CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals were AR grade, or otherwise specified.

The followings were products of Sigma Chemical Co.; standard -glutamyl hydroxamate, NADH, NADPH, snake venom phosphodiesterase, AMP, glucosamine-6-phosphate, and standard BSA.

Phenylalanine, isoleucine, leucine were purchased from Sigma Chemical Co., Glycine and tryptophan were from BDH Chemicals Ltd., and alanine from Fluka.

The chemicals for gel electrophoresis; acrylamide was purchased from Merck, SDS was from Bio-Rad laboratories, bis acrylamide and coomasie brilliant blue R-250 were from Sigma Chemical Co., TEMED and glycine were from BDH Chemicals,Ltd., and ampholine was from LKB.

Reactive blue 2-Sepharose CL-6B was purchased from Sigma Chemical Co., and Sepharose-4B was from Pharmacia.

Other chemicals; ATP, ADP and TCA from Fluka, phosphoric acid from Merck.

2.1.2 Bacteria:Klebsiella spp.R15

The bacteria was isolated from the rhizosphere of rice grown in paddy field at the Rangsit experimental site, Thailand by Harinsut and Boonjawat (26). The stock culture was maintained in 50% LB-glycerol medium at -70°C.

2.2 Preparation of bacterial media

2.2.1 N-Free medium (NF)

K_2HPO_4	0.05 g
KH_2PO_4	0.15 g
$CaCl_2.2H_2O$	0.01 g
$MgSO_4.7H_2O$	0.02 g
$NaMoO_4.2H_2O$	0.002 g
$FeCl_3$	0.01 g
$NaHCO_3$	0.01 g
Glucose	20.0 g
Distilled water	1.0 Liter

15.0 g agar for solid media

2.2.2 NFA100 medium (NFA100)

100mM NH_4Cl was added in NF medium

2.2.2 Luria broth medium (LB)

Tryptone	1.0 g
Yeast extract	0.5 g
NaCl	1.0 g

Distilled water 100 ml

adjust pH to 7.0 with NaOH,

1.5 g agar for solid media

2.3 Bacterial cultivation and crude enzyme preparation

Klebsiella spp.R15 was grown in LB broth by shaking at 100 stroke per min at 30°C, the growth was followed by measuring OD_{420}. When OD_{420} reached 1.2, the bacteria were seperately transfered into each 1 litre containing 250 ml of NF or NFA100 medium. The inoculum size was 3%. The cultures were further shaken on an orbital shaker (100 stroke per min), at 30°C until OD_{420} reached our required.

The cells were harvested by centrifugation at 30,000 g,4°C for 20 min and further washed three times with TME buffer (50 ml each). The washed cells were finally suspended in 3 ml of TME buffer and crushed broken with french pressure cell pressed at 1100 psi. The process was repeated three times before removal of the cell debris by centrifugation at 30,000 g for 20 min. The supernatant was referred to as "crude enzyme fraction" and was collected for GS, GDH and GOGAT assay and for further purification.

2.4 GS assay

In addition to catalyzing the synthesis of glutamine by the reaction shown in equation (1), purified GS also catalyzes the formation of -glutamyl hydroxamate by the 'biosynthetic'reaction (equation (2)) or the 'transferase'reaction (equation (3))(42).

$$\text{glutamate} + \text{ATP} + NH_4^+ \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{glutamine} + \text{ADP} + \text{Pi} \quad (1)$$

$$\text{glutamate} + \text{ATP} + NH_2OH \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \gamma\text{-glutamyl hydroxamate} + \text{ADP} \quad (2)$$

$$\text{glutamine} + NH_2OH \xrightarrow[ADP,\ AsO_4^{3-}]{Mn^{2+} \text{ or } Mg^{2+}} \gamma\text{-glutamyl hydroxamate} + NH_4^+ \quad (3)$$

GS activity (equation (1)) can be assayed by measuring the formation of ^{14}C-glutamine from ^{14}C-glutamate. Difficulties may be encounter. It is much easier to measure hydroxamate formation (equation (2) and (3)) spectrophotometrically after complexing the hydroxamate with $FeCl_3$.

2.4.1 Biosynthetic hydroxamate assay (42)

The activity obtained in the presence of Mg^{2+} was a suitable measure of the deadenylylated form of GS.

GS hydroxamate assay (0.7 ml total volume) contained 0.15 ml of 0.25 M imidazole-HCl buffer pH 7.0, 0.1 ml of 0.3 M sodium glumate pH 7.0, 0.1 ml of 0.3

M ATP, 0.05 ml of 0.5 M $MgSO_4$, and appropriate amount of the enzyme (0-0.3 ml).

The assay mixture was preincubated at 25°C for 5 min. The reaction was then started by the addition of 0.05 ml of hydroxylamine reagent (prepared just prior to use by mixing equal volume of 1.0 M NH_2OH-HCl with 1.0 M NaOH). The reaction was further incubated at 25°C for 15 min and stopped by the addition of 0.2 ml of $FeCl_3$ reagent (prepared by mixing equal volumes of 10% W/V $FeCl_3.6H_2O$ in 0.2 M HCl with 24% W/V TCA and 50% V/V HCl). Control assay was done by omitting hydroxylamine. After termination of the reaction, the assay mixture was centrifuged at 5,000 g for 10 min and the supernatant was measured for absorbance at 540 nm. Absorbances were converted into micromoles of γ-glutamyl hydroxamate using a calibration graph prepared from 0-0.3 ml of the 5 mM standard γ-glutamyl hydroxamate. GS activity was expressed as μmol γ-glutamyl hydroxamate formed/ min/mg protein.

2.4.2 Transfer hydroxamate assay

The transferase assay was used to measure the total amount of GS present, since both the adenylylated and deadenylylated forms of the enzyme were active in this assay.(43)

Method 1 (42)

The reaction mixture (for 80 assays) was prepared by mixing the followings: 2 ml of 0.5 M imidazole-HCl pH 7.0, 4 ml of 0.15 M glutamine, 0.6 ml of 0.1 M $MnSO_4$, 0.8 ml of 0.01 M ADP, 0.4 ml of 1.0 M sodium arsenate pH 7.0 and 2.2 ml of distilled water.

Transferase assay contained 0.125 ml of the reaction mixture described above and the enzyme solution to give a total volume of 0.225 ml. The mixture was preincubated at 25°C for 5 min. The reaction was then started by the addition of 0.025 ml of hydroxylamine solution (prepared by mixing equal volume of 1.0 M NH_2OH-HCl and 1.0 M NaOH just prior to use) and the reaction mixture was incubated at 25°C for 15 min. The reaction was stopped by the addition of 0.5 ml of the $FeCl_3$ reagent. (prepared by mixing 10% W/V $FeCl_3.6H_2O$ with 24% TCA, 6 M HCl and water in the proportions of 8:2:1:13). Control assays with minus ADP and arsenate was performed. Assay mixture was centrifuged at 5,000 g for 10 min and the absorbance of the supernatant was measured at 540 nm. Absorbances were converted into micromoles of γ-glutamyl hydroxamate using a calibration graph prepared from 0-0.1 ml of the 10 mM standard γ-glutamyl hydroxamate. Transferase activity was expressed as umole γ-glutamyl hydroxamate formed/ min/mg protein.

Method 2 (44)

The assay mixture was adapted from Shapiro and Stadtman (44). This procedure avoided the formation of precipitates and leaded to higher reproducibility of the assay. This transferase procedure was used in the studies of feedback inhibition and pH profile assays.

Reaction mixture (for 35 assays) was prepared by mixing the followings: 9.03 ml of water, 2.25 ml of 1.0 M imidazole-HCl, pH 7.15, 0.37 ml of 0.80 M NH_2OH-HCl, 0.045 ml of 0.10 M of $MnCl_2$, 1.5 ml of 0.28 M $K.AsO_4^{3-}$, 0.15 ml of 40 mM sodium ADP, pH 7.0. The solution was adjusted to various pH by using 1 M HCl or 2 M KOH and stored at 4°C.

This transferase assay contained 0.4 ml of the reaction mixture, and enzyme solution to give a total volume of 0.45 ml. The solution was equilibrated for 5 min at 37°C, and the reaction was started by the addition of 0.05 ml of 0.20 M L-glutamine (final concentration,20 mM). The reaction was further incubated at 37°C, 15 min and terminated by the addition of 1.0 ml of stop mix (prepared by dissolving 55 g of $FeCl_3.6H_2O$, 20 g of TCA and 21 ml of concentrated HCl with distilled water to a final volume of one liter). The sample was centrifuged to remove precipitate before absorbance at 540 nm was measured. One unit of GS activity was defined as

the amount of enzyme producing 1 umole of glutamyl hydroxamate per min.

2.5 GOGAT and GDH assays

The method of assays were adapted from Sabina, et al (45).

GOGAT and GDH activities were assayed spectrophotometrically at 30°C by measuring the initial rates of NADPH or NADH oxidation, respectively, at 340 nm. One unit of activity was the amount of enzyme which oxidises 1 umol of NADPH or NADH per min.

The standard assay mixture for GOGAT contained 50 mM HEPES-KOH buffer, pH 7.5, 5 mM L-glutamine, 2.5 mM 2-oxoglutarate, 0.2 mM NADPH and appropriate concentrations of the enzyme in a final volume of 1 ml.

The standard assay mixture for GDH contained 50 mM HEPES-KOH (pH 7.5), 100 mM NH_4Cl, 2 mM 2-oxoglutarate, 0.2 mM NADH, and appropriate amount of the enzyme in a final volume of 1 ml.

2.6 GS purification (46)

For each purification, 2 L of Klebsiella spp.R15 culture was used. Crude enzyme solution was prepared by the method described in 2.3. 50 mM Tris/HCl pH 7.5 was

used in replace of TME buffer and the cells were disrupted by sonicator(model W 375 from Heat System Ultrasonic Inc.) on pulsed input, 50% duty cycle. Completed cell breakage was estimated microscopically.

The crude enzyme solution (30,000 g supernatant) was heat-treated at 50°C for 15 min with constant stirring, then chilled in ice for 15 min and centrifuged at 30,000 g for 15 min. The supernatant was then loaded onto a Blue Sepharose CL-6B column (7.0X1.8 cm) preequilibrated with 10 mM Tris/HCl, 1 mM $MnCl_2$ buffer, pH 7.5. The column was then washed with about 250 ml of the previous buffer (approximate flow rate 10.9 ml/hr) until no significant absorbance at 280nm was observed. GS was eluted from the column with 2 mM ADP in the same buffer. Fractions with GS activity were pooled, dialysed overnight against the same buffer and concentrated to 2 ml on an Amicon-10 microconcentrator.

The concentrated solution was loaded onto the Sepharose-4B column (56.0X1.8 cm) preequilibrated with 50 mM Tris/HCl buffer, pH 7.5. The chromatography was performed with constant flow rate of 10.9 ml /hr and 2 ml fractions were collected. After assaying for GS activity, the active fractions were pooled.

2.7 Polyacrylamide gel electrophoresis

Electrophoresis in 7% discontinuous polyacrylamide gel both in the presence and absence of SDS were done according to David,B.J (47). For electrophoresis in denaturing condition, 0.1% sodium dodecyl sulfate (SDS) was included in both the electrode buffer and polyacrylamide gel.

The stock solutions were prepared as shown in table 1. The gel was formed between two 18X16 cm glassplates assembled with 1 mm thick spacer. The gel was overlayered with water. About one hour before electrophoresis was performed, the overlayered water was removed from the polyacrylamide gel and the comb was placed between the glass plates about 2 cm above the gel. Stacking gel solution (prepared as shown in table 1) was then poured on top. Complete polymerization took place within 30 min, after which the comb was removed, and the plates were clamped to the electrophoresis tank. Sufficient running gel buffer (prepared as in table 1) was supplied to the upper and the lower reservoirs, air trapped under the gel was removed before loading the gel with sample dissolving in 10 folds diluted "stock solution f" for normal gel or in 20% "stock solution g" for SDS-containing gel.

After electrophoresis, gels were stained for catalytic activity or protein. GS activity staining was accomplished by incubating gel in the transferase assay mixture at 25°C for 20 min. Color was developed after placing the incubatedgel in the $FeCl_3$ stop mixture. Gels for protein staining were stained with coomasie brilliant blue R and destained in 7% acetic acid, 5% methanol (shown in table 1).

Table 1. Formula for stock solutions, buffers, gels and reagents for polyacrylamide gel electrophoresis

A. Stock solution

a) 48 ml of 1 N HCl, 36.6 g Tris, 0.23 ml TEMED and distilled water to 100 ml.

b) 48 ml of 1 N HCl, 5.98 g Tris, 0.46 ml TEMED and distilled water to 100 ml, pH 6.7 (the pH is adjusted to exactly 6.7 with 1 N HCl).

c) 28.0 g acrylamide, 0.735 g bis-acrylamide and distilled water to 100 ml.

d) 10.0 g acrylamide, 0.25 g bis-acrylamide and distilled water to 100 ml.

e) 0.004 g riboflavin and distilled water to 100 ml.

f) 80.0 g sucrose and distilled water to 100 ml.

g) 3.88 g Tris-HCl, pH 6.7, 11.5% SDS,25% β-mercaptoethanol and 50% glycerol.

B.Working solutions prepared from stock solutions:

Running gel solution:1 part stock a, 2 parts stock c, 1 part stock e, and 4 parts distilled water.

Stacking gel solution:1 part stock b, 2 parts stock d, 1 part stock e, 4 parts distilled water.

C Electrode buffer for normal gel:6.0 g Tris, 28.8 g glycine and distilled water to 1 L, pH 8.3, diluted 10 folds before use.

D.Electrode buffer for denaturing gel:15.15 g Tris, 72 g glycine, 5 g SDS, and distilled water to 5 L, pH 8.3

E.Staining solution:Coomassie brilliant blue R 0.1%, methanol 50%, acetic acid 10%.

F.Destaining solution:Acetic acid 7% and methanol 5%

2.8 Isoelectric focusing

Isoelectric focusing was performed in a cylindrical tube gel (0.2X14 cm). The preparation of electrofocusing gel was perform according to O'Farrel,P.H. (48). The protein sample was dissolved in the polyacrylamide gel mixture which contained the chemical reagents shown in table 2. The completed gel mixture was then carefully loaded into the gel tube with a pasture

pipette (the tubes were set vertically with the lower end closed). Air-trapping in the gel tube should be avoided by dipping a pasture pipette into the gel while loading. The gel was overlayered with water and allowed to polymerization. The parafilm was removed and the gel tube was filled into the LKB 2001 Vertical Electrophoresis connected to a thermostat water-bath of LKB 2209 Multi Temp set at 15°C. The upper cathode chamber was filled with 0.02 N NaOH whereas the lower anode chamber was filled with 0.01 N H_3PO_4. Electrophoresis was carried out at 200 V for 30 min, 300 V for 30 min, 400 V for 12-16 hrs. (final~100 uA/gel) and finally 800 V for 1 hr. At the end of the run, all gel tubes were immediately removed and stained in staining solution (table 1). The stained gel was destained in destaining solution shown in table 1.

Table 2. Isoelectric focusing gel mixture (2ml)

urea	1.10 gm
10%NP40	0.40 ml
enzyme solution	0.40 ml
warm to dissolve urea	
28.4%acrylamide,1.6%Bis	0.20 ml
ampholyte pH 5-7	0.10 ml

5 g% $(NH_4)_2S_2O_8$ (freshly prepared)	0.01 ml
1/10 TEMED	0.005 ml

2.9 Determination of molecular weight

The molecular weight of the enzyme was determined by gel filtration on a Sepharose- 4B column (56.0X1.8 cm) equilibrated with 50 mM Tris/HCl buffer(pH 7.5). Using 16 mg thyroglobulin (669,000), 8 mg ferritin (440,000), 6 mg alcohol dehydrogenase (150,000) were used as marker proteins. The determination of subunit molecular weight was done by discontinuous gel electrophoresis in the presence of 0.1% SDS as described in 2.7, with the following protein standards: 20 µg amylase (55,000), 20 µg ovalbumin (45,000), 20 µg chymotrypsinogen (25,000).

2.10 Protein determination (49)

Preparation of reagent: 50 mg coomassie brilliant G-250 was dissolved in 25 ml of 95% ethanol. 50 ml of conc. phosphoric acid was slowly added to the solution. Final volume of 500 ml was adjusted with distilled water. The solution was then filtered through whatman NO.1

Assay: 5 ml of the reagent was added to 0.1 ml of protein solution. The absorbance was measured at 595 nm with Spectronic 2000 spectrophotometer (Bausch&Lomb).

Bovine serum albumin was used as standard protein.