CHAPTER III

METHODS OF EXPERIMENTS

3.1 Extraction of laccase from Haed Nangrom (*Pleurotus sapidus*)

It was extremely difficult to find a genuine crude latex of lacquer trees. It was then decided that the source of laccase would be the common cultivated mushroom available from Nakornpathom market. The mushrooms must be white fresh and as small and young as possible. A total of 1.5 kg of the mushroom was pounded in 300 ml. 0.01 M. sodium phosphate buffer, pH 6.0 using a blender. The homogenate was filtered through cheesecloth. Then separated other residuals by centrifugation at x8000 g. for 20 min 4°C using a refrigerated centrifuge. Discarded the precipitate and a final clear supernatant of 760 ml. was a precipitated by 80 % saturated ammonium sulfate and left overnight at 4°C. Precipitate was separated by centrifugation and then the buffer was added and adjust to a total volume to 245 ml. The excess salt and low molecular weight components were removed from the enzyme by dialysis. The dialysis tube used has a molecular weight cut off 12,000 daltons which does not allow molecules larger than 12,000 daltons to pass through. Dialysis was carried out overnight and the buffer was changed three times (Figure 3.1). To remove water from the swollen dialysis bag, the bag was put in a beaker then covered with PEG (M.W. 35,000-40,000), which absorbed water from the dialysis bag. The concentrate sample was subjected

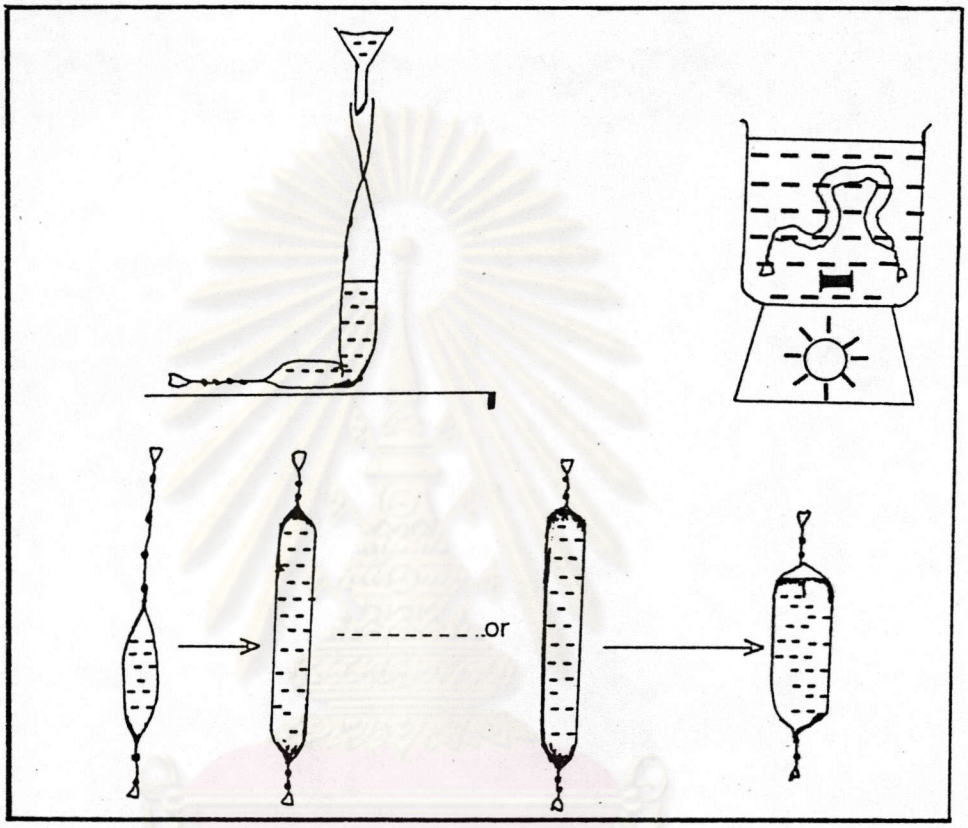

FIGURE 3.1: The use of the dialysis bag,
(a) filling with sample,
(b) agitation during dialysis,
(c) results of swelling due to osmotic forces.

to further purification using ion exchange chromatography.

3.2 Ion exchange chromatography using DEAE-Sephadex A-50

DEAE Sephadex A-50 was swollen in 0.01 M. sodium phosphate buffer, pH 6.0 for 2 hours in a boiling water bath with a change of fresh buffer several times during the swelling period. The swollen gel was exhaustively deaerated before packing in a column of 2.5x40 cm. The column was equilibrated with the same buffer before use. The concentrate laccase from dialysis was loaded onto a DEAE Sephadex A-50 column (2.5x40 cm) and washed with 3 bed volumes of 0.01 M. sodium phosphate buffer, pH 6.0. The eluting gradient was started with 0.1 M. sodium phasphate buffer to 0.5 M. sodium phosphate buffer, pH 6.0 gradients. The elution rate was 1.32 ml./min and a total volume of 5.5 ml.was collected in each fraction. Totally 80 fractions were collected. Estimation of protein in each fraction was done by measuring absorption at 280 nm and laccase activity was assayed as described in 3.5. The laccase activity peak from DEAE Sephadex A-50 column was pooled and then concentrated by ultrafiltration using membrane PM-10 having M.W. cut off 10,000. The volume of concentrated sample was further reduced using the dialysis bag/PEG system to a final volume of 10 ml.

3.3 Gel permeation chromatography of laccase using Sephadex G-100

Sephadex G-100 was swollen in 0.01 M. sodium phosphate buffer, pH 6.0 for 5 hours at 90°C. The swollen gel was deaerated before packing in a column of 1.6x100 cm. The column was equilibrated and washed with 0.01 M. sodium phosphate buffer, pH 6.0 before use. The

concentrated sample of laccase was mixed with blue-dextran before loading onto the Sephadex G-100 column. After loading the column was eluted with 0.01 M. sodium phosphate buffer, pH 6.0 and the chromatography was carried out at a flow rate of 4.9 ml./10 min. Fractions of 4.9 ml. were collected, protein profile was monitored by the measurement of the absorbance at 280 nm. The laccase activity was assayed using syringaldazine as a substrate. The laccase peaks were pooled then converted to a lyophilized power using Dynavac lyophilizer and kept at -20°C for later used. The overall scheme for purification is shown in Figure 3.2.

3.4 Polyacrylamide gel electrophoresis of laccase

Electrophoresis was performed according to the method of Williams and Reisfeld [40]. Using a disc gel consisting of 2 sections, namely, the separating gel and the stacking gel. The separating gel has a smaller pore size and consisted of 5 ml. of 30 % w/v polyacrylamide 0.8 % w/v N,N'-methylene bis acrylamide, 0.1 ml. of 10 % v/v of TEMED in 0.5 M. Tris HCl, pH 8.9 and 0.1 ml. of 5 % ammonium persulfate. The later was added to polymerize the gel. Capped the tubes at their lower ends with parafilm and inserted the tubes in the stand. Place the tubes upright, and level the polymerizing stand (Figure 3.3 A). Used a pasteur pipette to fill all tubes to approximately 7 cm from the bottom of the glass tubes taking care to avoid trapping air bubbles (Figure 3.3 B). Witha pipette carefully layer 0.2-0.5 ml. of distilled water over the gel (Figure 3.3 C) then allow polymerization to proceed at room temperature for at least 1-2 hours. After the separating gel was polymerized poured off

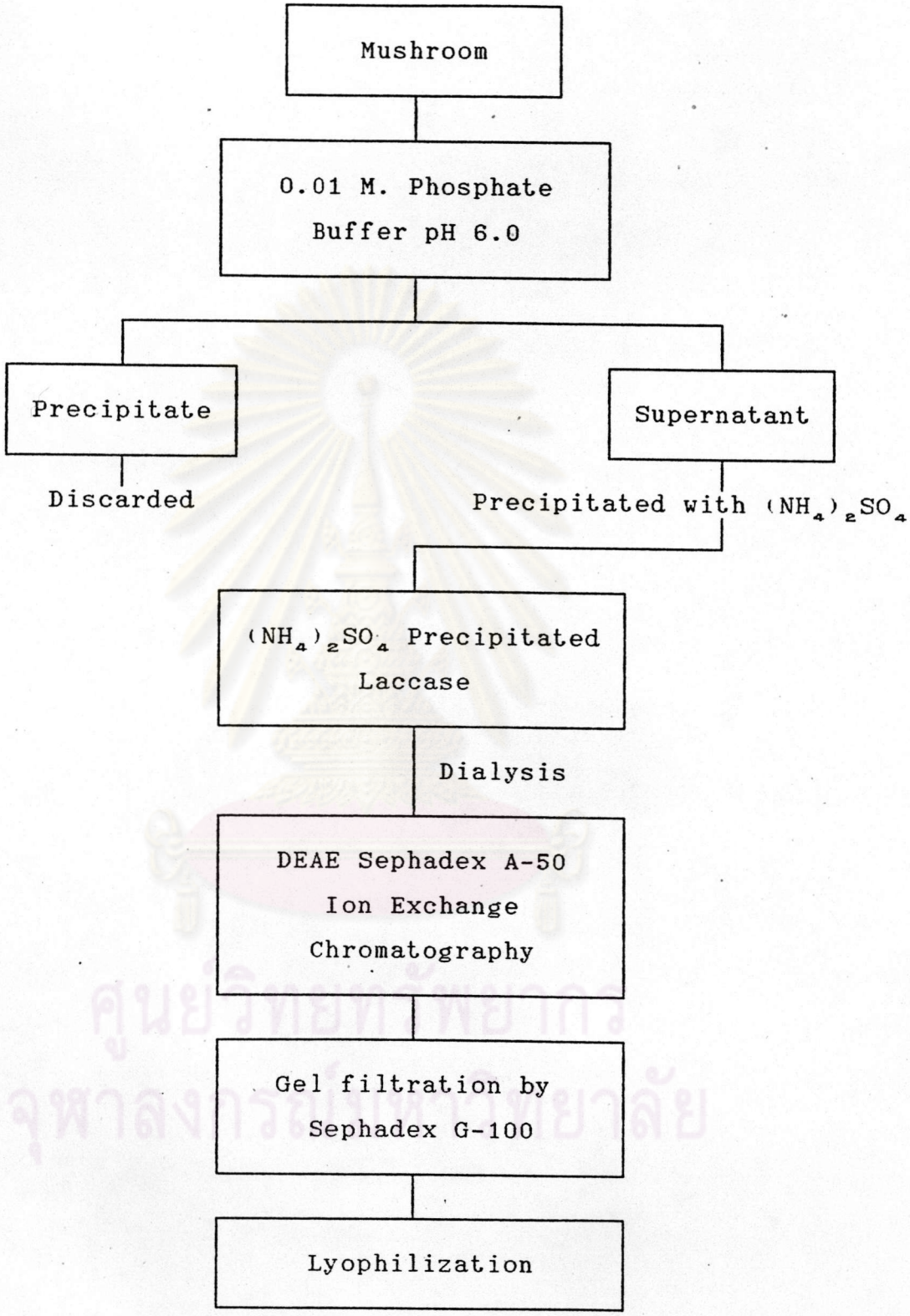

FIGURE 3.2: Flow chart outlining steps involved in the purification of laccase.

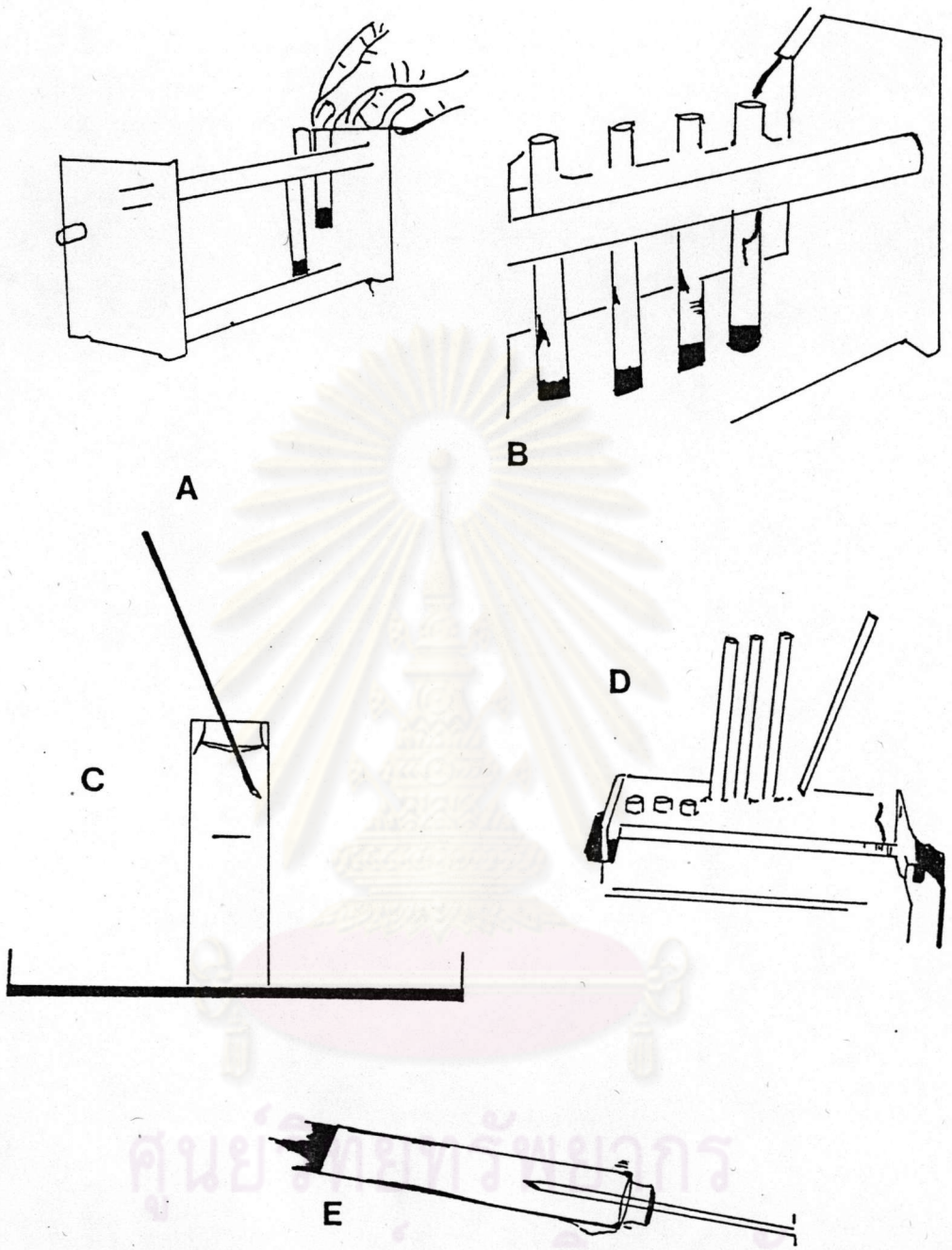

FIGURE 3.3: A-D Casting gel rods with gel rod casting stand.

the water layer above the gel then added the stacking gel. The stacking gel had a pore sizes and consisted of 0.625 ml. of 30 % w/v polyacrylamide. 0.8 % w/v N,N´-methylene bis acrylamide, 0.05 ml. of 10 % v/v of TEMED in 0.5 M. Tris HCl, pH 6.7, and 0.02 ml. of 5 % ammonium persulfate. The later was added to polymerize the gel. All gels were overlayed with distilled water (Figure 3.3 C) then allowed the polymerization to go to completion for at least 1-2 hours. Water was then poured off, carefully removed the parafilm from the end of the tubes. The gel was then ready to be used.

20 μl of the sample was mixed with 10 μl of 40 % sucrose having bromophenol blue as dye marker. Twenty microlitres of the mixtured was loaded onto the gels. The gels were electrophoresed at 4°C with constant current of 20 mA in a vertical electrophoresis apparatus using 0.5 M. Tris glycine as buffer. The electrophoretic migration proceeded from the anode towards the cathode.

After electrophoresis was completely removed the gels from the glass rods by rimming with water-filled syringe with a narrow gauge, long needle (Figure 3.3 D). The gel were stained for proteins using 0.1 % Commassie Blue R-250 in 25 % methanol - 10 % acetic acid (v/v/v) for 2 hours. The gel was then destained in the destaining solution (7 % acetic acid in distilled water) until the clear background was obtained. The duplicate gel was also stained for laccase activity using syringaldazine as substrate.

3.5 The assay for laccase

Prepared solutions of 0.081 mM syringaldazine (N,N´-bis-3,5-dimethoxy-4-hydroxybenzylidene) by adding

10 mg. of syringaldazine in 100 ml. of 95 % ethanol. Mixed 3 parts of the syringaldazine in 95 % ethanol with 7 parts of 0.01 M. sodium phosphate buffer, pH 6.0 just before use. The assay of laccase was started by adding 20 μl of each purified step into 3.5 ml. of syringaldazine substrate. Laccase activity was measured using the time-scanning mode of the Shimadzu UV-spectrophotometer model 240. The reaction was carried out at 25°C. The change in absorbant at 530 nm was continuously recorded and the initial velocity of the reaction was calculated from slope of the curve. One unit of enzyme activity is defined as a change in A_{530} of 0.001 mM per min. A typical curve for the assay of laccase is shown in Figure 3.4. The protein of enzyme samples were determined by lowry method [41].

3.6 The determination of protein by the lowry method

Protein concentration was determined by the method of Lowry et al [41] using bovine serum albumin as a standard. Prepared the following reagents for a routine use.

(A) 2% Na_2CO_3 in 0.1 M. NaOH.

(B) Mix equal parts of 1.0 % $CuSO_4.5\ H_2O$ and 2 % Sodium or potassium tartrate (stable for limited period only approximate 1 week).

(C) Mix 50 ml. of reagent A with 1 ml. of reagent B.

(D) Folin Ciocalteu Phenol Reagent.

(E) Mix 1 part of reagent D with 2 parts of distilled water.

(F) Standard protein, using bovine serum albumin (1 mg./ml.)

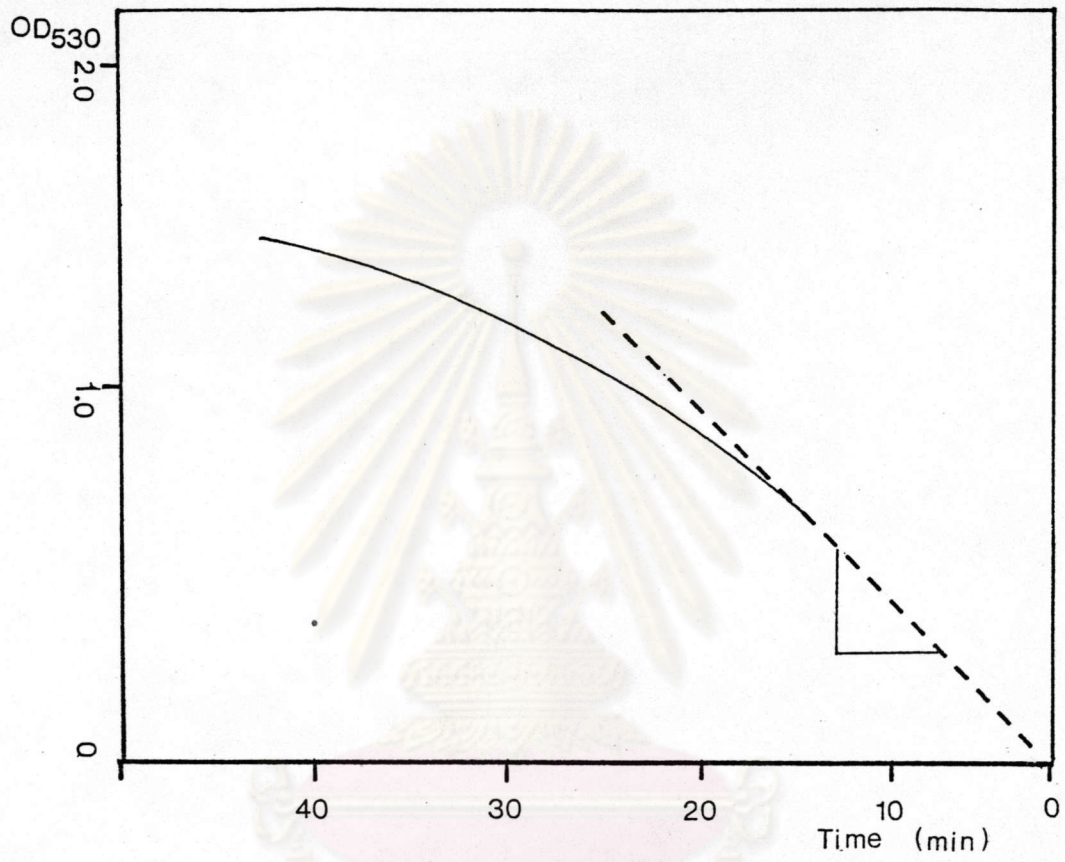

FIGURE 3.4: The continuous assay of laccase using the time-scanning mode of Shimadzu UV-visible spectrophotometer. The increase in absorbance was followed at 530 nm. The activity of laccase was calculated from the slope of the curve.

Prepare standard and unknowns in the range of 10 to 200 μg of protein. Adjusted the volume to 0.2 ml. with water. Added 1 ml. of reagent C, mixed well and allowed to stand for 10 min or longer at room temperature. Added 0.1 ml. of reagent E rapidly with immediate mixing. After 30 min read the absorbancies at 500 nm using the Jasco UV-visible spectrophotometer model Uvidec-4. The protein concentrations of the unknown samples were determined from a standard curve.

3.7 Spectralproperties of partially purified laccase and commercially available Pyricularia oryzae laccase.

Dissolved 1 mg. of laccase in 1 ml. of 0.01 M. phosphate buffer, pH 6.0. Then added 500 μl of dissolved laccase to 3.5 ml. 0.01 M. phosphate buffer, pH 6.0 in the sample cuvette. Use only buffer in the reference cuvette. Carried out the wavelength scan using the "sequential scanning" mode of the Shimadzu UV-visible spectrophotometer model 240.

3.8 Studies on partially purified laccase

3.8.1 The effect of temperature

The partially purified laccase was tested for heat stability by incubating the purified laccase at various temperatures from 4°C to 100°C for 2 hours. The remaining activity was assayed as described in section 3.5. The activities at each temperature were expressed as percentage of the activity of laccase observed at 4°C.

3.8.2 The determination of optimum pH for reaction

The experiment was performed by preparing syringaldazine is buffers having pH's ranging from 3 to 8.

The assays were carried out at 25°C using the continuous assay system as described in section 3.5. The activities at different pH's were expressed as percentage of the highest activity.

3.8.3 Catalytic actions of laccase on catechol and hydroquinone

The purified laccase was tested for its ability to catalysed the oxidation of hydroquinone and catechol as the followings. Prepare 1 mM. catechol by dissolving 0.011 gm. of catechol in 100 ml. of 0.01 M. sodium phosphate buffer, pH 6.0. Also prepared 0.001 mM. hydroquinone by dissolving 0.001×10^{-3} gm. of hydroquinone in 100 ml. of the same buffer. The reaction were started by adding 100 μl of Lyophilized laccase to 3.5 ml. of hydroquinone solution in a cuvette positioned in the sample compartment. Use buffer in the corresponding reference compartment.

The oxidation of hydroquinone was monitored through the wavelength sequential scanning mode of the Shimadzu UV-visible spectrophotometer model 240. The scannings were carried out after 20, 30, 40, 45 and 50 min of reaction. The oxidation of catechol was studied in a similar fashion but 500 μl of laccase was used. The extend of oxidation was checked at 5, 8, 11, 13 and 15 min.

3.9 Immobilize laccase on Amberite IRA-68

Two grams of Amberite IRA-68 (Weakly basis anion exchanger, capacity > 1.6 mequiv/ml.) was first mixed with 10 ml. of epichlorohydrin in MeOH-H_2O (1:1) and incubated with shaking water bath at 37-39°C for 15 hours to allow enough time for epichlorohydrin to react with the Amberite. The resins were washed with 2 volumes

of 20 ml. water then with 1 volume of 20 ml. phosphate buffer, pH 6.0 to remove any excess epichlorohydrin.

After the activation procedure the activated resins were suspended in 10 ml. phosphate buffer, pH 6.0. 2 mg. of crude laccase in 1 ml. (2 mg./1 ml.) was added into this suspension of activated resins and incubated at 37-39°C for 4 hours to allow laccase to be attached the epichlorohydrin-activated resin. The enzyme-bound resin was washed with 2 volumes of 20 ml. 0.01 M. NaCl to remove laccase that might associated with resin by other means. Finally washed with 2 volumes of 20 ml. of distilled water and then filtered off, the immobilized enzyme was ready for use.

3.10 Determination of the time-curve of reaction of immobilized laccase.

0.2 gm. of the enzyme-resin was added into 5 ml. of syringaldazine substrate and incubated at 37°C with continuous shaking in a water bath from 10-80 min. The oxidized product was measured spectroscopically at 530 nm. The absorbance at 530 nm was plotted against the time of reaction.

3.11 The test for the stability of immobilized laccase

3.11.1 The batch system

0.2 gm. of the immobilized laccase-resin was placed in a 25 ml. flask. Then 5 ml. of syringaldazine substrate was added to the enzymes. Incubation was carried out at 37-39°C for 40 min with continuous shaking. The coloured product was filtered off and measured at 530 nm. This some immobilized laccase-resin was washed with 2 volumes of 10 ml. of 0.01 M. phosphate buffer, pH 6.0 and

used to catalyse the reaction again and again. The process was repeated 8 times.

3.11.2 Continuous column system

Prepared 7 gm. of the immobilized enzyme-resin then added 10 ml. 0.01 M. phosphate buffer, pH 6.0. Loaded the suspension into the column of 10 ml. amd allowed the resin to pack in the column. The syringaldazine substrate was passed through the column with peristaltic pump connected through the bottom of the column. The solution was, therefore, forced against the gravity. The coloured product emerged from the top through a fine silicone tube. The product was measured at 530 nm. The set up of the continuous system is shown in FIGURE 3.5.

3.12 Immobilize laccase by entrapment within the polyacrylamide gel.

Another procedure for immobilizing enzymes has tested. This was based on the technique used in the conventional bead-polymerization process [42]. In this process an aqueous solution of the enzyme laccase and acrylamide monomers is dispersed in a hydrophobic phase before the polymerization is initiated.

In a typical experiment 60 mg. of crude laccases were dissolved together with 8.55 gm. of acrylamide and 0.45 gm. of N,N´-methylene bis (acrylamide) in 0.01 M. phosphate buffer, pH 6.0 having a total volume of 60 ml. After the addition of the catalyst system, namely, 0.5 ml. of TEMED and 0.25 gm. of ammonium persulfate dissolved in 0.5 ml. of the above buffer, the solution was poured into a 1000 ml. flask. The solution flask is contained 400 ml. of organic phase (toluene-chloroform, 290:110, v/v) [42] and 30 ml. of the stabilizer methocel F-50. The solution of

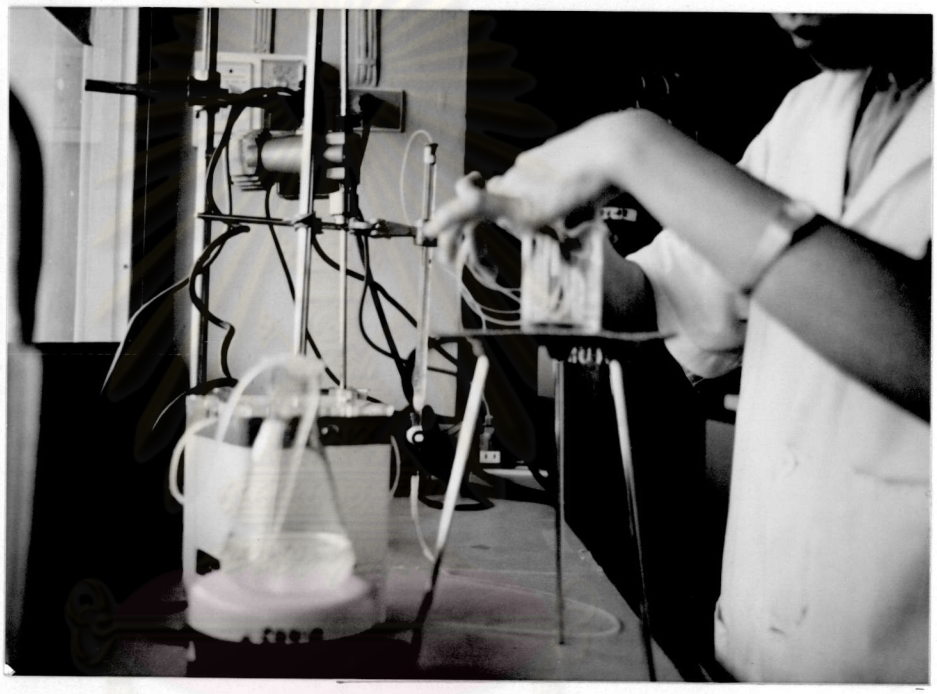

FIGURE 3.5: A set up of continuous column system for the assay of immobilize laccase-Amberite IRA-68. The substrate was forced through the column against the gravity by the use of a peristaltic pump.

methocel F-50 was prepared by adding 1 gm. of methocel F-50 in 30 ml. of boiling water. The solution was continuously stirred using a magnetic stirrer. It should be noted that the stirring with magnetic stirrer must be done prior to the addition of the monomer enzyme solution. The polymerization reaction was carried out at room temperature under N_2. The laccase-entrapped polyacrylamide beads formed after 20 min.

The gel beads was filtered on a buchner funnel and washed twice with toluene to remove traces of chloroform and were stirred for 30 min at 4°C, using the following solution in sequence 0.1 M. $NaHCO_3$, 1 mM. HCl, 0.5 M. NaCl and water. This was done to ensure the removal of laccase not properly entrapped. The "laccase beads" were then ready for use but could be freeze-dried until required. The freeze-dried polymer is pulverized in a motar and sieved. Particals passing 20 mesh (0.85 mm) to 30 mesh(0.5 mm) sieves were collected. And laccase-entrapped polyacrylamide beads should be stored in a desiccator at 4°C for later use. The entrapped laccase was tested for its stability using the batch system and the column system in similar ways those described for covalently-immobilized laccase.

The bead-polymerization procedure applied here is similar to that reported for the preparation of cross-linked polyacrylamide used in gel chromatography by Nilson [42].