PURIFICATION AND CHARACTERIZATON OF THE HYDROXYLAMINOBENZOATE LYASE FROM *PSEUDOMONAS PICKETTII* YH105, CLONED IN *ESCHERICHIA COLI*

by

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ABSTRACT OF THE THESIS

Purification and Characterization of the *p*-Hydroxylaminolyase from *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii*), cloned in *Escherichia coli*

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The hydroxylaminolyase enzyme of *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii*) removed the hydroxylamino group from *p*-hydroxylaminobenzoate with the concomitant formation of protocatechuate. In prior work, Brian A. Koller transferred the YH105 gene coding for hydroxyaminolyase into *Escherichia coli* now identified as BAK100. Although transferred, the hydroxyaminolyase enzyme was not characterized with respect to protein size and activity.

Utilizing samples of BAK100, the bacteria was grown on Luria-Bertani broth, induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), separated by centrifugation, washed and weighed. After suspension in morpholinopropane sulfonic acid (MOPS) buffer, the enzyme was released from the crude cells by processing through a French press. Following centrifugation to remove non-target cell fragments, protamine sulfate treatment was used to remove RNA. The resulting solution was concentrated and non-target protein removed utilizing ammonium sulfate treatment prior to gradient DEAE and Sepharose 6L-6B gel filtration. Other purification techniques evaluated and discarded included dialysis, hydroxylapatite treatment, polyethylene glycol precipitation, and pH precipitation. Overall, the protein was purified relative to the crude cell extract,

increasing by a factor of 2.7. The purification steps resulted in a substantial loss of activity. The overall yield was only 5%.

Several SDS electrophoresis gels all showed multiple prominent bands preventing the determination of enzyme size. Kinetic studies utilizing HPLC to quantifying protocatechuate formation provided a K_m of 0.079 mM and V_{max} of 0.16 μmol/min·mg for protamine sulfate treated crude cell extract.

Substrates other than *p*-hydroxylaminobenzoate were tested to evaluate enzyme specificity including *m*-hydroxylaminobenzoate, 3-methyl-4-hydroxylaminobenzoate and *p*-hydroxylamino phenyl acetic acid. Only 3-methyl-4-hydroxylaminobenzoate showed slight conversion, suggesting a high level of enzyme specificity.

Dedications

This dissertation is dedicated to Dr. Theodore Chase, Jr. whose patience and guidance made this work possible.

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I would like to thank Dr. Gerben J. Zylstra and Dr. Alan Antoine for serving as members of my committee.

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Table of Contents

List of Tables

List of Figures

1.0 Literature Review

1.1 Nitro Aromatics in the Environment: Potential for Biological Degradation

Nitroaromatic compounds are used in the manufacture of pesticides, dyes explosives and industrial solvents. They become part of industrial waste streams and can accumulate in the environment. The characteristic electron withdrawing nitro group contributes to reduction of electron density on the aromatic ring. The resulting electrophilic nucleus reduces the ease of oxidative electrophilic attack by oxygenases and the oxidative degradation of nitro aromatic compounds.

Researchers have shown that despite the recalcitrant nature of these compounds, microbes like *Comamonas acidovorans* NBA-10 (Groenewegen et al., 1992), *Pseudomonas* sp strain 4NT (Haigler and Spain, 1993), *Pseudomonas pickettii* YH105 (Yabannavar and Zylstra, 1995) and *Pseudomonas putida* TW3 (Hughes and Williams, 2001) can metabolize them and use them as a source of carbon and / or nitrogen.

1.2 Microbially Mediated Degradative Processes of Nitroaromatic Compounds

Spain et al. (1995) discussed the enzymes involved in the biodegradation of nitroaromatic compounds. The nitro group could be removed from the ring by either an oxygenase reaction leading to the release of nitrite or reduction to the amino derivative followed by the release of ammonia. Spain et al. (1995) mentioned a third mechanism involving the reduction of the ring and subsequent release of the nitro group from an intermediate Meisenheimer complex. Research by Groenewegen et al. (1992) and Spain

et al. (1995) resulted in the identification of a reductive pathway involving a hydroxylamino intermediate in the degradative pathway.

1.2.1 Elimination of Nitrate via Oxidation Reactions

Yabannavar and Zylstra (1995) reported on several oxidation reactions that eliminate nitrate from nitro aromatic compounds catalyzed by monooxygenases or dioxygenases. The authors indicate monooxygenases have been implicate in the direct removal of the nitro group the aromatic rings for *p*-nitrophenol, and *o*-nitrophenol degradation. They list dioxygenases involved with the direct removal of the nitro group from the aromatic ring during the degradation of 2,4-dinitrotoluene, 2,6-dinitrophenol, 2-nitrotoluene, nitrobenzene and *m*-nitrobenzoate. Zylstra et al. (2000) describes the research by Zeyer et al. (1986) where they demonstrated *Pseudomonas putida* B2 degrades *o*-nitrophenol via catechol with the release of nitrite. The initial oxygenase enzyme was shown to require 2 mol of NADPH as a cofactor for the reaction. Similarly, Spain and Gibson (1991) demonstrated *p*-nitrophenol is degraded by a *Moraxella* species though hydroquinone and β-ketoadipate also requiring 2 mol of NADPH as a cofactor.

Spain et al. (1991) reported on the biodegradation of 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol by *Pseudomonas* sp. DNT with a subsequent oxidation to the 2-hydroxy-5-methylquinone and release of nitrite. A dioxygenase-mediated attack leading to the mineralization of 2-nitrotoluene by a strain of *Pseudomonas* sp. strain JS42 was reported by Spain et al. (1994). Spain et al. (1994) identified a similar dioxygenase-mediated attack on 4-nitrotoluene by *Pseudomonas* sp. strain NT.

1.2.2 Hydrogenation of the Aromatic Ring

The hydrogenation of the aromatic ring was first discovered in *Rhodococcus erythropolis* HL24-2 by Lenke and Knackmuss (1992). The aromatic nucleus of 2,4,6-trinitrophenol is initially reduced by a hydride ion (H^-), yielding a hydride-Meisenheimer complex. Following the formation of the hydride-Meisenheimer complex, a proton adds on the aromatic ring and the nitro group is subsequently released as nitrite and 2,4-dinitrophenol is formed. Yabannavar and Zylstra (1995) mentioned the same hydride attack, and formation of a Meisenheimer complex was demonstrated by Vorbeck et al. (1994) to be involved in the removal of the nitro group from 2,4,6-trinitrotoluene.

1.2.3 Reduction of the Nitro Group

Spain et al. (1995) described two types of enzymes with nitroreductase activity distinguished on the basis of their ability to reduce nitro groups in the presence of oxygen. Oxygen sensitive (type II) enzymes catalyze a one-electron reduction of the nitro group which yields a nitro anion radical. The nitro anion radical reacts with oxygen to form superoxide and regenerate the parent nitro compound. This has been called the futile cycle. Oxygen-insensitive (type I) enzymes reduce the nitro group in a series of two-electron transfers to produce an intermediate nitroso or hydroxylamino group which can lead to the direct elimination of ammonia. Spain et al. (2003) reported that hydroxylamino intermediates are degraded via one of three different routes. In compounds with more than one nitro group, the intermediate is often reduced to the amine. In a second strategy, the hydroxylamino compound is converted to a catechol. A third strategy involves the rearrangement of the hydroxylamino intermediates to the corresponding aminophenols.

Groenewegen and De Bont (1992) and by Groenewegen et al. (1992) described the degradation of 4-nitrobenzoate by *Comamonas acidovaorans* NBA-10 through 4-nitrosobenzoate to 4-hydroxyaminobenzoate followed by further degradation to ammonia and protocatechuate, the hydroxylaminobenzoate lyase reaction..

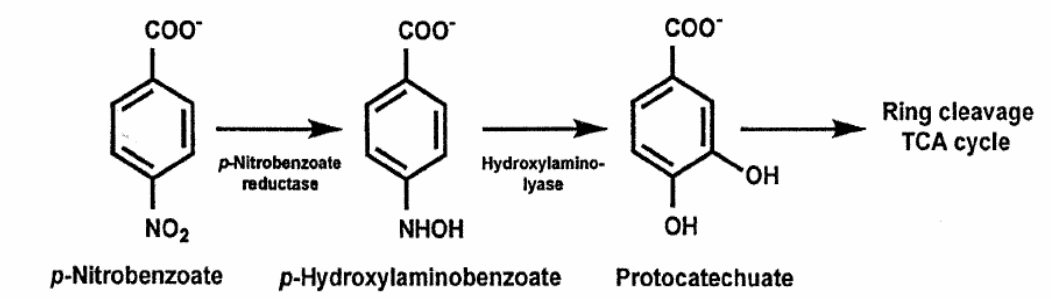
In one of the papers, (Groenewegen and DeBont, 1992) they indicated a loss of activity in cell extracts after dialysis which was restored by the addition of NADH or NADPH under anaerobic conditions. Under aerobic incubation conditions no restoration took place. Groenewegen evaluated other redox potential lowering compounds including ascorbate, cysteine and dithiothreitol. Only dithiothreitol restored enzyme activity under aerobic conditions.

Spain et al. (1993) proposed that *Pseudomonas* sp. strain 4NT was able to degrade 4-nitrotoluene through successive steps that included the formation of 4-hydroxylaminobenzoate, then protocatechuate. The research demonstrated the reductive elimination of the nitro group as ammonia from 4-nitrotoluene. This mechanism for nitro group elimination contrasts with the 2,4-dinitrotoluene degradation from *Pseudomonas* sp. strain DNT through a dioxygenase-mediated enzymatic reaction with removal of the nitro group as nitrite.

Spain et al. (2003) further elucidated the lyase mechanism by proposing an enzymatic conversion of the substrate to the imine intermediate produced from the intermolecular transfer of hydroxyl moiety from the hydroxylamino group to the adjacent position of the aromatic ring. The imine intermediate would then spontaneously hydrolyze to protocatechuate.

Yabannavar and Zylstra isolated and identified *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii* YH105) a bacterial strain capable of utilizing *p*-nitrobenzoate as the

sole carbon source. The research demonstrated the absence of nitrite and presence of ammonia in the culture media, which was indicative of a reductive route of degradation similar to that proposed by Groenewegen et al. (1992). Scheme 1 shows the degradative pathway as given by Yabannavar and Zylstra (1995).



Scheme 1: Reductive pathway for the degradation of p-nitrobenzoate

Yabannavar and Zylstra cloned the genes from *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii* YH105) with the analysis of the open reading frame suggesting a monomeric size of the hydroxylaminolyase in the range of 22 – 23 kDa and that the enzyme may consist of a dimer of identical subunits. The dimer size would then correspond closely with that of the hydroxylaminolyase isolated from *Comamonas acidovorans* NBA-10, which Groenewegen et al. (1992) reported to be 45 kDa. Hughes and Williams (2001) reported that the gene coding for the hydroxylaminolyase *pnb*B in *Pseudomonas pickettii* YH105 has been identified and isolated in *Pseudomonas* sp. YH102 and *Pseudomonas putida* TW3.

Brian A. Koller, an undergraduate student working in Dr. Zylstra's laboratory transferred the *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii* YH105) gene coding for hydroxyaminolyase into *Escherichia coli*. The strain is now identified as BAK100. Although the gene was transferred, the hydroxylaminolyase enzyme was not characterized with respect to protein size and activity.

1.3 Conclusion

Great progress has been made in understanding the degradative pathways of amino aromatic compounds. Similarities in mechanism between different microorganisms have begun to provide the foundation for a better definition of individual enzyme function, the intermediates formed by their action, the genes that code for the enzyme and the enzyme's size and activity.

1.4 Research Goals

The hydroxylaminobenzoate lyase enzyme activity has been defined for only one isolated enzyme from *Comamonas acidovorans* NBA-10. The V_{max}, K_m and enzyme size were determined and reported by Groenewegen et al. (1992). The availability of BAK100 provides the opportunity to determine a matching set of data for an enzyme whose genetic code was isolated from a different microorganism, *Pseudomonas pickettii* YH105 (now *Ralstonia pickettii* YH105). Further, this work can be conducted in an aerobic environment with the addition of dithiotreitol to retain enzyme activity under aerobic conditions. Finally, enzyme specificity will be tested by utilizing varying substrates.

2.0 Materials and Methods

2.1 Chemicals

All the substrates (*m* and *p*-hydroxylaminobenzoate, 3-methyl-4-hydroxylaminobenzoate, *p*-hydroxylamino phenyl acetate) were prepared by Dr. Theodore Chase, Jr. *p*-Hydroxylaminobenzoic acid was synthesized as described by Bauer & Rosenthal (1944); the other hydroxylamine substrates were synthesized by a similar procedure. Remaining chemicals used for enzyme activity and total protein determinations were purchased from Sigma Chemical, Aldrich Chemical, Fisher Biotech or Fisher Scientific. Sepharose CL-6B and diethylaminoethyl (DEAE) - Sepharose gels were purchased from Pharmacia.

2.2 Bacteria Strain and Media

Pseudomonas pickettii YH105 (now *Ralstonia pickettii*) is a wild strain capable of converting *p*-hydroxylaminobenzoate to protocatechuate, originally studied by Yabannavar and Zylstra (1995). The gene for *p*-hydroxylaminobenzoate lyase was cloned into *Escherichia coli* by Brian Koller, a former undergraduate student of Dr. Zylstra in Biochemistry, and the strain identified as BAK100 (Brian A. Koller, unpublished data). Cultures were grown from cells stored at -80^{o}C.

Dr. Chase grew the *E.coli* host expression strain on Luria-Bertani broth prepared from solid purchased from Fisher Scientific. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Invitrogen.

2.3 Bacterial Growth Conditions

Frozen BAK100 was used to inoculate two 250 ml culture flasks each containing 35 ml sterilized Luria-Bertani (LB) broth. After overnight growth, the cells were

centrifuged down, resuspended in 5 ml broth and used to inoculate 800 ml broth in 2.8 L Fernbach flasks. The cells were grown to an optical density of 0.6 at 600nm on a shaker table at 33^{o}C whereupon isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a concentration of 0.4 mM to induce enzyme formation and the growth continued for an additional 3 to 4 hours.

In the BAK100 clone the lacZ gene is replaced with the gene for hydroxylaminobenzoate lyase. The IPTG inhibits the lacZ repressor, inducing the formation of hydroxylaminobenzoate lyase. After 3.5 hours induction the cells were harvested by centrifugation, washed in 0.1 M potassium phosphate pH 6.8-0.2 M NaCl and stored at -20^{o}C.

2.4 Purification Procedure

All steps in the purification of BAK100 hydroxylaminobenzoate lyase were conducted between 4^{o}C and 7^{o}C unless otherwise noted. A 0.5 ml sample from each step was taken before measuring the final volumes. These retains were later assayed for total protein and specific lyase activity.

2.4.1 Crude Cell Lysate

Cells of about 7 gram wet weight were resuspended in 4 ml of 0.05 N morpholinopropane sulfonic acid (MOPS) pH 6.8 - 10% glycerol – 25 mM dithiothreitol (DTT) per gram of crude cell wet weight. The solution was loaded into a French press cell. Utilizing a hydraulic press, the solution was placed under 6,000 to 10,000 psig, then slowly bleed out into a small glass beaker. Cells were disrupted by this and two subsequent passes through the French press. After the French press, the solution was centrifuged (39,000 x g) for 30 minutes while maintaining the temperature at ~7^{o}C in a

Sorvall RC5B Plus centrifuge equipped with a SS-34 bowl. The supernate was decanted, retained and identified as crude cell extract.

2.4.2 Protamine Sulfate Treatment

A volume of 50 mg/ml protamine sulfate solution was added to the crude cell extract to yield a final concentration of 1 mg/ml. The resulting solution was stirred using a vortex mixer, allowed to stand on ice for 10 minutes and centrifuged (39,000 x g) for 10 minutes at ~7^{o}C. The supernate was drawn off and retained while the pellet was discarded.

2.4.3 Ammonium Sulfate Treatment

Protamine sulfate treated enzyme solution was combined with pH 7 adjusted saturated ammonium sulfate solution to yield a 37% saturated ammonium sulfate solution. After mixing, the mixture was allowed to stand in ice for 10 minutes and then centrifuged (31,000 x g) for 10 minutes at ~7^{o}C. The supernatant was decanted and the pellet discarded. Solid ammonium sulfate was ground in a mortar/pestle to increase surface area then added to the 37% saturated ammonium sulfate supernatant solution to achieve a final concentration of 55% saturated ammonium sulfate. After mixing, the solution container was allowed to stand in ice for 10 minutes and then centrifuged (31,000 x g) for 10 minutes at ~7^{o}C. The supernatant was decanted and discarded. The pellet was resuspended in 4 ml of 0.05 N-morpholinopropane sulfonic acid (MOPS) pH 6.8 -10% glycerol -25 mM dithiothreitol (DTT).

2.4.4 Gel Filtration Chromatography

A Sepharose CL-6B column (1.6 cm x 45 cm) located in a walk-in refrigerator maintained at 7^{o}C was equilibrated with 0.05 N-morpholinopropane sulfonic acid

(MOPS) pH 6.8 - 10% glycerol - 25mM dithiothreitol (DTT). The ammonium sulfate treated enzyme solution was then applied as a single volume. The same buffer solution used to equilibrate the column serves as the isocratic mobile phase. The column eluate was collected in 50 drop fractions (~2 ml) via a fraction collector for a total of 30 fractions. All eluate fractions had their absorbance at 280 nm measured. Select column fractions were assayed for hydroxylaminobenzoate lyase activity. Fractions showing the highest activity were combined.

2.4.5 Gradient Ion Exchange Chromatography

A diethylaminoethyl- (DEAE) Sepharose column (1.6 cm x 13 cm) located in a walk-in refrigerator maintained at 7^{o}C was equilibrated with 0.05 N-morpholinopropane sulfonic acid (MOPS) pH 6.8 - 10% glycerol - 25mM dithiothreitol (DTT). The combined Sepharose CL-6B eluate fractions were applied as a single volume. The same buffer solution used to equilibrate the column served as the solution for the mobile phase gradient, 50 ml without NaCl and 50 ml with 0.25 M NaCl. The selection of 0.25 M NaCl as the upper gradient limit represents a refinement based on several column runs that initially started with 0.5 M NaCl. The column eluate was collected in 50 drop fractions (~2 ml) via a fraction collector for a total of 46 fractions when the gradient was fully consumed. All eluate fractions had their absorbance at 280 nm measured. Select column fractions were assayed for BAK100 activity. Fractions showing the highest activity were combined.

2.5 Characterization of BAK 100 Hydroxylaminolyase

2.5.1 Assay Procedure

Enzymatic activity was determined by adding 20 µl – 40 µl enzyme solution to 0.05 M N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid) (HEPES) pH 6.8 – 25 mM DTT containing various concentrations of substrate for a final volume of 2 ml in a conical sample tube, preheated to 37^{o}C and mixed using a vortex mixer. Originally, the level of DTT utilized was five fold less until it was discovered that the enzyme would degrade over the several hours it was assayed even being kept on ice. To avoid any similar degradation in the assay's reaction period, the DTT was increased.

The mixture was preheated in a 37^{o}C constant temperature block (Fisher Scientific Dry Bath Incubator), the enzyme added and the mixture placed back in the constant temperature block for 1 minute then quenched by the addition of 3 µl glacial acetic acid. The use of glacial acetic acid was found to stop the reaction; however, the original addition of 20 µl was found to degrade the organic constituents in the reaction mixture. Trial and error was utilized to find a lower level that would stop the reaction but not degrade the reaction constituents.

Figure 1 shows a plot of enzyme activity versus assay time using crude cell extract. A one minute assay time was chosen as the longest time period that was in the linear part of the curve.

High performance liquid chromatography (HPLC) analysis of the reacted solution was performed with a Waters HPLC system consisting of a Waters WISP 710B auto injector set for a 100 µl injection volume and a Waters µBondapak C18 10µm 125 Å 3.9x300 mm reverse-phase column. An isocratic mobile phase of 5% methanol – 0.25%

acetic acid was pumped through the column at a rate of 2.0 ml/min. Yabannavar and Zylstra (1995) used a reverse phase column and a gradient of 0 to 100% methanol in water under acidic (0.1% acetic acid) conditions. Given the complication of utilizing a gradient with the available equipment, isocratic mobile phase conditions were developed which provided satisfactory separation of sample components. Typically, the minimum time between injections was set at eight minutes to avoid peak overlap.

Eluting compounds were detected using an ABI Analytical Spectroflow 783 UV-Vis detector set at $\lambda = 254$ nm. Peak areas were charted and integrated by a Hewlett Packard HP3396A Integrator using the setting: attenuation = 1, threshold = 2, peak width = 0.2 and chart speed = 0.25. Protocatechuate peak areas were compared with those from known standards to determine concentration. Figure 2 shows the protocatechuate calibration curve used for converting peak areas to molar concentration.

2.5.2 Total Protein Determination

At each step in the purification, a total protein determination was made using a modified Bradford assay. Zor and Selinger (1995) linearized the Bradford method of quantifying protein as published by Bradford (1976), thus the term modified.

A 5µl enzyme solution sample was diluted 1:50 with distilled water. Aliquots of 5µl, 10µl, 15µl, and 20µl diluted enzyme solution along with a blank were added to individual 13 x 100 mm glass tubes then distilled water was added to bring the total volume to 300µl. A 3 ml volume of Coomassie Blue stock solution was added to each tube, the tube mixed and allowed to sit 10 minutes for color development. A Cary 300 UV/VIS spectrophotometer was zeroed using deionized water in plastic cuvets. The

spectrophotometer was programmed to read at 595 nm and 466 nm and to calculate the ratio A_{595} nm / A_{466} nm.

A 1.0 mg/ml stock solution of ovalbumin was utilized as a standard reference to allow the results of the individual determinations to be quantified. The slope generated by plotting the individual absorbance ratios for each enzyme solution was divided by the slope of the line from plotting the absorbance ratio of the ovalbumin. The resulting value was multiplied by the ovalbumin concentration to obtain the protein concentration of the enzyme solution.

2.5.3 Kinetic Study

Characterization of the hydroxylaminobenzoate lyase focused on determining V_{max} and K_m for the enzyme. These values were determined under experimental conditions that ensured that the enzyme was in the linear range with respect to reaction time. This range was established prior to varying substrate concentration in the V_{max} and K_m determination.

2.5.4 Electrophoresis in Sodium Dodecyl Sulfate

SDS gel filtration was performed using a Bio-Rad Mini PROTEAN® 3 System following a standard procedure developed for the Biochemistry teaching lab using a 10% total acrylanitrile gel. Bio-Rad Precision Plus Protein dual color protein standard (catalog # 161-0374, effective range of 10 to 250 kilo Daltons) was used to identify the bands from the Sepharose CL-6B column fractions.

A 20 µl sample volume of the Sepharose CL-6B column eluate fractions was combined with a 13µl aliquot of 3x sample buffer (3% SDS – 15% 2-mercaptoethanol-3X Tris – Tricine buffer) and placed in a water/ethylene glycol filled tube located in the

Fisher Scientific Dry Bath Incubator and maintained at 100°C for 10 minutes. A 15µl volume of protein standard was similarly heated. Both sample and standard were cooled on ice. A 4 µl aliquot of tracking dye (0.1% bromophenol blue in 1 x Tris-Tricine buffer) with 50% glycerol was added to the sample and 2 µl to the standard.

A 20 µl aliquot of the denatured enzyme solution and a 15 µl aliquot of the denatured standard were added to individual but adjoining wells in the 10 well SDS gel. After running the SDS gel, it was removed from the cell and placed in a Coomassie Blue R staining solution overnight with gentle agitation. After removing the staining solution, the SDS gel was destained in 45% methanol – 10% acetic in water for several hours. This solution was removed and the SDS gel placed in a solution of 5% methanol and 7% acetic acid to reverse the gel shrinkage which occurred during destaining. The SDS gel was photographed and inspected for prominent bands to aid in enzyme size determination.

2.5.5 Alternative Substrates

Alternative substrates were evaluated by running standard assays to determine if the enzyme was able to convert these various substrates to its substrate specific product. The substrates included *m*-hydroxylaminobenzoate, 3-methyl-4-hydroxylaminobenzoate, and *p*-hydroxylamino phenyl acetate (as Tris salts).

3.0 Results and Discussion

3.1 Purification of BAK 100 Hydroxylamino Lyase

3.1.1 Crude Cell Extract

Wet crude cells weighting a total of 6.37 grams yielded a 27 ml volume of extract from processing the cells, suspended in buffer solution, through a French press followed by centrifugation and decanting the supernatant. The resulting enzyme solution was assayed for enzyme activity and total protein concentration. Table 1 provides the assay results for the several steps of purification. Table 2 provides the data points used to determine the total protein and the resulting slope calculated by the Microsoft® Excel function "slope".

3.1.2 Protamine Sulfate Treatment

Crude cell extract treated with protamine sulfate to remove nucleic acids yielded a total solution volume of 27.5 ml. The resulting enzyme solution was assayed for enzyme activity and total protein concentration (see values in Table 1 and Table 2). The observed reduction in total protein was expected relative to the crude cell extract. However, the slight increase in enzymatic activity was unexpected. It may be that a soft pellet formed during the crude cell centrifugation allowed some carry over in the crude cell extract which interfered with the enzymatic action. The protamine sulfate pellet is much denser and the supernatant draw off is less turbid.

3.1.3 Ammonium Sulfate Treatment

Enzyme solution treated with ammonium sulfate yielded a total solution volume of 5.5 ml. The resulting enzyme solution was assayed for enzyme activity and total protein

concentration. Ammonium sulfate treatment resulted in a significant loss in activity. One possible explanation is that the loss of hydration associated with driving the protein out of solution destabilized the protein. Oxidation of the enzyme is also a distinct possibility given the mixing and handling involved. Additionally, the choice of lower and upper ammonium sulfate concentrations may have resulted in some enzyme being discarded with the pellet and supernatant respectively.

In an attempt to avoid this significant loss of activity, two alternative approaches to concentrating the protamine sulfate treated crude cell extract were attempted. First, the ammonium sulfate step was eliminated and the DEAE column was run first followed by the Sepharose CL-6B column. The rationale for this was the inherent sample concentration that occurs in a gradient column provided some volume reduction before loading the sample onto the Sepharose CL-6B column. This resulted in extremely long column run times as the sample had limited processing before loading it onto the DEAE column. Long column runs allowed for significant enzyme degradation.

Second, a dialysis step vs. poly(ethylene glycol) was substituted for the ammonium sulfate treatment. The dialysis did result in a concentration of the enzyme solution; however, it did not produced the desired level of concentration and the overnight period in the refrigerator allowed significant degradation of the enzyme.

The lower percent ammonium sulfate value of 37% was based on the recommendation of Dr. Chase. Even lower levels were tried but resulted in very little precipitate. The upper level of 55% was established based on balancing recovery with purity. Analysis of the supernatant from the 55% saturated ammonium sulfate treatment showed no enzyme activity. After resuspending the 55% saturated ammonium sulfate

treatment pellet, the resulting enzyme solution was assayed for enzyme activity and total protein concentration (see values in Table 1 and Table 2).

3.1.4 Gel Filtration Chromatography

The ammonium sulfate treated enzyme solution was loaded onto the Sepharose CL-6B column. A total of 44 fractions, ~2.0 ml each, were collected and the absorbance at 280nm measured. Select fractions were assayed for enzyme activity. Those containing the highest activity levels were combined for a total volume of 5.5 ml and the resulting enzyme solution was assayed for enzyme activity and total protein concentration.

Prior column runs provided a good understanding of what fractions will contain the enzyme. Figure 3 provided a graph of the A_{280} (determined in a 1 cm cell) and enzyme activity for the fractions analyzed. It suggests that the enzyme solution was well fractionated and that this step had the desired effect of separating the various protein constituents of the ammonium sulfate treated crude cell extract. However, the column run takes several hours and even with 25 mM DTT, significant degradation occurred. After combining the factions with activity, the resulting enzyme solution was assayed for enzyme activity and total protein concentration (see values in Table 1 and Table 2).

3.1.5 Gradient Ion Exchange Chromatography

The Sepharose CL-6B combined fractions was loaded onto the DEAE column. A total of 44 fractions, ~2.0 ml each, were collected and the absorbance at 280nm measured. Select fractions were assayed for enzyme activity. Those containing the highest activity levels were retained for later analysis. Prior column runs suggested what fractions will contain the enzyme. Figure 4 provided a graph of the A280nm and enzyme activity for the fractions analyzed.

Figure 4 shows that the enzyme solution was not well fractionated and this step did not separate the various protein constituents. In considering the possible reason for this lack of separation, one possible cause could have been that the salt containing buffer was added to the front gradient bottle instead of the rear. This would result in a high salt concentration early on in the run. It would have flushed out all the proteins almost immediately. This possibility was ruled out for two reasons: first, this type of mistake was never made previously in over a dozen column runs; and second, the protein peak came early but still there were several fractions of low protein content at the beginning.

Another possible cause is poor stirring in the gradient maker and the high salt solution flowed across the bottom of the mixing chamber, eluting the protein almost immediately.

The most likely cause of the premature passing of the protein was the column had just been repoured. Once before, when the column had been repoured, the column separation effectiveness was impacted initially. The DEAE was supplied in ethanol which was drawn off after allowing the DEAE to settle in a beaker. MOPS buffer was added, allowed to settle and decanted off to remove any residual ethanol. Additional MOPS was added and the pH adjusted to 6.8 before pouring the DEAE solution into the column. Additional MOPS was run though the poured column and the pH of the eluate checked to ensure that it was at pH 6.8. Based on the care taken in preparing the column and the observation of poor retention each time the column is repoured lead to speculation that the column must be further acclimated by actually running protein through it in order to ensure that the level of separation, demonstrated on numerous occasions, takes place.

The DEAE column run was shorter than the Sepharose CL-6B column and as a result the degradation of enzyme activity was less. The protocatechuate area used to populate

Table 1 was the average values from DEAE column eluate fractions #10 and #11. Table 2 total protein value for the DEAE step was based on eluate fraction #10.

	BAK 100 Activity with 4-hydroxylaminobenzoate						
	Volume (ml)	**Total Protein (mg/ml)**	**Total Protein (mg)**	**Enzyme Activity (µmole/min·ml)**	**Enzyme Activity (µmole/min·mg)**	**Total Enzyme Activity Units (µmole/min)**	**Yield (% recovery)**
Crude Cell Extract	27.0	81	2199	10.46	0.128	282.40	100%
Protamine Sulfate	27.5	79	2164	11.34	0.144	311.82	110%
Ammonium Sulfate	5.5	200	1101	20.49	0.102	112.68	40%
Sepharose CL-6B	5.5	14	74	3.60	0.267	19.82	7%
DEAE – Sepharose	5.5	8	43	2.74	0.351	15.09	5%
	BAK 100 Activity with 3-methyl-4 hydroxylaminobenzoate						
Crude Cell Extract	27	81	2187	0.70	0.009		

Table 1. BAK100 Hydroxylaminobenzoate Lyase Protein Purification

BAK 100 Total Protein Determination

	Abs (595nm/466nm) Ratio						
Protein Volume (ml)	**Crude Cells**	**Protamine Sulfate**	**Ammonium Sulfate**	**Separose CL-6B**	**DEAE**	**Ovalbumin (1.0 mg/ml)**	**Ovalbumin (1.0 mg/ml)**
0.000	0.5460	0.6125	0.5873	0.4965	0.5116	0.5530	0.4841
0.005	0.6437	0.6177	0.7167	0.5069	0.5164	0.6299	0.5232
0.010	0.6861	0.6662	0.9194	0.5187	0.5186	0.6430	0.5769
0.015	0.7373	0.7674	1.0673	0.5263	0.5243	0.7105	0.5973
0.020	0.8215	0.8131	1.2860	0.5403	0.5386	0.7316	0.6449

	Abs Ratio vs Protein Vol Least Sq Slope	**Total Protein (mg/ml)**
Crude Cells	12.89	81
Protamine Sulfate	13.748	79
Ammonium Sulfate	34.96	200
Separose CL-6B	2.14	14
DEAE	1.238	8
Ovalbumim	8.736	
Ovalbumim	7.914	

Table 2. Total Protein Determination for BAK 100 Hydroxylaminobenzoate Lyase Purification Steps

3.2 Characterization of BAK 100 Hydroxylaminobenzoate Lyase

3.2.1 Enzyme Kinetics

The research intent was to determine the enzyme kinetics of the purest protein solution which should have been the DEAE combined fractions. Unfortunately, the fractions containing the enzyme were kept in the refrigerator instead of the freezer for a week and all the enzymatic activity was lost prior to determining the V_{max} and K_m. Instead, the protamine-sulfate treated crude cell extract sample retained was utilized.

Figure 5 shows the fit of assay data generated by determining the enzymatic activity at varying substrate levels to the Michaelis-Menten equation. The Kaleidoscope® graphics software packaged was used to fit the data. The resulting kinetic values are provided in Table 3 along with the *Comamonas acidovorans* NBA-10 values reported by Groenewegen and DeBont (1992). The V_{max} values are remarkably different with the value for BAK 100 hydroxylaminobenzoate lyase, much lower than the reported value for *Comamonas acidovorans* NBA-10. Much of the difference could be attributed to the purity of the enzyme solution used for the kinetic study. The BAK 100 hydroxylaminobenzoate lyase protamine sulfate treated sample contained 79 mg/ml protein as shown in Table 2 while the *Comamonas acidovorans* NBA-10 enzyme sample contained only 1.2 mg/ml protein as reported by Groenewegen and DeBont (1992)

3.2.2 Protein Size Determination

Protein size determination consistently yielded SDS gels with multiple bands of similar intensity. The inability to produce a single distinct band can be traced to a number of challenges of this particular enzyme.

Hydroxylaminobenzoate Kinetic Data Using Protamine Sulfate Treated Crude Cell Extract

HAB (ml)	HAB (mM)	PC Area	PC (µMole/min)
0	0	0	0
0.02	0.191	59276	0.054
0.04	0.382	88673	0.081
0.1	0.955	154698	0.141
0.12	1.145	166123	0.151

	BAK100	*Comamonas acidovorans* NBA-10
Vmax, µM/min*mg	0.159	13.5
Km, mM	0.079	1.3

Note: *Comamonas acidovarans* NBA-10 Vmax and K_m values reported by Groenewegen and De Bont (1992)

Table 3. BAK 100 Hydroxylaminobenzoate Lyase Kinetic Data

First, during cell growth, consistent induction was not achieved resulting in a wide range of crude cell extract enzyme levels. Starting with low levels of enzyme relative to the total protein content challenged the purification methodology.

Second, the enzyme was found to precipitate out over a wide range of % ammonium sulfate saturation levels forcing the inclusion of more unwanted protein in order to not lose too much of the desired protein.

Third, enzyme degradation during the ammonium sulfate treatment and column runs forced the inclusion of a greater number of eluate fractions than ideal. These marginal eluate fractions included unwanted protein.

Fourth, attempting additional separation steps including pH precipitation, poly(ethylene glycol) dewatering, hydroxylapatite adsorption and a second DEAE column run came with significant loss of enzyme activity.

3.2.3 Substrate Specificity

Alternative substrates were evaluated by running standard assays to determine if the enzyme was able to convert these various substrates to its substrate specific product. The substrates included *m*-hydroxylaminobenzoate, 3-methyl-4-hydroxylaminobenzoate, and *p*-hydroxylamino phenyl acetate. Substrate purity was quite good as all three showed only one main peak. An additional small peak was observed for both *m*-hydroxylaminobenzoate and 3-methyl-4-hydroxylaminobenzoate eluting from the HPLC runs. However the additional peak was too small for area integration suggesting a very low concentration. No additional peaks were observed for the *p*-hydroxylamino phenyl acetate.

A 20µl volume of crude cell extract containing 2.28 mg protein was used in the assay. Of the three, only the assay using 3-methyl-4-hydroxylaminobenzoate was observed to demonstrate a very slight conversion to the associated catechol. The crude cell extract used demonstrated enzyme activity of 1,194 µmole/min·mg using 4-hydroxylaminobenzoate as a substrate and only 79 µmole/min·mg using 3-methyl-4-hydroxylaminobenzoate as a substrate. The lack of conversion of these compounds suggests a high level of enzyme specificity.

4.0 Conclusion and Future Work

Conducting part-time research on a labile enzyme has proven to be a challenge. Fundamental improvements in enzyme production and purification would be required in order to obtain a sample sufficiently pure to make a molecular weight determination. As the literature pointed out, the hydroxylaminobenzoate lyase degrades under aerobic conditions. The process of resuspending crude cells, passing them through a French press, mixing in the protamine sulfate and ammonium sulfate, resuspending pellets and other mixing and handling of the enzyme solution provides a substantial amount of aeration. DTT somewhat counters this by providing a reducing environment. A main problem will remain with purification steps taking several hours as the enzyme degrades even in the 4^{o}C to 7^{o}C range at which all the purification steps were maintained. The ammonium sulfate precipitation step reduces the protein content, however the enzyme activity falls in the same proportion. Switching to a short DEAE preparation column where the enzyme could be retained and removed quickly could provide reasonable protein separation in advance of the Sepharose CL-6B column without having to apply to large a volume of sample onto the Sepharose CL-6B column. A shorter Sepharose CL-6B column may prove to be a better approach, as the lower separation may be more than offset by the lower loss of enzyme activity. With less loss of enzyme activity, fewer individual samples would need to be pooled in order to have sufficient enzyme activity for the next purification step.

Collectively, more must be done to improve the yields. The primary improvement would be to purify the enzyme under a more reducing environment by employing a nitrogen blanket or utilizing a more effective reducing agent. Currently, approximately

20 hours are required to process the crude cells through the various steps. If this could be cut in half and the significant loss associated with the ammonium sulfate precipitation avoided, a much improved purification process would result.

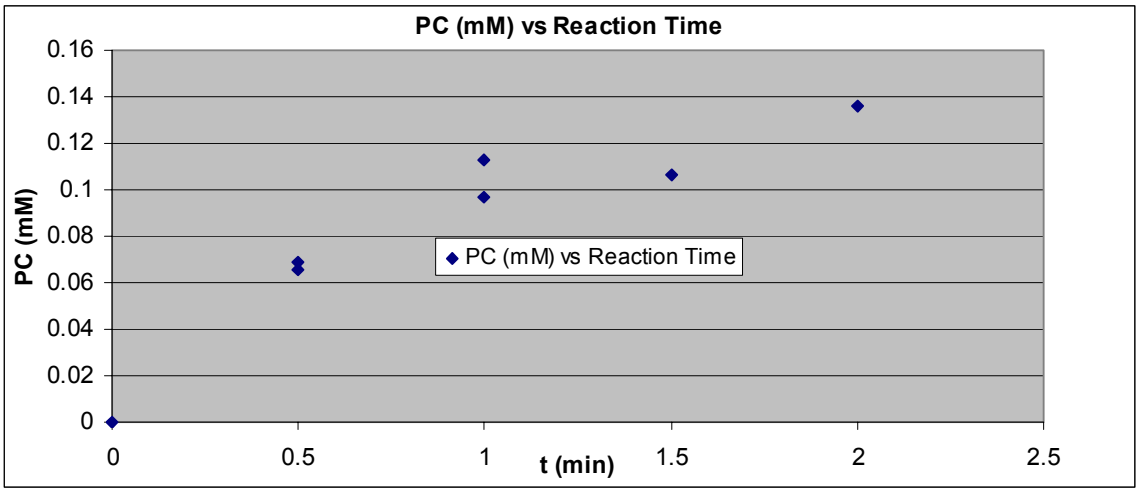


Figure 1. Determination of Linear Reaction Rate Range

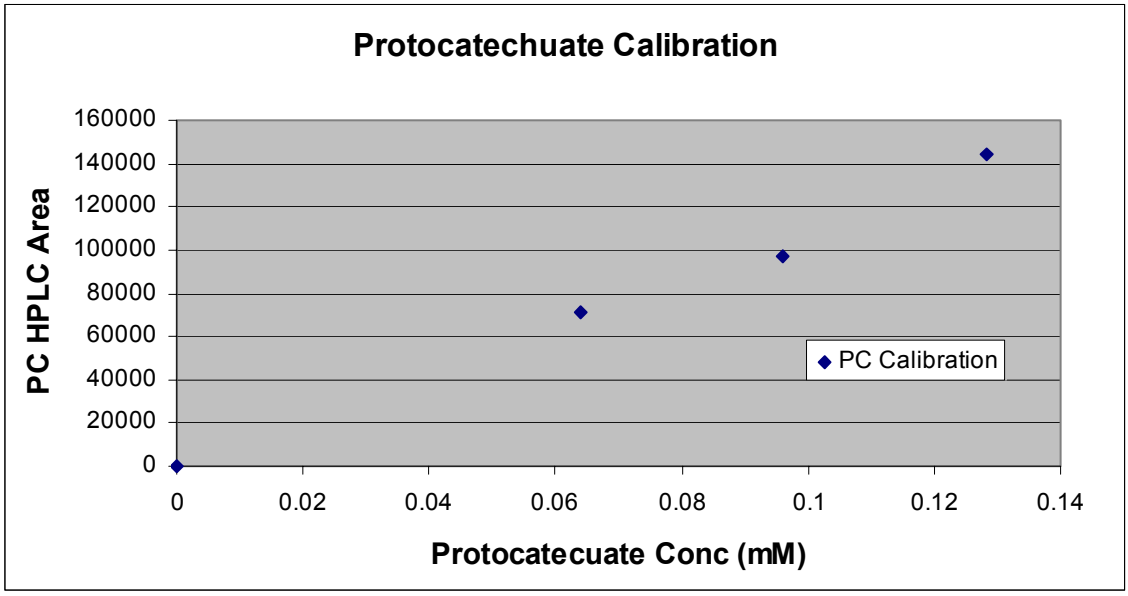


Figure 2. Protocatechuate Calibration Curve

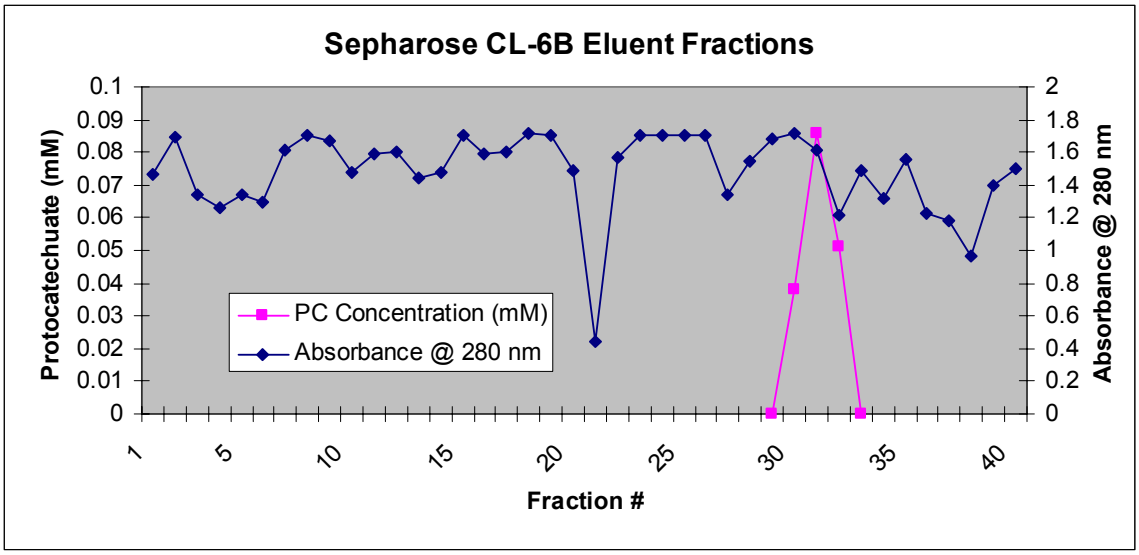


Figure 3. Sepharose CL-6B Eluate Fraction Analysis

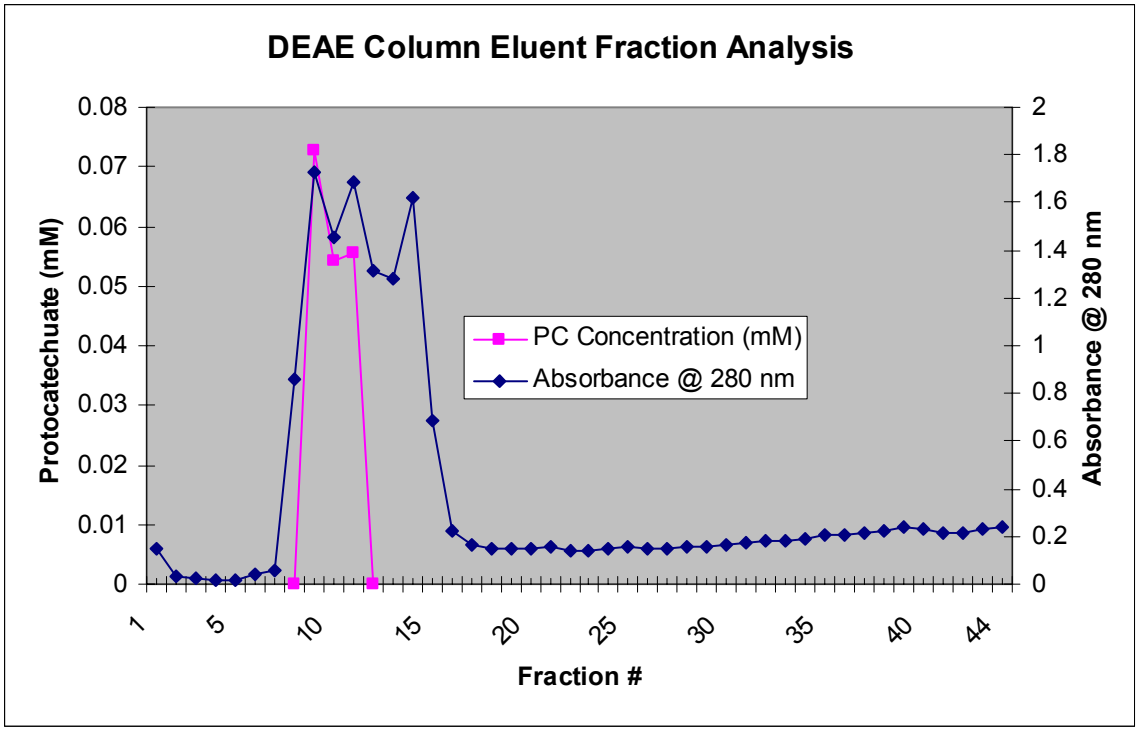


Figure 4. DEAE-Sepharose Eluate Fraction Analysis

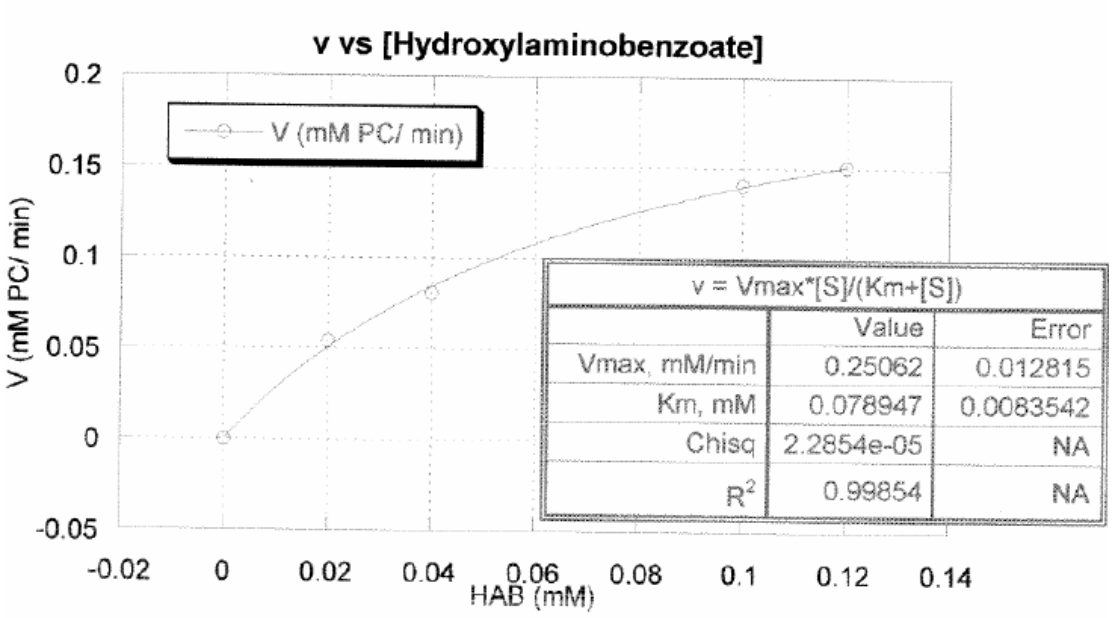


Figure 5. Reaction Rate versus Hydroxylaminobenzoate Concentration

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