CHAPTER THREE

BIOCHEMICAL STUDIES ON LACCASE

3.1 Introduction

Laccase, a class of copper-dependent phenol oxidases, is produced by plants and fungi (Thurston, 1994). It was first characterized from the latex of the Japanese lacquer tree *Rhus vernicifera*. A notable number of fungi are known to produce this enzyme. The most studied laccase appear to be from *Agaricus bisporus, Podospora angerina, Rhizoctonia practicola, Polyporus versicolor, Pleurotus ostreatus* (Von Hunolstein *et al.*, 1986) and *Neurospora crassa* (Germann *et al.*, 1988). Laccase is a glycoprotein. According to Zonazi *et al.* (1987), the carbohydrate moiety of the enzyme may constitute 86-91% of the enzyme by weight.

The pH optimum, pH stability, molecular weight, and isoelectric point of laccase obtained from Novo Nordisk was studied. Kinetic studies were conducted using the crude extract, and one of the partially purified forms of the enzyme from the isoelectric focussing experiment.

MATERIALS AND METHODS

3.2 Materials

Experimental Material

A sample of crude laccase preparation was obtained from Novo Nordisk, Kuala Lumpur, Malaysia.

Instrumentation

Centrifugation was done in a Hercule Model, 2K 401, superspeed centrifuge. Flow rates of chromatographic columns were regulated using a microtubing pump, EYELA pump, Tokyo Rikakai Co. Ltd. (Model MP-3). Fractions eluted from these columns were collected on a Bio-RAD 2110 Fraction collector.

Routine enzyme assays were carried out spectrophotometerically on a Shimadzu Spectrophotometer Model UV-160.

pH determination was carried out using a Hanna microprocessor pH meter (Model HI-8521).

Isoelectric focussing was conducted on an all Glass LKB 8101 column of capacity, 110ml.

Quartz and plastic cuvettes were used to measure absorbance values at ultra violet or visible wave lengths respectively.

Glass chromatographic columns were obtained from Whatman Limited Company.

Chemical Reagents

All chemicals and reagents were of analytical grade and were used without further purification. The list of chemicals is in Appendix A.

3.3 METHODS

General Methods

3.3.1 Preparation of buffers

For routine enzyme assay the buffer system used was 0.1M. sodium citrate buffer pH 5.5

Gel filtration chromatography was carried out in 0.05 M sodium citrate buffer pH 5.5

3.3.2 Preparation of substrate

0.1mM syringaldazine was dissolved in 50% ethanol (Harkin and Obst, 1973). It took about 13 hours to dissolve 0.1mM syringaldazine completely in 50% ethanol with constant stirring.

3.3.3 Standard enzyme assay method

The assay method for the enzyme was adapted from Harkin and Obst (1973) and Leonowicz and Grzywnowicz (1981).

The enzyme activity was assayed by mixing 0.1 ml of a diluted crude extract (25 times dilution) with 0.1M sodium citrate buffer pH 5.5 and 0.1 ml of 0.1mM syringaldazine in ethanol. Total volume of the reaction mixture was 3.0ml. The mixture was mixed well and the rate of change in absorbance at 527nm per min was measured.

One unit of enzyme activity is defined as the amount of enzyme producing an OD change of 1 unit/min/g substrate at 527 nm.

A Typical Laccase Assay

Solutions	Test	Blank (ml)
Buffer 0.1M	2.8 ml	2.9 ml
0.1M syringaldazine	0.1 ml	0.1 ml
Enzyme	0.1 ml	-
Total Volume	**3.0 ml**	**3.0 ml**

3.3.4 Protein Estimation

In the course of this study, two techniques for the estimation of protein were used:

3.3.4.1 Protein estimation by the absorbance at 280 nm

Routine estimation of the protein content of chromatographic fractions were made by measuring the absorbance of each fraction at 280nm. The protein content of each fraction was then estimated by reference to a standard curve constructed from a series of diluted BSA solution from the stock solution of 1 mg/ml.

However, no attempt was made to correct any absorbance due to nucleic acids, phenols, free amino acids, pigments etc. that might be present.

3.3.4.2 Protein determination by the protein dye binding method of Bradford (1976)

The protein reagent was prepared by dissolving Coomassie Brilliant Blue G-250 in 50ml 95% ethanol. To this solution, 10ml 85% (w/v) orthophosphoric acid was added and the resulting solution was diluted to a final volume of 1 litre.

A stock solution of bovine serum albumin (BSA) of concentration 1mg/ml was prepared in distilled water. Appropriate dilutions were made to construct a standard curve of 0-1 mg/ml. To 0.1ml of this diluted BSA stock, 5ml of Bradford reagent was added and the contents mixed thoroughly. Absorbance in 3ml cuvettes against a reagent blank at 595nm was measured after two minutes, but not later than 1hour.

The required dilutions were made to the sample in order to accommodate the absorbance reading within the range of the calibration curve.

3.3.5 Preparation of the crude enzyme

1ml of the crude laccase was diluted with 5.0 ml of 0.1M sodium citrate buffer at pH 5.5. The extract was centrifuged at 13,000 rpm for 15 minutes and the supernatant decanted. The clear reddish pink supernatant obtained (referred to as the crude enzyme hereafter) was subsequently used for all further studies.

3.4 Characterization

All studies were carried out with the crude enzyme.

3.4.1 Effect of pH on laccase activity: pH optimum and pH stability.

pH optimum: Aliquots of the enzyme were assayed for activity using 0.1M buffers of pH values : 3.5 to 6.5 sodium citrate and 7.0 to 9.00 Tris – HCl

pH stability: Equal volumes of crude enzyme extract and buffer solutions of pH ranging from 4.5 to 6.5 were incubated for various intervals of time (0 min, 15 min, 30 min, 60 min, every hour for the first day and every 24 hrs for the next 14 days). The enzyme was assayed at pH optimum of 5.5 in 0.1M citrate buffer.

3.4.2 Temperature stability

The effect of temperature on laccase activity was studied. The enzyme was incubated at 5°C and room temperature (25°C) for two weeks.

3.4.3 Isoelectric focussing

The method of Vesterberg and Svensson (1966) was followed, using an LKB 8101 analytical column.

The 110ml column was mounted vertically and water at 5°C was circulated through the water jacket from a thermostated water bath. A 1% ampholine solution of pH range of 3.5 – 10.00 was used in a sucrose density gradient of 0-50% (w/v).

The surcose density gradient was prepared by mixing different volumes of dense (sucrose) solution and less dense solution. The enzyme sample was used as the less dense solution. In this way, a total of 24 fractions of 4.6 ml each were prepared and transferred to the column by pipetting them in succession starting with the most dense solution. To prevent the carrying ampholites from anodic oxidation and cathode reduction, the electrodes were surrounded by acid (H_2SO_4) and base (ethanolamine) respectively.

Electrofocussing was carried out for 48 hours, at constant voltage of 300V. At the end of the experiment, fractions of 2.0 ml were collected by using a fraction collector. The pH of each fraction was measured immediately at 4^0C, the temperature at which the electrofocussing was conducted. The protein profile was determined by measuring the absorbance at 280 nm of each fraction.

3.4.4 Molecular Weight Determination

The molecular weight of the crude laccase was determined by gel filtration chromatography on Sephadex G-100.

The gel was packed into a vertical glass column (diameter 2.5cm , length 100cm) and equilibrated with starting buffer, 0.05M citrate buffer pH 5.5 at 5^0C $\pm$ 1^0C using descending flow of 13.5ml per hour and collecting 4.5ml fractions. Standard proteins of known molecular weights were eluted through this column and their elution volumes were used to plot the calibration curve. The proteins used to calibrate the column for apparent molecular weight determination were bovine

serum albumin (69,000), cyctochrome-c (12,300), carbonic anhydrase (29,000) and transferrin (81,000). Blue dextran was used to determine the void volume of the column.

The following formula was used:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where,

K_{av} represents the fraction of the stationary gel volume which is available for diffusion of a given solute species.

V_t = the bed volume of the gel.

V_e = the elution volume of the sample

V_o = the void volume

K_{av} was plotted against log molecular weight of each standard protein to obtain a calibration curve. The elution volume of laccase obtained in this column was used to calculate its K_{av} value and its molecular weight read off from the calibration curve.

3.4.5 Kinetic Studies

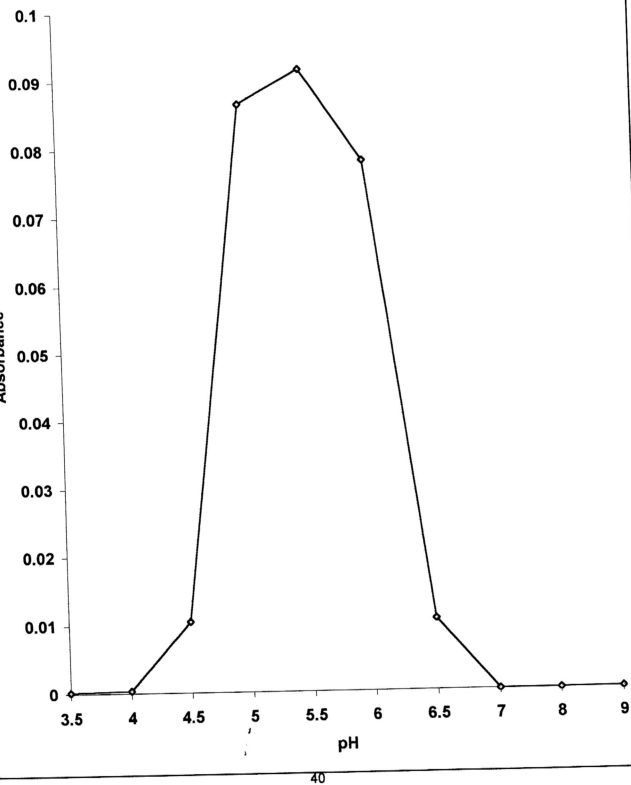
Kinetic studies were carried out on the crude and partially purified peaks from isoelectricfocussing in different concentrations of syringaldazine ranging from 0.6μM to 6.6μM at a temperature of $25^0C \pm 1^0C$ in 0.05M citrate buffer pH 5.5.

3.5 RESULTS AND DISCUSSION

pH Optimum

The crude supernatant prepared as described in the text was used to estimate the optimum pH. When activity of laccase was estimated between pH 3.5 to 9.0 maximum activity was detected at pH 5.5 as shown in the Fig. 3.1. This pH is almost close to the pH optimum of laccase obtained from the white rot basidiomycete *Trametes villosa* (Yaver *et al.*, 1996). The three isoforms of this crude laccase have optimal activities at pH 5.0 to 5.5 using syringaldazine as a substrate. The pH optimum studies using the above substrate was also performed for the laccase in *Botrytis cinerea* (Dubernet *et al.*, 1977). The optimum pH for the oxidation of 4-methyl catechol and quinol was 4.7 and oxidation of a mixture of anthocyanins extracted from wine showed an optimum at pH 4.0. Most laccases from the fungi are unstable at alkaline pH. The optimum pH of laccase from *Rhus* depending on the species shows quite a considerable variation, being between 6.4 to 7.5 (Omura, 1961). The optimum pH of *Ganoderma* laccase was around 5.4 (Froehner and Eriksson, 1974). The optimum pH of laccase from *Chaetomium thermophilium* was 6.0 (Chefetz *et al.*, 1998). In contrast to most fungal laccases, the *Chaetomium thermophilium* laccase was found to be active and stable in alkaline pH values between 5 to 10.

Fig 3.1 : Optimum pH of laccase



pH Stability at 5°C and 25°C

Since the pH optimum for this enzyme was 5.5, the stability of this enzyme was investigated between pH 4.5 and 6.5 at both 5^0C and 25^0C. In this study the enzyme was preincubated in buffers ranging from 4.5 to 6.5 and aliquots removed at the specified time intervals and the enzyme activity was assayed at optimum pH. When the enzyme was preincubated in pH 4.5 at 25°C, it retained about 10% activity four days and it gradually declined with time (Fig. 3.2). At 5^0C, the enzyme retained about 30% activity after four days of incubation. From Fig. 3.3, it is shown that at 5^0C, pH 5.0 the enzyme lost almost 90% of its activity after four days. Further, for the same pH at 25^0C, the enzyme lost about 90% of its activity after four days. In buffer of pH 5.5, the enzyme lost almost 90% after four days at both 5^0C and 25^0C as shown in Fig. 3.4.

In pH 6.0 the enzyme lost all its activity after four days at 25^0C as shown in Fig. 3.5.

It lost about 80% activity after 4 days and gradually decreased at 5^0C. In pH 6.5, the

enzyme lost 95% of its activity by the fourth day in 25^0C. At 5^0C, for the same

period of time the enzyme lost about 70% of its activity as shown in Fig. 3.6. The enzyme had highest activity within twelve hours after thawing at room temperature. At pH 5.5, it retained about 70% of its activity after 24 hours. It lost about 95% of its activity at pH 6.5 after 96 hours (4 days) at room temperature. This is also true for pH 5.0 and 5.5. At 5°C, the enzyme appeared to lose about 70%

Fig 3.2 : Stability of Laccase at 25°C and 5°C in 0.05M sodium citrate buffer pH 4.5

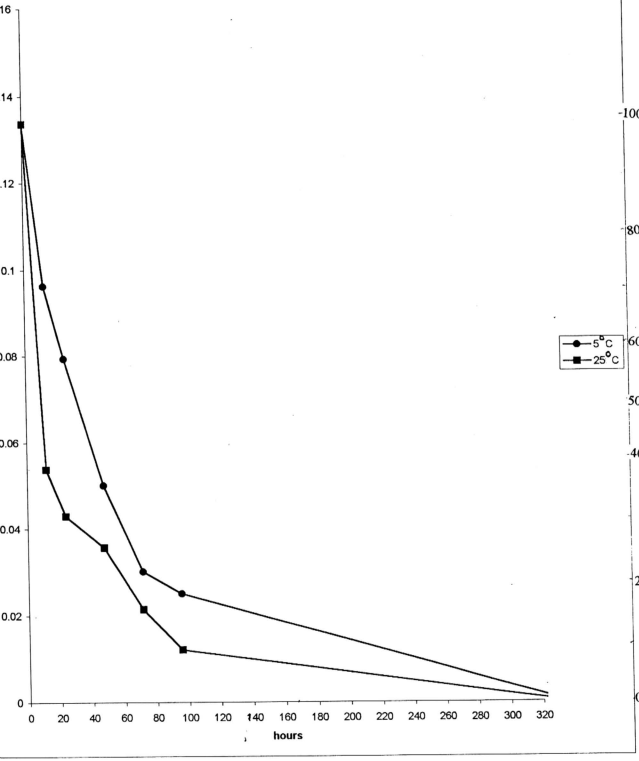


Fig 3.3 : Stability of laccase at 25°C and 5°C in 0.05M sodium citrate buffer pH 5.0

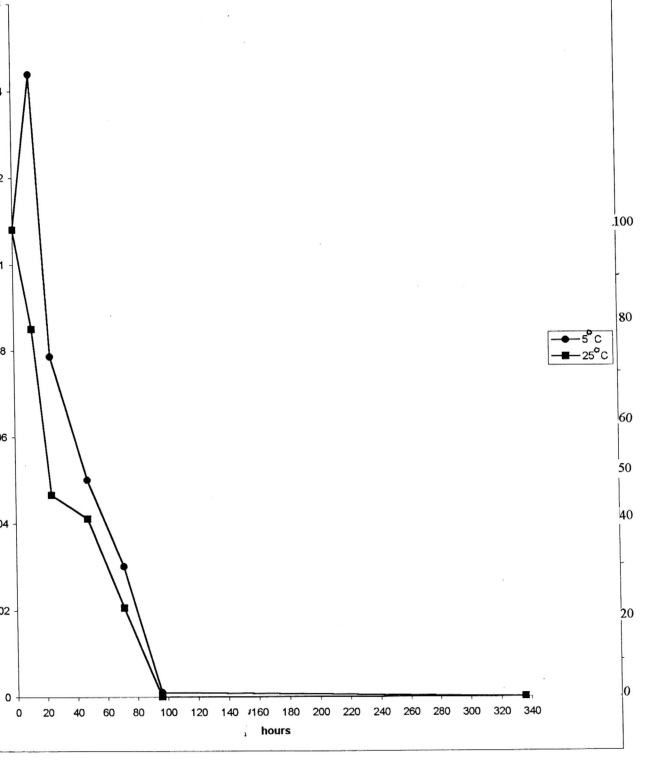
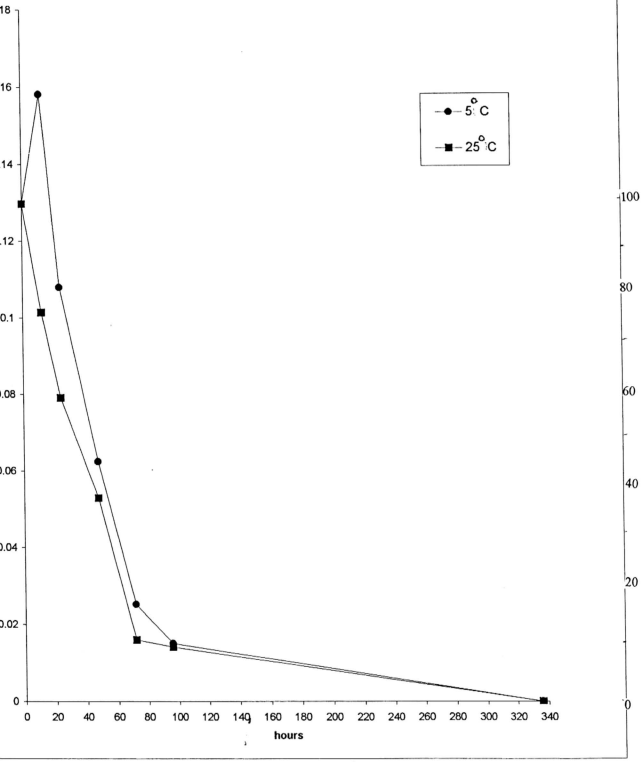


Fig 3.4 : Stability of laccase at 25°C and 5°C in 0.05M sodium citrate buffer pH 5.5



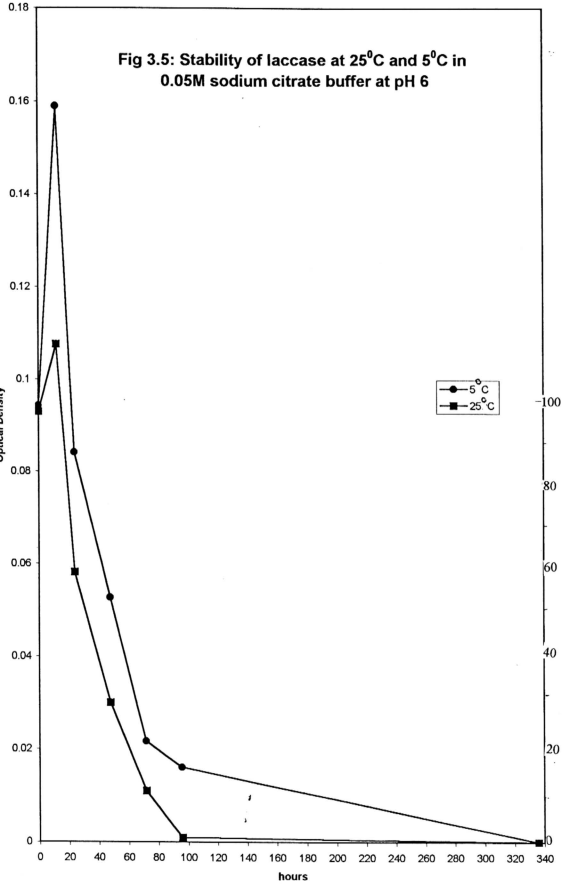
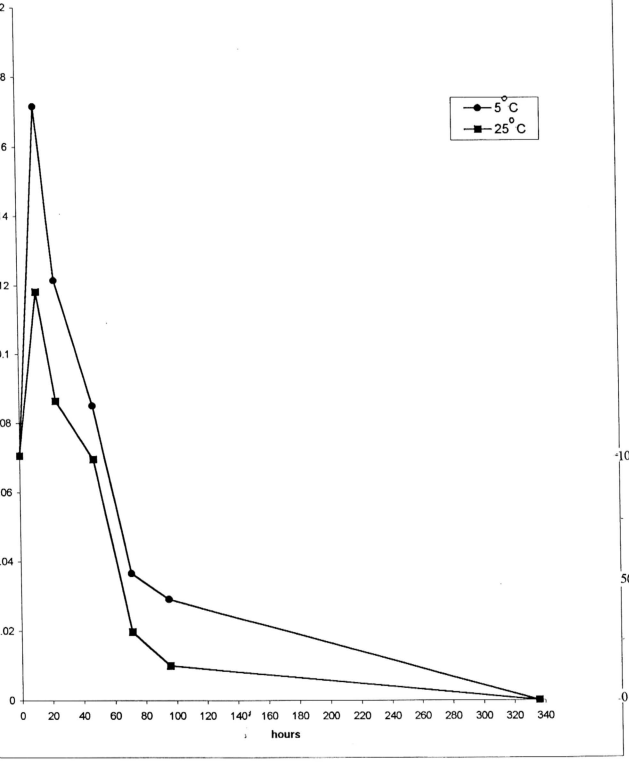


Fig 3.5: Stability of laccase at 25°C and 5°C in 0.05M sodium citrate buffer at pH 6
Optical Density
% relative activity
hours
5°C
25°C
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
100
80
60
40
20
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340

Fig 3.6: Stability of laccase at 25^{0}C and 5^{0}C in 0.05M sodium citrate buffer pH 6.5



80% of activity after four days. After 2 weeks the enzyme was inactive at 5°C as well as room temperature condition.

Unlike most fungal lacasses which are generally active and stable in low pH values (Slomezynski *et al.*, 1995), the *C. thermophilium* laccase exhibited stable activity at pH 5 to pH 10 (Chefetz *et al.*, 1998). This laccase is produced during the thermophilic stage of composting and can remain active at alkaline pH values.

Molecular weight Determination by Gel Chromotography, G-100

Calibration of a column (diameter 2.5cm, length 100cm) was done using BSA, cyctochrome-C, carbonic anhydrase and transferrin. The single peak laccase activity had an apparent molecular weight of 79,000 Daltons as shown in Fig. 3.7. The crude laccase was prepared as described in the text. The apparent molecular weight of the laccase was determined by gel filtration. About 2.0 ml of the concentrated homogenate was applied to a G-100 gel column (Diameter 2.5cm , length 100cm , bed volume 410.0 ml). The column was equilibrated with citrate buffer pH 5.5 at 5°C + 1°C, using descending flow at rate of 13.5ml per hour and collecting 4.5ml fraction. The proteins used to calibrate the column were bovine serum albumin (69,000), cyctochrome-C (12,300), carbonic anhydrase (29,000) and transferrin (81,000). Blue dextran was used as the void volume marker.

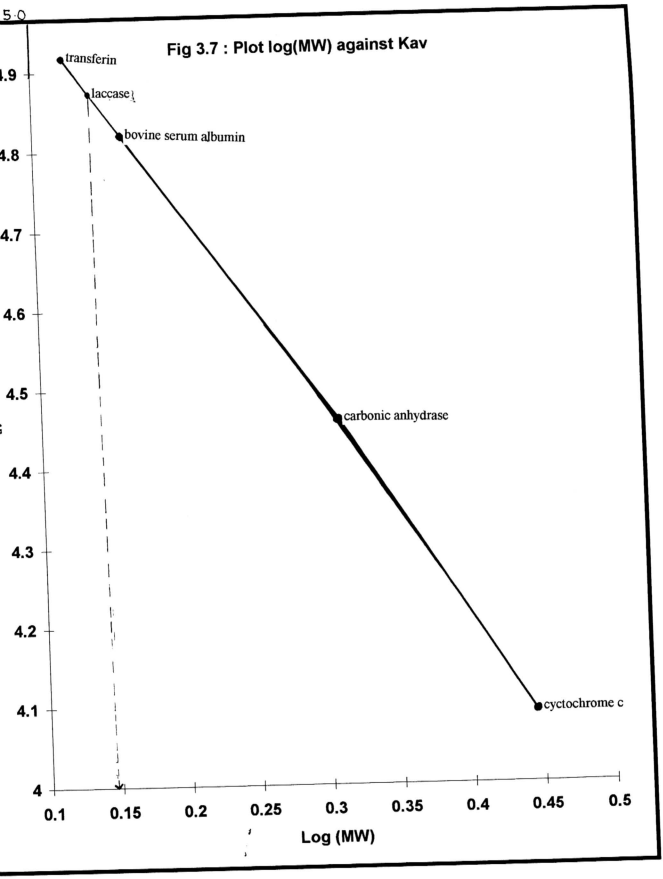
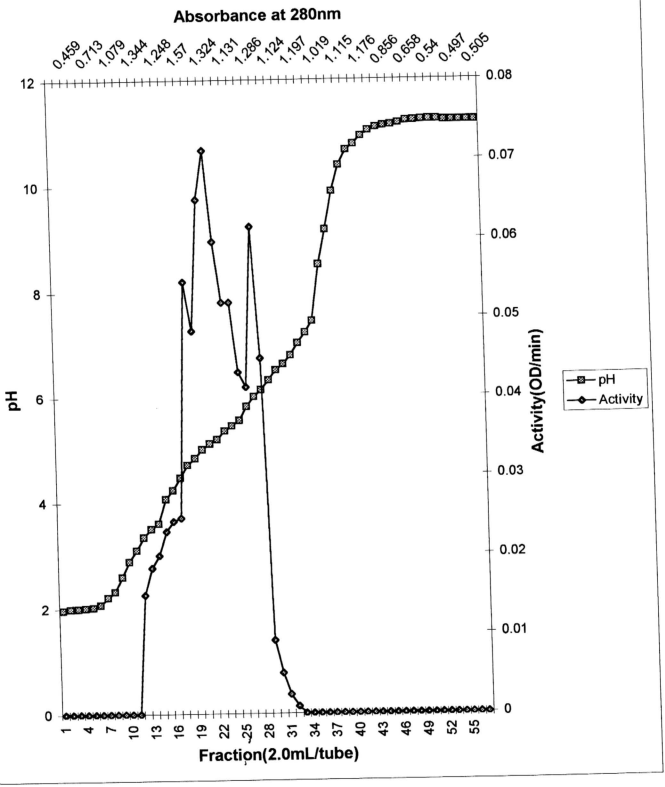


Fig 3.7 : Plot log(MW) against Kav
transferin
laccase
bovine serum albumin
carbonic anhydrase
cyctochrome c
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
Log (MW)

the crude extract which was brownish pinkish in colour. The molecular weight of this single peak was around 79,000 Daltons.

Laccases are a heterogenous group of glycoproteins having a basic structural unit of between 50–70,000 Daltons melecular weight which can undergo aggregation to give larger units. The laccase I of *Podospora* contains four such subunits (Molitoris *et al.*, 1972). The molecular weight of laccases from various sources differ considerably. Laccase from the latex of the Japanese lacquer tree (*Rhus vernicifera*) was reported to be close to 120,000 Daltons by Reinhammar (1970). A majority of laccases from basidiomycete fungi were reported to have molecular weight in the range of 55,000 Daltons to 72,000 Daltons (Yaropolov *et al.*, 1994), but several forms of laccases from the white rot fungus *Trametes versicolor* were reported to be 43,000 Daltons to 66,000 Daltons (Milstein *et al.*, 1989) and five isoforms of *Schizophyllum commune* laccase ranged in molecular weight from 36,000 Daltons to 48,000 Daltons (De Vries *et al.*, 1986). Laccase from peach was reported to have a molecular weight of 73,500 Daltons by Lehman *et al* (1974) while the molecular weight of *Neurospora* laccase is 64,800 Daltons (Froehner *et al.*, 1974). The molecular weight of laccase from *Lentinus edodes* a commercially cultivated mushroom has a molecular weight of 100,000 Daltons (Leatham and Stahmann, 1981). However, it is not known if this enzyme is a monomer or a dimer. Dedeyan *et al.* (2000) have characterized the laccase from

Fig 3.8: Isoelectric points of laccase



Marasmius quercophilus to be a monomeric glycoprotein of approximately 63,000 Daltons.

Isoelectric Focussing Studies

When a sample of crude enzyme was electrofocussed in ampholine of pH range from 3.5 to 10, three peaks were obtained (Fig. 3.8) and the pI were estimated to be 4.7, 5.2 and 6.0.

The laccase partially purified from the fungus *Botrytis cinerea* however, had a very low isoelectric point of 2.5 (Dubernet *et al.*, 1977)

Laccase from the white rot basidiomycete *Trametes villosa* was purified and was found to have three forms. Purified laccase forms 1 and 3 have pIs of 3.5 and 6.0 to 6.5 respectively. The second form of laccase has a pI in the range of 5 to 6 (Yaver *et al.*, 1996). These laccases were found to have optimal activity at pH 5 to 5.5 with syringaldazine. The isoelectric point of laccase from *Chaetomium thermophilium* was 5.1 (Chefetz *et al.*, 1998). Laccase from *Marasmius quercophilus* was found to have an isoelectric point of 3.6 (Dedeyan *et al.*, 2000).

K_m studies on crude and partially purified peaks from isoelectrifocussing

Kinetics studies were conducted on both the crude enzyme and the partially purified peaks from isoelectricfocussing. The effect of substrate concentration on laccase activity was expressed as Lineweaver- Burk plots.

The K_m for the crude enzyme was 5.0μM (Fig. 3.9) while that for the partially purified enzyme was 2.0μM (Fig. 3.10). It is interesting to note that the K_m value

cinerea (Dubernet *et al.*, 1977) did not change during purification. The crude laccase from *Marasmius quercophilus* (Dedeyan *et al.*, 2000) had a K_m of 7.1 μM.

In this study it is possible that an endogenous inhibitor was removed in the crude preparation during electrofocussing and therefore the partially purified enzyme was able to function better in its absence. Further work is necessary to confirm this. Unfortunately it is difficult to do similar kinetic studies on immobilized enzyme or entrapped enzymes as the quantity of enzyme in each enzyme assay cannot be precisely determined. More work is necessary to determine if immobilization or entrapment using purified enzyme preparation will improve its catalytic efficiency.

Fig: 3.9
Lineweaver Burk plot for crude enzyme

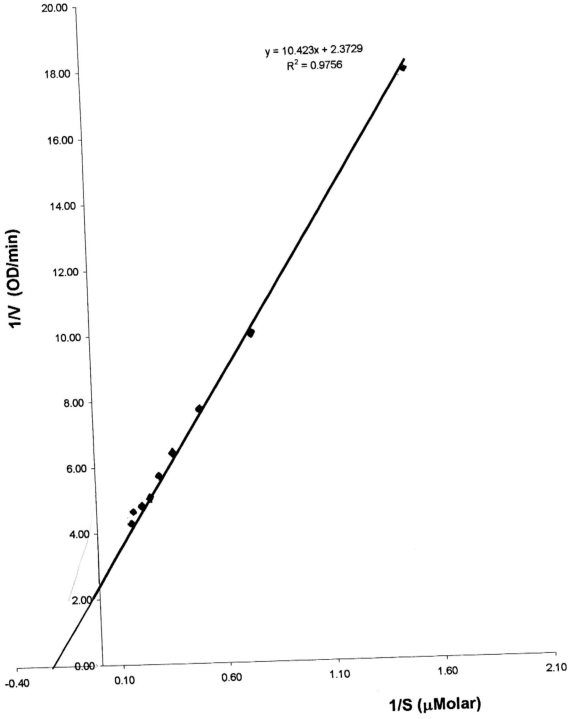


Fig: 3.10

Lineweaver Burk plot for partially purified enzyme from IEF