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# Purification and Characterization of Catalase From Enterococcus Faecalis

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# THESIS APPROVAL

The abstract and thesis of Zhenghong Zhu for the Master of Science in Biology were presented 12 February, 1997, and accepted by the thesis committee and the department.

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# ABSTRACT

An abstract of the thesis of Zhenghong Zhu for the Master of Science in Biology presented 12 February 1997.

Title: Purification and Characterization of Catalase from *Enterococcus faecalis*

Catalase is an important biological enzyme that most organisms employ during aerobic growth to catalyze the decomposition of hydrogen peroxide, a toxic reduction product of oxygen, into water and molecular oxygen. Traditionally, the bacterium *Enterococcus faecalis* is considered to have no cytochrome system and to be catalase negative. However, subsequent studies have shown that certain strains of *E. faecalis* are capable of producing catalase when grown aerobically in a medium containing hematin.

The goals of this study were to purify and characterize catalase from a clinical strain of *E. faecalis*. The organisms were grown under aerobic conditions in a hematin supplemented medium. The catalase

of *E. faecalis* was purified to homogeneity with procedure that yielded a final 120-fold purification and a recovery of 17% of the original activity. The apparent native molecular weight was found to be 250,000±10,000 daltons, and only one subunit type with a molecular weight of 64,000±2000 daltons was present. The purified catalase shows pH optima from 6.0 to 7.8. The optical spectrum exhibits an absorption peak at 406*nm*. The enzyme is sensitive to the heme poison azide and contains iron, a typical property of the heme catalase. The purified catalase from *E. faecalis* characterized in this study is a typical heme-iron catalase, rather than an atypical nonheme pseudocatalase which contains manganese. Thus, the catalase of *E. faecalis* is similar to most of other typical catalase enzymes in many important characteristics. This study of catalase from *E. faecalis* may provide knowledge necessary to investigate the mapping and cloning of the gene encoding the catalase enzyme.

# PURIFICATION AND CHARACTERIZATION OF CATALASE FROM *ENTEROCOCCUS FAECALIS*

by

ZHENGHONG ZHU

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# Chapter 1

# Introduction

Catalase is an important enzyme that catalyzes the decomposition of toxic hydrogen peroxide into water and molecular oxygen. It is ubiquitously present in nature, having been found in animals, plants and microorganisms. Catalase activity from various living species has been thoroughly studied because most organisms use catalase to eliminate hydrogen peroxide formed during aerobic growth. The enzymes from mammalian tissues (1-4) were purified for most studies of catalase, and the detailed structure of beef liver catalase has been reported (5-6). Reports of catalase activity from eucaryotic sources (7-10) and procaryotic sources (11-17) have indicated that these enzymes appear to be more or less alike in their basic structure and catalytic properties.

Biochemical and genetic studies have revealed that some organisms such as *Escherichia coli* (11-12), *Bacillus subtilis* (13-14) and *Saccharomyces cerevisiae* (7-8) can synthesize more than one catalase enzyme, whereas others such as *Proteus mirabilis* (16-17) and *Neurospora crassa* (10) synthesize just one catalase enzyme. Most catalase

enzymes closely resemble the catalase enzyme from bovine liver. They are hemoproteins with four identical subunits; each contains one heme prosthetic group, and all have a combined molecular weight in the range of 240,000 daltons. The enzymes show a broad range of pH optima, and are sensitive to both azide and cyanide. The optical spectrum exhibits an absorption band at 406*nm*.

However, some catalase enzymes with different properties are found. For example, catalase HP-1 of *E. coli* has a molecular weight of 337,000 daltons; it has four larger subunits and only two heme prosthetic groups(11). Catalase HP-2 of *E. coli* and the catalase of *N. crassa* are hexamers, have larger subunits than bovine liver catalase, and have a heme-d-like component (10,12). The catalase enzyme from *Aspergillus niger* has a higher molecular weight and, unusually, lacks sensitivity to cyanide (9). Despite the fact that some catalase enzymes display different structural properties, all catalase enzymes have the same main physicochemical properties.

In recent years, catalase enzymes from various bacteria have been isolated, purified, and characterized. As early as 1948, Herbert and Pinsent (15) isolated the catalase enzyme from *Micrococcus lysodeikticus,* and it is the best-known representative of the typical procaryotic catalase enzymes. In contrast, catalase enzyme from

*Enterococcus faecalis* has not been purified and characterized.

*E. faecalis* is one of the lactic acid bacteria which is a facultative anaerobe. Traditionally, the lactic acid bacteria are considered to contain no cytochrome system and to be catalase negative because they cannot synthesize iron-porphyrins. They are also thought to be incapable of respiration since no functional transport phosphorylation can be formed. Thus, these organisms are characterized as bacteria that can carry out only fermentation as a means of energy generation (either heterofermenters or homofermenters) even when grown aerobically.

However, recent studies demonstrated that certain strains of *E. faecalis* could synthesize a functional, membrane-bound cytochrome electron transport system when grown aerobically if the medium contained hematin (18-21). Oxidation of NADH by electron transport to oxygen is catalyzed by heme carriers and may be chemiosmotically coupled to phosphorylation by the proton ATP-ase. These bacteria cannot synthesize heme but have the genetic determinants to synthesize a membrane-bound cytochrome electron transport chain under appropriate conditions when hematin is added to the growth medium.

Hydrogen peroxide is formed biochemically during the respiratory process by a two-electron reduction of oxygen.

This process is generally mediated by flavoproteins. When hydrogen peroxide is produced in any cell system, it must be eliminated for bacterial growth to occur. The catalytic decomposition of the peroxide in aerobic bacteria is done by catalase ($H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$) or NADH peroxidase ($NADH + H_2O_2 + H^+ \rightarrow 2H_2O + NAD^+$). Aerotolerant anaerobic bacteria produce only peroxidase to decompose hydrogen peroxide. Obligate anaerobic bacteria do not produce catalase or peroxidase. If they are exposed to oxygen, it is thought that the toxic oxygen forms destroy the cell. Hence, the presence of catalase or peroxidase is essential to all bacteria growing under aerobic conditions since their enzymes will allow the cells to eliminate potentially damaging products of oxygen. Most lactic acid bacteria are classified as aerotolerant anaerobes which are not sensitive to oxygen, can grow in its presence as well as in its absence, and are considered to possess only peroxidase activity.

Further studies of lactic acid bacteria, however, provided evidence to show that some are capable of producing catalase. A review of the literature on hydrogen peroxide decomposing activity of lactic acid bacteria shows that there are many reports of proteins in lactic acid bacteria that have catalase-like activity (22-32).

An early study by Feton et al. in 1953 (22) reported that some strains of pediococci showed catalase activity. Other investigators (23-26) also observed catalase-like activity in certain lactic acid bacteria grown under specific conditions. Whittenbury (27-28) examined certain lactic acid bacteria for catalase-like activity when the bacteria were grown on heated-blood agar and on basal medium. He reported hydrogen peroxide formation and the presence of two different kinds of catalase activity in some lactic acid bacteria. Some lactobacilli, pediococci, and leuconostocs possessed a vigorous hydrogen peroxide destroying system that was insensitive to acid, but which was formed only when an external hematin source was added. This catalase activity was inhibited by azide and cyanide. Other strains of leuconostocs and pediococci, possessed a much weaker catalase activity that was acid-sensitive and had no heme content. This catalase activity (named pseudocatalase activity) was insensitive to azide and cyanide. The two types of catalase activity were very different in sensitivity to pH, azide and cyanide (heme poisons) and the requirment for hematin in the growth medium. Hence, there are two kinds of catalase which can be isolated from various genera of lactic acid bacteria, one being the typical heme catalase enzyme and the other being an atypical nonheme catalase enzyme.

Whittenbury (28) also found correlation between hydrogen peroxide production and the ability of lactic acid bacteria destroy it. Catalase production, when cells were grown under aerobic conditions, was clearly beneficial to bacteria, and these bacteria showed a diminished hydrogen peroxide-splitting activity when grown anaerobically.

In 1965, Johnston and Delwiche (29) showed that certain strains of lactobacilli and pediococci were capable of incorporating the heme component from blood or purified hemin to produce azide- and cyanide-sensitive catalase activity in crude extracts. In addition, these bacteria in the same culture medium also produced another type of catalase activity which was cyanide- and azide-insensitive. He found that other strains of streptococci and leuconostocs, however, could produce only the inhibitor-insensitive catalase activity.

Jones (31) revealed that catalase activity of *S. faecalis* was enhanced by aeration and the addition of ferric, manganic and zinc ions into the growth medium. An iron-porphyrin group could not be detected in the crude catalase preparation, and neither azide nor cyanide inhibited catalase activity. Catalase activity in cells was slowly lost by culturing the strain in stationary

broth tubes, but activity could be maintained if the cells were grown under continuous aerobic culture.

As described above, some strains of lactic acid bacteria have been shown to have some type of catalase activity without a hematin source in the growth medium (31). Kono and Fridovich (30) reported that the catalase enzyme isolated from *Lactobacillus plantarum* was a homohexameric manganic enzyme with a native molecular weight of 172,000±4000 daltons. It did not show the absorbance peak at 406*nm* associated with hemoproteins. Manganese served as a functional replacement for iron, the cofactor for classical catalase enzyme. The optical spectrum of Mn-containing catalase was similar to that of the manganese superoxide dismutase, but the Mn-containing catalase did not exhibit superoxide dismutase activity; conversely the Mn-containing superoxide dismutase did not exhibit catalase activity (30). Since this catalase enzyme is nonheme and contains Mn, it is called a Mn catalase or pseudocatalase ($H_2O_2 + 2H^+ + Mn^{III} \rightarrow Mn^{VI} + 2H_2O$) to distinguish it from the classical heme-iron catalase.

Kono and Fridovich (32) also indicated that although the pseudocatalase-negative strain of *L. plantarum* grew somewhat more rapidly aerobically and achieved a slightly higher cell density than did the Mn catalase-positive

strain, during the stationary phase of growth the cell visibility of pseudocatalase-positive strain was well maintained whereas the pseudocatalase-negative strain rapidly died off. This correlated with the finding that the pseudocatalase-negative strain accumulated $H_2O_2$ in the growth medium but the pseudocatalase-positive strain did not. When the growth medium of pseudocatalase-positive strain was treated with hydroxylammine ($NH_2OH$), which irreversibly inactivates pseudocatalase, the cells died at the same rate as the pseudocatalase-negative strain. Therefore, the pseudocatalase has the same biological utility as heme-iron catalase enzyme.

In 1984, the fecal streptococci were first transferred to the genus *Enterococcus* by Schleifer and Kilpper-Balz (44). Chemotaxonomic studies now refer to *E. faecalis* instead of *S. faecalis* in the classification.

Interest in the purification and characterization of catalase activity from various sources has been high in recent years because catalase is a required enzyme for any organism to survive aerobically. The activity of *E. faecalis* is of considerable practical importance to humans because *E. faecalis* can be opportunistic pathogen. Medical differentiation of *Enterococcus and Staphylococcus* often depends on the lack of catalase activity in *Enterococcus*. The strain used in this study was a

clinical strain of *E. faecalis* that was catalase positive when grown on blood agar. Thus, a detailed study of physical properties of its catalase may provide a reference to study the mapping and cloning of the genes encoding the catalase enzyme.

The goals of this study were to purify and to characterize the catalase enzyme from *E. faecalis*. In this study the catalase enzyme of *E. faecalis* was purified to homogeneity with a procedure that yielded a final 120-fold purification. To characterize the catalase enzyme from *E. faecalis*, I investigated a number of properties using the purified catalase. They included molecular weight, number of subunits, pH optimum, heme content and effect of azide on enzyme activity. The catalase of *E. faecalis* was found to be similar to bovine catalase in many important charateristics. The experimental results suggest that this enzyme may be regarded as identical with the typical heme-iron catalase enzyme.

Chapter 2 describes the materials and methods that were used in this study. Experimental results are presented in Chapter 3. Discussion of the expermental results is presented in Chapter 4, and conclusion of the study is presented in Chapter 5. Knowledge required for calculating the unit of catalase activity upon oxygen evolution is described in Appendix A.

# Chapter 2

# Materials and Method

## 2.1 Materials

DEAE-Sephadex A-50, streptomycin sulfate, hematin (bovine blood), trizma base, horseradish peroxidase (type VI), 3,3'-diaminobenzidine (tetrahydrochloride, grade II), purified bovine liver catalase, bovine serum albumin, sodium azide, SDS molecular weight markers for denatured molecular weight analysis and the most common biochemicals were obtained from Sigma Chemical Co. Bio-Gel HTP and N,N'-methylene bisacrylamide were obtained from Bio-Rad. Acrylamide was obtained from IBI. Ammonium sulfate, hydrogen peroxide and common salts were obtained from Mallinckrodt. High molecular weight markers (electrophoresis calibraton kit) for native molecular weight analysis was obtained from Pharmacia Fine Chemicals.

## 2.2 Bacterial Strain

The bacterium used was a clinical strain of *E. faecalis* obtained from Emanuel Hospital, Portland, Oregon.

This strain was isolated from an endocarditis infection and had initially been mistaken as *Staphylococcus* at a rural hospital. This identification error was due to the hospital norm of culturing pathogen bacteria on blood agar, and the bacteria exhibited a positive catalase test. This strain was characterized physiologically by the API test strip. Other identification techniques included Gram-stain (positive), microscopy (coccus in pairs or short chains) and staph O/F test (facultative). For the catalase test, this strain was inoculated onto a TGYE plate with hematin and a TGYE plate without hematin, respectively.

## 2.3 Culture Medium

The bacteria were maintained by daily transfer on TGYE agar plates containing (g-1): tryptone 5.0, yeast extract 2.5, glucose 2.0, and supplemented with hematin to give a final concentration $20\mu g/ml$. For catalase purification, cells were grown on a medium (33) containing the compounds shown in Table 1. Concentrated solutions of salts, vitamins, and purines were prepared as four separate stock solutions and stored at $-20^{o}C$. For norite treatment of casamino acid and casitone, $2.3g$ of norite was added to a solution ($50ml$) containing $5g$ of casamino

acid and 10*g* of casitone. This mixture was stirred for 20 min at room temperature and stored at 4°C overnight. The solution was centrifuged at 16,000 x *g* for 30 min, and the resulting supernatant was centrifuged at 35,000 *x g* for 30 min.

The culture medium was adjusted to pH 6.8 and sterilized at 121°C. Glucose was prepared as a 50% solution, sterilized separately, and added to the sterile medium. Hematin was prepared by dissolving it in 0.2*N* NaOH (5*mg/ml*) and sterilized by membrane-filtration (0.45$\mu m$ pore size) (40). The hematin was added to the sterile medium just before inoculation with culture.

## 2.4 Growth Condition

An initial 100*ml* of the culture medium was inoculated with *E. faecalis* grown on a hematin supplemented TGYE agar plate. The bacteria were grown overnight with shaking on a New Brunswick Gyrotory Shaker (G-25) at 200 rpm at 30°C. A 10*ml* of overnight culture was inoculated into each of ten 1000*ml* culture media in 2-L flasks. Large cultures were grown with constant shaking at 200 rpm at 30°C for 18 hours. Bacterial growth was monitored by measurement of turbidity using a Klett-Summerson colorimeter with a 660 filter. The cells were harvested in late log phase when

the cell density reached approximately 230 Klett units. Cells were collected by centrifugation at 16,300 x g for 20 min. The total yield was 54*g* of a cell mass (wet weight) from 10*L* of the culture medium. The cells were stored at -20°C until used.

To establish culture purity, samples from each of ten culture flasks were examined by plating onto TGYE agar, Gram-stain, microscopy and testing catalase activity on the bacterial colonies.

In addition, in order to determine the concentration of hematin required for maximal catalase activity, *E. faecalis* was cultured in ten flasks containing 100*ml* of the same medium but containing different concentrations of hematin as follows: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100*μg/ml*. A flask without hematin was used as a control.

## 2.5 Purification of Catalase

Catalase enzyme from *E. faecalis* was purified using modified schemes of Claiborne and Fridovich for the purifications of catalase HPI from *E. coli* B (11), and Loewen and Switala for catalase HPII from *E. coli* K12 (12) and catalase-1 from *B. subtilis* (14). All steps followed were performed at 4°C. Potassium phosphate buffer used in the procedure was sterilized at 120°C for 20 min.

Step 1: Crude extract

The cells (54g) were suspended in 150*ml* of 0.1*M* potassium phosphate buffer (pH 7.0) using a Sorvall Omni-mixer. To break the cells, the suspension was passed through a French Pressure Cell (SLM Instruments Co.) using Power Laboratory Press (American Instruments Co., Silver Spring, MD) at 20,000 p.s.i.. The extract was collected in an ice bath and clarified by centrifugation at 27,300 x *g* in a Sorvall centrifuge (Model RC2-B) for 60 min. To assure breakage of any remaining cells, the pellet was resuspended in 100*ml* of same buffer, and the breakage step was repeated. The extract was centrifuged as described above and the supernatants were combined. The pellet was discarded. The supernatant had a total volume of 200*ml*. This solution was called the crude extract. The solution was stored at -20°C until used.

Step 2: 2.5% Streptomycin sulfate

Streptomycin sulfate was added to the thawed crude extract to give a 2.5% final concentration. The mixture was stirred gently for 60 min and stored overnight at 4°C to ensure complete precipitation. The precipitate was removed by centrifugation for 60 min at 29,000 x *g* in a Sorvall centrifuge (RC2-B). The pellet was discarded and

the supernatant was recentrifuged in a Beckman (L-2) ultracentrifuge at 105,000 x g for 90 min to remove any remaining cellular debris. The pellet was discarded and the supernatant solution was 200*ml*.

Step 3: Fractionation of ammonium sulfate

The supernatant solution (pH 6.2) was adjusted to pH 7.0 with 0.1*M* dibasic potassium phosphate. Ammonium sulfate fractionation was done by addition of solid ammonium sulfate to give 45%, 50%, 55%, 60% and 65% saturated fractions. Each fractionation was stirred gently for 30 min to ensure complete dissolution and precipitation. Each precipitate was collected by centrifugation at 29,000 x *g* for 60 min in a Sorvall centrifuge (RC2-B) and the supernatant from that fraction was further fractionated with additional ammonium sulfate. Each pellet was dissolved in 20*ml* of 0.1*M* potassium phosphate buffer (pH 7.0), and assayed for catalase activity. It was found that all pellets had some catalase activity, but most catalase activity was present in the 55% and 60% saturation pellets.

Step 4: Dialysis

The 55% and 60% pellets which were each dissolved in 20*ml* of 0.1*M* potassium phosphate buffer (pH 7.0) were

dialyzed overnight against 4*L* of 50*mM* potassium phosphate buffer (pH 7.0). The solutions were mixed to give a total 42*ml* of the dialyzed solution.

Step 5: DEAE-Sephadex A-50

The dialyzed solution from step 4 was loaded on a Buchner funnel (diameter 4.5*cm*) containing 2*cm* of DEAE-Sephadex A-50 previously equilibrated with 200*ml* of 50*mM* potassium phosphate buffer (pH 7.0). After absorption for 1 hour, the proteins were eluted with each of following step preparations: 0.05*M*, 0.1*M*, 0.15*M*, 0.2*M*, 0.25*M*, 0.3*M*, 0.35*M*, 0.4*M*, 0.45*M*, and 0.5*M* NaCl in 50*mM* potassium phosphate buffer (pH 7.0). At each step, a 15*ml* of eluting solution was loaded onto the DEAE. The solution was allowed to empty by gravity and the gel was dried with suction. Approximately 15*ml* of solution was obtained from each fraction, and each fraction was assayed for catalase activity and protein content. The data are shown in Fig. 1. The fractions (No.6-8) with the peak of catalase activity were pooled to give a total 45*ml* of enzyme solution. This enzyme solution was dialyzed overnight against 4*L* of 5*mM* potassium phosphate buffer (pH 7.0).

Step 6: Bio-Gel HTP

The dialyzed enzyme solution was applied to a Buckner funnel (diameter 4.5*cm*) containing 2*cm* of Bio-Gel HTP, previously equilibrated with 200*ml* of 5*mM* potassium phosphate buffer (pH 7.0). The catalase was eluted by each of following step concentrations: 0.005*M*, 0.05*M*, 0.1*M*, 0.15*M*, 0.2*M*, 0.25*M*, 0.3*M*, 0.35*M*, and 0.4*M* potassium phosphate buffers (pH 7.0). At each step, a 10*ml* of eluting solution was loaded onto the Bio-Gel HTP. The solution was allowed to empty by gravity and the gel was dried with suction. Approximately 10*ml* of solution was obtained from each fraction, and each fraction was assayed for catalase activity and protein content. The data are shown in Fig. 2. The fractions (No. 2-3) with peak of catalase activity were pooled to give a final 20*ml* of enzyme solution. This enzyme solution was dialyzed overnight against 4*L* of 50*mM* potassium phosphate buffer (pH 7.0). The dialyzed solution was stored at -20°C. This enzyme solution was used in following as the "purified enzyme".

## 2.6 Catalase Assay

Catalase activity was determined by measuring the rate of oxygen evolution upon the breakdown of hydrogen

peroxide as described by Dempsey et al. (34). An oxygen monitor (Yellow Spring Instruments Model 53) with a oxygen probe (Yellow Spring Instruments Model 5331) and a specially constructed cylindrical glass cell were used to measure oxygen evolution. The assay mixture contained 3*ml* of 0.33*M* hydrogen peroxide in 0.067*M* potassium phosphate buffer (pH 7.0). The reaction was initiated by injecting 10$\mu l$ of appropriately diluted enzyme sample. Oxygen evolution was monitored by a Sargent Welch recorder (Model Srlg) for 3 min at 25°C. Only the linear portion was used in the activity calculations. One unit of catalase is defined as the amount of protein that liberates one micromole of oxygen per minute at 25°C (Appendix A). Specific activity of catalase is defined as units of catalase activity per milligram of protein.

Catalase activity in colonies on agar plates was determined by applying one drop of 3% $H_2O_2$ to the colonies. Colonies with catalase activity vigorously evolved oxygen.

## 2.7 Protein Determination

The amount of protein present in samples was estimated by the method of Lowry (35) using Folin phenol reagent. Bovine serum albumin was used as the primary

standard. A standard curve was prepared, containing from 25$\mu g$ to 250$\mu g$ of protein in 1$ml$ of 0.1$M$ potassium phosphate buffer (pH 7.0). A plot of the standards was made by plotting the Klett units against the protein concentration. The procedure was performed as the following steps. 1$ml$ of sample was added to 5$ml$ of alkaline copper reagent, which contained 2% sodium carbonate in 0.1$M$ sodium hydroxide and 0.01% cupric sulfate·$5H_2O$ in 0.02% potassium tartrate. The solution was mixed and incubated at room temperature for 10 min; 0.5$ml$ of 1.0$M$ folin-ciocalteus phenol reagent was added rapidly, and mixed immediately. After 60 min, the mixture yielded a blue color and was then read using a Klett-Summerson colorimeter with 660 filter. In comparison with the plot of standard, the amount of protein in the catalase samples was calculated.

## 2.8 Native molecular Weight Analysis

The molecular weight of native enzyme was determined by the method of Hedrick and Smith (36). The purified enzyme sample was mixed with an equal volume of 40% sucrose. This solution (40$\mu l$) was loaded onto a series of acrylamide gels with various acrylamide concentrations from 6% to 10% in the separation gel (pH 8.3) with a 3%

stacking gel (pH 6.9) (14x14*cm* slab gel and 1.5*mm* in thickness). The separation gel consisted of 4.575% Tris, 0.0003% TEMED, 0.0175% ammonium persulfate and 6% to 10% acrylamide. The stacking gel consisted of 0.7125% Tris, 0.3125% Bis, 0.0005% riboflavin, 5% sucrose and 3% acrylamide. Electrophoresis was run using Tris-glycine buffer system (pH 8.3) with 0.0001% bromothymol blue in upper buffer only. It was performed for 9 hours at room temperature at 100*V* initial voltage, increased to 200*V* after the dye front had moved into separation gel.

After electrophoresis, the gels were stained for protein bands using 0.125% Coomassie blue R250 in 10% acetic acid-50% methanol and destained using 10% acetic acid-50% methanol. The $R_f$ of the protein relative to the bromphenol blue tracking dye was determined for each different percentage gel. The retardation coefficient for each protein was determined from the slope of a graph of $100 log 100 R_f$ against acrylamide concentration. The retardation coefficient was then plotted against the protein molecular weight. The proteins used as molecular weight standard were as follows: thyroglobulin 669,000, ferritin 440,000, catalase 232,000, lactate dehydrogenase 140,000, and bovine serum albumin 67,000.

## 2.9 Denatured Molecular Weight Analysis

Samples were denatured with an equal volume of sample buffer containing 0.1*M* potassium phosphate (pH 7.0), 1% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, 12% glycerol and 0.001% bromphenol as the dye. The solution was boiled for 3 min, then centrifuged at 16,300 x g for 2 min. This solution (40$\mu l$) was loaded onto a SDS discontinuous 14x14*cm* slab gel according to technique of Laemmili (37). The concentrations in stacking gel (pH 6.8) were as follows: 4% acrylamide, 8% Tris, 0.1% SDS, 0.1% ammonium persulfate and 0.06% TEMED, and the separation gel (pH 8.8) consisted of 8% acrylamide, 0.0075% Tris, 0.1% SDS, 0.1% ammonium persulfate and 0.1% TEMED. The running buffer system (pH 8.3) was 0.3% Tris, 1.44% glycine and 0.1% SDS. Electrophoresis was run at room temperature at 80*V* until the bromphenol blue marker dye reached the bottom of the gel. Protein bands were stained with Coomassie blue as described previously. The $R_f$ of the protein relative to the bromphenol blue tracking dye was determined, and then the logarithm molecular weight was plotted against the $R_f$. The proteins used as molecular weight standard were as follows: trypsin inhibitor 20,100, trypsinogen 24,000, carbonic anhydrase

29,000, glyceraldehyde-3-phosphate dehydrogenase 36,000, ovalbumin 45,000 and albumin bovine 66,000.

## 2.10 Visualization of Catalase Activity on Polyacrylamide gel

After electrophoresis of native protein on a 8.5% polyacrylamide gel run as described previously, catalase activity was visualized by the method of Clave et al. (38). The gel first was soaked in horseradish peroxidase (50*μg/ml*) dissolved in 50*mM* phosphate buffer (pH 7.0) for 45 min at room temperature. Then $H_2O_2$ was added to the peroxidase-gel solution to give a concentration of 5.0*mM* and the gel was incubated for 10 min. The gel then was rapidly rinsed twice with water and placed into a solution of 3,3'-diaminobenzidine (0.5*mg/ml*) in 50*mM* phosphate buffer (pH 7.0) until staining was complete. The catalase band was visible as a light green band against a grey-yellow background.

## 2.11 Optimal pH

The optimal pH for catalase activity was determined using the following buffers: 0.1*M* sodium acetate/acetic acid and 0.1*M* dihydrogen potassium phosphate/monohydrogen potassium phosphate. PH values from 4.2 to 5.6 were

obtained from 0.1*M* sodium acetate buffers, and pH values from 5.8 to 8.0 were obtained from 0.1*M* potassium phosphate buffers. The enzyme sample was diluted into each of the different buffers from pH 4.2 to 8.0 and incubated at 4°C for 6 hours without $H_2O_2$. Reactions were started by injecting 10*µl* of the diluted enzyme sample (protein concentration at 3*µg/ml*) into 3*ml* each different pH buffer in 0.5*M* $H_2O_2$ and the catalase activity was measured by oxygen evolution using same method as described previously.

## 2.12 Effect of Sodium Azide

For determination of the activity of the purified catalase in the presence of sodium azide, the following different concentrations were used: 0.002*mM*, 0.01*mM*, 0.02*mM*, 0.05*mM*, 0.1*mM* and 0.2*mM* sodium azide in 0.05*M* potassium phosphate buffer (pH 7.0). The enzyme was incubated with each inhibitor solution at room temperature for 1 min prior to assay for catalase activity. To 3*ml* of inhibitor solution in 0.5*M* $H_2O_2$, 10*µl* of the diluted enzyme sample was added to assay for activity as described previously.

## 2.13 Hemochromogen Characterization

An optical spectrum was obtained using a spectrophotometer (8452A, Hewlett Packard). The enzyme was present at 2.2*mg* protein per milliliter in 0.05*M* potassium phosphate buffer (pH 7.0). The absorbance was recorded at room temperature versus a blank containing only the buffer. For determination of metal content, the purified enzyme was sterilized by membrane filtration (0.2*um* pore size) and was analyzed by neutron activation spectrometry using 0.05*M* potassium phosphate buffer (pH 7.0) and water as the blanks. Water and 0.05*M* potassium phosphate buffer were sterilized by membrane filtration.

Table 1. Growth medium for *Enterococcus faecalis*

| Component | Amt per liter |
|---|---|
| Norite-treated vitamin-free Casamino Acids | 5.0 g |
| Norite-treated Casitone | 10.0 g |
| Sodium acetate | 10.0 g |
| Glucose (autoclaved separately) | 20.0 g |
| $K_2HPO_4$ | 0.5 g |
| $KH_2PO_4$ | 0.5 g |
| Hematin (filter sterilized) | 20.0 mg |
| Tween 80 | 1.0 ml |
| Salts stock (a) | 10.0 ml |
| Vitamin stock A (b) | 10.0 ml |
| Vitamin stock B (c) | 2.0 ml |
| Purine stock (d) | 10. ml |

**a.** Salts stock: Sodium citrate 0.25g; $MgSO_4 \cdot 7H_2O$ 0.2g; NaCl 0.01g; $MnSO_4 \cdot 4H_2O$ 0.01g.

**b.** Vitamin stock A: Vitamin B-12 2.0µg; Pyridoxal HCl 0.25g; Thiamine HCl 1.0mg; Calcium pantothenate 1.0mg; Riboflavin 1.0mg; Niacin 1.0mg; *p*-Aminobenzoic acid 0.5mg; Pyridoxine HCl 2.0mg.

**c.** Vitamin stock B: Folic acid 0.25mg; Biotin 5.0µg.

**d.** Purine stock: Adenine HCl 5.0mg; Guanine HCl 5.0mg; Xanthine, sodium salt 3.0mg.

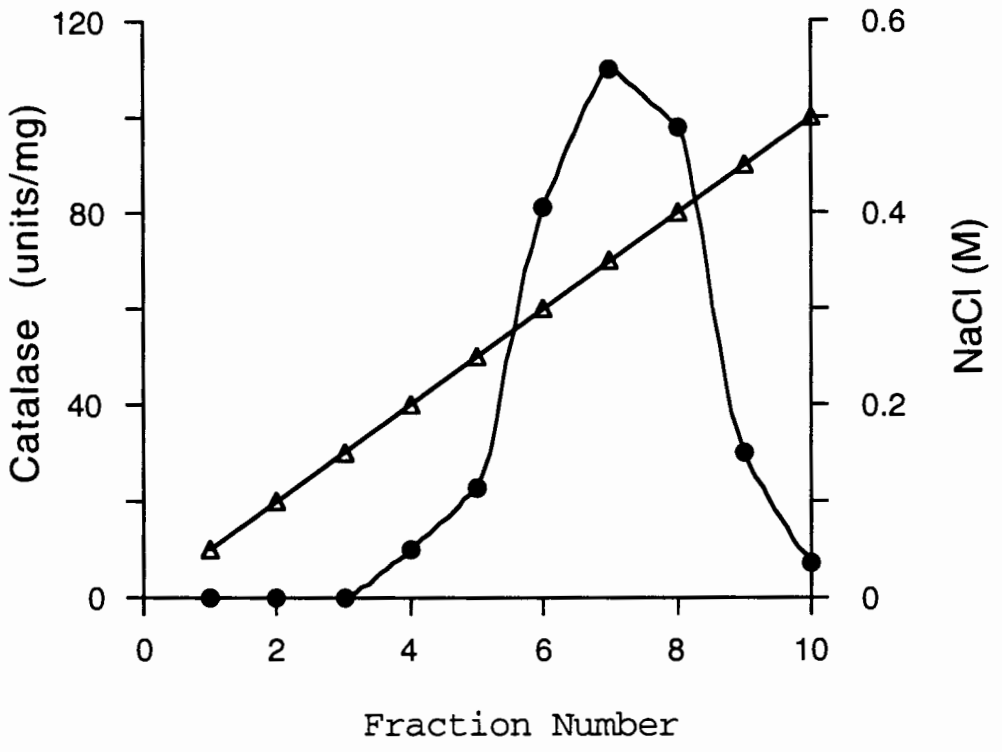


Figure 1. DEAE-Sephadex A-50 chromatography of *E. faecalis* catalase. The enzyme sample was eluted with a step NaCl gradient (0.05-0.5*M*) in 50*mM* potassium phosphate buffer (pH 7.0). Catalase activity (filled-circles) and NaCl concentration (open-triangles) are here plotted as a function of fraction (15*ml*) number. Enzyme activity is reported as specific activity in fraction number.

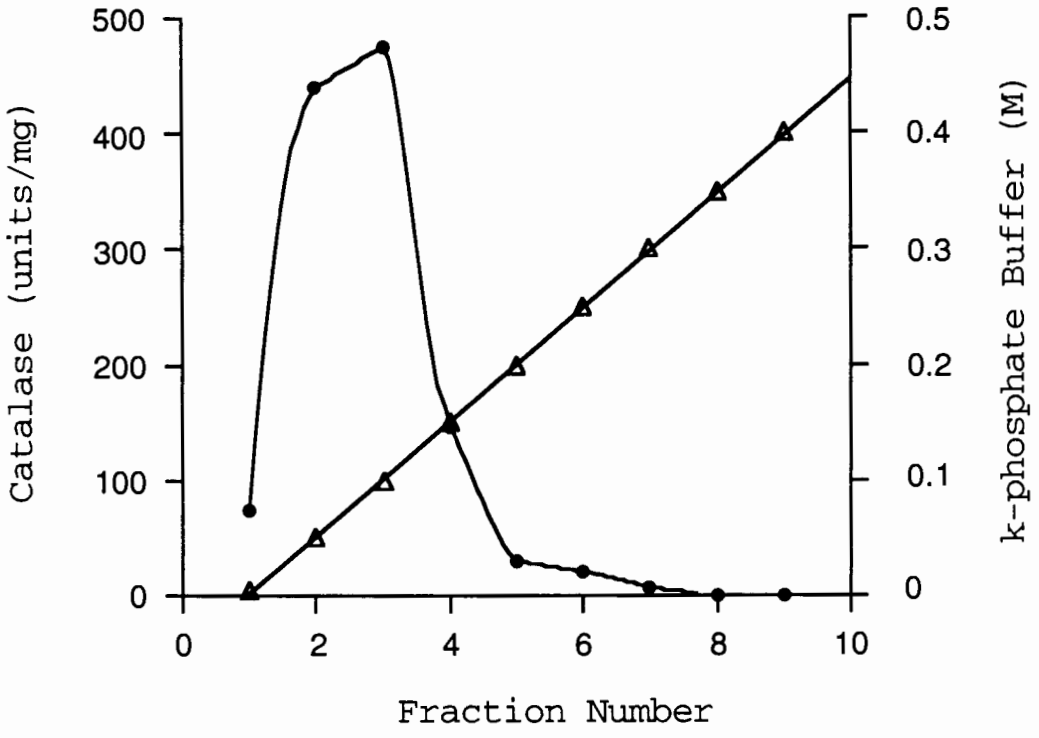


Figure 2. Bio-Gel HTP chromatography of *E. faecalis* catalase. The enzyme sample was eluted with a step gradient (0.005-0.4M) potassium phosphate buffer (pH 7.0). Catalase activity (filled-circles) and potassium phosphate concentration (open-triangles) are here plotted as a function of fraction (10ml) number. Enzyme activity is reported as specific activity in fraction number.

# Chapter 3

# Results

## 3.1 Growth condition

Catalase induction in *E. faecalis* has been shown to occur under aerobic conditions (31). In this study bacterial cultures were grown aerobically by shaking to ensure optimum aeration during bacterial growth. Catalase production of *E. faecalis* has been reported to depend upon the presence of hematin in growth medium (30, 40). The strain of *E. faecalis* used in this study exhibited a positive catalase test on the plate with hematin, whereas a negative catalase test on the plate without hematin. Also, this strain of *E. faecalis* did not produce catalase activity until the medium contained hematin as shown in the results from effect of hematin on catalase activity (Table 2). Crude extracts were prepared from the control flask cells and cells grown at each hematin concentration tested. Catalase activity was estimated as oxygen evolution as described in the materials and method. It was observed that catalase activity was dependent upon the presence of hematin in growth medium, and specific

activity of catalase was close at the different hematin concentrations, although greater specific activity was noted at 30*μg/ml* of hematin. Increasing the amount of hematin in the growth medium did not increase the amount or specific activity of catalase. In addition, earlier work in the laboratory showed that the addition of manganese instead of hematin had no effect on catalase activity.

In addition, the bacterial growth phase is an important factor in determining the amount of catalase produced. Loewen et al. (41) found that when the growth reached late log phase, the catalase concentrations were 15- to 30-fold higher than the basal level in mid-log growth of cells. In this study, the bacterial growth was monitored by an increase in turbidity and cells were havested just as the cells entered late logarithmic growth (230 klett units). The total cell yield of *E. faecalis* was 54*g* of cell mass from 10*L* of medium. Total units of catalase activity in the crude extract was 12320*μmol* $O_2$/min.

### 3.2 Enzyme purification

The catalase of *E. faecalis* was purified by removal of nucleic acids using 2.5% (final concentration)

streptomycin sulfate, protein fractionation using ammonium sulfate, chromatography on DEAE-Sephadex A-50 and Bio-Gel HTP as described in the materials and method. The results from the purification of catalase from *E. faecalis* are summarized in Table 3. It can be seen that a final catalase purification of approximately 120-fold was achieved with a recovery of 17% of the initial activity (with respect to crude extract). This purification is higher than that obtained for the catalase enzyme from *E. coli* B (11), but it is similar to that obtained for the catalase HPII from *E. coli* K12 (12). The specific activity of the purified enzyme was approximately 467*μmol* of oxygen liberated per minute per milligram protein. This catalase activity of *E. faecalis* shows a lower specific activity when compared with other bacteria (11,12,14). The reason for this may be that *E. faecalis* enzyme has lower turnover number.

The purified catalase enzyme could be stored at -20°C for 6 months, and retained activity after several freezing and thawing cycles. This compared favorably with the observations for other catalase enzymes (17, 42).

Electrophoresis of the native catalase on a 8.5% polyacrylamide gel was run to assess the purity of the purified enzyme (Fig. 3). The purified enzyme sample was run in duplicate. After electrophoresis, the gel was

split into two parts; one part was stained for protein and the second part was stained for catalase activity. Only a single band of protein was revealed and its migration was identical with the band of catalase activity.

Samples taken from each step of the purification were denatured with SDS and electrophoresed on SDS-polyacrylamide gel (Fig. 4). The progressive simplification of the protein band pattern culminated with the final product, which exhibited a main band and a weak band on the denatured gel. The weak band might be contaminating material (or a dimer).

### 3.3 Native size determination

The method of Hedrick and Smith (36) was used to determine the molecular weight of the purified native catalase enzyme. The rate of migration of a protein on a series of different percentage acrylamide gels is directly correlated to molecular weight. An apparent molecular weight of 250,000±10,000 daltons for the purified catalase enzyme was estimated (Figs.5,6 and 7). This molecular weight is similar to the molecular weight of catalase enzymes from various other sources (5,15,17).

### 3.4 Subunit size determination

The purified enzyme was denatured with SDS and electrophoresed together with a number of known molecular weight subunit proteins on SDS-polyacrylamide gel. The results are shown in Fig. 8. In this preparation, the purified catalase protein migrated as one major band and two minor bands. On most preparations, only the major band and one minor band at approximately 90,000 daltons were seen; this minor band may be comtaminating material (or a dimer). It is assumed that the minor band at approximately 45,000 daltons is an artifact. The $R_f$ of the major band was compared to that of the molecular weight standards (Fig. 9). The subunit size of catalase of *E. faecalis* was determined to be 64,000±2,000 daltons. The value suggests that the catalase from *E. faecalis* is composed of four subunits of equal size. The subunits are associated entirely by noncovalent forces. A similar phenomenon has been shown by other purifications of catalase enzymes(15, 17).

### 3.5 Optical spectrum and metal content

The spectrum of the purified catalase enzyme from *E. faecalis* exhibited a absorption band at 406*nm* (Fig. 10). The spectrum is similar to those of other classical

catalase enzymes (11,14,17). The purity index, measured as the ratio of absorbance at 406*nm* to that 280*nm*, was 0.78 for *E. faecalis*. A ratio of 1.0 would be expected for pure crystalline protein. In comparison with other classical catalase enzymes, this ratio is higher than the ratio for catalase from *E. coli* and *P. mirabilis* (11,17), but lower than the ratio for catalase from *B. subtilis* (14).

To determine iron content, the purified catalase was analyzed by neutron activation spectrometry using 0.05*M* potassium phosphate buffer (pH 7.0) and water as the blanks. Iron was revealed in the purified catalase from *E. faecalis*, whereas Mn, Cu, Co and Zn were not present at detectable levels.

### 3.6 Optimal PH

The pH optimum of the purified enzyme was assayed in a range of pH from 4.2 to 8.0. The results from the experiment showed that the purified enzyme retained activity over the entire range from pH 4.2 to 8.0, and had a pH optimum with little change in a range of pH 6.0-7.8 (Fig.11). Two maximal rates of catalase activity were noted: one at pH 6.2 and a second at 7.6. These pH optima are very similar to the result reported for the catalase

enzyme from *P. mirabilis* (17). However, this pH profile is slightly narrower than that exhibited by catalase enzymes of *E. coli* (12) and *B. subtilis* (14) where the activity was observed up to pH 12.

### 3.7 Effect of azide

Azide is a known specific inhibitor for heme-containing enzymes, including catalase. It reacts with heme groups of the enzyme to block activity of catalase. Catalase of *E. faecalis* was found to be sensitive to low concentrations of azide (Fig. 12). Data have been expressed as percentage of initial activity remaining. In the presence of 0.002*mM* azide, the catalase lost 50% of activity; with 0.02*mM* azide, the activity loss was at 78%; and complete inactivity was found at 0.2*mM* azide. The catalase enzyme from *E. faecalis* appears to be more sensitive to azide than the catalase from *P. mirabilis* (16), but it is similar to one shown for *B. subtilis* (14).

Table 2. Effect of hematin on the catalase activity in *E. faecalis*.

| **Hematin** | **Total Protein** | **Total Catalase** | **Specific activity** |
|---|---|---|---|
| (µg/ml) | (mg) | (units) | (U/mg) |
| 0 | 21 | 0 | 0 |
| 10 | 23 | 120 | 5.22 |
| 20 | 22 | 153 | 6.98 |
| 30 | 18 | 133 | 7.38 |
| 40 | 28 | 200 | 7.15 |
| 50 | 19 | 133 | 7.02 |
| 60 | 24 | 133 | 5.56 |
| 70 | 27 | 153 | 5.68 |
| 80 | 28 | 180 | 6.44 |
| 90 | 11 | 67 | 6.07 |
| 100 | 20 | 120 | 6.01 |

Table 3. Purification of catalase from *E. faecalis*.

| Step | Volume (ml) | Total Protein (mg) | Total units (µmol $O_{2}$/min) | Specific activity (U/mg) | Purification (n-fold) | Recovery (%) |
|---|---|---|---|---|---|---|
| 1.Crude extract | 200 | 3,180 | 12,320 | 3.9 | 1.0 | 100 |
| 2. 2.5% Streptomycin sulfate | 200 | 2,180 | 9,240 | 4.2 | 1.1 | 75 |
| 3.$(NH_4)_2SO_4$ fractionation | | | | | | |
| 55% pellet | 20 | 56 | 1,478 | 26.4 | 6.8 | 12 |
| 60% pellet | 20 | 108 | 4,928 | 45.6 | 11.7 | 40 |
| 4. Dialysis combined 55+60% | 42 | 142 | 6,037 | 42.5 | 10.9 | 49 |
| 5. DEAE-Sephadex A-50 | 45 | 30 | 3,049 | 101.6 | 26.1 | 25 |
| 6. Bio-Gel HTP | 20 | 4.4 | 2,053 | 466.6 | 119.6 | 17 |

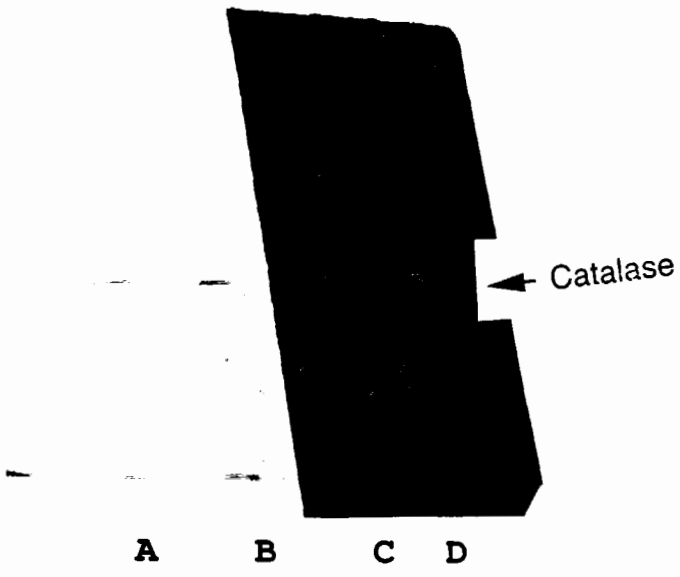


Figure 3. Electrophoresis of the purified catalase on 8.5% nondenatured polyacrylamide gel. Lanes A and C contained 3.3$\mu g$ of protein, lanes B and D contained 6.6$\mu g$ of protein. Lanes A-B were stained for protein bands and lanes C-D were stained for catalase activity bands. The bands of protein migrated coincident with the bands of catalase activity.

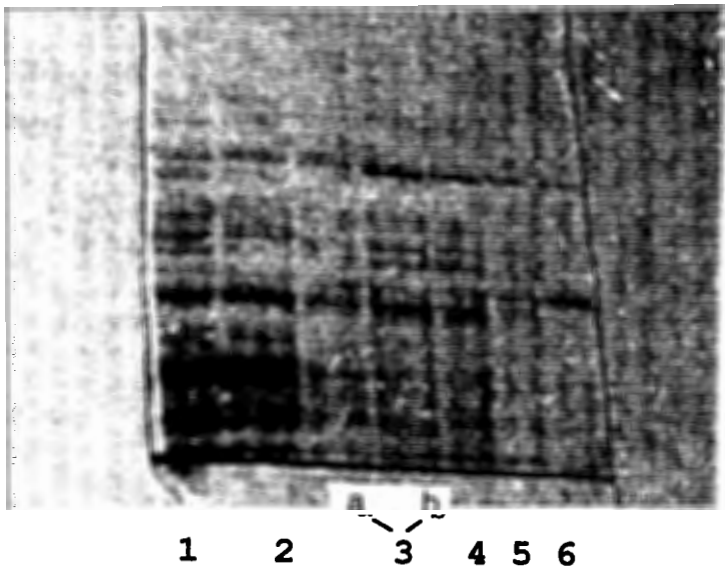


Figure 4. SDS-polyacrylamide gel electrophoresis of enzyme preparations at each step of purification. Lanes 1-6 on gel correspond to the purification steps 1-6, as shown in Table 3. Lanes 1-6 contained 318, 218, 108, 68, 13.4, and 4.4$\mu g$ of protein, respectively. The progressive simplification of the pattern of protein bands was shown on the gel.

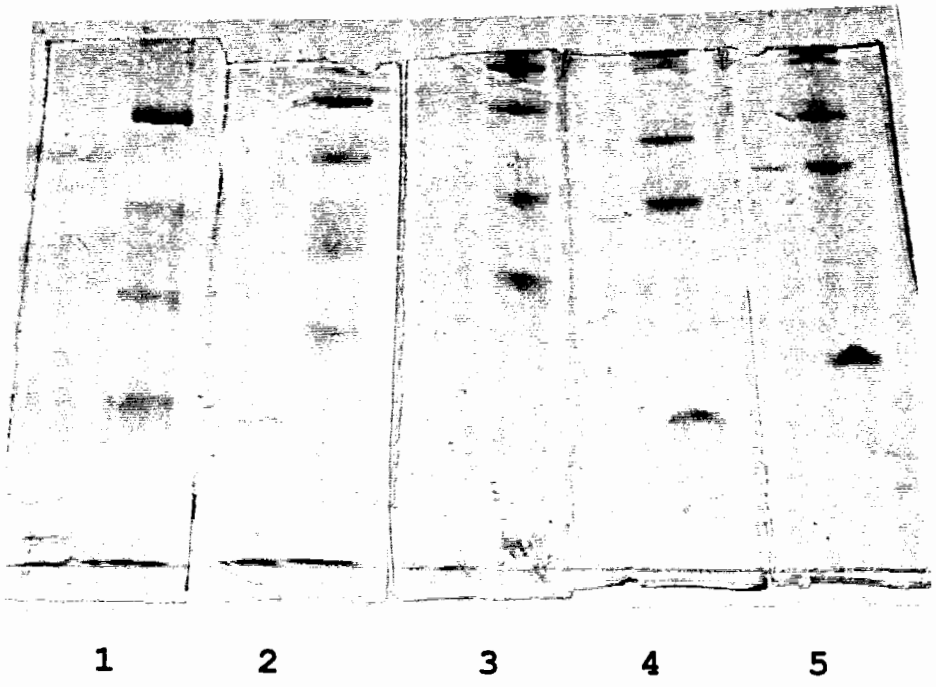


Figure 5. The purified catalase was electrophoresed on different concentrations of nondenatured polyacrylamide gels alongside molecular weight standards. Gels 1-5 correspond to the concentrations of acrylamide 6%-10%, respectively.

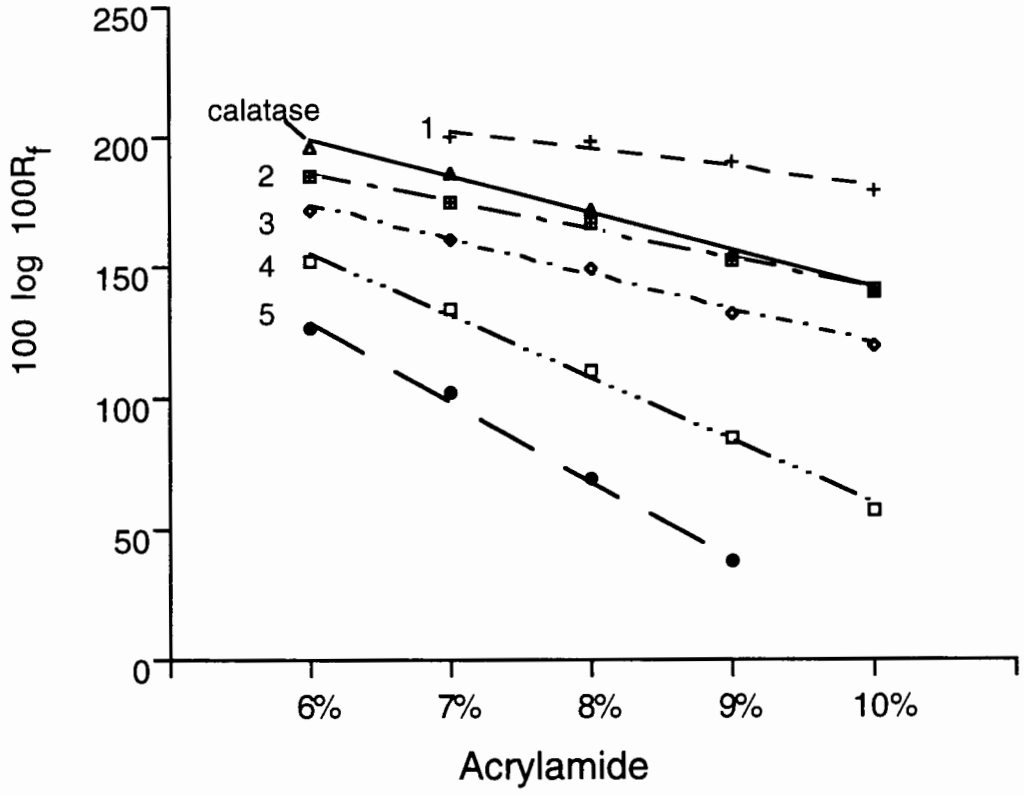


Figure 6. Effect of different acrylamide concentrations on the mobility of molecular weight standards and the purified catalase. The apparent molecular weight of catalase was determined by electrophoresis on native polyacrylamide gels with different concentrations from 6% to 10%.

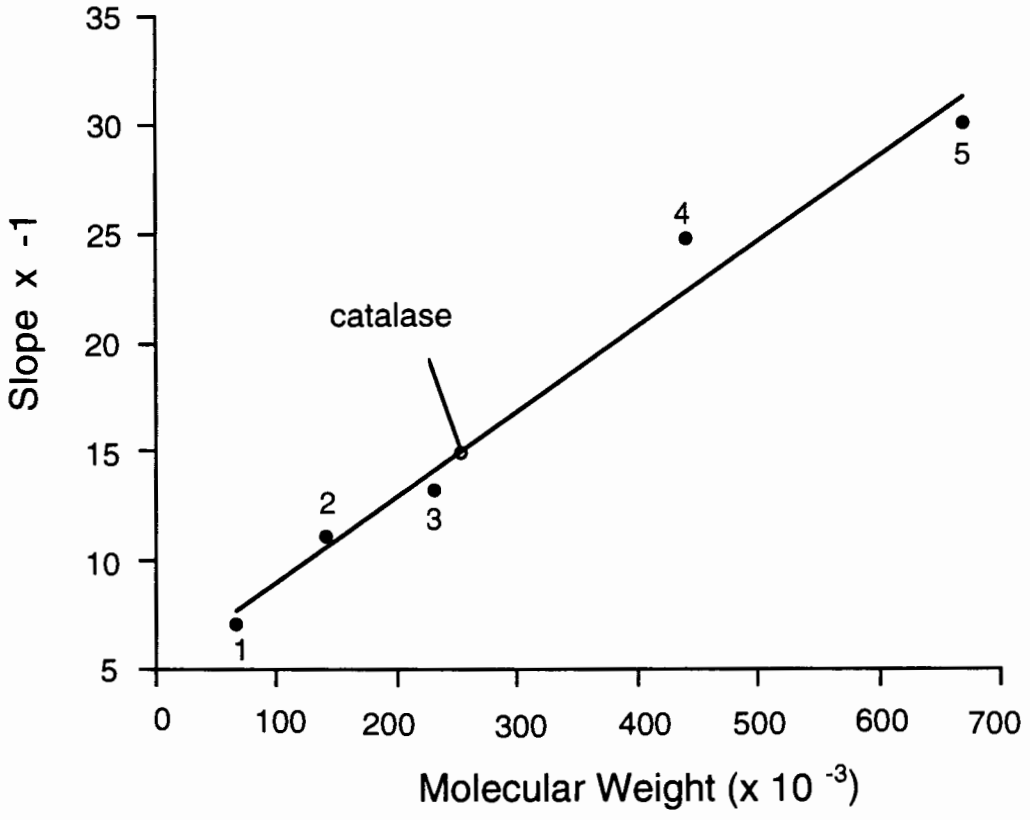


Figure 7. Determination of the molecular weight of the purified catalase from the slope determined in Figure 6. The proteins used as molecular weight standards were as follows: (1) bovine serum albumin 67,000; (2) lactate dehydrogenase 140,000; (3) catalase 232,000; (4) ferritin 440,000, and (5) thyroglobulin 669,000. The molecular weight determined for the purified catalase was 250,000±10,000.

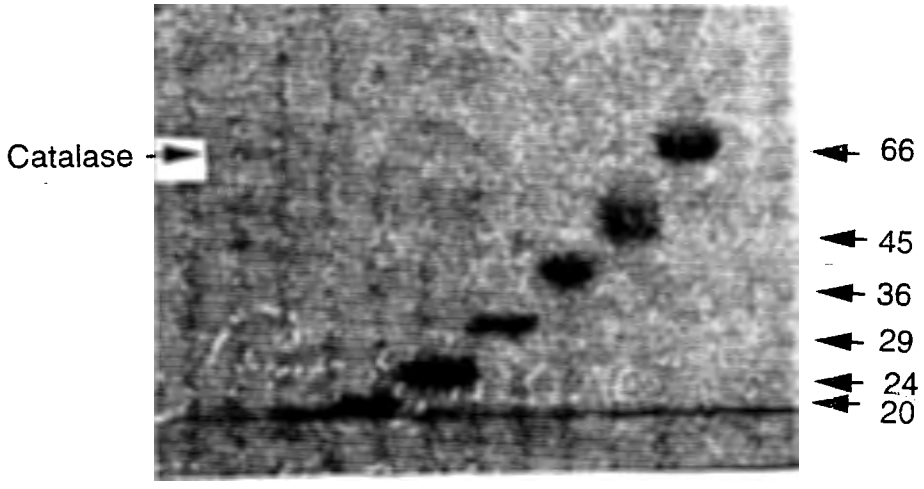


Figure 8. Electrophoresis of the denatured purified catalase on 8% SDS-polyacrylamide alongside molecular weight standards.

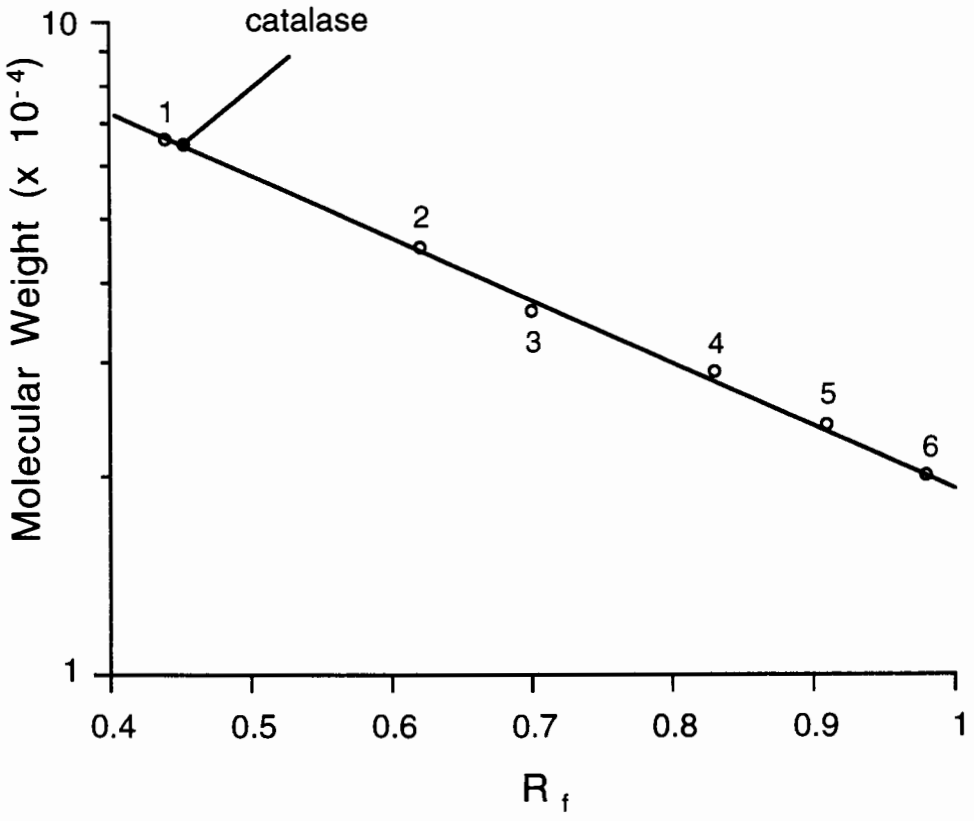


Figure 9. Determination of the subunit molecular weight of the purified catalase. The enzyme and molecular weight standards were electrophoresed on denatured SDS-polyacrylamide gel. Log molecular weight was plotted as function of mobility relative to the dye front (Rf). The proteins used as molecular weight standards were as follows: (1) albumin 66,000, (2) ovalbumin 45,000, (3) glyceraldehyde-3-phosphate dehydrogenase 36,000, (4) carbonic anhydrase 29,000, (5) trypsinogen 24,000, (6) trypsin inhibitor 20,100. The subunit molecular weight determined for catalase was 64,000±2,000.

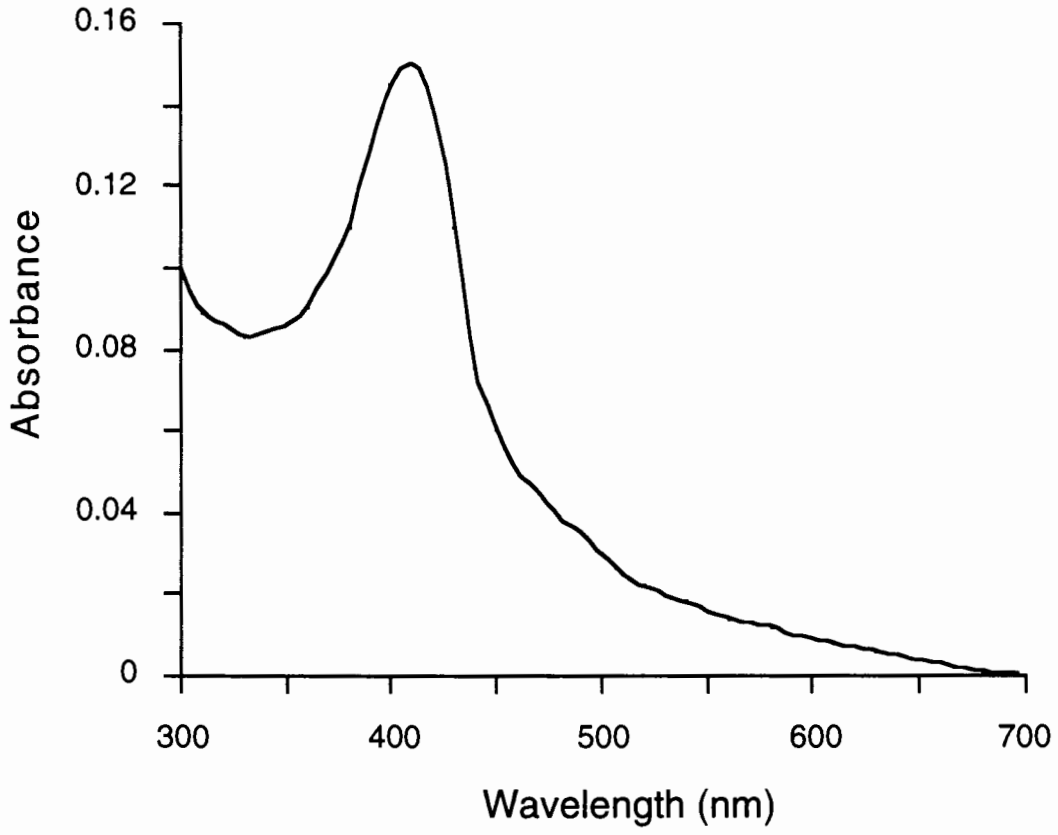


Figure 10. Absorption spectrum of the purified catalase. The enzyme was present at 2.2*mg/ml* in 50*mM* potassium phosphate (pH 7.0). The absorbance was recorded versus a blank containing only the buffer, using a 8452A, Hewlett Packard spectrophotometer.

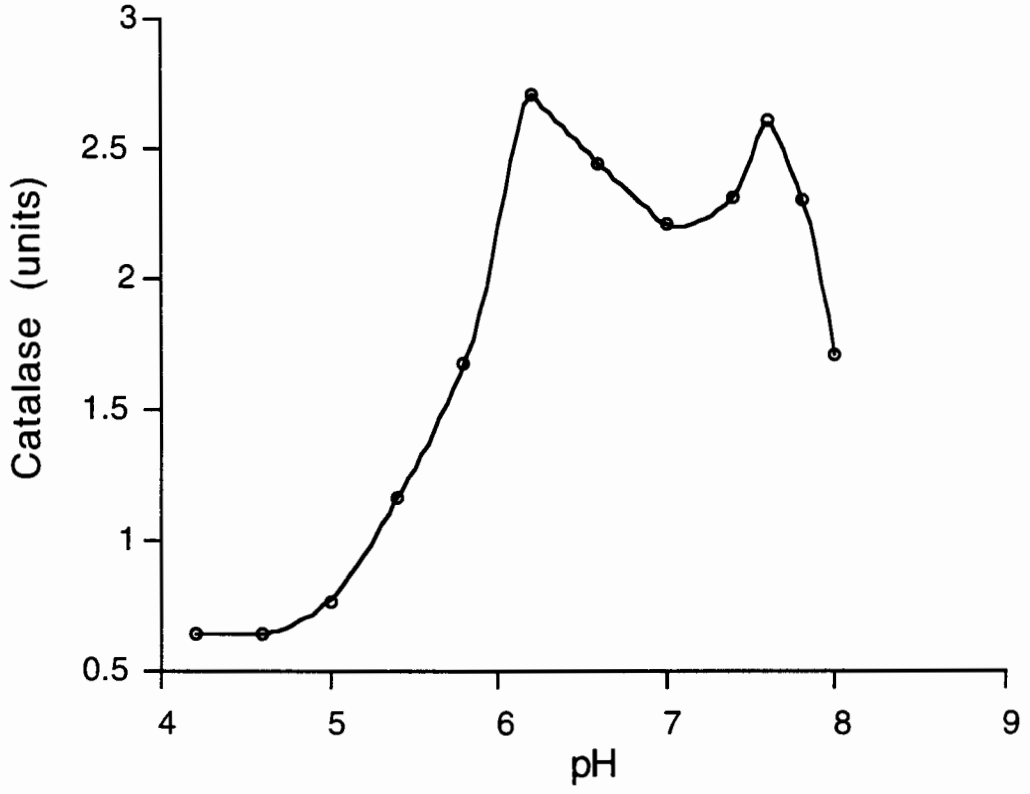


Figure 11. Effect of pH on the activity of the purified catalase. The enzyme ($3\mu g/ml$) was incubated at $4^{o}C$ for 6 hours in each of buffers at various pH values without $H_2O_2$, and then assayed by adding above enzyme solutions into each buffer with different pH in $0.5M$ $H_2O_2$.

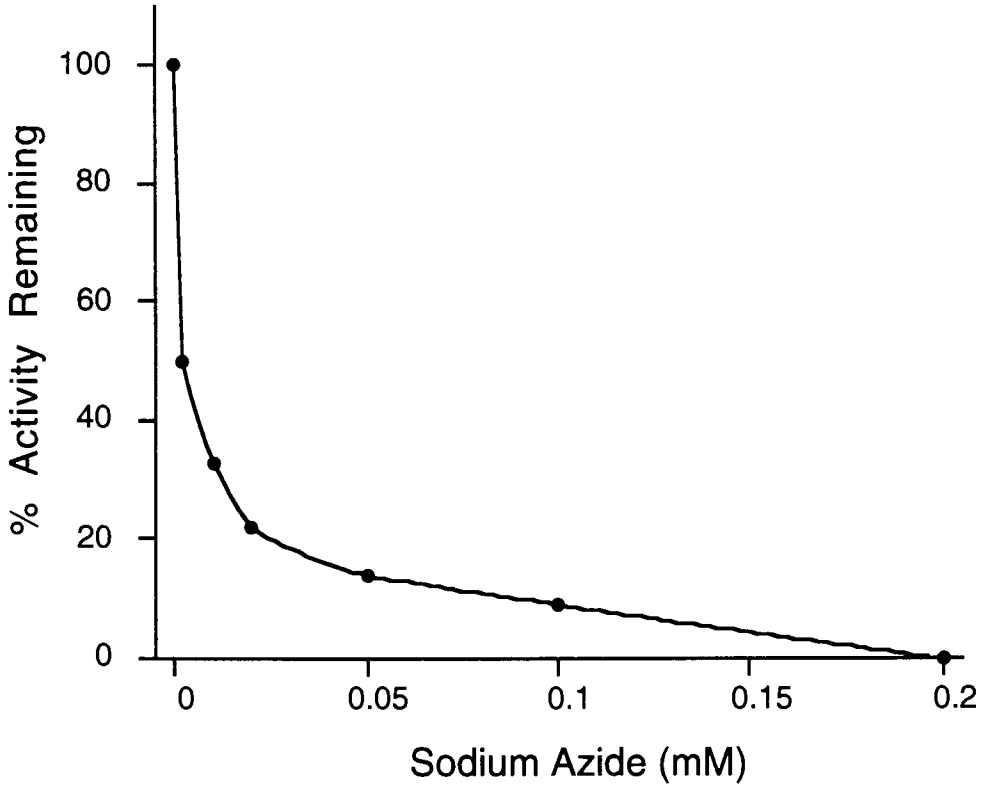


Figure 12. Effect of sodium azide on the activity of purified catalase. The enzyme was incubated with different concentrations of inhibitor in 50*mM* potassium phosphate buffer (pH 7.0) at room temperature for 1 min and then assayed by adding above enzyme solutions to the reaction mixture containing $H_2O_2$ and various amounts of inhibitor. Data have been expressed as percentage of initial activity remaining.

# Chapter 4

# Discussion

The purified catalase enzyme from *E. faecalis* described here represents the major part of the catalase activity in *E. faecalis*. This positive result confirms previous observations that *E. faecalis* can synthesize a classical catalase enzyme when grown in the medium supplemented with hematin. This is in contrast to other lactic acid bacteria, which produce pseudocatalase containing manganese. Evidence obtained in this study indicates that the production of catalase in *E. faecalis* occurs only under aerobic growth conditions and is dependent upon a exogenous hematin source, as has been reported previously (40,43). The presence of catalase in *E. faecalis* is a physiological property which is advantageous for the organisms' response to the selection pressures imposed by an aerobic environment, and it acts as a protective mechanism for bacterial survival. Oxygen must be present for induction of synthesis of the catalase, but hematin is a required cofactor for production of catalase. Most lactic acid bacteria are catalase-negative when grown in heme-free media

(27,28,39,40), although some species of lactic acid bacteria form hemeless pseudocatalases. The catalytic activity of classical catalase enzymes is directly dependent on the hematin content. Since lactic acid bacteria cannot synthesize heme or protoporphyrin (a final iron-free compound in biosynthetic pathway of heme) for the production of catalase, they thus have to obtain hematin from exogenous source (39).

An important step in the purification of *E. faecalis* catalase in this system was the use of small percentage increase steps for ammonium sulfate precipitation, which resulted in a twelvefold purification. Although the ammonium sulfate fractionation was only performed once, it achieved the same result as that done by twice fractionating (12, 14). Moreover, these results showed that catalase activity was not lost as it was during a second ammonium sulfate fractionation.

The last two steps of catalase purification proved to superior to all other purification techniques employed (Table 3). The use of chromatography on DEAE-Sephadex and subsequent chromatography on Bio-Gel HTP gave much higher separation of the proteins. As the molecules passed through the bed at different rates, they emerged from the outlet of the funnel separated from each other and the interaction of protein with DEAE-Sephadex or Bio-Gel HTP

was strongly reduced by the elution. Therefore, the results were increases in specific activity and good recovery of enzyme units. Although other procedures were attempted (for example, Bio-Gel A-1.5 M), they failed to resolve the enzyme to give satisfactory purification.

Although the presence of catalase activity in *E. faecalis* had been demonstrated previously, the characterization of catalase was not investigated completely. Some properties mentioned in differences and main different properties were found in sensitivity to heme poisons (i.e., azide and cyanide) and heme content. The catalase activity reported by Jones et al. (31) and Johnston and Delwiche (29) were cyanide- and azide-resistant, and the iron-porphyrin coenzyme of classical catalase was not detected. This suggests that the activity reported by them for *S. faecalis* was pseudocatalase. However, Clarke and Knowles (40) showed that *S. faecalis* var. *zymogenes* grown in a hematin-containing medium produced a cyanide- and azide-sensitive catalase, and it contained heme. In this study, the catalase from the clinical strain of *E. faecalis* was very sensitive to azide. Iron was revealed in the purified catalase preparation. The optical spectrum indicated the presence of a heme by the absorption at 406*nm*, a usual property of classical heme catalase enzymes. The azide-

sensitivity, the iron content, and the typical absorption band at 406*nm* suggest that the heme prosthetic group exists in the purified catalase from *E. faecalis*, and it plays an important role in the activity of this enzyme.

Like most of typical classical catalase enzymes, the purified catalase exhibited a broad pH range activity. The pH optimum over a range in pH 6.0-7.8 is similar to that of crude catalase from *S. faecalis* reported previously (40). Interestingly, two peaks of pH activity of the purified catalase were found. This feature is different from that reported for the pseudocatalase of *S. faecalis* (31), but it is similar to that classical catalase enzymes of other bacteria (12,14,17).

The purified catalase of *E. faecalis* has the subunit molecular weight of 64,000 daltons and the native molecular weight of 250,000 daltons. This also is similar to classical catalase enzymes isolated from other sources, whether from eucaryotes or procaryotes. A comparison of the native and subunit sizes suggests that the purified catalase of *E. faecalis* exists as a tetramer, and these subunits are associated entirely by noncovalent forces, a common structure for most catalases previously characterized. Also, a great deal of similarity between *E. faecalis* catalase enzyme and most of other classical catalase enzymes can be deduced from the sensitivity to

azide, the heme content, the spectral properties, molecular weight and subunit structure. Thus, the purified catalase from *E. faecalis* exhibits properties usually found for classical heme-iron catalase rather than Mn-pseudocatalase.

Only one protein band with catalase activity was found. It is not known, however, whether more than one catalase species exists in some *E. faecalis*, as has been shown in *E. coli* (12). It is possible other catalase activity may have been lost during the purification process. This remains for further investigation.

# Chapter 5

# Conclusion

The objectives of this study were two: to purify the catalase from *E. faecalis* and to characterize this enzyme. The catalase of *E. faecalis* has been purified to homogeneity. This study has revealed the following important properties: (1) it has a native molecular weight of 250,000±10,000 daltons, and is composed of four identical, noncovalently associated subunits with molecular weight of 64,000±2,000 daltons; (2) the optical spectrum exhibits a absorption band at 406*nm* that is a typical property of the heme-iron catalase, and it contains iron content; (3) other metals were not present at detectable levels; (4) the pH activity shows a broad activity range from pH 4.2 to 8.0 with two peaks of activity at pH 6.2 and 7.6; and (5) it is sensitive to azide.

According to the results presented here, it is remarkable that these characteristics of the purified catalase from *E. faecalis* are shared by other classical catalases, including its optical spectrum, the presence of heme, sensitive to azide, the broad pH range, molecular

weight and the more usual identical tetramer structure. Therefore, it is concluded that the purified catalase from *E. faecalis* is a typical classical heme-containing and azide-sensitive catalase. It is not an atypical nonheme and azide-insensitive pseudocatalase as is present in most lactic acid bacteria. The study of the physiology of the catalase from *E. faecalis* may provide knowledge necessary to study the mapping and cloning of the gene encoding the catalase.

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# Appendix A

Catalase activity was determined by measuring the rate of oxygen liberation upon the breakdown of hydrogen peroxide. It was employed that the measuring rate of oxygen pressure was converted into actual amount of oxygen present with time. The calculation is based on the solubility of oxygen for the reaction solution being used, the temperature and barometric pressure.

The reaction solution was calculated as if it was distilled water saturated with air at 25°C. 1*ml* of distilled water at this temperature contains 0.01708*ml* of air and 33.82% oxygen in the dissolved air (Winkler 1904, 1921). Since 3*ml* of the reaction solution was used, the oxygen contained in the solution would be to a total 0.017328*ml* of volume. This value is equal to 0.77*μmol* of oxygen. Although the assay system is sensitive to barometric pressure changes, this factor was ignored because the assays always were done at the same laboratory and the under same conditions. Thus, the oxygen content of the sample can be determined as

$$C\ (\mu mol) = 0.77 \cdot P$$

Where C is the actual oxygen liberated in the sample and P is the measured oxygen pressure. If the oxygen liberation

of the sample was increased in a span of X chart units on the recorder, above equation then becomes

$$c' \ (\mu mol) = \frac{0.77 \cdot P}{X}$$

Where C' is oxygen liberation per chart unit. When assays for enzyme activity are read as a recorded rate in chart units per minute, the actual oxygen liberated per minute is calculated as

$$c'' \ (\mu mol) = \frac{0.77 \cdot P \cdot R}{X}$$

Where C" is the actual oxygen liberated per minute and R is the recorded rate in chart units per minute.

If enzyme activity was assayed at other reaction solution, temperature and baromtric pressure, the calculation for actual oxygen liberation should be changed.