CHAPTER III

MATERIALS AND METHODS

List of Equipment

1.	Balance	L2200P and A200S, Sartorius, USA
2.	pH meter	pH meter 240, Corning, USA
		pH meter CG 812, Schott, USA
3.	Autoclave	Kokusan, Japan
4.	Laminar flow	ISSCO BVT-124, International Scientific Supply, USA
5.	Shaker	Gyrotory shaker G10, New Brunswick Scientific, USA
		Incubator shaker G27, New Brunswick Scientific, USA
		Innova 4330 refrigerated incubator shaker, New Brunswick Scientific, USA
		Incubator digital series incubator, Contherm Scientific, New Zealand
		Memmert, Germany
		Hereaus B5050E, Hereaus, Germany
6.	Photometer	Spectronic 21, Bausch & Lomb, USA
		UV-160A, Shimadzu, Japan
		Uvikron 860, Kontron, Germany
7.	Centrifuge	Refrigerated centrifuge J2/21, Beckman Instrument, USA
		M/E centrifuge J14/21, Beckman Instrument, USA
		M/E centrifuge J10/25/5, Beckman Instrument, USA
		Minifuge T, Heraeus, Germany
		Biofuge A, Heraeus, Germany
		Bench-top centrifuge D-7200, GS, Germany
		Bench-top centrifuge KM-15200, Kubota, Japan
9.	Oven	Digital series oven, Contherm Scientific, New Zealand
10.	Ultrasonicator	Ultrasonicator bath RK100, Bandelin Electronic, Germany
		Ultrasonicator bath FS4000, Decan Ultrasonics, England
		Bransonic 5200, Branson Ultrasonics, USA

11. Vortex mixer — G-560E, Scientific Industries, USA
12. Sonication — Branson sonifier 450, Branson Ultrasonics, USA
13. Water Bath — Tokyo Rikakikai, Japan
14. Evaporator — Rotary vacumn evaporator N-N, Tokyo Rikakikai, Japan
    Centrifugal vaporizer CVI-200P, Eyela, Japan
15. TLC — Silica gel 60 F_{254} TLC aluminium sheet 20×20 cm., Merck, Germany
    Silica gel 60 F_{254} PLC aluminium sheet 1×20×20 cm., Merck, Germany
16. HPLC — LC-3A, Shimadzu Scientific, Japan
    - UV/visible detector SPD-2A, Shimadzu, Japan
    - Chomatopac C-R1A, Shimadzu, Japan
    - Microsyringe MS-R50, Exmire, USA
    - 4.6×150 mm. Senshu Pak Pegasil ODS column, Senshu Scientific, Japan

    10 ADVP, Shimadzu Scientific, Japan
    - LC-10ADVP Pump
    - SIL-10ADVP Autosampler
    - SPD-10ADVP UV/Visible detector
    - 4.6×150 mm. Senshu Pak Pegasil ODS column, Senshu Scientific, Japan

    LKB Bromma, Germany
    - HPLC Controller 2152, LKB Bromma
    - HPLC Pump 2150, LKB Bromma
    - Variable wavelength detector 2155, LKB Bromma
    - Fraction collectors Superfrac, Pharmacia, USA
    - Q Sepharose fast flow, Pharmacia, USA
    - Superdex G200 prep grade, Pharmacia, USA
    - Hydroxyapatite, BioRad, USA
    - Phenyl Sepharose, Pharmacia, USA
    - Blue Sepharose, Pharmacia, USA
17. GC-MS — HP 5890 GC System, HP, USA
    - Mass selective detector 5970 series
    - HP5 Capillary column

18.	MS	Mass spectrometry Trio 2000, Fisons Instruments, England
19.	Electrophoresis	Power supply, 500V, 400 mA, Biotech Fischer, Germany
20.	Membrane filtration unit	0.2 μM DISMIC-13JP Filter unit, Tokyo Roshi Kaisha, Japan 0.2 μM Millex LG filter unit SLLG013SL, Millipore, USA 0.5 μM FH Filter unit, Tokyo Roshi Kaisha, Japan 0.45 μM DISMIC-25CS Filter unit, Tokyo Roshi Kaisha, Japan
21.	Ultrafiltration	Ultrafiltration set, Amicon Millipore, USA
22.	Micropipette	20, 100, 200, 1000 and 5000 μl micropipettes, Drummond Scientific, USA Disposable micropipettes, E. Merck, Germany
23.	Pasteur pipette	Becthai Bangkok Equipment & Chemical, Thailand
24.	Pipette	1, 2, 5 and 10 ml pipettes, Gilson, France
25.	UV Lamp	215-250 nm ultraviolet lamp UVGL-15, UVP, USA

Chemicals

All chemicals, solvents, bacteriological media and antibiotics used in these experiments were obtained from Carlo ERBA (Italy), Nacalia tesque (Japan), Difco Laboratories (USA), BDH Chemicals (Australia), AJEX Chemicals (Australia), May & Baker (England), Merck (Germany), J. T. Baker (USA), Fluka (Germany), Sigma (Germany), Serva (Germany), BioRad (Germany), Gibco (Germany) and Pharmacia (Germany).

PAHs; acenaphthylene, fluorene, fluoranthene, dibenzofuran, pyrene, acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, 2,5-dihydroxybenzoic acid and 1-naphthoic acid were purchased from Kanto Chemical, Tokyo, Japan. Acenaphthene and phenanthene were obtained from Sigma Chemicals, USA. All chemicals and solvents were of the highest purity commercially available.

Methods

3.1 Bacterial strains and growth conditions

Rhizobium sp. CU-A1, isolated from petroleum contaminated soil from Thailand (Paengthai, 2000), was cultured on Luria-Bertani (LB) agar then transferred to mineral medium (MM) agar with acenaphthylene being placed on the lid of an inverted petridish as a sole source of carbon. The plate was incubated at 30°C for 3 days. A single colony was then transferred to liquid mineral medium containing 600 mg/L of acenaphthylene, from a stock solution of acenaphthylene prepared in dimethyl sulfoxide (DMSO), and grown on gyrotory shaker at 200 rpm, 30°C for 3 days. Then, glycerol was added to the culture to 30% final concentration. After mixing, 1 ml aliquots were stored at -20°C.

Rhizobium sp. CU-A1, Tn*5* transpson mutants A53, B1 and E11, defective in acenaphthylene degradation as shown in Table A.1 of Appendix A constructed by Kriangkripipat (2001) were cultivated in MM agar supplemented with 1 g/L protocatechuic acid and 30 mg/L kanamycin at 30°C for 3 days. Then, single colony was transferred to MM liquid medium containing 1 g/L protocatechuic acid and 30 mg/L kanamycin and shaked at 200 rpm, 30°C for 3 days. For long term storage, all cultures suspended in 30% glycerol were kept at -20°C.

3.2 Substrates utilization

Inoculum preparation of mutant strains was performed by transferring bacterial culture to MM agar containing 1 g/L protocatechuic acid and 30 mg/ml kanamycin. It was incubated at 30°C for 3 days. A single colony was then transferred to 5 ml liquid mineral medium containing 1 g/L protocatechuic acid and 30 mg/ml kanamycin and cultured on shaker at 200 rpm and 30°C for 24 h. The inoculum was subsequently transferred to 100 ml liquid mineral medium supplemented with 1 g/L protocatechuic acid and 30 mg/ml kanamycin in 250 ml-Erlenmeyer flask and incubated on shaker at 200 rpm and 30°C for 24 h. Then, the cell pellet was harvested by centrifugation at room temperature, 10,000 rpm for 10 min, the cells were washed

thee times with 0.85% sodium chloride solution, resuspended in 5 ml MM broth to an optical density (A_{600}) of 1.0 and starved for 6 h prior to the addition of acenaphthylene and kanamycin to the final concentrations of 600 mg/L and 30 mg/L, respectively. The incubation was conducted on shaker at 200 rpm and 30°C. The samples were collected everyday for 14 days and determined for bacterial growth, substrate utilization and intermediates compared with those found in wild type strain CU-A1, grown in MM broth in the same manner as described above except in the absence of kanamycin. For using acenaphthylene degrading intermediates as a substrate, the experiment was carried out in the same manner as described above except 300 mg/L of identified intermediates was substituted for 600 mg/L acenaphthylene and the samples were collected at 2 day-interval for 10 days. The % remaining of substrate was determined by HPLC.

3.2.1 Bacterial cell number determination

Growth was verified by demonstrating an increase in bacterial cell number and concomitant with a decrease in substrate concentration. Bacterial cell number was determined by using viable plate count technique. The samples were serially diluted prior to spreading on MM agar containing 1 g/L protocatechuic acid and 30 mg/L kanamycin for mutant strains or without kanamycin for wild-type strain. Bacterial colonies were counted after incubation at 30°C for 3 days.

3.2.2 Extraction of the remaining substrate in the medium

The remaining substrate in each sample was extracted from the culture with ethyl acetate. After incubation, whole cell cultures were acidified to pH 2.0-3.0 with 1N HCl, extracted twice with equal volume of ethyl acetate. The solvent was then dehydrated by adding anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure at 25°C. The residue was redissolved in 0.5 ml methanol, filtered through 0.2 μm PTFE filter unit and stored in glass vial at -20°C for further determination of remaining substrate and intermediates formation by TLC and HPLC.

3.2.3 Determination of acenaphthylene and its intermediates concentration by HPLC

Twenty microlitres of the samples from 3.2.2 were injected into the reversed phase HPLC equipped with 4.6 × 150 mm ODS column. For acenaphthylene determination, HPLC was operated under the following conditions: column temperature 40°C, flow rate 1.0 ml/min, mobile phase 80% aqueous methanol solution and detection by UV detector at 275 nm. Peak area from the HPLC chomatogram was calculated from the calibration curve. Acenaphthylene calibration curve was generated by adding acenaphthylene to MM medium to a final concentration of 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 mg/L. Then, the medium were extracted as described in 3.2.2 and analyzed by HPLC. The calibration curve of acenaphthylene was shown in Appendix C, Fig. C.1. For intermediates determination, the column was eluted with a linear gradient of 40-80% aqueous methanol for 30 min then with 80% aqueous methanol for another 15 min. Calibration curve for each compound was prepared in a similar manner as described for acenaphthylene.

3.2.4 Determination of substrate degradation profile by TLC

Analysis of substrate degrading profile by Thin-Layer chromatography was performed with 8 × 8 cm silica gel 60 F_{254} TLC plate. Five to ten microlitres of the extract from 3.2.2 was spotted onto the TLC plate. The developing solvent system used for TLC was 90:25:1 (volume/volume) toluene: dioxane: acetic acid. Compounds on chomatograms were visualized by UV light at 215-250 nm.

3.3 Extraction and purification of acenaphthylene degradation intermediates

3.3.1 Isolation of accumulated intermediates from acenaphthylene grown mutants

Each mutant was first enriched by growing in MM broth supplemented with 1 g/L protocatechuic acid and 30 mg/L kanamycin as described in 3.2. Then, the inoculum was transferred to 1000 ml MM to an A_{600} of 1.0 prior to the addition of acenaphthylene and kanamycin to the final concentrations of 600 mg/L and 30 mg/L, respectively. The culture was incubated on shaker at 200 rpm and 30°C for a suitable period of time (3-5 days). Then, the culture was extracted by ethyl acetate in the same manner as described in 3.2.1 and kept for further purification.

3.3.2 Purification of intermediates by preparative-TLC

Purification of intermediates was initially carried out by preparative thin-layer chromatography (TLC). Two hundred microlitres of the samples from 3.3.1 dissolved in methanol were applied as a single line on a silica gel precoated TLC plate, 20 × 20 cm (L × W) and 1 mm thickness, at 2 cm from the lower edge. The plate was developed in TLC chamber equilibrated with hexane : ethyl acetate : acetic acid (10:10:1, v/v/v) as the developing solvent, then air dried and visualized under UV light at 215-250 nm. The desired spot was scraped off the plate, eluted twice with 250 ml water-saturated ethyl acetate. The solvent was then dried by passing through column filled with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 25°C by rotary vacumn evaporator. The residue was redissolved in 5 ml methanol, filtered through 0.2 μm PTFE filter unit and stored in glass vial at -20°C. The sample was analyzed for its purity by HPLC as described in 3.2.3 and by TLC as described in 3.2.4.

3.3.3 Purification of intermediates by silica gel column chromatography

The resulting intermediates from 3.3.2 were further purified by silica gel column chromatography. The column (1 × 30 cm) packed with 10 g of 63-70 mesh silica gel 60 was prepared by placing a cotton at the bottom of column, then filling the column with hexane and slowly adding a slurry of silica gel suspended in hexane. The sample was mixed with 3 g of silica gel, dried by rotary vacumn evaporator and resuspended in small volumn of hexane. Then, the sample was loaded

onto the column and stepwise-eluted with 100 ml of 0-100% ethyl acetate in hexane with 10% increment for each step as follows;

100% hexane
90% hexane in ethyl acetate
80% hexane in ethyl acetate
70% hexane in ethyl acetate
60% hexane in ethyl acetate
50% hexane in ethyl acetate
40% hexane in ethyl acetate
30% hexane in ethyl acetate
20% hexane in ethyl acetate
10% hexane in ethyl acetate
0% hexane in ethyl acetate (100% ethyl acetate)

Each fraction was dried over anhydrous Na_2SO_4 ,evaporated to dryness in vacuo at 25 °C, redissolved in methanol, filtered through 0.2 μm PTFE filter unit and stored in glass vial at -20°C. The intermediates were analyzed for its purity by HPLC as described in 3.2.3 and by TLC as described in 3.2.4.

3.3.4 Purification of intermediates by HPLC

The intermediates were purified by reversed phase HPLC using model 10ADVP chromatography, Shimadzu Scientific, Japan. The HPLC using ODS column was operated under the following conditions: column temperature 40°C, flow rate 1.0 ml/min, linear gradient of 40-80% aqueous methanol as a mobile phase and detection by UV detector at 275 nm. Two to five hundred microlitres of the sample was injected into the column and the desired intermediate was collected after monitoring by UV detection. The fractions which contained the desired intermediate were pooled, dried over anhydrous Na_2SO_4, evaporated to dryness in vacuo at 25 °C, redissolved in methanol, filtered through 0.2 μm PTFE filter unit and then analyzed for its purity by HPLC as described in 3.2.3 and by TLC as described in 3.2.4.

3.4 Identification of purified intermediates by MS or GC-MS

The purified intermediates were identified by mass spectrometry or gas chromatography-mass spectrometry. MS experiment were performed using a Trio 2000 mass spectrometry (Fisons Instruments, England). The mass spectrometer was operated at 70 eV of electron ionization energy.

Gas chromatography-mass spectrometry was performed using an HP 5890 GC system, connected to mass selective detector (5970 series). Compounds were separated on an HP-5 capillary column (30 m by 0.25 mm) with helium as the carrier gas (linear velocity 36.2 cm/s). The column temperature was held isothermally at 50°C for 1 min and then increased to 290°C at a rate of 10°C/min. The mass spectrometer was operated at an electron ionization energy of 70 eV. Injector and analyzer temperatures were set at 290 and 310°C, respectively. Intermediates were identified by comparison of their electron impact (EI) MS spectra with those obtained from authentic compounds. Moreover, instrumental library searches were also used for identification of metabolites.

3.5 Enzyme assays

3.5.1 Preliminary detection of acenaphthylene dioxygenase activity in the crude cell free extract

Acenaphthylene dioxygenase activity in the cell free extract was initially detected by measurement of the amount of acenaphthylene or other tested substrates decreased after incubation with the cell free extract supplemented with NADH. The assay was performed in 50 mM sodium phosphate buffer pH 7.5 at 35°C. The reaction was initiated by adding 90 µl of 0.3 mg/ml acenaphthylene or other tested substrates to 5 ml sodium phosphate buffer containing cell free extract at the final protein concentration of 0.1 mg/ml and 2 mM NADH. Samples were collected at every hour-interval for 3 h, and the enzyme reaction was stopped by adding 1 N HCl to pH 2-3. The reaction mixture was extracted twice with equal volume of ethyl acetate, dehydrated by adding anhydrous Na_2SO_4, and evaporated to dryness under

reduced pressure at 25°C. The residue was dissolved in methanol and the remaining substrate was then determined by HPLC as described in 3.2.3. Boiled cell free extract was used as a control. The reaction products were purified by HPLC as described in 3.3.4 and identified by gas chromatography-mass spectrometry as described in 3.4. Intermediates were identified by comparison of their electron impact EI-MS spectra with those obtained from authentic compounds and from instrumental library.

3.5.2 Preliminary assay for acenaphthylene dioxygenase activity

The reaction mixture (4.0 ml) contained 10 mM of acenaphthylene and appropriately diluted enzyme in 50 mM sodium phosphate buffer pH 7.5 to give about 1 mg of protein, NADH, FAD and $FeSO_4$ at the concentration as indicated in the results. Reaction was started by adding the substrate and incubation was carried out at 35°C for 2 h. Samples were collected every 30 min and the enzyme reaction was stopped by adding HCl to pH 2-3. The remaining substrate was extracted from the reaction mixture by ethyl acetate, dehydrated by adding anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure at 25°C. The residue was dissolved in methanol, analyzed for the remaining substrate by HPLC using a calibration curve of 0, 200, 400, 600, 800, 1,000, 1,200, 1,400, 1,600 nmole of acenaphthylene in 20 µl methanol as shown in Appendix C, Fig. C.2. Boiled cell free extract was used as a control. One unit activity was defined as the amount of enzyme that catalyzed the conversion of 1.0 nmole acenaphthylene in 1 min under the assay conditions.

Optimization for assay conditions was performed with the crude cell free extract as follow:

3.5.2.1 Effect of pH on the enzyme activity was determined as described in 3.5.2 except at various pH by using 50 mM acetate buffer pH 4-6, 50 mM phosphate buffer pH 6-8 and 50 mM Tris-HCl buffer pH 8-10.

3.5.2.2 Effect of buffer concentration on the enzyme activity was determined as described in 3.5.2 except various concentrations of Tris-HCl and sodium phosphate buffer pH 7.5 ranging from 25 to 100 mM were used.

3.5.2.3 Effect of temperature on the enzyme activity was determined as described in 3.5.2 except at various temperatures between 20 and 60°C.

3.5.2.4 Effect of co-factors on the enzyme activity was determined as described in 3.5.2 except NADH or NADPH was added to the reaction mixture to a final concentration of 0.5, 1, 2 and 3 mM, exogenous $FeSO_4$ was added to the reaction mixture to a final concentration of 100, 200 or 300 μM and FAD or FMN was also added to the reaction mixture to a final concentration of 1, 2 or 3 μM.

3.5.2.5 Effect of NaCl or KCl on the enzyme activity was determined as described in 3.5.2 except in the presence of NaCl or KCl at the final concentration between 0-1.0 M.

3.5.3 Assay for acenaphthylene dioxygenase under the optimal conditions

The reaction mixture (4.0 ml) contained 50mM sodium phosphate buffer pH 7.5, 2 mM of NADH, 2 μM of FAD, 200 μM of $FeSO_4$, 10 mM of acenaphthylene, and an appropriate amount of enzyme, and incubation was carried out at 35°C for 2 h. The remaining substrate at 30 min-intervals were determined as described in 3.5.2.

3.5.4 Cytochrome *c* reductase activity

The enzymatic reduction of cytochrome *c* was measured spectrophotometrically at 25°C as described by Peterson et al. (1966) with minor modifications. The reaction mixture (1.0 ml) contained 87 nmole of cytochrome *c*, 0.3 mM of NADH, 1 μM of FAD and 50 mM Tris-HCl buffer, pH 7.5, and appropriately diluted enzyme. The reaction was started by the addition of NADH in 20 μl of reaction buffer, and cytochrome *c* reduction was measured as an increase in optical density at 550 nm. Concentrations of cytochrome *c* were calculated from a molar extinction coefficient of $E = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$. One unit of NADH dependent

cytochrome *c* reductase activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmole of cytochrome *c* per min under the assay conditions.

3.6 Protein determination

Protein concentration was determined by Lowry (1951) or Bradford method (1976). Bovine serum albumin (BSA) was used as a protein standard.

For Lowry method, 5 ml of solution C (Appendix B, Number 15) was added to 1 ml of sample. Following mixing, the solution was kept at room temperature for 15 min. Then, 0.5 ml of solution D (Appendix B, Number 15) was added to the mixture and kept at room temperature for another 30 min prior to the measurement of the absorption 660 nm. Bovine Serum Albumin (BSA) was used as a calibration curve in the range of 0-50 μg/ml.

For Bradford method, the mixture containing 50 μl of 1 N NaOH, 1 μl Bradford reagent (Appendix C, Number 16) and 20 μl of protein sample was well mixed and kept at room temperature for 15 min. Thereafter, absorption at 595 nm was measured and the concentration was interpolated from a calibration curve using BSA in the range of 0-50 μg/ml as a protein standard.

3.7 Purification of acenaphthylene dioxygenase from *Rhizobium* sp. CU-A1

All purification procedures were performed at room temperature unless otherwise stated. The enzyme purification scheme was summarized in Fig. 3.1.

3.7.1 Crude cell free extract preparation

Rhizobium sp. CU-A1 was enriched in mineral medium containing 1 g/L protocatechuic acid as a sole carbon source. The culture was incubated with shaking at 200 rpm, 30°C for 18 h. Acenaphthylene was then introduced to the culture to a final concentration of 10 mM, and the culture was allowed to incubate for another

6 h before being harvested. Cells were harvested by centrifugation at room temperature at 10,000 rpm for 10 min, and washed three times with 0.85% NaCl. The cell pellet was weighed and stored at -20°C until required.

For the preparation of crude cell free extract, one part of frozen cell pellet (g) was thawed and suspended in 2 parts (v) of 50 mM sodium phosphate buffer pH 7.5 containing 1 mM dithiothreitol (DTT). Fifteen milliliters of cell suspension were sonicated by Branson sonifier 450 using ultrasonic horn with a tip diameter of 5 mm, output control at 7 and 50% duty cycle for 6 cycles including 2 min for disruption and 2 min for cooling on NaCl-ice for each cycle. Subsequently, cell debris was removed from the supernatant by centrifugation at 10,000 rpm, 4°C for 20 min. The supernatant was collected and used as a starting material for purification of acenaphthylene dioxygenase.

3.7.1.1 Effect of N-ethylmaleimide (NEM)

N-ethylmaleimide was introduced to an aliquot of the cell free extract prepared as described in 3.7.1 to the final concentration of 0.2 to 2 mM and incubated at 4°C for 15 min. The remaining enzyme activity was determined as described in 3.5.1.

3.7.1.2 Effect of dithiothreitol (DTT)

Dithiothreitol was added to the aliquot of the cell free extract from 3.7.1 to the final concentration of 0.2 to 2 mM. The mixture was incubated at 4°C for 15 min. The enzyme activity was determined as described in 3.5.1. Effect of DTT to stabilize the enzyme was also studied by incubating cell free extract from 3.3.1 with DTT at a final concentration of 0.5 to 2 mM. The incubation was performed at 4°C for 48 h and the samples were collected every 6 h and determined for their remaining activity as described in 3.5.1.

Effect of DTT to restore the activity of enzyme after the treatment with NEM was also tested. The cell free extract was incubated with NEM at

final concentration of 0.4 mM at 4°C for 15 min. Then, DTT was introduced to the extract to the final concentration ranging from 0.5 to 5 mM. Following the incubation at 4°C for 15 min, the activity was determined as described in 3.5.1.

3.7.2 Ammonium sulfate fractionation

The crude extract was prechilled to 4°C and ammonium sulfate powder was added to 40% saturation with constant stirring by magnetic stirrer at 4°C. The suspension was equilibrated for 1 h, followed by centrifugation for 30 min at 10,000 rpm, 4°C. The supernatant was collected and stepwisely precipitated with 60-100% saturation of ammonium sulfate with 20%-increment for each step. The pellet for each step was washed twice with equivalent salt saturated phosphate buffer pH 7.5 and then resuspended in small volume of 50 mM phosphate buffer pH 7.5 containing 1 mM DTT and dialyzed overnight in the same buffer at 4°C. Then, each fraction was determined for protein content and the enzyme activity. For the purification of the enzyme, the extract was fractionated with the suitable salt saturation obtained from the previous step. The ammonium sulfate precipitate was washed twice with equivalent salt saturated phosphate buffer pH 7.5 and then resuspended in 50 mM phosphate buffer pH 7.5 containing 1 mM DTT and dialyzed overnight in the same buffer at 4°C. Then, it was determined for protein content and the enzyme activity.

3.7.3 Q Sepharose fast flow column

Anion exchange was performed using FPLC Pharmacia LKB-VWM 2141 (Pharmacia, Germany) equipped with Q Sepharose fast flow column (Amersham Pharmacia, Germany). The dialyzed sample from 3.7.2 was loaded onto the column previously equilibrated with 50 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT (starting buffer). After washing unbound proteins with starting buffer, the bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in starting buffer for 60 min then with 1.0 M NaCl in the same buffer for another 15 min at a flow rate of 1 ml/min. The 1.0 ml-fractions were collected, desalted and concentrated by ultrafiltration with Amicon YM-10 membrane before assay for the activity. The fractions with activity were then pooled and concentrated by

ultrafiltration with Amicon YM-10 membrane and determined for protein content and the enzyme activity and kept for further use.

3.7.4 Superdex G200 column

The enzyme preparation from 3.7.3 was loaded onto a hiload Superdex G200 preparative gel filtration column (2.6 × 60 cm), equilibrated with 50 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT and then eluted with the same buffer at a flow rate of 1 ml/min. Fractions of 3.0 ml were collected. Fractions from each protein peak (A, B and C) were pooled, concentrated and assayed for the enzyme activity either separately or as a combination.

3.7.5 Hydroxyapatite column

The concentrated component A from 3.7.4 was then applied to a hydroxyapatite column (1.6 × 10 cm), preequilibrated with 20 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT. After washing with the same buffer, the enzyme was eluted from the column with a linear gradient of 20-200 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT. The 5-ml fractions were collected, concentrated by ultrafiltration with Amicon YM-70 membrane and analyzed for the activity in the presence of component B and C. Pooled fractions with activity were concentrated by ultrafiltration with Amicon YM-70 membrane and then measured for volume, protein content and the enzyme activity.

3.7.6 Phenyl Sepharose column

The concentrated enzyme from 3.7.5 was added with ammonium sulfate powder to a final concentration of 1 M and loaded onto a phenyl Sepharose column (1.6 × 10 cm) previously equilibrated with 50 mM sodium phosphate buffer pH 7.5 containing 1.0 M ammonium sulfate and 1 mM DTT. After washing with starting buffer, bound proteins were eluted with a linear descending gradient of ammonium sulfate (1.0-0 M) in starting buffer. The 1 ml-fractions were collected, dialyzed and concentrated by ultrafiltration with Amicon YM-70 membrane and analyzed for the activity in the presence of component B and C. The fractions with

activity were pooled, dialyzed and concentrated by ultrafiltration with Amicon YM-70 membrane and then measured for volume, protein content and the enzyme activity and stored at -70°C.

3.7.7 Blue Sepharose column

Component B from step 3.7.4 was further loaded onto Blue Sepharose column. After elution of unbound proteins by 50 mM sodium phosphate buffer pH 7.5 containing 1 mM DTT, the column was eluted with a linear gradient of 0-2.0 M NaCl in the same buffer for 60 min and with 2 M NaCl in the same buffer for another 10 min. A flow rate of 1 ml/min was used and the 1 ml-fractions were collected. Each fraction was concentrated and desalted by ultrafiltration with Amicon YM-30 membrane and assayed for cytochrome *c* reductase activity. The fractions with activity were pooled and concentrated by ultrafiltration and then measured for volume, protein content and the enzyme activity and stored as a source of component B at -70°C.

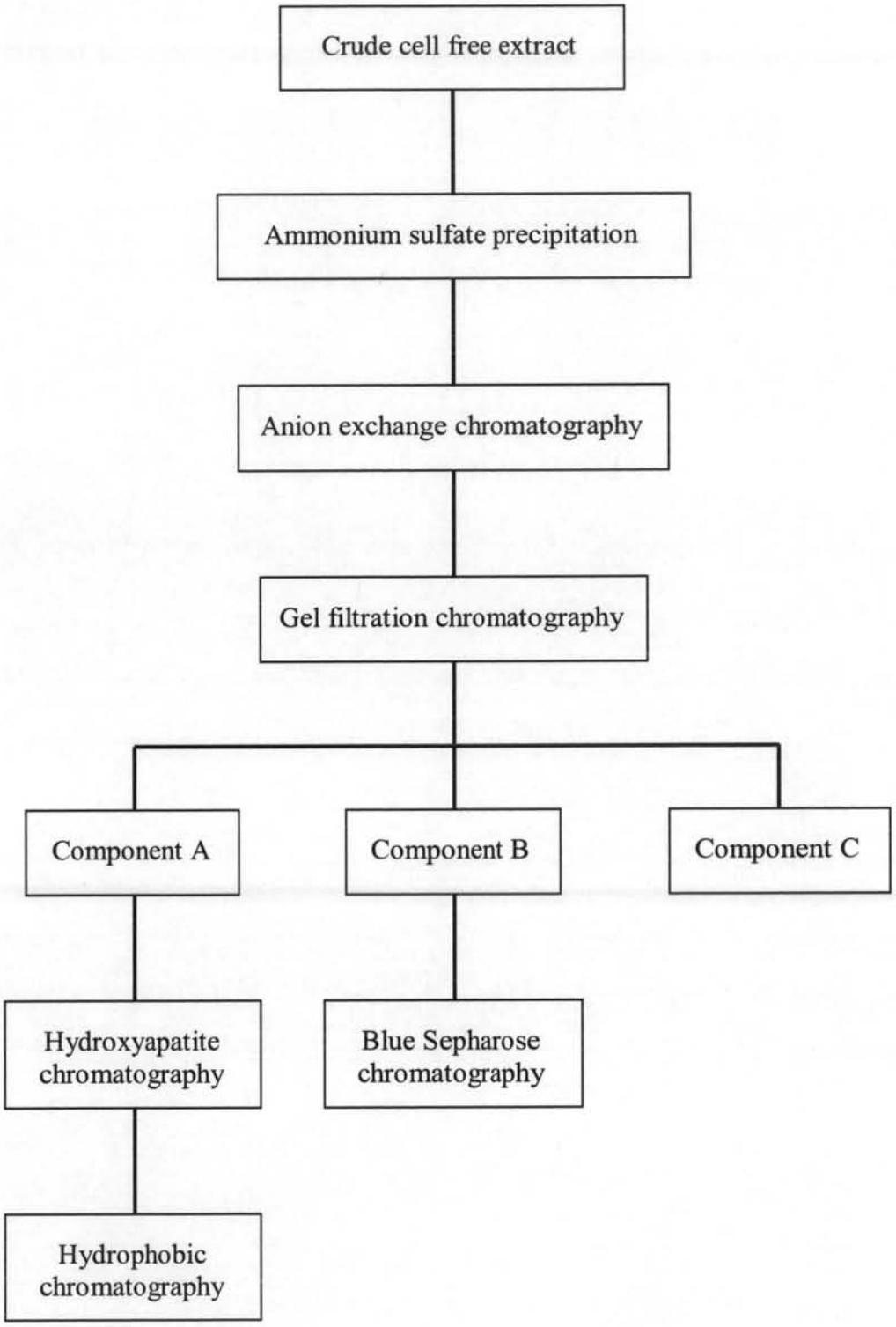

Fig. 3.1 Acenaphthylene dioxygenase purification scheme.

3.8 Polyacrylamide gel electrophoresis

Clean glass plates were assembled and 5 ml of 12% resolving gel, prepared according to Appendix B, Number 17, Table B.2 was poured between two glass plates. Subsequently, the surface was overlaid with *iso*-propanol and the gel was allowed to solidify for 1 h. After polymerization was completed, *iso*-propanol was removed and the surface of the gel was rinsed with distilled water and drained. Then, 2 ml of 5% stacking gel (Appendix B, Number 17, Table B.2) was filled on top of the resolving gel. A comb was immediately inserted into the gel carefully avoiding the formation of air bubbles and allowed to polymerize for 1 h. Then, the comb was removed and the loading wells were rinsed with electrophoresis buffer (Appendix B, Number 17). The gel was attached onto the electrophoresis apparatus and the buffer was poured to the top and bottom reservoirs.

One part of the protein samples with the same concentration of 10 μg was mixed with one part of loading buffer, prepared according to Appendix B, Number 17. Then, 10 μl of each sample was applied into the well using 25 μl microsyringe. The electrophoresis unit was then connected to an electric power supply. Initial voltage was set at 40 V and increased to 80 V after the dye front reached the resolving gel. The unit was turned off after the dye front had reached the end of the resolving gel. Then, the gel was removed from the glass plates and stained with coomassie staining solution (Appendix B, Number 17) for 30 min. The gel was then destained of the excess dye by an excess destaining solution (Appendix B, Number 17) for overnight under gentle shaking until the clear protein bands were observed.

3.9 Molecular mass determination

3.9.1 Molecular mass determination by using Superdex G200 gel filtration column

The molecular weight of the native protein was estimated by using Superdex G200 H26/60 column under the same condition for protein purification in 3.7.4. A mixture of standard proteins including dextran (2000.0 kDa), ferritin (440.0 kDa), bovin serum albumin (66.0 kDa), cytochrome *c* (13.2 kDa) and riboflavin (1.2

kDa) was loaded and eluted from the column. The calibration curve was plotted between logarithm of molecular weight and K_{av} value of each protein, calculated according to an equation shown in the result and Appendix C, Number 4.

3.9.2 Molecular mass determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in the same manner as described in 3.8 except SDS was introduced to the resolving gel, stacking gel, loading buffer and electrophoresis buffer according to an Appendix B, Number 17. The Low-Molecular-Weight protein markers, obtained from Amersham, were used to determine the molecular weights of the protein bands based on the migration of the proteins.

3.10 Characterization of the purified enzyme

3.10.1 Determination of temperature and pH optima

Optimal temperature for dioxygenase activity was determined by assaying in 50 mM phosphate buffer pH 7.5 at different temperatures from 15 to 90°C. For determination of optimal pH, the enzyme activity was determined at different pH values from 4-10 by using 50 mM acetate buffer pH 4-6, 50 mM phosphate buffer pH 6-8 or 50 mM Tris-HCl buffer pH 8-10 and at the optimal temperature.

3.10.2 Determination of temperature and pH stability

Temperature and pH stability were determined by preincubating the enzyme complex in the absence of substrate in closed vial at different temperatures from 15°C to 90°C or at different pHs from 4 to 10. At time interval, aliquots were withdrawn from the preincubation mixture and the residual enzyme activity was determined under the optimal conditions.

For temperature and pH stability of individual component, each component was preincubated as described above and the residual activity was determined under standard assay conditions as described in 3.5.2

3.10.3 Determination of substrate specificity and kinetic parameters

Acenaphthylene dioxygenase activity was determined under standard conditions as described in 3.5.2 except using naphthalene, acenaphthene, phenanthene, fluorene, anthracene, fluoranthene, pyrene, acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, 1-naphthoic acid, 1,2-dihydroxynaphthalene, salicylic acid or gentisic acid as a substrate at the final concentration of 5 mM. Kinetic characterizations were determined under standard conditions at 35°C using acenaphthylene, acenaphthenequinone, 1-naphthoic acid or naphthalene as a substrate at concentrations ranging from 0.5-10 mM. One unit of the enzyme represents the amount of enzyme required to catalyze the conversion of 1.0 nmole substrate in 1 min under the assay conditions. The K_m and V_{max} values were determined from Lineweaver-Burk plot.

3.10.4 Effects of metal ions and chemicals on enzyme activity

The effect of different metal ions on enzyme activity was measured by adding metal ions into the reaction mixture to the final concentration of 100, 500, 1,000 or 5,000 μM. The metal ions used in this study were $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $NiCl_2$ and $ZnSO_4$. The effect of metal ion was expressed as relative activity with that of in the absence of metal ion. For determination of the effect of some chemicals, the enzyme activity was determined under standard conditions in the presence of diphenyliodonium, phenylhydrazine, aminobenzotriazole, EDTA or iodoacetate at the final concentration of 1, 5 or 10 mM. The results were expressed as relative activity with that of in the absence of these compounds.

3.10.5 Spectral properties

Spectral properties of components A, B and C were measured spectrophotometrically between 180-600 nm in one cm-pathlength cuvette at a scan speed of 50 nm per minute.

3.10.6 $^{18}O_2$ incorporation experiment

The reaction mixture of 2 ml containing 50 mM phosphate buffer pH 7.5, NADH (2.0 mM), FAD (2.0 μM), $FeSO_4$ (200 μM), substrate, acenaphthylene or acenaphthenequinone (10 mM), and appropriated amount of the purified enzyme complex from 3.7 was put in a round bottom flask connected with an open valve. The open valve was then connected to a vacumn pump and the air was partially evacuated. Then $^{18}O_2$ (99% isotopic purity, Stohler Co., Germany) was introduced into the flask. Reaction was carried out with constantly stirring at 35°C for 2 h. Reaction was terminated by the addition of 1 N HCl to pH 2-3 and the products were extracted from reaction mixture as described in 3.2.2. The residue was dissolved in methanol and ^{18}O incorporation into the product was analyzed by GC-MS as described in 3.4. The experiment conducted in the normal atmorshere was used as a control.