1981). *Chironomid* larvae are important primary consumers and are often a significant portion of the diet of predatory invertebrates and fishes. They play a primary role in the bioaccumulation and transport of contaminants. Furthermore, chironomid*s* have a relative short life cycle in comparison to other organisms that may serve as biological indicators such as fish or birds. Additionally, bloodworms in water are exposed to contaminants during the longest and most critical stage of their life cycle, the larval stage. All the energy required to complete the life cycle is built up in the larval stage because the adults, with few exceptions, do not feed (Oliver, 1971). Hence, *chironomid* larvae have excellent potential as aquatic biomonitoring organisms.

1.4.3 Sources of bloodworms

C*hironomid* larvae can be grown in chicken manure (Shaw et al., 1980). The yield is about $28gm^{-2}week^{-1}$ that is much lower than the yield of 250 to $375gm^{-2}week^{-1}$ obtained by Yashouv (Yashouv, 1970) who grew *chironomid* larvae on chicken manure in pans in a greenhouse with aeration. Horse manure has also been used to fertilize the pool for bloodworm culture, but the average yield of the best pools was $11gm^{-2}week^{-1}$ which was only a fraction of the maximum yields obtained from other

midge culture systems (McLarney et al., 1974).

To produce blood worms in large quantities is expensive. Food processing factories' by-products are suitable for this purpose. The attempt to rear bloodworms with various by-products such as wheat bran, rice bran, soyabean meal and coconut refuse have been carried out with satisfactory results (Koh et al., 1980; Teo et al, 1984).

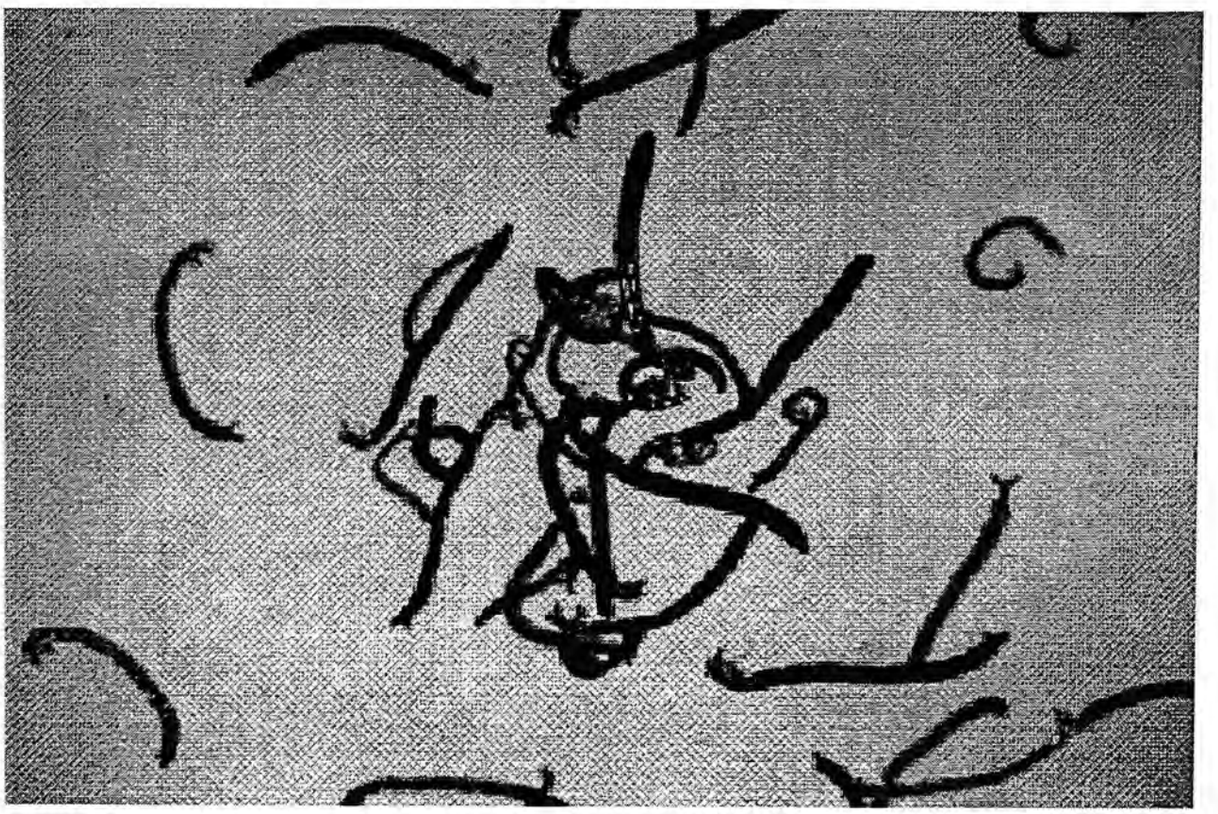

Figure 1.6 Bloodworms bought from local market.

1.5 Aim of the project

In this study, catalase was purified from *chironomid* larvae since catalase has been linked to the anti-oxidative effect of organisms. Bloodworms, the larvae stage of *Chironomidae*, were used to prepare catalase. Bloodworms are exposed to contaminants during the longest and most critical stage of their life cycle. Also, there is a transition that involves an adaptation to a comparatively oxygen rich environment during its life cycle. Hence, catalase may play an important role in modulating of oxidative stress and the adaptation to the adverse living environments. The objective of my research is to investigate the biochemical properties of the purified catalase in *Chironomidae*.

1.6 Application of the project

Chironomidae was widely distributed and abundant in freshwater. The number of species was found to be about 15,000. The occurrence of *Chironomidae* was regarded as a pest problem. Pest control of *Chironomidae* is concerned in different countries. In order to solve the pest problem involved with *Chironomidae*, a better understanding of *Chironomidae* could provide a solution. It was reported that the length of insect's life span might relate to the expression of catalases (Deisseroth et al., 1970). The biochemical, physical & structural properties of the catalase could provide useful information in pest problem. Hence, purification and characterization

of catalase in bloodworms was carried out in this project.

CHAPTER 2
MATERIALS AND METHODS

2.1 Protein determination

The amount of protein was measured by the method of Bradford (1976) using bovine serum albumin (BSA) (Sigma Chemical co.) as the standard. The assays were done by mixing diluted protein samples with Bio-Rad protein assay reagent. The mixtures were allowed to stand for 10 minutes at room temperature. The absorbance at 595nm was recorded on a Beckman DU 650 spectrophotometer. A standard curve was established using different concentrations of BSA (figure 2.1). The amounts of protein in unknown protein samples were determined.

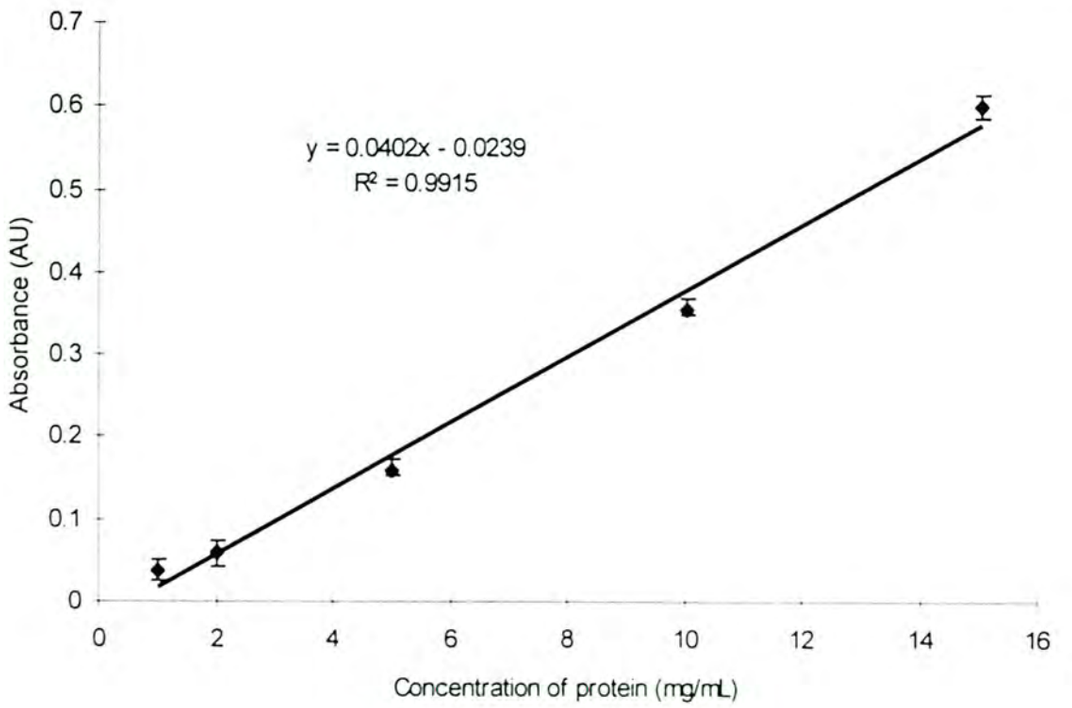

Figure 2.1 The calibration curve using BSA standard.

2.2 In vitro activity assays

Two assays were carried out to detect the presence of the purified enzyme containing both catalase and peroxidase activity.

2.2.1 Catalase activity assay

Catalase activity was measured spectrophotometrically at 25^{0}C by following the decrease in absorption at 240nm of 13mM hydrogen peroxide (Sigma Chemical co.) in 50mM potassium phosphate buffer pH 7.0 (Sigma Chemical co.) (Aebi, 1984). The absorbance at 240nm was recorded on a Beckman DU 650 spectrophotometer. A complete assay mixture without enzyme was used as a control. One unit of activity was defined as the amount of enzyme that catalyzed the decomposition of 1 μmol of hydrogen peroxide per minute. Specific enzyme activity was defined as U/min/mg of protein. Results represented averages and standard deviations from experiments performed four times using three independent samples.

2.2.2 Peroxidase activity assay

Peroxidase activity was measured by following the rate of oxidation of σ-dianisidine (BDH AnalaR®) at 460nm (Calera et al., 2000). The reaction mixtures contained 50mM potassium phosphate pH 7.0, 1.0mM hydrogen peroxide and 0.05% σ-dianisidine. The absorbance change was recorded on a Beckman DU 650 spectrophotometer at 25^{0}C. One unit of enzyme activity was defined as the amount

of enzyme that catalyzed the oxidation of 1μmol of σ-dianisidine per minute. The specific enzyme activity was defined as U/min/mg of protein. Similarly, the results represented the averages and standard deviations from experiments performed four times using three independent samples.

2.3 Screening of catalase in different subcellular fractions

All preparation procedures were carried out at 4^0C unless otherwise specified.

2.3.1 Preparation of mitochondrial fractions

Bloodworms which were bought from local market were washed with distilled water. They were homogenized using homogenizer (KIKA Labortechnik) in 50mM sodium acetate pH 5.0 buffer (Sigma Chemical co.) containing 1mM ethylenediaminetetraacetic (EDTA) (USB). The homogenate was centrifuged twice at 1,000g for 10 minutes using a Beckman ADVANTi™ J-30i centrifuge. The supernatant obtained was filtered through glass wool and cheesecloth. It was then centrifuged twice at 20,000g for 30 minutes using a Beckman ADVANTi™ J-30i centrifuge. The resulting pellets containing mitochondria were redissolved by mixing with 50mM sodium acetate buffer containing 5mM digitonin (Sigma Chemical co.) for 30 minutes.

2.3.2 Preparation of microsomal fractions

The homogenate of bloodworms was centrifuged twice at 27,000g for 20 minutes using a Beckman ADVANTi™ J-30i centrifuge. The supernatant was filtered through glass wool and cheesecloth., followed by centrifugation twice at 105,000g for 60 minutes using a Beckman Optima™ XL-100K ultracentrifuge. The resulting pellets of microsomes were resuspended in 50mM sodium acetate buffer containing 250mM sucrose (Sigma Chemical co.) and 300mM EDTA.

2.3.3 Preparation of cytosolic fractions

The homogenate of bloodworms was centrifuged twice at 27,000g for 20 minutes. The resulting supernatant was filtered through glass wool and cheesecloth, followed by centrifugation at 105,000g for 60 minutes. The supernatant obtained was also filtered through glass wool and cheesecloth.

2.3.4 Preparation of post-mitochondrial fractions

The homogenate of bloodworms was centrifuged twice at 27,000g for 20 minutes. The resulting supernatant of post-mitochondrial fractions were filtered through glass wool and cheesecloth.

2.4 Purification of post-mitochondrial catalase

All purification procedures were carried out at 4^0C unless otherwise specified.

2.4.1 Preparation of post-mitochondrial fractions

The post-mitochondrial fractions were prepared as described in section 2.3.4. It was dialyzed against 50mM sodium acetate pH 5.0 overnight.

2.4.2 Ethanol-chloroform precipitation

The dialyzed post-mitochondrial fractions were vigorously mixed with ethanol (BDH AnalaR®) and chloroform (BDH AnalaR®) in the volume ratio 10:4:1 (post-mitochondrial fraction: ethanol: chloroform) for 20 minutes with stirring. The resulting mixture was then centrifuged at 20,000g for 30 minutes. The supernatant obtained was filtered through 3mm chromatography paper (Whatman®). The filtered supernatant was dialyzed against 50mM sodium acetate buffer pH 5.0 overnight. After dialysis, the sample was centrifuged at 20,000g for 30 minutes. The collected pellets were redissolved in 50mM Tris(hydroxymethyl)aminomethane (TRIS) buffer pH 8.5 (USB).

2.4.3 Affinity chromatography

The resuspended protein was applied onto a blue sepharose (Amersham Pharmacia Biotech) 25/15 column that was equilibrated with 50mM TRIS buffer pH 8.5 overnight. The column was washed successively with 4-5 bed volumes of 0 and1.5M sodium chloride (USB) in the same buffer (2mL/min flow rate). The active fractions were eluted by 3 bed volumes of 50mM TRIS buffer pH 8.5 containing 2M

sodium chloride and 2mM nicotinamide adenine dinucleotide (NAD^+) (Sigma Chemical co.) (2mL/min flow rate). The salt concentration of the active sample was reduced by dilution and concentration by ultrafiltration. The ultrafiltration was performed using Amicon stirring cell fitted with PM 10 membrane of 10kDa molecular weight cutoff. The sample was subsequently dialyzed against 20mM sodium acetate buffer pH 5.0 overnight.

2.4.4 Cation exchange chromatography

The sample collected from the affinity chromatography described in the section 2.4.3 was purified using fast protein liquid chromatography (FPLC) (Amersham Pharmacia Biotech). It was applied onto a Mono S HR 5/5 column (Amersham Pharmacia Biotech) that was equilibrated with 20mM sodium acetate buffer pH 5.0. The column was washed with a 40mL buffer with increasing linear gradient from 0 to 0.4M sodium chloride in the same buffer. The fractions containing catalase were diluted and concentrated as before. They were then applied onto a Mono S HR 5/5 column that was equilibrated with 20mM sodium acetate buffer pH 5.0 for rechromatography. The column was washed with a 50mL buffer with increasing linear gradient from 0 to 0.4M sodium chloride in the same buffer. The fractions were diluted and concentrated using ultrafiltration. The purity of the fraction was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE).

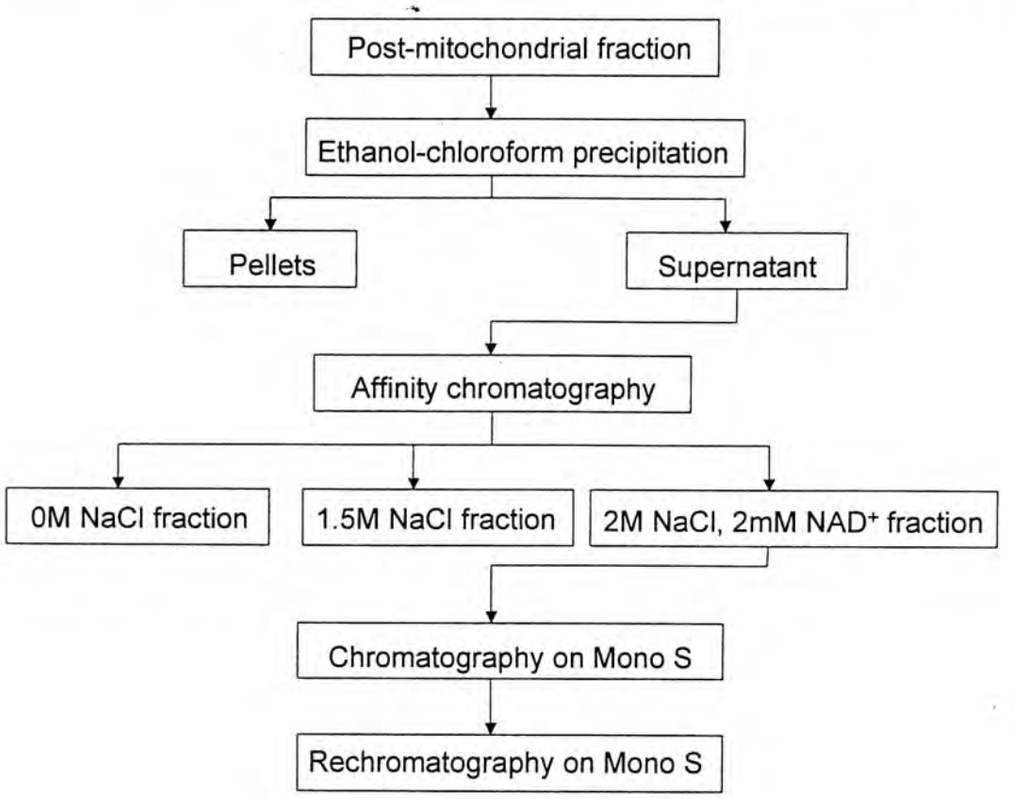

Figure 2.2 Purification scheme of post-mitochondrial catalase from *chironomid* larvae.

2.5 Molecular mass determination

The native and subunit molecular mass of the purified enzyme was determined using size exclusion chromatography and SDS-PAGE, respectively.

– Native molecular mass

The native molecular mass of the purified enzyme collected in the section 2.4.5 was determined using Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) in FPLC system. The flow of FPLC gel filtration column was set at a constant flow rate of 0.7mL/min. To determine the native molecular mass of the purified enzyme, the gel filtration column was calibrated using standard proteins. The standard proteins (Sigma Chemical co.) used for column calibration were shown in table 2.1. The native molecular mass of the purified enzyme was calculated from the standard curve.

– Subunit molecular mass

The subunit molecular mass of the purified enzyme collected in the section 2.4.5 was estimated by SDS-PAGE. The molecular mass of the purified sample was determined by performing electrophoresis with standard proteins (Sigma Chemical co.). Table 2.2 showed the standard proteins used in the SDS-PAGE.

The SDS-PAGE was prepared by the method of Laemmli's buffer system (Laemmli, 1970). Protein samples which were applied to the SDS-PAGE were mixed

with sample buffer in the volume ratio 1:1 and were heated at 95^{0}C for 5 minutes prior to loading. Composition of the separation gel, stacking gel and sample buffer were shown in table 2.3, 2.4 and 2.5, respectively. The SDS-PAGE was performed at constant voltage (150V) until the bromophenol blue tracking dye ran at the bottom of the gel with the Bio Rad Mini-Protean II system (Bio-Rad Laboratories).

The polyacrylamide gel was fixed and stained with staining solution (0.1% Coomassie Brilliant Blue R-250 (Boehringer Mannheim Corp.), 10% acetic acid (BDH AnalaR®) and 40% ethanol) for at least two hours. It was then destained with solution (10% acetic acid, 40% ethanol) until the background was clear.

Protein	Native molecular mass (Da)
Blue dextran (BD)	2,000,000
Thyroglobulin (TH)	669,000
Apoferritin (AP)	443,000
Beta-amylase (BA)	200,000
Alcohol dehydrogenase (ADH)	150,000
Bovine serum (BS)	66,000
Carbonic anhydrase (CA)	29,000

Table 2.1 Standard proteins of gel filtration column.

Protein	Molecular mass (Da)
Lysozyme, egg white	14,300
β-Lactoglobulin, bovine milk	18,400 (subunit)
Trypsinogen, bovine pancreas, PMSF treated	24,000
Pepsin, Porcine stomach mucosa	34,700
Albumin, egg (ovalbumin)	45,000
Albumin, bovine plasma	66,000

Table 2.2 Standard proteins for SDS-PAGE.

Separating gel, in 0.375M Tris buffer, pH 8.8				
	7%	10%	12%	15%
Distilled water	5.1mL	4.1mL	3.4mL	2.4mL
1.5M TRIS pH 8.8	2.5mL	2.5mL	2.5mL	2.5mL
Acrylamide (USB)/ Bis-acrylamide (Sigma Chemical co.) (30%/ (w/v) 0.8% (w/v))	2.3mL	3.3mL	4.0mL	5.0mL
20% (w/v) SDS	0.05mL	0.05mL	0.05mL	0.05mL
10% (w/v) ammonium persulfate (Sigma Chemical co.)	0.05mL	0.05mL	0.05mL	0.05mL
N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma Chemical co.)	0.005mL	0.005mL	0.005mL	0.005mL
Total volume	10.005mL	10.005mL	10.005mL	10.005mL

Table 2.3 Composition of separation gel with acrylamide at different concentrations.

Stacking gel, 4% gel, 0.125M Tris, pH 6.8	
Distilled water	3.075mL
0.5M TRIS pH 6.8	1.25mL
20% (w/v) SDS	0.025mL
Acrylamide/ Bis-acrylamide (30%/0.8% w/v)	0.67mL
10% (w/v) ammonium persulfate	0.025mL
TEMED	0.005mL
Total volume	5.05mL

Table 2.4 Composition of stacking gel.

Sample buffer	
Distilled water	4.0mL
0.5M TRIS pH 6.8	1.0mL
Glycerol (USB)	0.8mL
10% SDS	1.6mL
β-mercapoethanol (Sigma Chemical co.)	0.4mL
0.05% (w/v) bromophenol blue	0.2mL

Table 2.5 Composition of sample buffer.

2.6 Isoelectric focusing

The isoelectric point (p*I*) of the purified enzyme was measured by the method of isoelectric focusing (IEF) (Robertson et al., 1987). The gel was cast 1.5mm thick from the following mixture: 7mL water, 2mL acrylamide mixture (30% (w/v) acrylamide, 1% (w/v) bis-acrylamide), 2.4mL 50% glycerol and 0.6mL ampholyte (pH range 3 – 10) (Amersham Pharmacia Biotech). These components were mixed and degassed. An aliquot of 50μL of 10% (w/v) ammonium persulfate and 20μL TEMED were added.

The cathode solution was 25mM sodium hydroxide solution (Sigma Chemical co.) and the anode solution was 20mM acetic acid. These solutions were cooled to 4^0C prior to electrophoresis. After polymerization was complete, the comb was removed and the wells were rinsed and filled with cathode solution. Protein standards and samples were mixed with an equal volume of 60% (v/v) glycerol and 4% (v/v) ampholyte of the same pH range used to prepare the gel. Electrophoresis was performed at 4^0C for 1.5 hours at 200V constant voltage and increased to 400V constant voltage for an additional 1.5 hours with the Bio Rad Mini-Protean II system.

After electrophoresis, the gel was fixed in 10% trichloroacetic acid (TCA) (Sigma Chemical co.) for 10 minutes and 1% TCA for 2 hours successively for the removal of ampholyte. The gel was stained and destained as described in the

section 2.5.2.

The standard curve of isoelectric point was established using standard proteins (Amersham Pharmacia Biotech). The pI of standard proteins and the calibration curve was shown in table 2.5 and figure 3.6, respectively. The pI of the purified enzyme was determined using the calibration curve.

Protein	p*I*
Amylglucosidsae	3.50
Methyl red (dye)	3.75
Trypsin inhibitor	4.55
β-Lactoglobulin	5.20
Carbonic anhydrase B (bovine)	5.85
Carbonic anhydrase B (human)	6.55
Myoglobin, acidic band	6.85
Myoglobin, basic band	7.35
Lentil lectin, acidic	8.15
Lentil lectin, middle	8.45
Lentil lectin, basic	8.65
Trypsinogen	9.30

Table 2.6 The standard proteins of IEF gel.

2.7 Kinetic studies of the purified enzyme

2.7.1 Optimal pH

The effect of pH on the purified enzyme was assayed from pH 2.5 to 11.0 using different 100mM buffer systems (phosphoric acid pH 2.5-3.0 (Sigma Chemical co.), chloroacetate acid (Sigma Chemical co.) pH 3.0-3.5, acetic acid pH 3.5-5.5, sodium monohydrogen phosphate pH 5.5-7.5 (Sigma Chemical co.), TRIS buffer pH 7.5-9.0 and N-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) (Sigma Chemical co.) pH 9.0-12.0). Assays were conducted as described in the section 2.2.1.

2.7.2 Thermal stability

The thermal stability of the purified enzyme was studied by pre-incubation of the purified enzyme at desirable temperatures for 15 minutes. The purified enzyme was dissolved in 50mM potassium phosphate buffer pH 7.0 containing 10mM dithiothreitol (DTT) (Sigma Chemical co.) and 10mM EDTA in order to prevent the oxidative inactivation. After pre-incubation, the catalase activity was measured at 20^0C and the assay was recorded as described in the section 2.2.1.

2.7.3 K_m and V_{max}

The K_m and V_{max} of the purified enzyme was studied by measuring the enzyme activity at different concentrations of hydrogen peroxide solution (1 to 28mM). The assay was performed at 40^0C in 50mM potassium phosphate buffer pH 7.0

containing different concentrations of hydrogen peroxide. The decrease in absorbance at 240nm was recorded on a Beckman DU7500 spectrophotometer. The apparent K_m and V_{max} were calculated from the experimental results of this section using Lineweaver-Burk plot.

2.7.4 Inhibition studies

The activity of the purified enzyme was assayed with addition of some inhibitors such as KCN, NaN_3 and 3-amino-1,2,4-triazole (3AT).

2.7.4.1 3-amino-1,2,4-triazole

The purified enzyme was allowed to be pre-incubated with 3AT that was a specific inhibitor of monofunctional catalase (Margoliash et al., 1960; Bhuyan et al., 1977). The purified enzyme could be classified as monofunctional catalase if catalase activity was reduced. The purified enzyme was allowed to pre-incubate with different concentrations of 3AT at 25^{0}C for 10 minutes. After pre-incubation, the assay was done as described in the section 2.2.1.

2.7.4.2 Potassium cyanide and sodium azide

The purified enzyme was allowed pre-incubated with different concentration of potassium cyanide and sodium azide at 25^{0}C for 10 minutes that were common inhibitors of catalase. After pre-incubation, the assay was done as described in the section 2.2.1.

2.8 Spectroscopic analysis

Spectroscopic analysis of the purified enzyme was done by scanning the ultraviolet/visible spectrum of samples (200 to 800nm) at 25^{0}C using a Beckman DU 7500 spectrophotometer, to show whether the purified enzyme was heme protein or not. Also, the structural change of the purified enzyme could be observed in the analysis.

2.8.1 Native enzyme

The purified enzyme was dissolved in 100mM potassium phosphate buffer pH 7.0. It was allowed to stand at 25^{0}C for 10 minutes. After the pre-incubation, the spectroscopic analysis was done as described in the section 2.8.

2.8.2 Denatured enzyme

Common catalase inhibitors (KCN and NaN_3) were used in this project. The purified enzyme was dissolved in 100mM potassium phosphate buffer pH 7.0 with addition of enzyme inhibitor and the volume ratio of the enzyme solution to inhibitor solution was 1:1. The concentration of the enzyme inhibitor was 2mM. The mixture was pre-incubated at 25^{0}C for 10 minutes. After the pre-incubation, the spectroscopic analysis was done as described in the section 2.8.

2.8.3 Determination of pyridine hemochrome

The heme content of the purified catalase was determined using the

modified method by Berry (Berry et al., 1987). The concentration and class of heme was determined by the absorption spectrum. For the spectroscopic analysis of the purified catalase, 3mL of 50mM NaOH, 20% pyridine and 6μL of 0.1M $K_3Fe(CN)_6$ were added to 100μL protein sample with known concentration. Spectra were then recorded before and at intervals after reducing with solid sodium dithionite (Sigma Chemical co.). Spectra were corrected by subtracting with a baseline recorded with pyridine/NaOH solution and ferricyanide in the cuvette before adding the purified catalase. The ratio of the heme/protein was calculated using the extinction coefficient of 33.88 at 556nm.

2.9 N-terminal amino acid sequence analysis for blotted protein

The sample was prepared using gel electrophoresis followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane (Millipore) in this project.

2.9.1 Semi-dry electroblotting

The purified sample for N-terminal amino acid sequence was transferred from SDS-PAGE to PVDF membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories). The electrophoresis was run using 10% separating gel as described in the section 2.5.2. The electrophoresis was stopped

when the desirable band running in the lower half of the gel. After electrophoresis, the gel was soaked in transfer buffer (25mM TRIS, 190mM glycine and 10% methanol (BDH AnalaR®)) for 15 minutes with gentle shaking. One sheet of PVDF membrane and four sheets of 3MM chromatography papers were cut to the same size as the gel. The PVDF membrane was immersed in methanol for 10 seconds followed by equilibration in transfer buffer for 5 minutes. The 3MM chromatography papers were also equilibrated in transfer buffer for 3 minutes. A gel/PVDF/blotting paper sandwich as for western blotting was prepared with exclusion of all air bubbles. The blotting was carried out at constant voltage (20V) for 2 hours.

2.9.2 Protein staining on PVDF membrane

The PVDF membrane was washed in transfer buffer for 10 minutes with shaking after the semi-dry electroblotting. It was stained with staining solution (0.1% Coomassie Brilliant Blue R-250, 40% methanol) for 5 minutes and destained with destaining solution (50% methanol, 10% acetic acid) for 2-5 minutes until the membrane remaining a faint blue color (Christiansen et al., 1992). The membrane was washed twice in deionized water (3x10 minutes, shaking) and was allowed to air-dry. It was stored at -20^{0}C until sequencing.

2.9.3 N-terminal amino acid sequence analysis

The N-terminal sequencing was done based on the method developed

during 1950s by Pehr Edman. The sequence of amino acids in protein was determined by sequential chemical degradation from the N-terminus of the protein in the Edman chemistry cycle. The Edman chemistry cycle consisted of three stages. They were called coupling, cleavage and conversion. At the end of each cycle of Edman degradation, the PTH-amino acid is separated from reaction by-products and identified, typically by HPLC chromatography and UV absorbance, respectively. In this project, the amino acid sequencing was carried out using the HP G100A Edman degradation unit an HP1090 HPLC system. However, there was no signal detected in the sample. It was believed that the N-terminus of the protein was blocked.

2.9.4 N-terminal deblocking of protein bound on PVDF membrane

The N-terminal deblocking was carried out using the modified method of Wellner (Hirano et al., 1993; Tsunasawa et al., 1993). The protein bound PVDF membranes were incubated with saturated trifluoroacetic acid (TFA) vapor at 65^{0}C for 1.5 hours. Nitrogen gas was used to dry the PVDF membranes after incubation. The amino acid sequence analysis was carried out using the dried PVDF membranes immediately.

2.9.5 BLAST® search

BLAST® (Basic Local Alignment Search Tool) was a set of programs designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships (Altschul et al., 1990). The amino acid sequence obtained in this project was matched with other available sequences in databases using the BLAST®.

CHAPTER 3
RESULTS

3.1 Catalase in different sub-cellular fractions

Catalase and catalase peroxidase activities were detected in different sub-cellular fractions of *chironomid* larvae. The activities were shown in the table 3.1.

Sub-cellular fraction	Mitochondria	Microsomes	Cytosols
Catalase activity (U/min)	0.06	0.24	0.88
Catalase activity (U/min/mg)	3.14	11.97	43.88
Peroxidase activity (U/min)	0.05	0.06	0.07
Peroxidase activity (U/min/mg)	0.53	0.12	0.68

Table 3.1 Catalase activity and catalase peroxidase activity of different sub-cellular fractions.

The activity was measured as described in the section 2.2.1 and 2.2.2.

3.2 Purification of post-mitochondrial catalase

Differential centrifugation, ethanol-chloroform precipitation, affinity chromatography and Mono S chromatography were employed in purification of post-mitochondrial catalase in bloodworms. Catalase activity and catalase peroxidase activity was measured in each purification step as described in the section 2.2.1. The purification and recovery of the enzyme was shown in table 3.2.

3.2.1 Ethanol-chloroform precipitation

The collected post-mitochondrial fractions were centrifuged after mixing with ethanol and chloroform. Catalase activity was detected in the supernatant. The supernatant was collected for dialysis against 50mM sodium acetate pH 5.0 for 24 hours. No catalase activity was detected in pellets and so pellets were discarded. After dialysis, the sample was centrifuged at 20,000g for 30 minutes. Catalase activity wasn't detected in the supernatant but it was detected in pellets that were resuspended in 50mM TRIS pH 8.5. On the other hand, catalase peroxidase activity could not be detected in both supernatant and pellets.

3.2.2 Affinity chromatography

A step gradient elution using NaCl and NAD^+ was carried out to elute catalase from the affinity column. Catalase was bound to the affinity column and so it was not eluted using 50mM TRIS buffer pH 8.5 containing 1.5M NaCl. However, it

was eluted by 50mM TRIS buffer pH 8.5 containing 2M sodium chloride and 2mM NAD^+. The elution profile was shown in figure 3.1.

3.2.3 Cation exchange chromatography

The sample collected in affinity chromatography was further purified by Mono S HR 5/5 column. The elution profile of the Mono S column was shown in the figure 3.2. Catalase was eluted at about 23.5mS/cm using a linear NaCl gradient. When the sample was checked using SDS-PAGE, a small amount of contaminants was observed in the gel. Hence, fractions of the peak apex were collected and were applied onto Mono S HR 5/5 column again for the removal of contaminants. The elution profile of the rechromatography was shown in the figure 3.3.

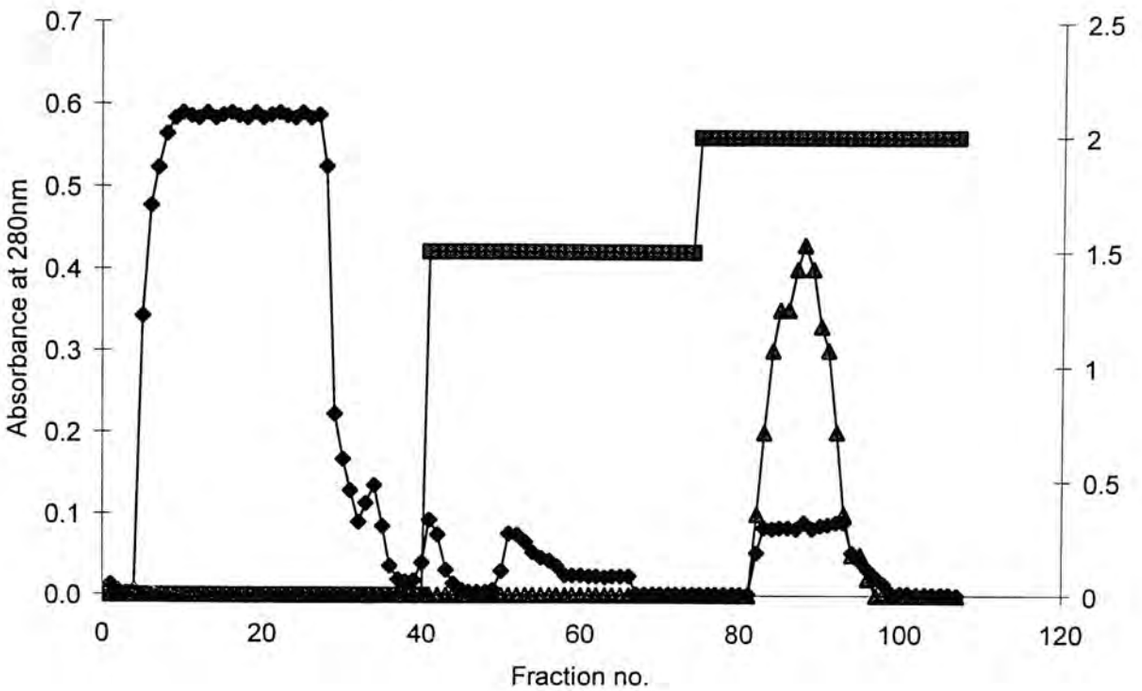

Figure 3.1 The elution profile of the supernatant from ethanol chloroform precipitation onto the Affinity column.

Legend: (-♦-) Absorbance at 280nm, (-) Concentration of NaCl, (- -) Relative enzyme activity.

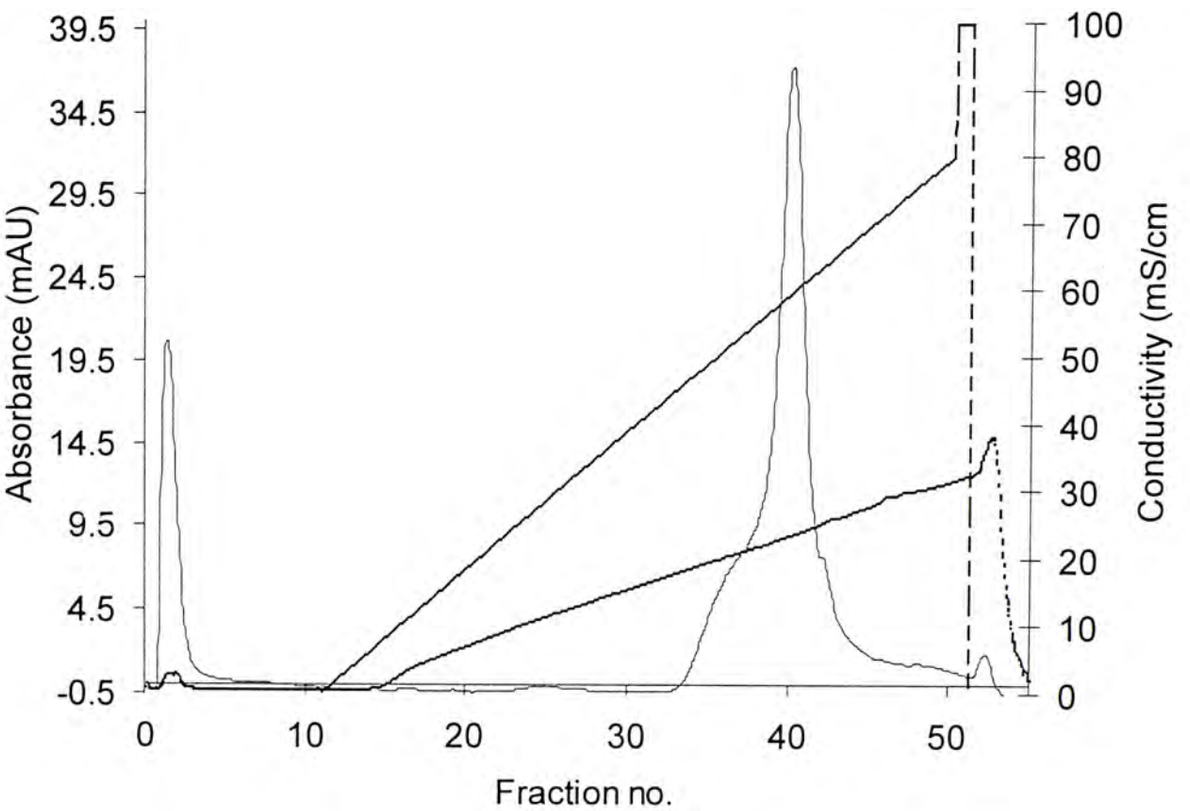

Figure 3.2 The elution profile of sample from affinity column onto the Mono S HR 5/5 column.

Legend: (-) Absorbance at 280nm, (— —) Percentage of Buffer B, (--) Conductivity (mS/cm).

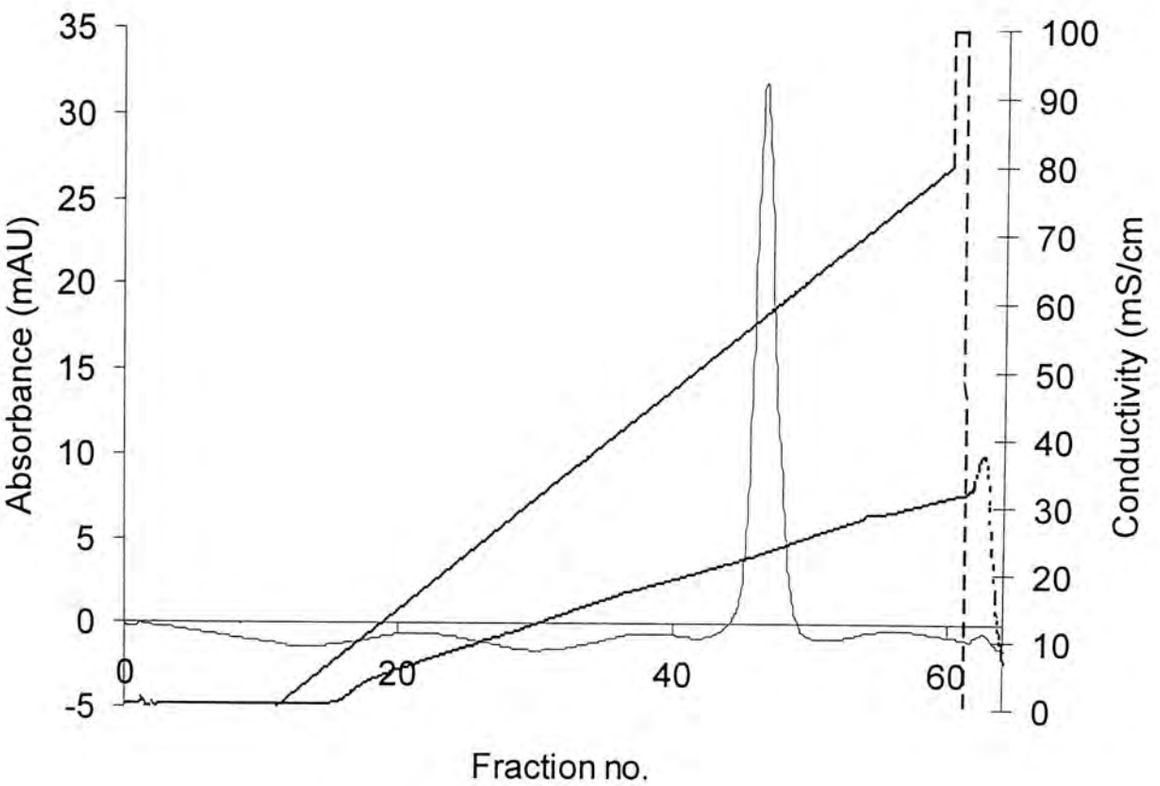

Figure 3.3 Rechromatography of semi-purified catalase onto the Mono S HR 5/5 column.

Legend: (-) Absorbance at 280nm, (— —) Percentage of Buffer B, (--) Conductivity (mS/cm).

Steps	Total Protein (mg)	Total Activity (Unit)	Specific Enzyme Activity (Unit/min/mg)	Recovery (%)	Purification (Fold)
Post-mitochondrial fraction	9785.76	536147	54.79	100.0	1.00
ETOH-$CHCl_3$ precipitation	45.13	381960	8463.38	71.2	154.47
Affinity chromatography	2.58	130528	50670.62	24.3	924.84
Mono S	1.54	75535	49112.49	14.1	896.40
Rechromatography on Mono S	0.43	24261	56030.61	4.5	1022.67

Table 3.2 Purification of catalase from bloodworms.

3.3 Determination of molecular mass

- Native molecular mass

The native molecular mass of the catalase was calculated using the calibration curve of standard proteins. The elution profile of the purified catalase in the calibrated gel filtration column was shown in figure 3.4. The molecular mass of the catalase was about 205kDa.

- Subunit molecular mass

The subunit molecular mass of the catalase was estimated using the SDS-PAGE. The fraction collected from the rechromatography was homogenous in the SDS-PAGE. The size of the band was about 53.2kDa (figure 3.5).

3.4 Determination of isoelectric point

The isoelectric point (p*I*) of catalase was determined by IEF-PAGE as described in the section 2.6. The p*I* of the catalase was estimated to be about 9.23. The calibration curve of the standard proteins and the IEF gel was shown in the figure 3.6 and 3.7, respectively.

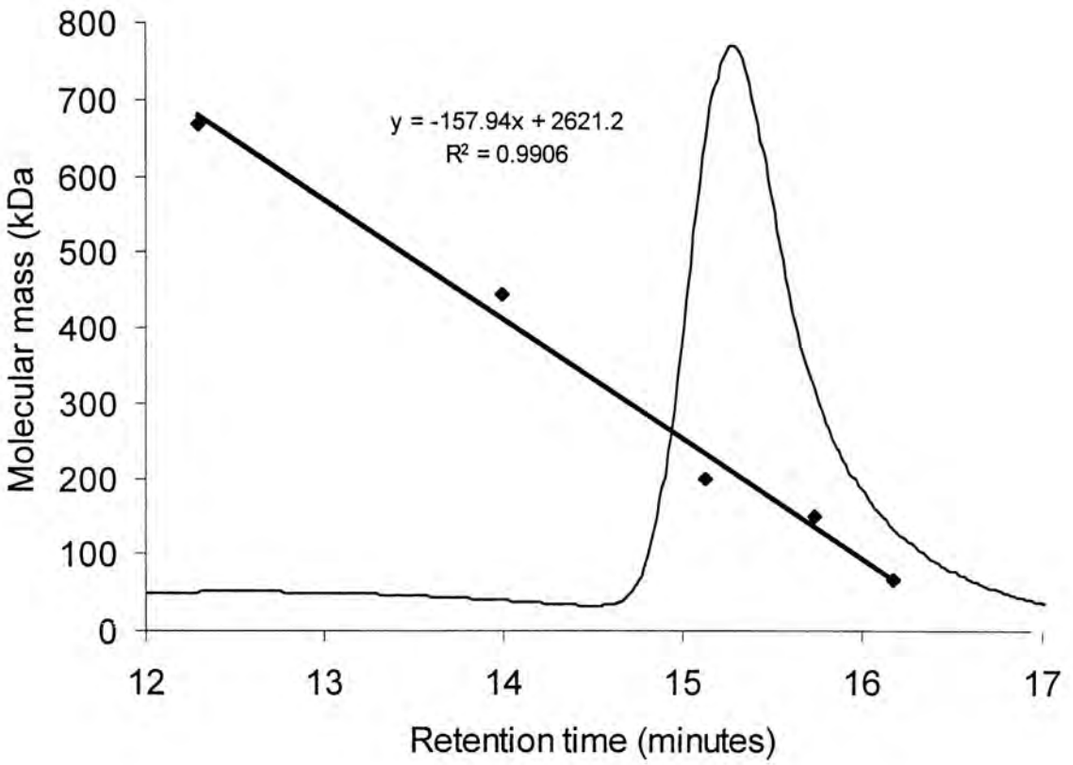

Figure 3.4 The calibration curve of the standard proteins using the Superose 6 HR 10/30 column.

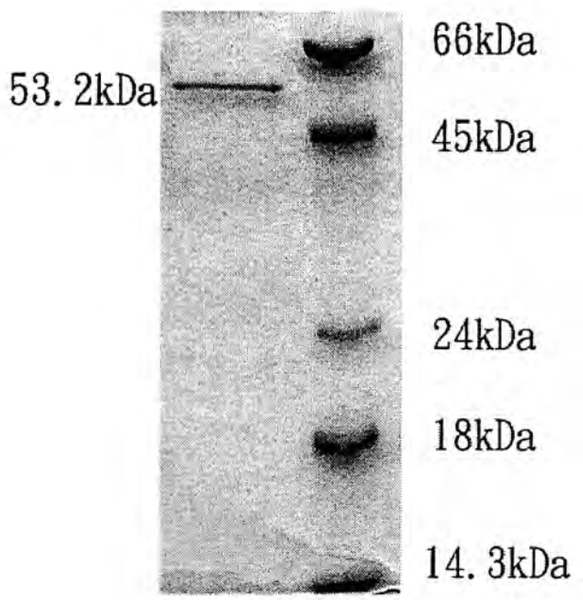

Figure 3.5 SDS-PAGE of the purified catalase.

The sample was collected from rechromatography using Mono S. It was prepared as described in the section 2.5.2. The gel was run using 15% acrylamide and stained with Coomassie Brilliant Blue after electrophoresis.

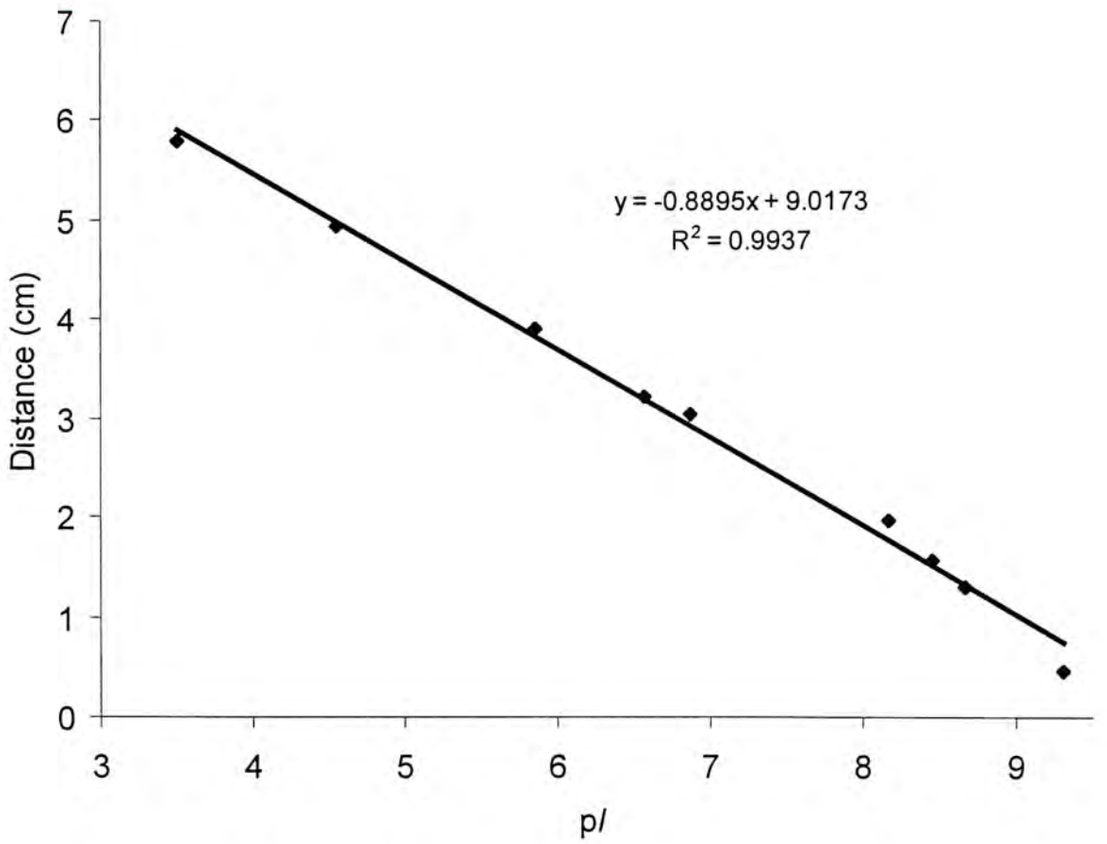

Figure 3.6 The calibration curve of standard proteins in IEF-PAGE.

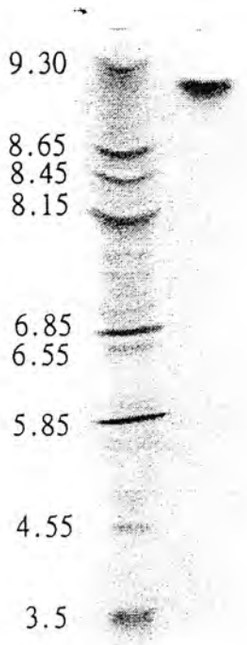

Figure 3.7 The IEF-PAGE of the purified catalase.

The protein sample was prepared as described in the section 2.6.

The gel was stained with Coomassie Brilliant Blue after electrophoresis.

3.5 Kinetic studies of the catalase

3.5.1 Optimal pH

The effect of pH on the catalase was assayed as described in the section 2.7.1. The effect of pH on the catalase was shown in the figure 3.8. The catalase activity at the optimal pH was expressed at 100%. The result showed that the enzyme was active in a broad range of pH (Hochman et al., 1987). The optimum pH of the catalase was between 7 and 8.5. The enzyme was inactive in extreme pH. The catalase activity had over 60% activity when the pH was between 5.5 and 10.

3.5.2 Thermal stability

The thermal stability of the catalase was assayed as described in the section 2.7.2. The result was shown in the figure 3.9. The catalase activity of the control was expressed to be 100%. The enzyme was quite stable at temperature up to 40^{0}C and could maintain 70% activity up to 60^{0}C. Above 60^{0}C, the enzyme activity was sharply decreased as the temperature increased.

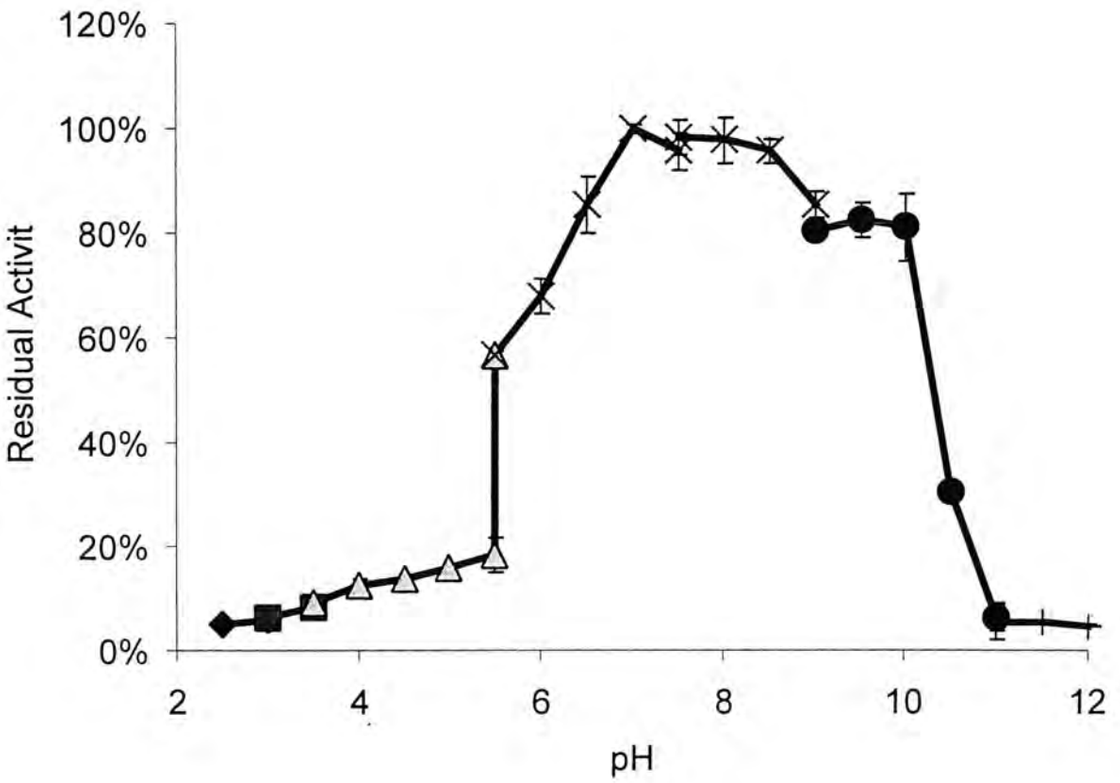

Figure 3.8 Effects of pH on catalase activity.

The activity at the optimal pH was expressed as 100%. Different buffer systems were used in this assay, (-▲-) 100mM phosphoric acid pH 2.5-3.0, (-■-) 100mM chloroacetate acid pH 3.0-3.5, (-x-)100mM acetic acid pH 3.5-5.5, (-－-) 100mM sodium monohydrogen phosphate pH 5.5-7.5, (-●-) 100mM TRIS buffer pH 7.5-9.0 and (-｜-)100mM CAPS pH 9.0-12.0.

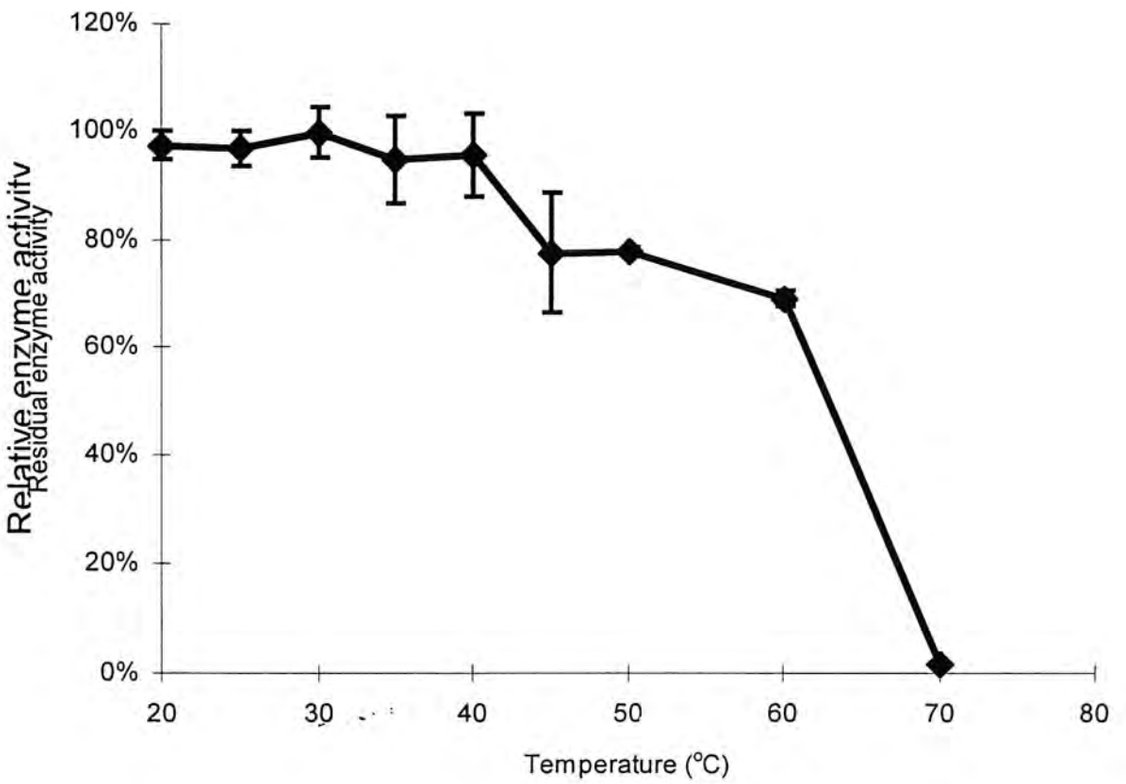

Figure 3.9 Thermal stability of the purified catalase.

3.5.3 K_m and V_{max}

K_m and V_{max} were obtained using the assay method described in the section 2.7.3. Effect of hydrogen peroxide on the catalase activity was shown in the figure 3.10. The results showed that the catalase activity increased as the concentration of hydrogen peroxide. The catalase activity would level off at higher concentration of hydrogen peroxide. Based on the result shown in the figure 3.10, the kinetic data were computed in figure 3.11 by Lineweaver-Burk plot. The value of V_{max} and K_m was calculated using the inverse of the Michaelis-Menton equation. The apparent V_{max} and K_m was about 125,000 U/mg of protein and 25mM, respectively.

3.5.4 Inhibition studies

3.5.4.1 3-amino-1,2,4-triazole

3AT was a specific inhibitor of monofunctional catalase. The effect of 3AT on the catalase was shown in the figure 3.12. The purified catalase was found to be inhibited using 3AT. The catalase activity was decreased as the concentration of 3AT was increased.

3.5.4.2 Potassium cyanide and sodium azide

Potassium cyanide and sodium azide were known inhibitors of catalase. The effect of potassium cyanide and sodium azide on the catalase activity was shown in the figure 3.13 and figure 3.14, respectively. The catalase activity was decreased

as the concentration of potassium cyanide/sodium azide was increased.

3.5.5 Catalase peroxidase activity

The purified enzyme was assayed with catalase peroxidase activity as described in the section 2.2.2. The horseradish peroxidase was used as control in this assay. The result of the catalase peroxidase activity was shown in the figure 3.15.

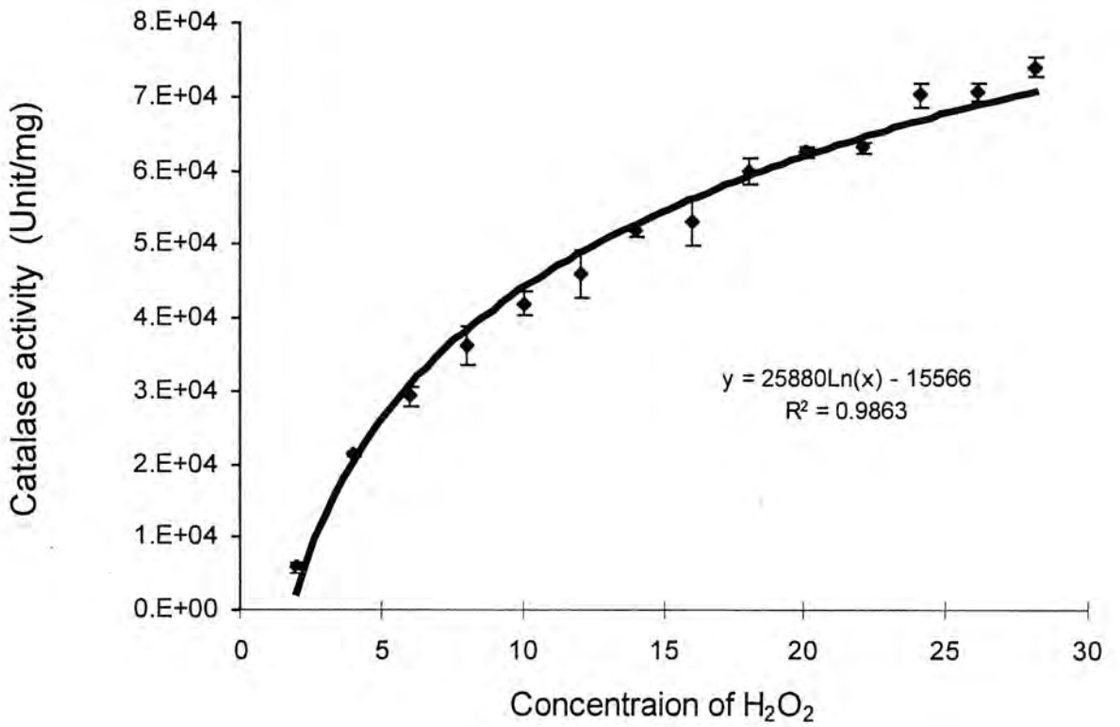

Figure 3.10 Effects of hydrogen peroxide on catalase activity.

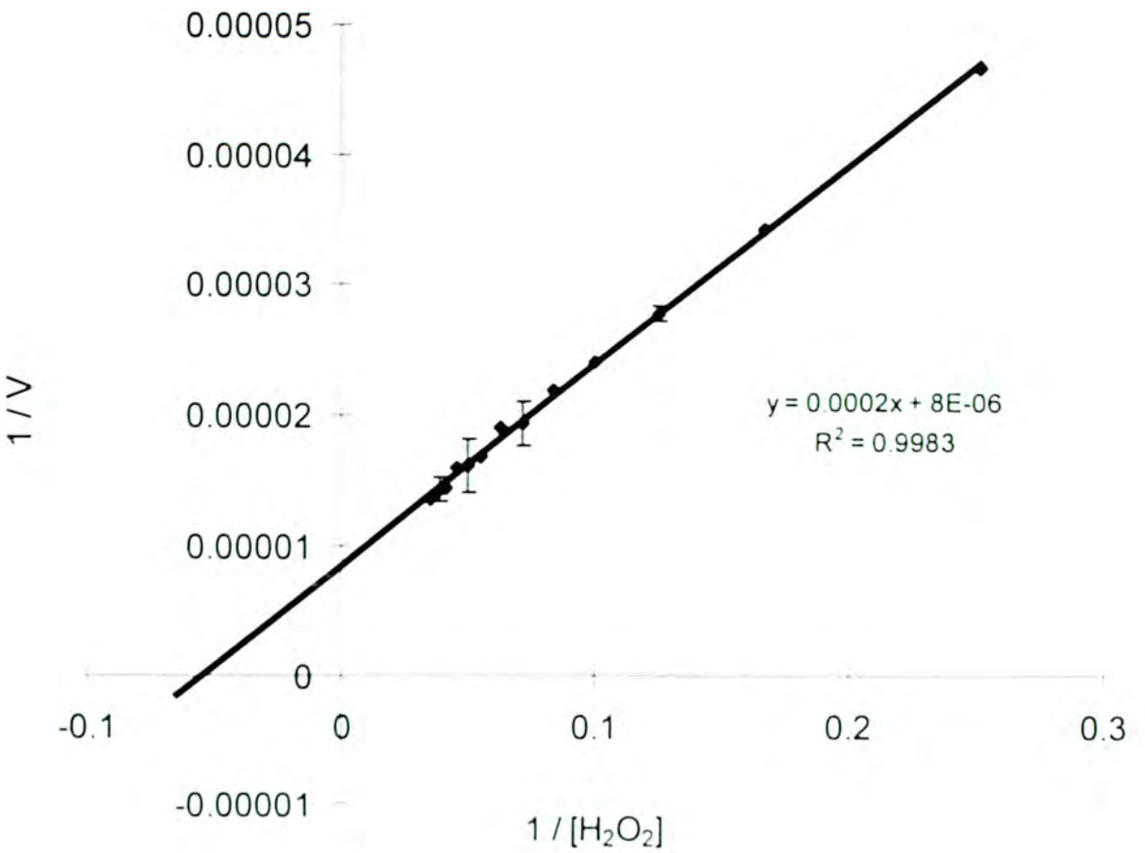

Figure 3.11 Lineweaver-Burk plot of catalase activity.

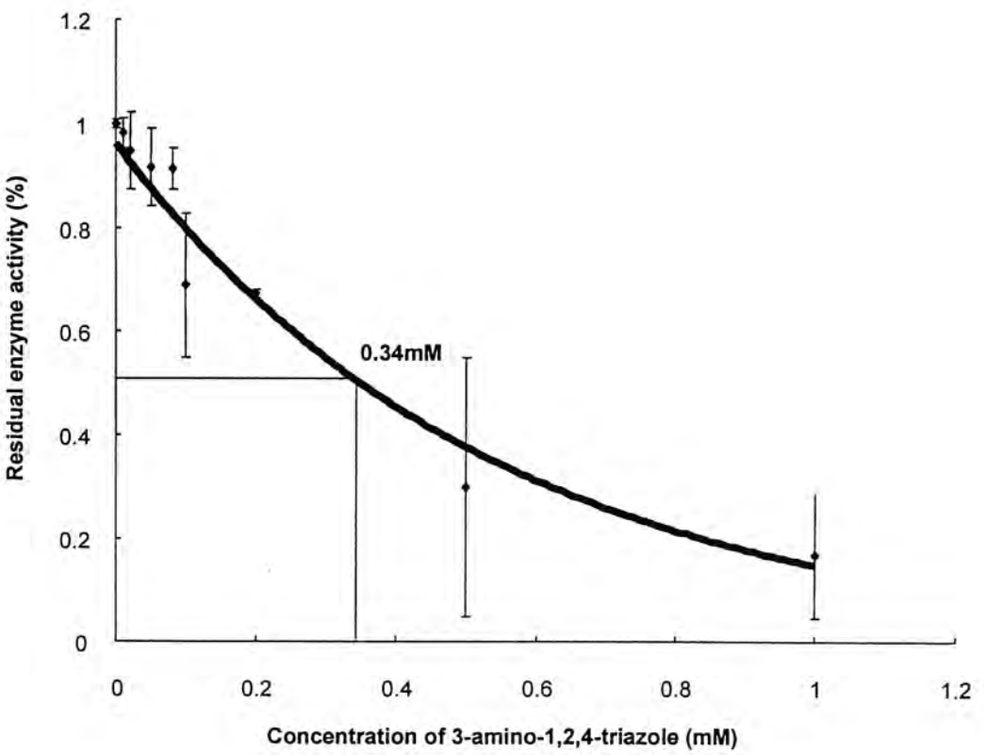

Figure 3.12 Inhibition of catalase by 3-amino-1,2,4-triazole.

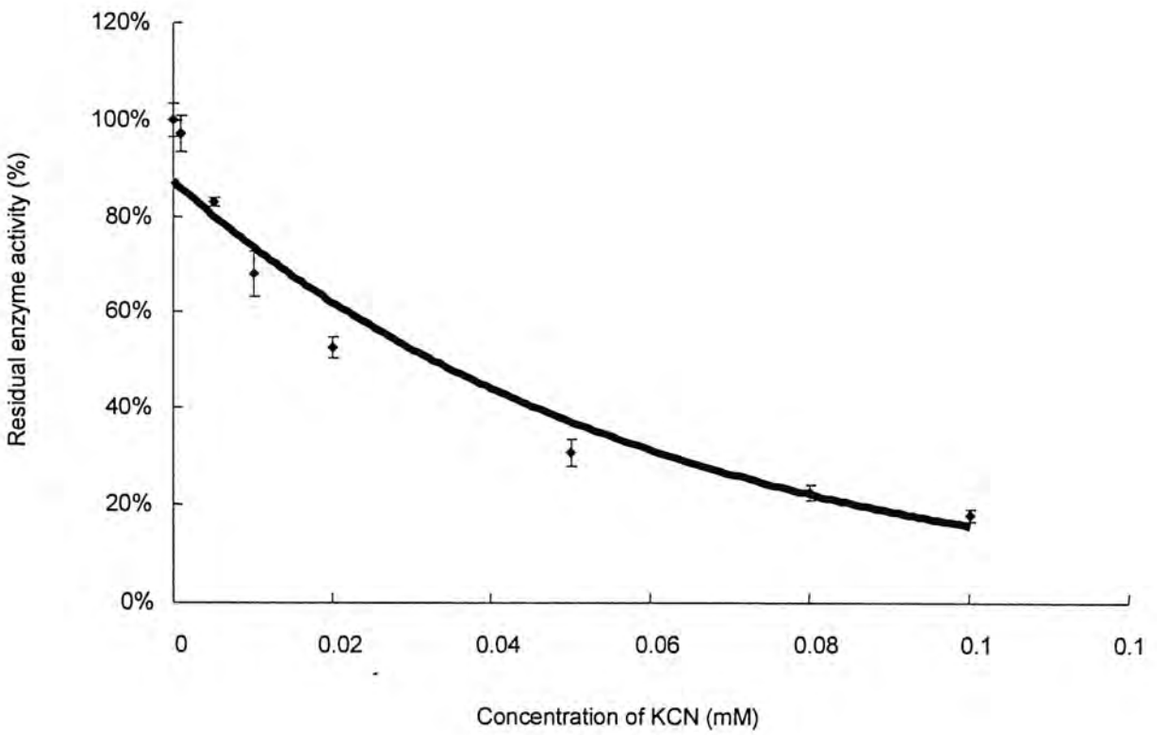

Figure 3.13 Inhibition of catalase by potassium cyanide.

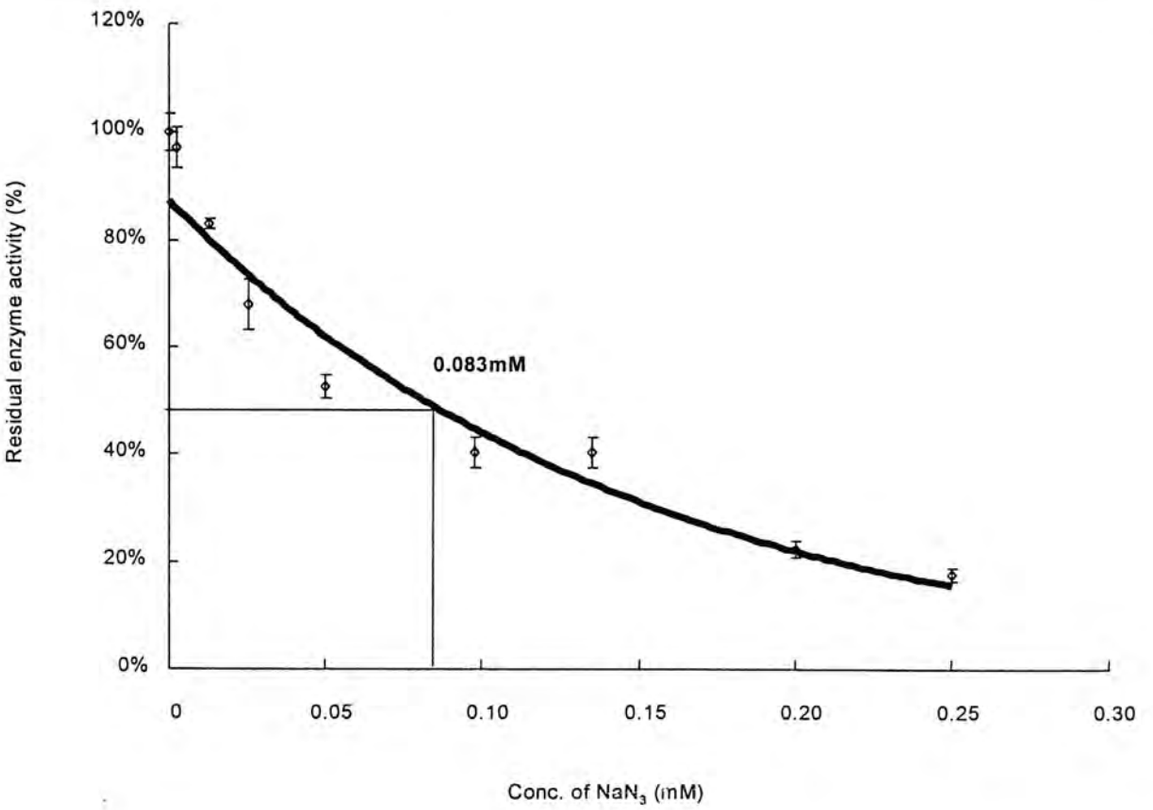

Figure 3.14 Inhibition of catalase by sodium azide.

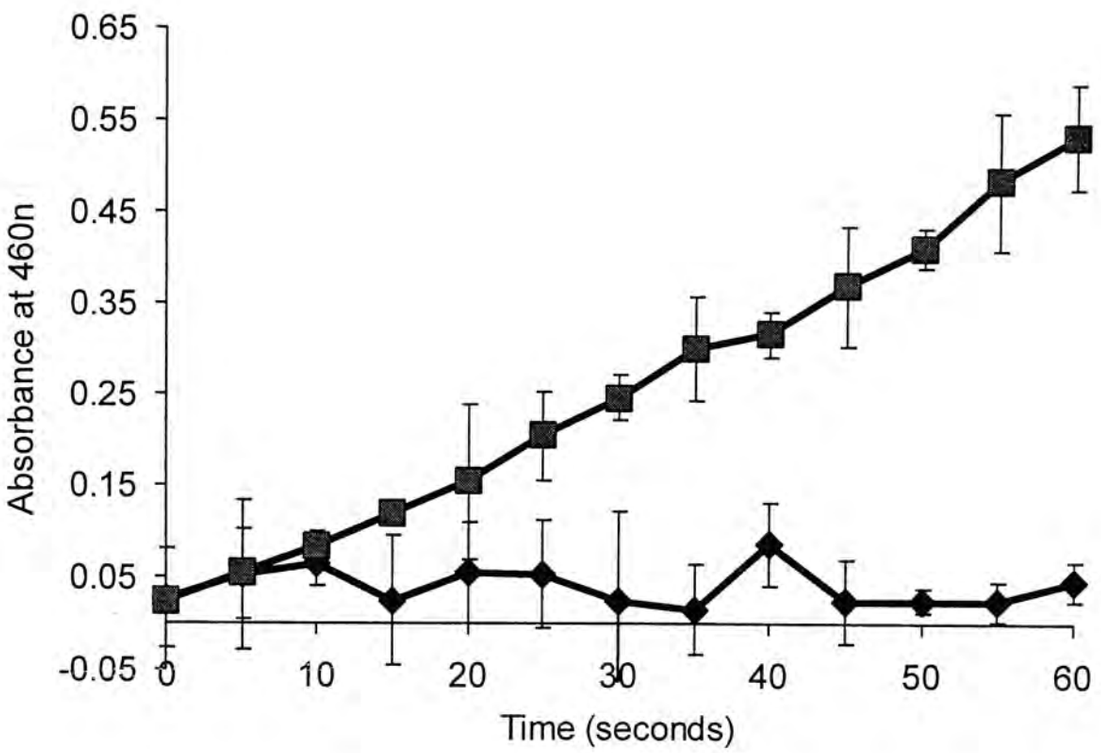

Figure 3.15 Catalase peroxidase activity of the purified enzyme. Horseradish peroxidase was used as control. Legend: (■) horseradish peroxidase, (◆) purified catalase.

3.6 Spectroscopic analysis

3.6.1 Native enzyme

The ultraviolet/visible spectrum of the native enzyme was shown in figure 3.16. Two absorption peaks were detected at about 280 and 406nm. A Soret peak at about 406nm showed a typical feature of ferric heme proteins (Hochman et al., 1987). The ratio of A_{406}/A_{280} was about 0.97. It indicated that the heme of the catalase did not dissociate during the purification process.

3.6.2 Denatured enzyme

3.6.2.1 Potassium cyanide

The spectroscopic effect of potassium cyanide on catalase was shown in figure 3.17. The Soret band at 406nm was shifted to 425nm upon addition of potassium cyanide. Similar shifts had also been described for other catalases (Hochman et al., 1987; Goldberg et al., 1989; Brown-Peterson et al., 1995).

3.6.2.2 Sodium azide

Similarly, the Soret band at 406nm was shifted to 415nm upon the addition of sodium azide and the result was shown in figure 3.18.

3.6.3 Pyridine hemochrome characterization

The absorption spectrum of pyridine hemochrome was shown in figure 3.19. The absorption peak at 556nm was a characteristic of pyridine hemochrome b

(protoheme IX) (Berry et al., 1987). The number of mole of heme per protein molecule was about 0.97.

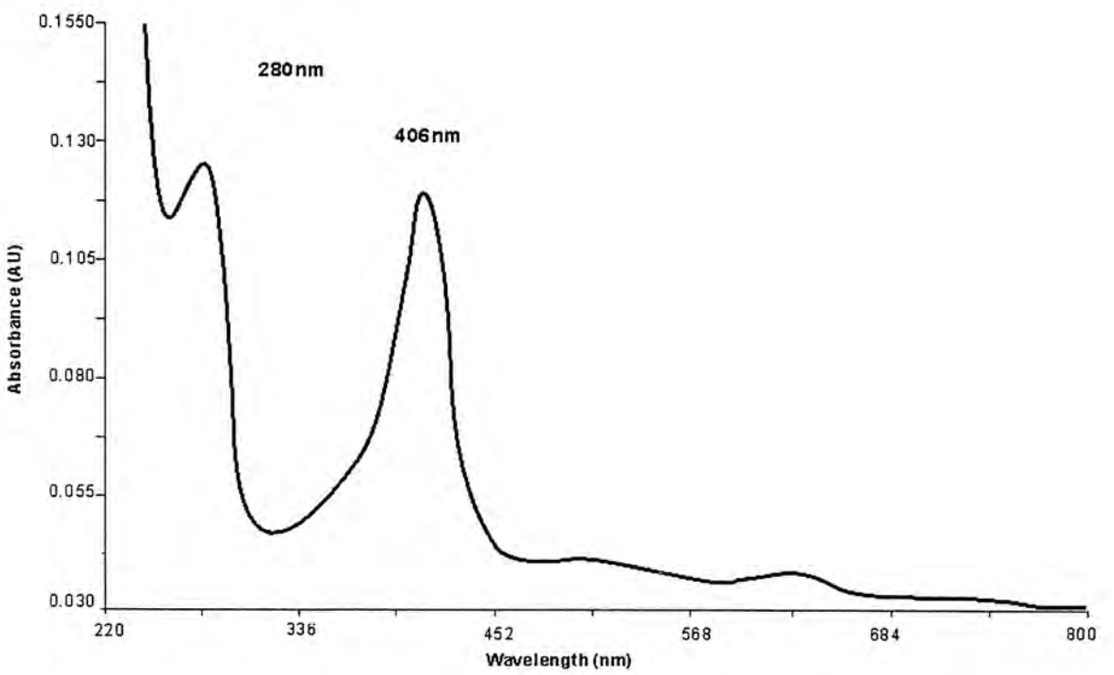

Figure 3.16 The ultraviolet/visible spectrum of the catalase.

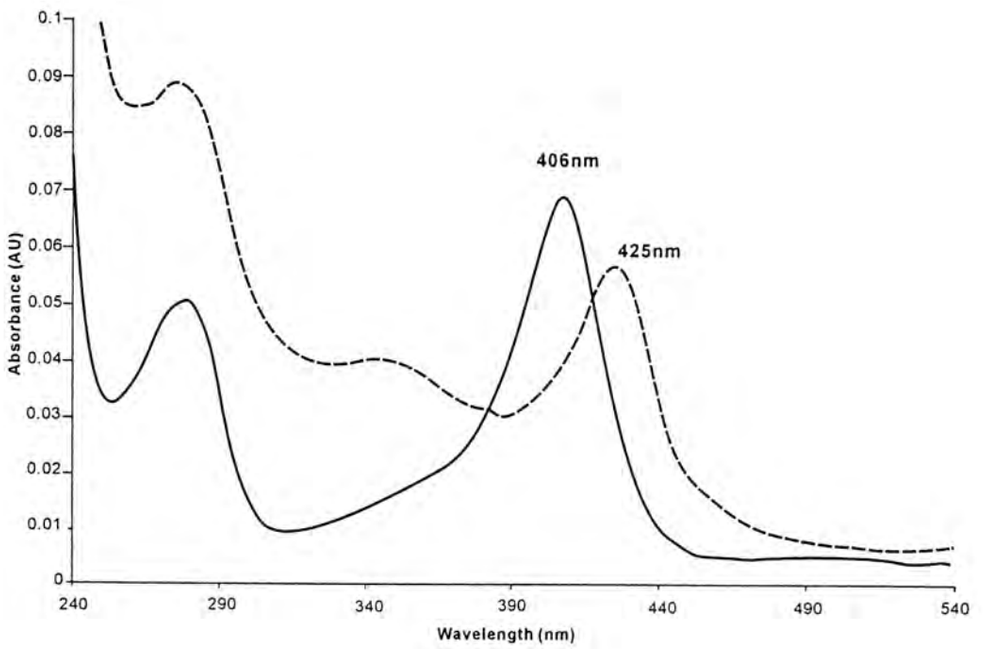

Figure 3.17 The spectroscopic effect of potassium cyanide on catalase.

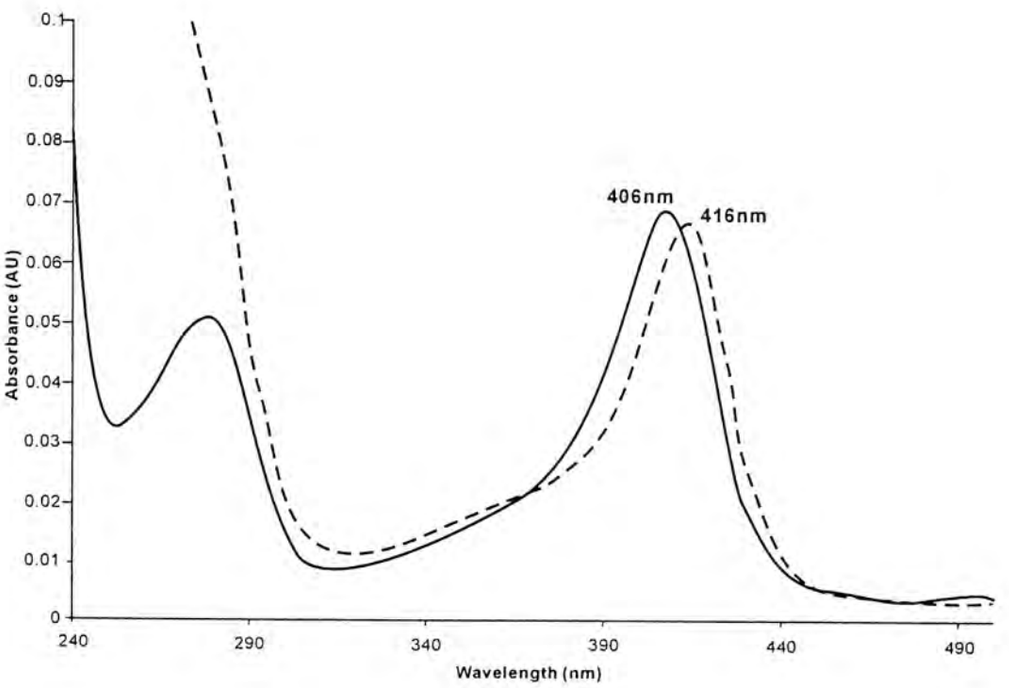

Figure 3.18 The spectroscopic effect of sodium azide on catalase.

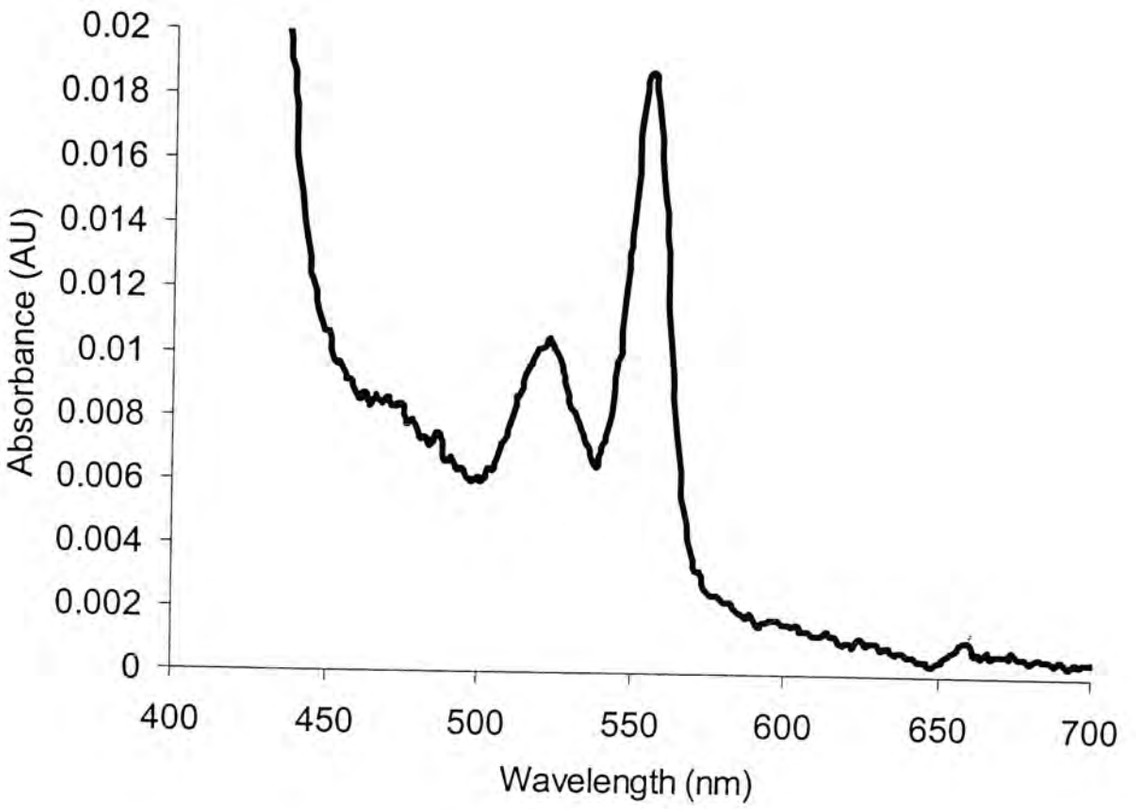

Figure 3.19 The ultraviolet/visible spectrum of pyridine hemohrome.

3.7 N-terminal amino acid sequence analysis

The N-terminal sequence of the catalase was found to be blocked in the preliminarily trial. Deblocking of the protein bound on PVDF membrane was then carried out as described in the section 2.9.4. Due to the low sequencing yield obtained, only 10 cycles could be run after deblocking. The sequence obtained was then matched with other known sequences in the SWISS-PROT sequences database (Altschul et al., 1997) and Brookhaven Protein Data Bank (Berman et al., 2000) using the BLAST® (Bairoch et al., 2000). The sequence of the purified catalase was found to match with other known catalases. The searched results were shown in table 3.3.

Sequence segment		Source	Classification	Identity (%)	Positives (%)
1	G R K F A V F F Y T 10	Chironomid larvae	Insecta		
147	R G F A V K F Y T 155	*Pseudomonas putida*	Bacteria	77	77
145	R G F A V K F Y T 153	*Pseudomonas aeruginosa*	Bacteria	77	77
128	R G F A V K F Y T 136	*Bacillus halodurans*	Bacteria	77	77
139	R G F A V K F Y T 147	*Oyster mushroom.*	Eukaryota	77	77
144	R G F A V K F Y T 152	*Xanthomonas oryzae*	Bacteria	77	77
120	R G F A V K F Y T 128	*Oryza sativa*	Eukaryota	77	77
120	R G F A V K F Y T 128	Castor bean	Eukaryota	77	77
120	R G F A V K F Y T 128	Bread wheat	Eukaryota	77	77
120	R G F A V K F Y T 128	Para rubber tree	Eukaryota	77	77
129	R G F A V K F Y T 137	*Bos taurus*	Bos taurus	77	77
130	R G F A V K F Y T 138	Human erythrocyte	Eukaryota	77	77
109	R F F A L K F Y T 117	*Proteus mirabilis*	Eubacteria	66	77

Table 3.3 Results of BLAST® search using SWISS-PROT sequences database and Brookhaven Protein Data Bank.

CHAPTER 4
DISCUSSION

4.1 Subcellular locations of catalase in bloodworms

Catalase is normally located in peroxisomes where many hydrogen peroxide producing enzymes are present (Van Den et al., 1992). In this project, catalase in *chironomid* larvae is found mainly in the soluble cytosolic and microsomal fractions. However, the amount of catalase in post-mitochondrial fractions is much higher than that of mitochondrial fractions. About ninety-five percent of catalase is found in post-mitochondrial fractions.

With reference to literatures, insect catalase is unusual in that the activity is not restricted to the microsomal fraction that contains the peroxisomes, but is also present in the cytosol and mitochondria (Ahmad et al., 1988b). The wide intracellular distribution of catalase in insects is an apparent evolutionary adaptation to the absence of selenium-dependent glutathione peroxidase (Ahmad et al., 1988a). Similarly, catalase in guinea pig is present predominantly in the soluble cytosolic fraction of liver (Himeno et al., 1993).

4.2 Purification of post-mitochondrial catalase

In this project, catalase purified from bloodworm has not been reported before. Several purification steps, such as differential centrifugation, ethanol-chloroform precipitation, affinity chromatography on blue sepharose CL-6B and cation exchange chromatography on Mono S were carried out to isolate the catalase for characterization.

The whole red bloodworm was used instead of parts of the body or certain organelles in purification of catalase, because there are some advantages as follows:

- The size of red bloodworms is very difficult for dissection and subsequent tissue processing.
- The location of antioxidant enzymes is unknown and the enzymes may not concentrate in specialized organelles.
- The contamination problems of gut symbiont of red bloodworms can be eliminated during the purification procedures.

Hence, catalase is purified using the whole bloodworms.

In preparing post-mitochondrial fractions, the supernatant was required for filtering through glass wool and cheesecloth. This filtering step was used to remove fats. Bloodworms usually contained about 14% crude fat (Wissing et al., 1971; Driver et al., 1974). The removal of crude fat was helpful in preventing column from blocking in the subsequent purification steps. The ethanol-chloroform precipitation could remove undesirable proteins. The amount of protein was decreased from 9.79g to 45mg in a purification step. The stability of the enzyme in ethanol and chloroform was a characteristic of monofunctional catalase (Nadler et al., 1986; Terzenbach et al., 1998). In affinity chromatography on blue sepharose CL-6B, the enzyme was found to be bound tightly on the column. The binding of the enzyme to the affinity column suggests that there could be a NAD^+ binding site on the surface of the enzyme.

Similarly, catalases of *Proteus mirabilis* (Gouet et al., 1995), bovine liver (Kirkman et al., 1984) and human erythrocyte (Kirkman et al., 1987) were also found having affinity for NAD^+. Using the cation exchange chromatography on Mono S, a major peak was detected in the elution profile that was shown in the figure 3.2. However, contaminants were detected using SDS-PAGE. Fractions at the peak apex were collected for rechromatography in order to obtain a purified catalase. The characteristic of the purified catalase was investigated by SDS-PAGE, IEF-PAGE and different enzymatic assays.

Finally, the N-terminal sequence of catalase was determined by Edman degradation. The result indicated that the purified catalase showed similar N-terminal sequence with other catalases in SWISS-PROT sequences database (Altschul et al., 1997) and Brookhaven Protein Data Bank (Berman et al., 2000).

4.3 Physical properties of the purified enzyme

4.3.1 Native and subunit molecular mass

As determined from gel filtration chromatography and SDS-PAGE, the native and subunit molecular mass was about 205kDa and 53.2kDa, respectively. The ratio of native molecular mass to subunit molecular mass was approximately equal to 4:1. Since the purified catalase showed a homogenous band in the SDS-PAGE, the native catalase was probably a homotetramer. Monofunctional catalases usually exist as homotetramers with molecular mass ranging from 200 to

350kDa (Deisseroth et al., 1970).

4.3.2 Isoelectric point

The p*I* of the purified catalase was estimated about 9.23 using IEF-PAGE. It was slightly higher than the normal range of heme-containing catalase. The normal range of p*I* of heme-containing catalase was from 5.5 to 8.3 (Deisseroth et al., 1970).

4.4 Kinetic properties of the purified enzyme

4.4.1 Optimal pH

Change of the enzyme activity with pH was studied in a pH range of 2.5 to 12.0 (figure 3.8). The purified catalase was active in a broad pH range (pH 5.5 to 10.0). The optimal pH range was observed from pH 7 to 8.5. In comparison with other catalses (table 4.1), they are active in a broad range of pH. The broad optimal pH range was a characteristic of monofunctional catalase (Schonbaum et al., 1976).

4.4.2 Thermal stability

The purified catalase and other catalases were quite stable at temperature up to 40^{0}C. They could maintain 70% activity up to 50^{0}C. Above 50^{0}C, the catalase activities were sharply decreased as the temperature increased. In comparison with other catalases (*V. rumoiensis* S-1^T, *M. luteus* and bovine liver), catalase of bloodworms was found having a greater thermal stability (Yumoto et al., 2000). The thermal stability of catalases was shown in figure 4.1.

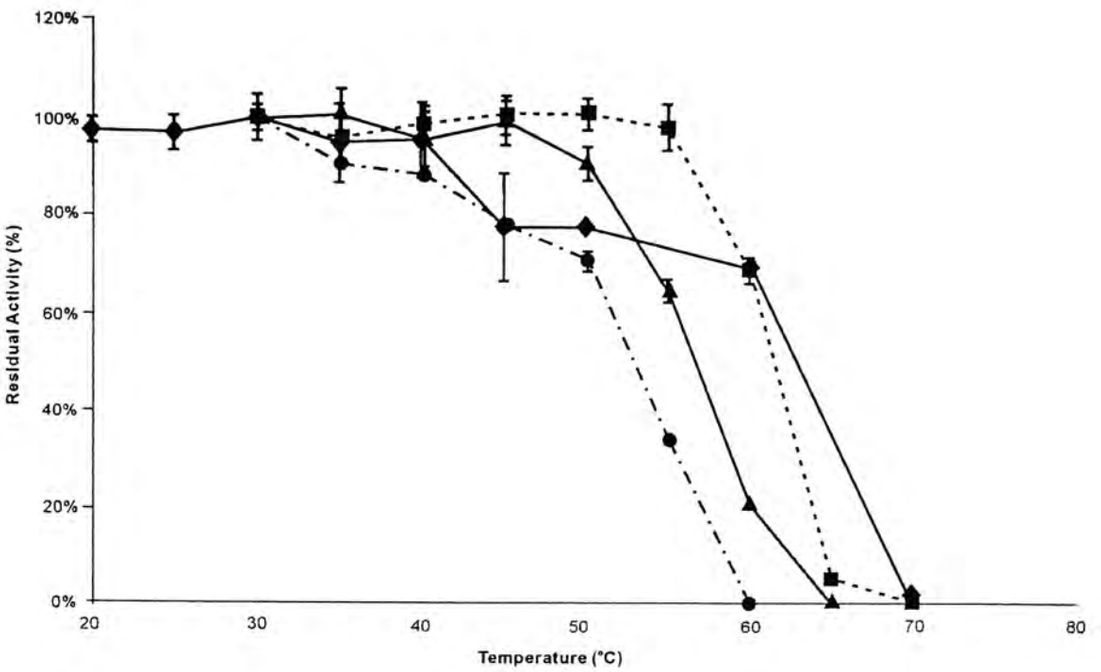

Figure 4.1 Thermal stability of catalases.

Legend: catalase from (●) *V. rumoiensis* S-1^T, (■) *M. luteus*, (▲) bovine liver, (◆) *Chironomid* larvae.

4.4.3 K_m and V_{max}

The apparent V_{max} and K_m of the purified catalase was determined to be 125,000U/mg of protein and 25mM, respectively. The specific activity was similar to the other monofunctional catalases as shown in table 4.1. The specific activities of the monofunctional catalases were usually higher than that of the catalase peroxidase (table 4.1).

4.4.4 Inhibition studies

The effect of 3AT, KCN and NaN_3 on the catalase activity was studied as described in the section 2.7.4. The catalase activity was decreased when the concentration of inhibitors was increased. 3AT was a specific inhibitor of monofunctional catalase. KCN and NaN_3 were common inhibitors of catalases. The order of inhibitory effect was KCN > NaN_3 > 3AT. On the other hand, the inhibitory effect of 3AT on the purified enzyme showed that the purified enzyme had a characteristic of monofunctional catalase. Similarly, 3AT can also inhibit other monofunctional catalases (table 4.1).

4.4.5 Catalase peroxidase activity

The purified catalase was assayed for catalase peroxidase as described in the section 2.2.2. No catalase peroxidase activity was detected in the purified enzyme (figure. 3.15). It indicated that the purified enzyme did not belong to the group of catalase peroxidase. Similarly, other monofunctional catalases were found

not to have catalase peroxidase activity (table 4.1).

4.5 Spectroscopic analysis

4.5.1 Native and denatured enzyme

Spectroscopic analysis of native enzyme and denatured enzyme was studied as described in the section 2.8. The ultraviolet/visible spectrum of the purified enzyme indicated the presence of a heme prosthetic group (figure 3.15). The native enzyme had a Soret peak at 406nm. It was reported that the position of the Soret peak varied among catalases (table 4.1). The Soret peak of the purified catalase was shifted to 425nm upon addition of potassium cyanide (figure 3.16). These are characteristic properties of monofunctional catalase (Brown-Peterson et al., 1995; Terzenbach et al., 1998). Similarly, the Soret peak was shifted to 415nm upon addition of NaN_3 (figure 3.17). The A_{406}/A_{280} ratio of 0.97 was common for typical catalases. It indicated that the heme of the enzyme did not dissociate at all during the purification process (Shima et al., 1999).

4.5.2 Pyridine hemochrome characterization

Upon incubation of the purified enzyme with NaOH and pyridine, a typical pyridine hemochrome spectrum that could indicate the presence of protoheme IX was recorded (figure 3.18). The spectrum was not reducible by dithionite. The calculated value of 0.93mol of protoheme IX was present per native enzyme. These were the characteristic properties of monofunctional catalases (Williams et al., 1964).

However, a partial loss of heme during purification process was also recorded (Hochman et al., 1991; Nadler et al., 1986; Yumoto et al., 2000) and the ratio of heme to enzyme molecule of some catalases was shown in table 4.1.

4.6 N-terminal amino acid analysis

In the preliminary trial of amino acid sequencing, the sample was found to be N-terminal blocked as no signal was detected. As a result, N-terminal deblocking of the sample was carried out. There were several kinds of N-terminal modifications of amino acids such as, acetylation, formylation, methylation and pyroglutamic acid. Using the deblocking method described in the section 2.9.4, the N-terminal amino acid sequence of the purified enzyme was determined. The results also indicated that the N-terminal amino acid of the purified enzyme was acetylated.

Based on the result of BLAST® search, the sequence of the purified enzyme was matched with other catalases (table 3.3). Similarly, the matched catalases in BLASR® search and the purified enzyme in this project were also heme-containing enzymes and existed as homotetramers. The sequence identity and positive of the purified enzyme towards the matched catalases was greater then 66% and equal to 77%, respectively. Hence, it was believed that the purified enzyme belonged to the group of catalases.

4.7 Conclusions

In this project, catalase in post-mitochondrial fraction of bloodworms has

been purified and characterized. The catalase of *Chironomidae* larvae has not been reported before. No isoform of the catalase was found. However, the presence of isoforms was recorded in plants only. The reduction of oxidative stresses by plants is different from that of other eukaryotes.

The catalase of bloodworm was purified using a specific and relatively efficient method by ethanol-chloroform precipitation, affinity chromatography and cation exchange chromatography. The purification process is different from the existing methods. The affinity chromatography employed in this project is seldom used in purification of the known catalase. It is because the occurrence of NADPH-binding site on the enzyme surface is uncommon. Up to now, the number of catalase found to have NADPH-binding site is limited to a few. Also, the commonly used hydrophobic interaction chromatography (HIC) is not employed in this project because the purified enzyme was found having an unusually strong interaction with the functional groups (e.g. phenyl group & octyl group). In the preliminary trials, the yield of the purified enzyme using HIC was very low and the results of the HIC were inconsistent with different batches of samples. Hence, the HIC was not employed in this project.

In term of the physical properties, the purified enzyme probably exists as a homotetramer. It is because the size of the enzyme was estimated to be about 205kDa and 53.2kDa in gel filtration chromatography and SDS-PAGE, respectively.

The size of purified catalase was relatively small in comparing with other catalases because the size of catalases was found ranging from 200 to 350kDa (Deisseroth et al., 1970). The result of amino acid sequencing indicates a positive relationship between the purified enzyme and the other known catalases. The relative percentage identity and positive of the purified enzyme to the other known catalases is low. It is because sequences of other insect catalases have not been published in the NCBI database before and the purified insect catalase can only match with catalases from other organisms, such as bacteria, plants and mammals.

Besides, the purified enzyme was characterized by standard assays of catalase. The results of the kinetic studies were similar to those of the other known catalases. The study of the characteristics of the purified catalase suggest it is a monofunctional catalases. For example, it was inhibited by 3-amino-1,2,4-triazole and it possessed a heme prosthetic group that was not reducible by dithionite. According to the experimental results, the purified insect catalase is classified as monofunctional (HPII) catalase and exists as a homotetramer.

Characteristic	*Chironomid* larvae catalase[a]	Eukaryotic catalase[b]	*V. numoiensis S-1*[T] catalase[c]	*R. capsulatus* catalase peroxidase[d]
Molecular mass	205000	240000	230000	236000
No. of subunits	4	4	4	4
Heme/tetramer	4	4	2.9	2.5-4
Soret peak (nm)	406	405	406	403
Specific activity (U/mg of protein)	125000	97000	396900	7800
CN^- and N_3^- inhibition	+	+	+	+
Aminotriazole inhibition	+	+	+	-
Optimum pH range	broad	broad	Broad	narrow
Peroxidative activity	-	-	-	+

Table 4.1 Comparisons of different catalases.

[a] Data are from this study. [b] Data are from references (Deisseroth A et al. 1970; Nadler et al. 1986; Schonbaum et al. 1976). [c] Data are from reference (Yumoto et al., 2000). [d] Data are from references (Hochman et al., 1991; Nadler et al., 1986).

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