CHAPTER IV

DISCUSSION

4.1 Purification and characterization of GS from *Klebsiella* R15

Purification of GS from *Klebsiella* R15 according to Boontariga (1988) by using affinity column chromatograph, (Blue Sepharose CL-6B) which bind specifically with many proteins or enzymes that require adenylyl group as cofactor; such as NAD(H) or NADPH. Thus, adenylyl group analogue, of the gel binds specifically to the adenylyl - binding site on GS molecular, and only the deadenylylated or slightly adenylylated GS can strongly bind to the column while fully-adenylylated form can not. So ADP is used as eluent to with the adenylyl - binding site and elute GS from the column successfully with 53-fold purification and 181% recovery. After gel filtration on Sepharose 4B chromatography other adenylyl - binding proteins have been removed. The purification fold increased to 86, with 46 % recovery, and the purity of GS is evident by a single band on SDS-PAGE (Figure 3.1, lane 3).

Boontariga Wongwaitayankul(1988) reported that purified GS from *Klebsiella* R 15 is likely to be an octameric enzyme (430 Kda of identical subunit (54 Kda by molecular sieve chromatography an Sepharose 4B and denaturing gel electrophoresis. But in this research. The native GS activity staining on PAGE of *Klebsiella* R15 was revealed as a single band of similar high molecular weight as GS in the crude extract from *E. Coli* and *K. pneumoniae* M5a1 (Figure 3.3a), and confirmed by immunoblot in denaturing gel to contain similar subunit molecular

weight as in *E.Coli* and *K. pneumoniae* M5a1. since native GS is a large complex about 540 Kda, the heat treatment used in previous purification of *Klebsiella* R15 GS might degrade the native molecular weight of R15 resulting in smaller apparent molecular weight of 430 Kda and different subunit molecular weight of 54 Kda, which is smaller than 59 Kda reported in this research. And the molecular weight of *Klebsiella* R15 GS was higher than that of rice root cytosolic GS (GSr) as it moved relatively slower in SDS-PAGE of 7.5 % T(Figure 3.7a). The molecular weight of Gsr was estimated by polyacrylamide gel electrophoresis about, 330 Kda(Hirel and Gadal, 1980) .

4.2 Characteristics of GS antibody

Antisera against GS from *Klebsiella* R15 was produced from only one rabbit because the yield of purified GS protein was very limited. However, high titer of 64 was obtained after 4 months, and the important characteristic of purified GS antibody is the cross reactivity with GS from other enteric bacteria such as *E. coli* and *K. pneumoniae* M5a1 (Figure 3.5b). This result is similar to previous report on *E.Coli* antibody GS which can cross react with the GS from a number of Gram-negative bacteria(Tronick, et al, 1973). It does not cross react with rice GS when protein of equal were loaded lane 1, 3 (Fig 3.7b). This allows the specific quantitation of *Klebsiella* R15 GS by ELISA, even in associative condition of *Klebsiella* R15 with rice. Anti GS is able to react with denatured GS antigen caused by SDS as shown in Figure 3.4, in immunoblot analysis with very high sensitivity of 3 ng pure GS antigen. By indirect ELISA the sensitivity of the method is better (0.5 ng) with %CV lower than 8.0 in the intra-assay and lower than 14.0% in

among inter-assay.

4.3 Regulation of GS activity and synthesis in free-living and associative *Klebsiella* R15

GS activity in free-living *Klebsiella* R15 is regulated by NH_4 (Boontariga Wongwaitayakul, 1988). In NH_4-excess condition (100 Mm) GS activity both biosynthetic and transferase activity decrease concurrently with GOGAT activity, while glutamate dehydrogenase (GDH) activity is very low. In N_2-fixing condition GS activity is highest in the late log phase (OD_{420} = 0.7), together with GOGAT activity, Therefore the GS-GOGAT pathway is the primary assimilation of ammonia in the free-living state. The regulation of GS activity is under control of adenylylation-deadenylylation system through a cascade of α-ketoglutarate, glutamine, ATP, and UTP. The adenylylation system responds to the ratio of α-ketoglutarate to glutamine, high ratio of α-ketoglutarate to glutamine, characteristic of cells growing in an ammonia-deficient medium will stimulate adenylylation of GS and conversely a low of those, characteristic of cells growing with an excess of ammonia, will stimulate its adenylylation (Stadtmam, 1991). In *K. pneumoniae* cells growing in excess-ammonium are largely present in adenylylated form, and when NH_4 has been depleted GS will be changed to adenylylated form Streicher et. al., 1974). For regulation of GS synthesis, high-level of transcription of *glnA* requires activation of by the product of *ntrC* gene (NRI). In N-limited condition, transcription of *ntrBC* is from P2 promoter and in N-excess condition, transcription of *ntrBC* is from P3 promoter (Cannon et al., 1985)

Free-living *Klebsiella* R15 grown in excess ammonium leads to the

repression in GS total activity (Boontariga Wongwaitayakul, 1988) as GS is in adenylylated forms. So GS of *Klebsiella* R15 is controlled by NH_4 in the environment, and NH_4 also regulates GS activity by adenylylation-deadenylylation system as in *K. pneumoniae* (Nagantani et al., 1971 ; Streicher et al., 1974).

Symbiotic *Rhizobium* is a specialized ammonium producer, but poor user of NH_4 produced by nitrogenase and thus contributes in fixing of nitrogen to the host plant (Brawn and Dilworth, 1975 ; Ludwig, 1978). In contrast to enteric bacteria, *Rhizobium* GS becomes repressed in N_2-fixing bacteroid at very low ammonium concentration by adenylylation mechanism. Recently, three distinct GS enzymes and corresponding genes (*glnA*, *glnII*, and *glnT*) have been identified in *Rhizobium,* and their control are different from enteric bacteria (de Brunijn et al., 1989). In this research GS of associative *Klebsiella* R15 in the rice rhizosphere show higher specific activity than free-living *Klebsiella* R15, which is in opposite direction to Rhizobium GS in symbiotic form. While the rice GS, which is root specific and resides in cytosol (Hirel and Gadal, 1980) seems to remain unchanged or slightly decreased (Table 3.7). In contrast *Phaseolus vulgaris*, pea and soybean nodule-specific GS have shown to be induced by bacterial symbiont (Hirel et. al., 1987; Miao et. al., 1989).

This higher bacterial GS activity in *Klebsiella*-rice association indicates that ammonia produced from nitrogenase should be partially assimilated in associative *Klebsiella* R15 and are not released totally as ammonia. Because nitrogenase activity increases 400-500 fold, but GS specific activity increases 7-9 fold in associative *Klebsiella* R15. So the level of GS activity may not sufficient to assimilate all NH3 produced during N_2-fixation. However GS specific activity in rice root

inoculated with *Klebsiella* R15 is slightly decreased, possibly because products of N_2-fixation are exported to plant cells as glutamine or another nitrogenous compounds.

In *Nostoc-Anthrocerose* symbiotic system, the first product of assimilation of N_2 was NH_4 and assimilated by plant (Meek et. al., 1985). This was evident by a decrease in specific activity of *Nostoc* GS 3-4 fold lower in symbiotic condition with *Anthrocerose* than in free living Nostoc. The activity of Nostoc GS was regulated by post-translational mechanism.

In *Anabaena-Azolla* symbiosis, the decrease in GS activity in symbiotic Anabaena has been shown to be due to a decrease level of GS protein (Haselkorn, 1980; Orr and Haselkorn, 1982). In this symbiotic system the cyanobiont releases fixed-nitrogen as NH_3 which assimilated by eukaryotic partner (Ray et al., 1978 ; Stewert et al., 1983 ; Meek et al., 1985).

Based on ELISA the amounts of GS protein in associative *Klebsiella* R15 is also 3-5 fold higher than in free-living condition, which implies that GS synthesis should increase together with GS specific activity. As confirmed in Figure 3.8, Western blot analysis of washing fraction containing rhizospheric *Klebsiella* R15 or in rice root pellet-fraction which contains endorhizospheric *Klebsiella* R15, the increasing amount of GS protein can be observed as higher intensity GS-band in associative condition comparing the free-living *Klebsiella* R15 where equal amount of total protein have been loaded.

These data indicate that the level of GS activity and GS protein in associative *Klebsiella* R15 have been increased in the presence of rice root, and also implies that the regulation of GS in associative *Klebsiella* R15 is at the transcriptional level as well as post-

translational level.

4.4 Induction of nitrogenase and GS activity in free-living and associative *Klebsiella* R15

GS has been determined before and after nitrogenase induction. R15 was shifted from NF medium to associate with RD7 and GS activity was measured. At D0, GS specific activity was 0.37+0.16 nmol -glutamyl hydroxamate (mg protein^{-1} min^{-1}, while nitrogenase was very low and not detected by ARA (Figure 3.9). GS specific activity in associative *Klebsiella* R15 has been increasing 7-9 fold of free-living *Klebsiella* R15 on D7 or D8 (N_2-grown) while nitrogenase activity has become significantly greater than that of free-living *Klebsiella* in the same condition without rice at the same time (Figure 3.9). By assuming that when nitrogenase is expressed, the ammonium products will activated GS activity and *glnA ntrBC* operon. The presence of *glnA* and *ntr* BC operon has been found in *Klebsiella* R15 by using *gln* A probe from *K. pneumoniae* (Suthisukon, personal communication). Therefore the *ntr* system should be involved in the regulation of *Klebsiella* R15 as in *K. pneumoniae.* Different situation exist in Rhizobium GSI and GSII are present at very low levels whenever fixation occurs and therefore it fails to assimilate ammonium produced by nitrogenase. In *Rhizobium* unadenylylated GSI mediates nitrogenase derepression (Ludwig, 1980). When nitrogenase appears, it produces ammonium which stimulates adenylylation of GSI and leads to repression of both GSI (slightly) and GSII (completely).

From these results of regulation of GS and induction of nitrogenase and GS activity suggest that the difference in NH_3 regulation on GS protein is the key step of controlling ammonium assimilation to be restricted in the bacterial cell or shifted to the

plant cytosol. The interesting questions are: 1) Can we make rice GS to be more sensitive to ammonia like leguminous nodule specific GS? And 2) Can we repress *Klebsiella* R15 GSI in associative condition, if so the associative *Klebsiella* R15 should become poor user of ammonia produced from N_2, and contribute more to the host plant.

Conclusion

1. The GS activity in the associative *Klebsiella* 15 is 7-9 fold higher than that in free-living condition. This increase is apparently due to regulation at level of GS synthesis rather than GS activity (post-translation).

2. The nitrogenase and GS activity is concurrently changed by increasing about 400-500 fold for nitrogenase activity and about 7-9 fold for GS activity after inoculation of rice cv. RD7 with *Klebsiella* R15 for 7 day. This result suggests that GS is induced by NH_4 produced from nitrogenase and it may be involved in derepression of nitrogenase regulon in associative condition.

3. GS specific activity of rice root is constant or slightly decreased in the rice roots that have been inoculated *Klebsiella* R15.

These results imply that products of fixed-dinitrogen(NH_4) by *Klebsiella* R15 when they associate with the rhizosphere of rice should not be transferred to rice plant directly, but should be changed to glutamine or the other nitrogenous compounds before taken up by rice plant.