CHAPTER III

RESULTS

3.1 Purification of GS

GS was purified 86-folds from *Klebsiella* R15 as summarized in table 3.1 with column chromatography using Blue Sepharose CL-6B and Sepharose-4B as described by Wongwaithayakul (1988). The specific activity increased from 0.8 to 42.78 (μmol γ-glutamyl hydroxamate)·(mg protein)$^{-1}$·min^{-1} after elution from Blue Sepharose CL-6B affinity column by ADP which compete for adenylyl-binding site of GS and other adenylyl-binding proteins resulting in an increase in total activity about 2-folds of the crude extract. The other adenylyl-binding proteins were removed by gel filtration on Sepharose 4B column,resulting in per cent final recovery of 45.6 units and the final purification fold of 69 - fold. The purity of GS was shown by a single band on SDS-PAGE (Figure 3.1a) and the subunit molecular weight was estimated (Figure 3.1b) by comparing the electrophoretic migration with standard molecular weight markers to be 59 Kda.

3.2 Preparation of GS antibody.

Antisera against GS from *Klebsiella* R15 was produced in the female New Zealand White rabbit using the immunization scheme shown in Figure 3.2a. Bleeding started two weeks after the first injection and the serum titre was determined by observing the precipitin line under double immunodiffusion method (Figure 3.2b). The serum titer remained

Table 3.1 Purification of *Klebsiella* R15 GS.

Enzyme purification and enzyme assay were described in 2.4 and 2.5. All purification steps were performed at 4 °C. One enzyme unit is defined as µmol γ-glutamyl hydroxamate produced min^{-1}

Step	Volume (ml)	Total cytosolic protein(mg)	Total activity (units)	Recovery (%)	Specific activity (**)	Purification (fold)
Crude Tris-HCl pH 7.5 extract	30	57.00	45.44	100	0.80	
Blue Sepharose CL-6B	9	1.93	82.56	181.7	42.78	53.5
Sepharose 4B	12	0.30	20.72	45.6	69.07	86.3

** : (µmol γ-Glutamyl hydroxamate) ·(mg protein)$^{-1}$·min^{-1}

Figure 3.1a Gel electrophoresis of purified *Klebsiella* R15 glutamine synthetase was performed on discontinuous SDS-PAGE of 12 % T.

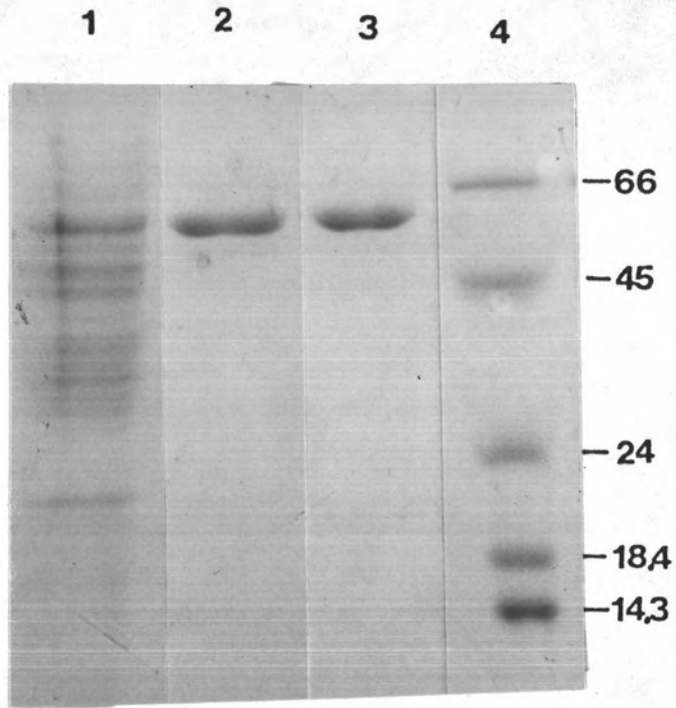
Lane 1 Crude extract, 10 µg

lane 2 Purified GS from blue Sepharose CL-6B, 2 µg

lane 3 Purified GS from Sepharose 4B, 2 µg

lane 4 Standard markers of albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), ß-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa)

Figure 3.1b Determination for the molecular weight of the GS subunit by SDS-PAGE of 12 % T. Standard proteins were albumin (66 kDa) ovalbumin (45 kDa), trypsinogen (24 kDa), ß-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).



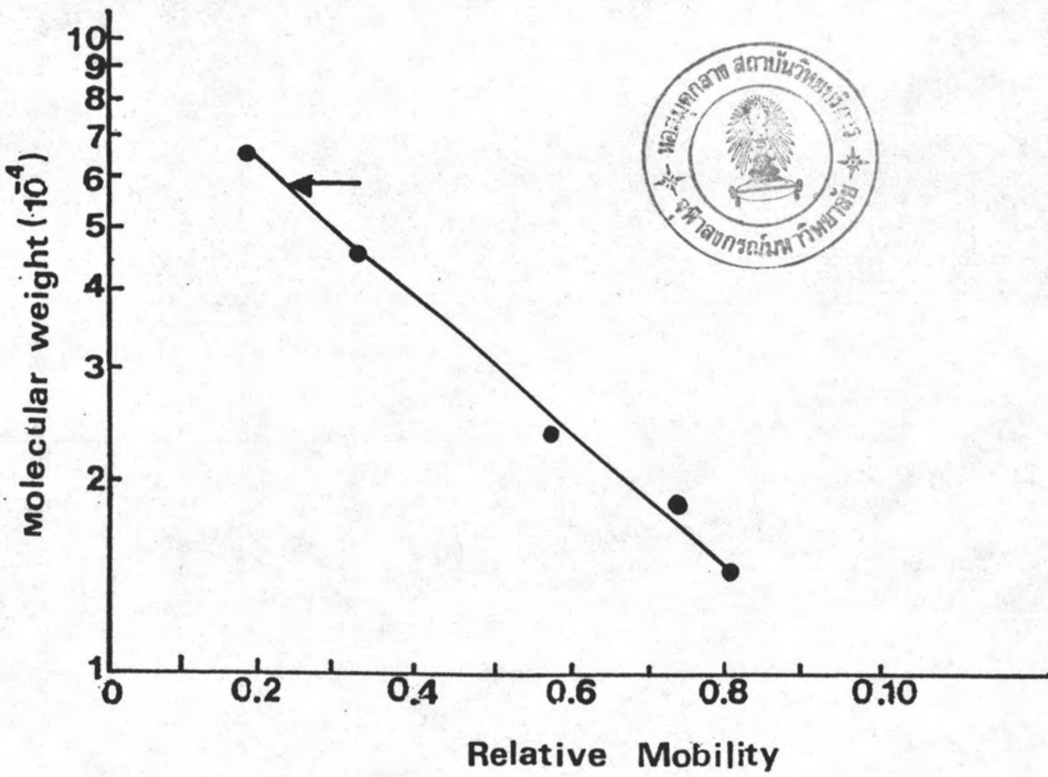


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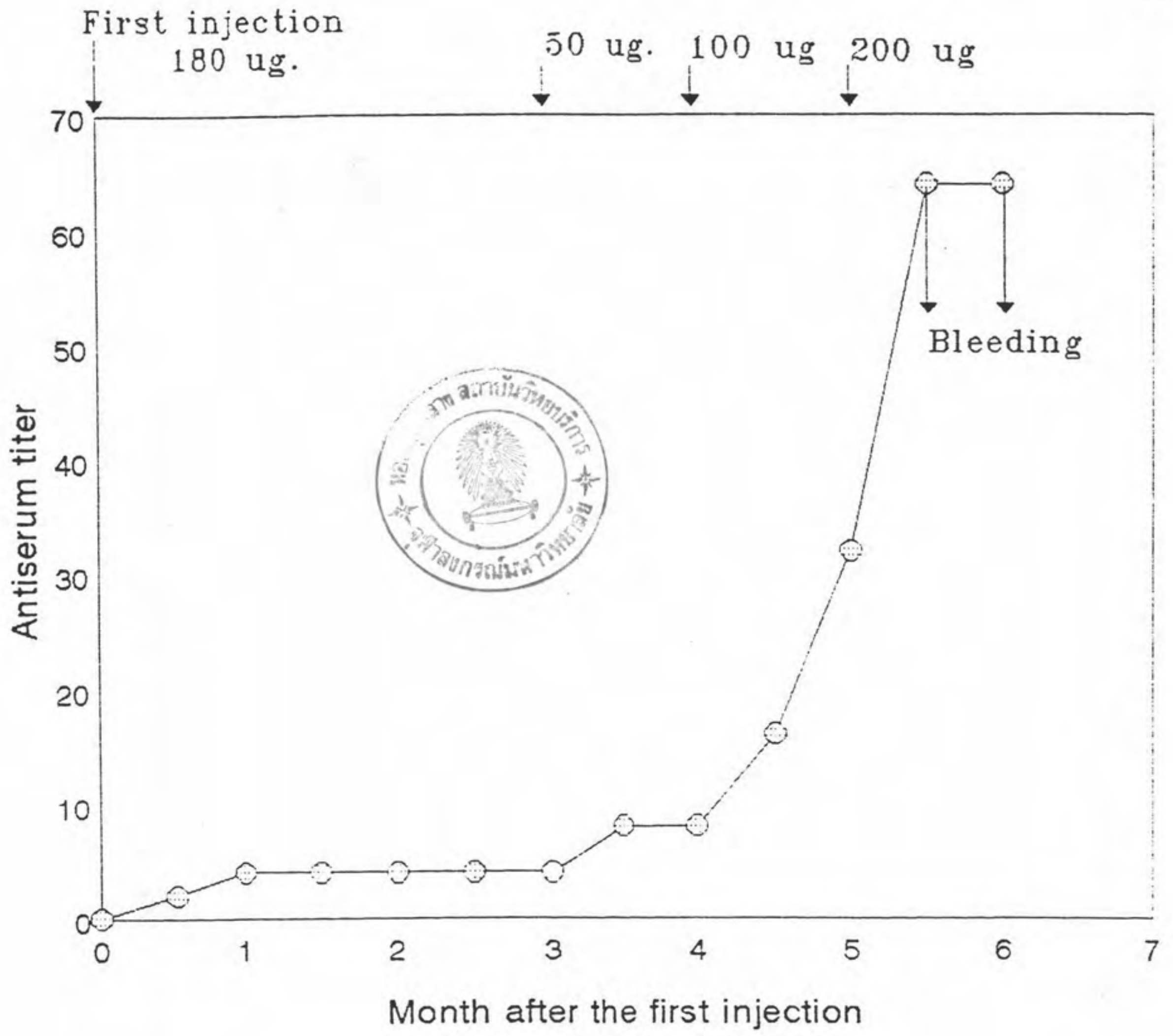
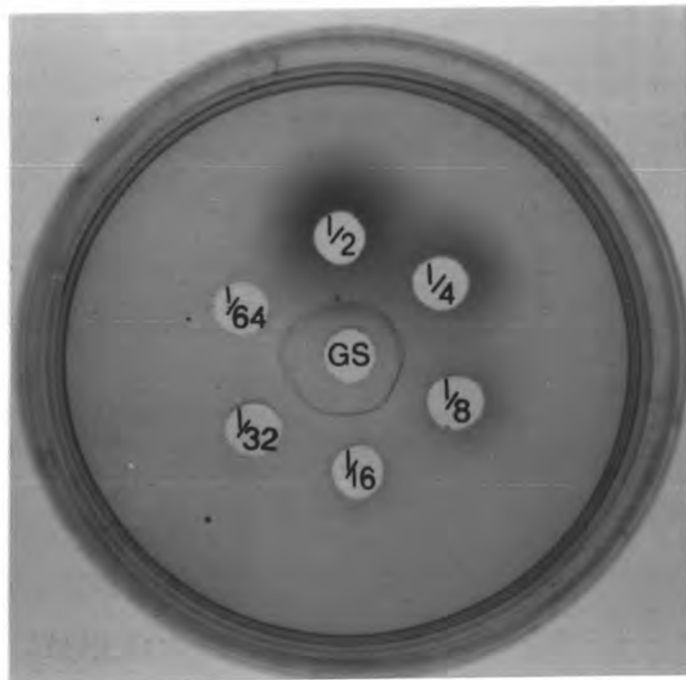
Figure 3.2a Immunization scheme and antiserum titer against GS.

Figure 3.2b Double immunodiffusion of purified GS (1 μg) reacted with serial dilution rabbit antiserum against GS.



First injection
180 ug.
50 ug.
100 ug
200 ug
Bleeding
Antiserum titer
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
Month after the first injection



1/2
1/4
1/64
GS
1/8
1/32
1/16

constant at 4 about three months after the first injection. The following booster injections with 50,100 and 200 µg GS protein resulted in the highest titer of 64. At this time bleeding of 10 and 20 ml blood were collected.

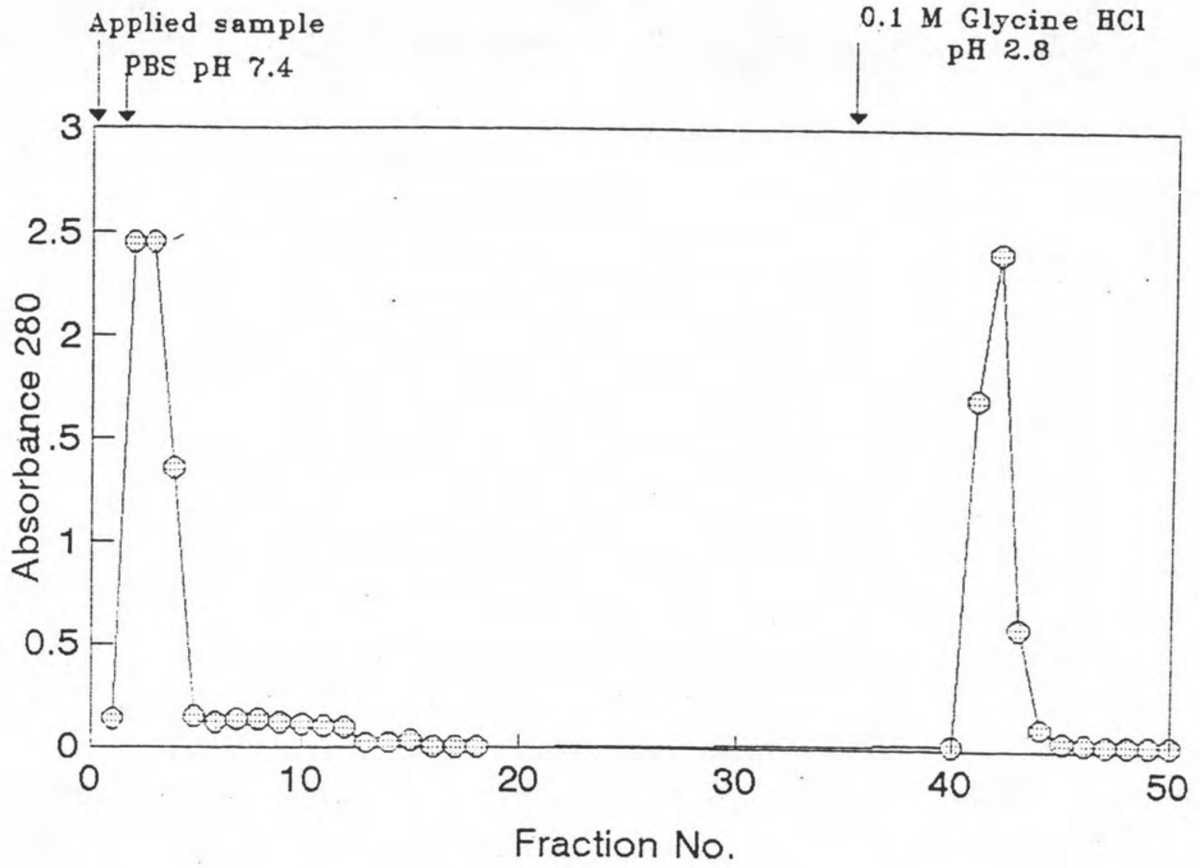
Purification of antiserum against GS (2.5 ml) on an affinity column of immobilized Protein A (2.5 ml) has removed other proteins as shown by the elution profile (Figure 3.3a). After neutralization and desalting on an excellulose GF 25 column, the immunoglobulin fraction was concentrated to 2.0 mg/ml. The purity of GS antibody was checked by SDS -PAGE as shown in Figure 3.3b lane 3,4 and 5 to be nearly free from other serum proteins.

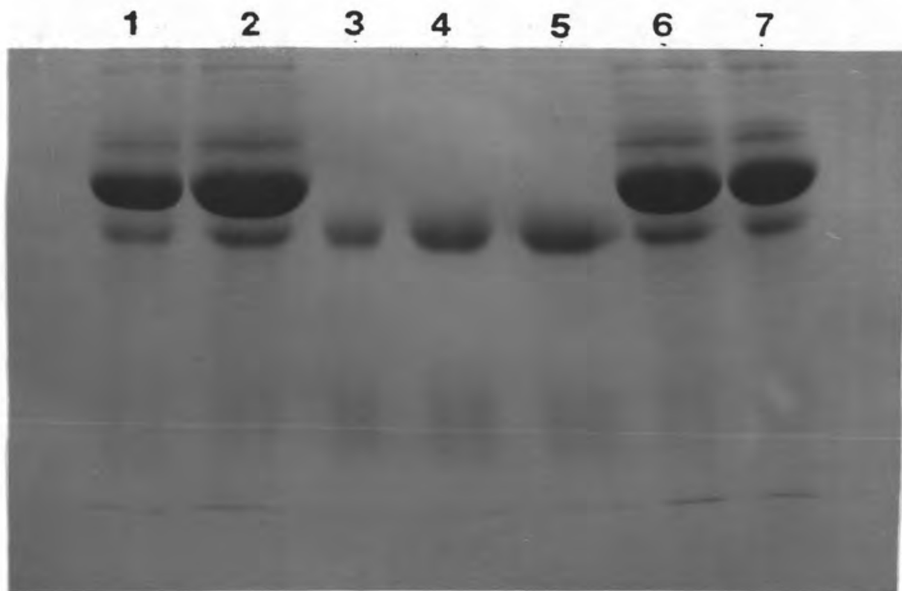
3.3 Quantitation of GS protein by Western blot analysis.

To determine the sensitivity of purified GS antibody in the detection of GS antigen, increasing amount of GS protein 3-21 ng were loaded on SDS-PAGE, and investigated by immunoblotting procedure described in Method 2.10. Purified GS antigen can be detected in every lane (Figure 3.4) but sharp band were observed at an amount of 15 ng protein or larger.

3.4 Homology between GS from *Klebsiella* R15 and other enteric bacteria

By activity staining of GS from R 15 , *E.Coli* and M5a1 in non-denaturing polyacrylamide gel and immunoblotting after SDS-PAGE, Figure 3.5 a and b indicate for the similarity of GS among the 3 strains in term of molecular weight, subunit-molecular weight and immunological cross-reactivity. By using crude extracts preparation, the results also


Applied sample
PBS pH 7.4
0.1 M Glycine HCl
pH 2.8
Absorbance 280
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
Fraction No.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6
7

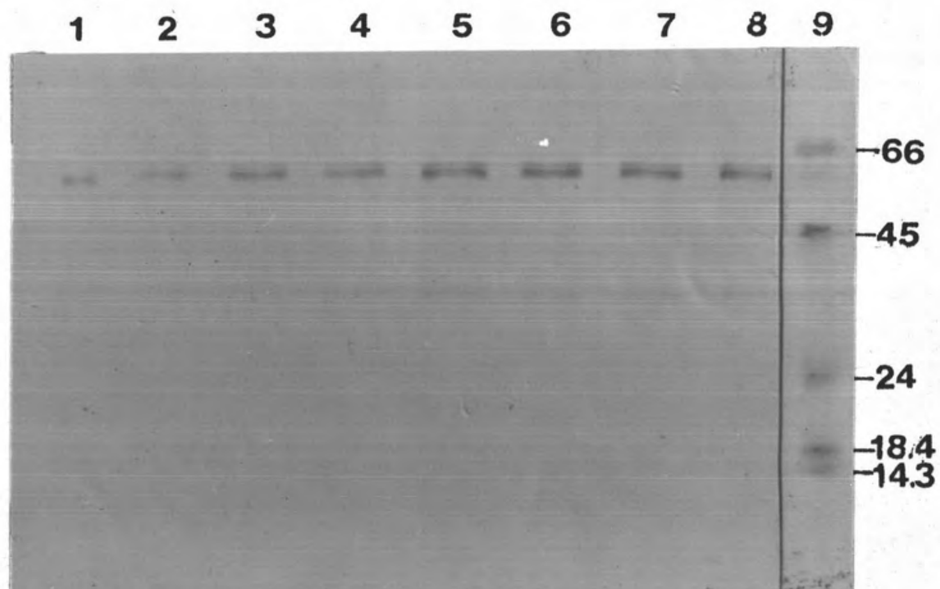


Figure 3.4 Western blot analysis of purified GS, the purified GS from *Klebsiella* R15 were run on 12 % SDS-PAGE and blot to nitrocellulose (NC) membrane. Then it was stained with anti-GS antiserum(1:800) using HRP-goat anti-rabbit IgG(1:3,000). Lane 1-8, purified GS of 3, 6, 9, 12, 15, 18, 21 and 21 ng respectively.

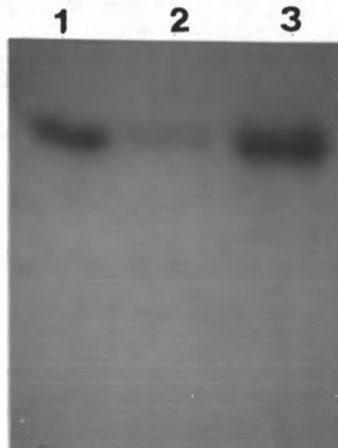


Figure 3.5a Comparison of GS activity staining of E.Coli M5a1 and *Klebsiella* R15 crude extract were performed on PAGE of 7.5 % T.

Lane 1-3 , 8 µg of M5a1 crude extract, *E.Coli* crude extract and *Klebsiella* R15 crude extract respectively.

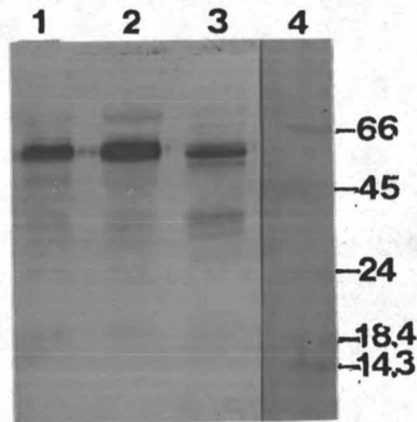


Figure 3.5b Determination for the cross-reactivity between GS from *E.coli* and M5a1 crude extract with anti-GS antiserum were done on NC membrane after separating on SDS-PAGE of 12 % T.

Lane 1-3, 3 µg of *Klebsiella* R15 crude extract, M5a1 crude extract and *E.coli* crude extract respectively.

Lane 4 Standard molecular weight markers.

confirm the specificity of GS-antisera to only GS protein (Figure 3.5b).

3.5 Development indirect ELISA for GS

3.5.1 Precision and accuracy for ELISA method.

After preliminary study to determine the appropriate dilution of the first and second antibody, the precision and accuracy of the ELISA were done as shown in Table 3.2 and Figure 3.6. The distribution of absorbance values which exhibited the variation of standard deviation in the intra-assay was lass than 8 %, whereas the variation among inter-assay was higher(ranging 5-14 %). So the precision of ELISA in intra-assay was high. The accuracy of the low level GS antigen(0.5-2.0 ng) were higher than the high level antigen(2.0-5.0 ng) in the inter assay. The sensitivity of this method to detect the GS protein was 0.5 ng.

3.5.2 The stability of GS protein

GS protein in *Klebsiella* R15 crude extract were assayed by at various times after breaking cells by sonicator, Table 3.3 shows that GS protein contents obtained were very consistence at 46.0-47.8 ng/mg protein for at least 16 weeks.

3.5.3 Examination for the nonspecific binding

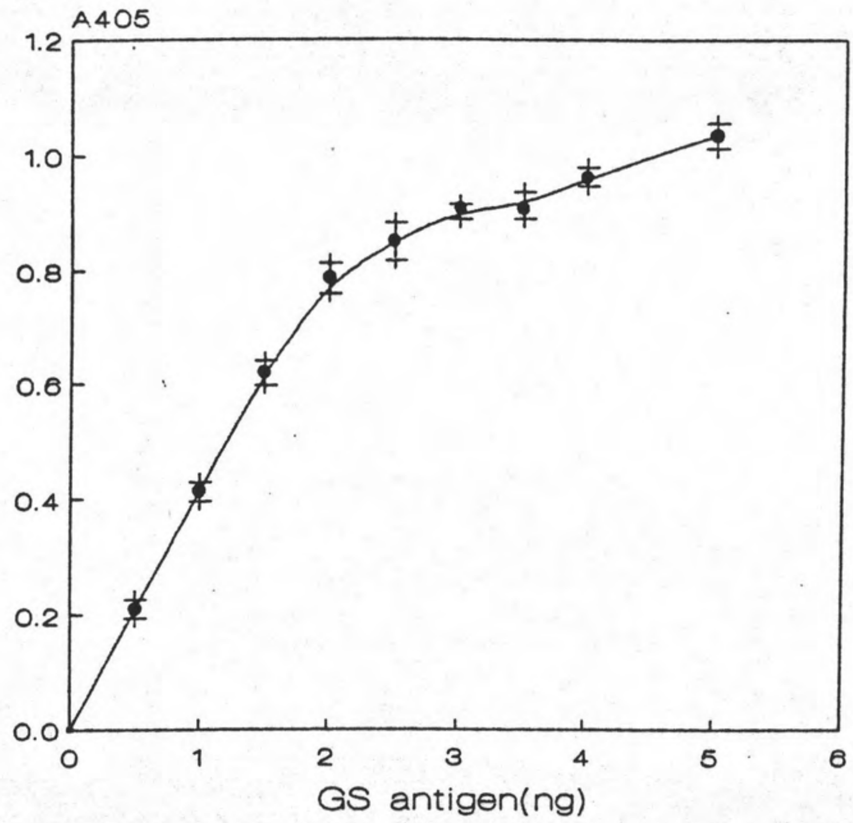
Preimmune serum was dilute to 1:10000 and was added in place of antibody against GS to determine nonspecific binding, resulting in zero values of the absorbance at 405 nm whereas immune serum yielded absorbance at 405 nm. Nonspecific binding of GS protein to the second antibody was also non detectable.

Table 3.2 Precission of the ELISA method.

GS protein ranging from 0.5-5.0 ng/well showed the per cent coeffience of variation(% CV) in intra-assay less than 8 % and inter-assay of ELISA less than 14 %.

Varience	GS antigen (ng)								
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0
Intra-assay									
Mean A_{405}	0.210	0.412	0.619	0.786	0.850	0.907	0.909	0.963	1.035
SD	0.016	0.017	0.022	0.028	0.033	0.018	0.028	0.016	0.022
n	6	6	6	6	6	6	6	6	6
% CV	7.62	4.13	3.55	3.56	3.88	1.99	3.08	1.66	2.13
Inter-assay									
Mean A_{405}	0.219	0.431	0.672	0.800	0.908	0.988	1.079	1.087	1.126
SD	0.011	0.025	0.083	0.044	0.126	0.138	0.139	0.135	0.142
n	5	5	5	5	5	5	5	5	5
% CV	5.20	5.90	12.31	5.50	13.84	13.99	13.22	12.42	12.64

a)



b)

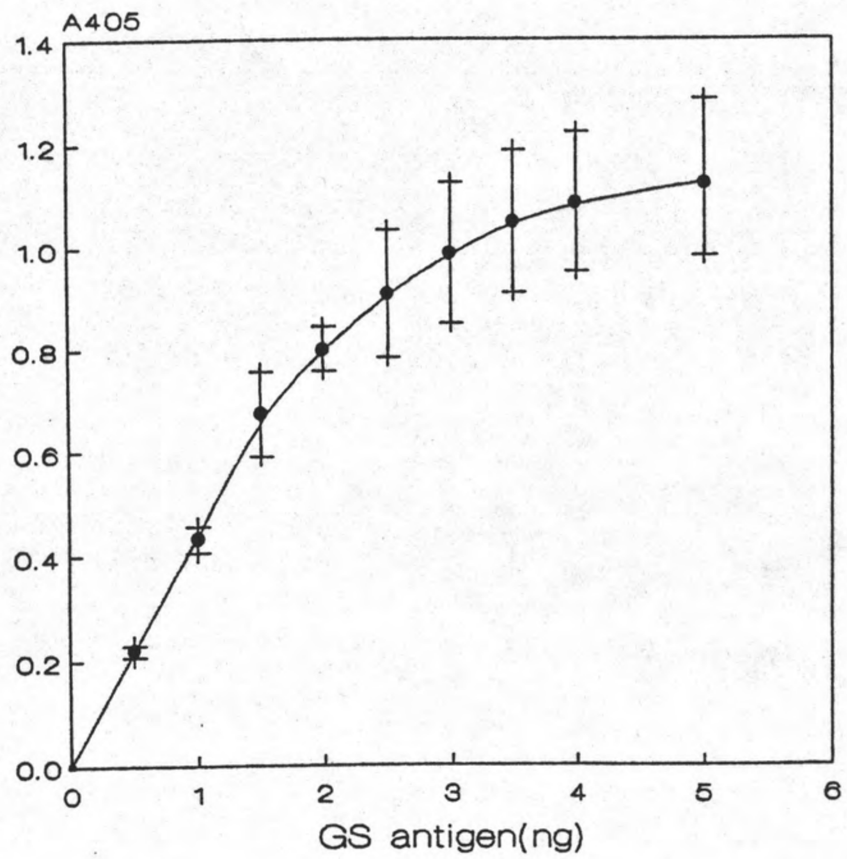


Table 3.3 Stability of GS protein in *Klebsiella* R15 crude extract was determined by ELISA method.

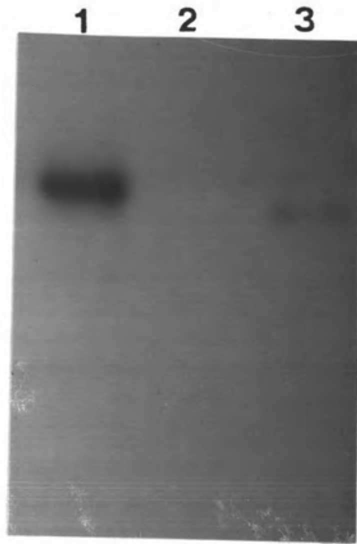
Week	GS protein (nG mg $protein^{-1}$)
1	47.7
2	47.8
3	47.6
7	46.0
16	47.0

3.6 No cross-reactivity between bacterial GS and cytosolic GS of rice root.

Native PAGE by activity staining of crude extract from free living *Klebsiella* R15 grown in N_2 - fixing condition and rice root showed difference in the molecular size (Figure 3.7a) corresponding to the known information that enteric bacterial GS 600 Kda) is larger than the octameric plant GS (340 Kda). Rice root cytosolic GS appears as smeared faint bands by Western blot indicating that rice GS did not cross-react with anti-GS (Figure 3.7b, lane 1,4 and 5). This result confirms that anti-GS of *Klebsiella* R15 obtained is specific to bacterial GS. In case of bacterial crude extract plus rice root pelletted fraction bacterial GS band can be observed at the same position as in *Klebsiella* R15 crude extract (Figure 3.7b, lane 6).

3.7 Induction of bacterial growth in associative condition

In the comparative study of GS specific activity of *Klebsiella* R15 just added on D0 and following associate on with rhizosphere of rice(D8). The total number of viable *Klebsiella* R15 at that time was estimated by spread plate method along with GS determination as shown in Table 3.4. A significant increase in active bacterial cells in associative condition implies that both nutrients and energy sources are provided by root exudate.


1
2
3

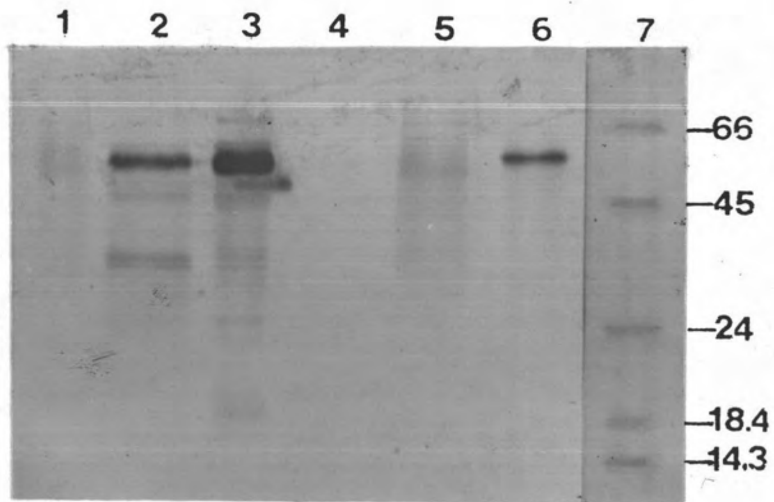

1
2
3
4
5
6
7
66
45
24
18.4
14.3

Table 3.4 Total viable number of bacteria in free-living versus associative condition(150 rice seedlings)

Time/condition	Number of colony ($X 10^9$)
a) D0/*Klebsiella* R15 free-living	30
a) D8/*Klebsiella* R15 free-living	3
b) D8/*Klebsiella* R15 rhizosphere of rice	200
b) D8/*Klebsiella* R15 endorhizosphere of rice	7

3.8 GS antigen levels in *Klebsiella* R15-rice association

The GS protein in free-living *Klebsiella* R15 and associative *Klebsiella* R15 condition were determined by indirect ELISA as shown in Table 3.5. GS protein of *Klebsiella* R15 in associative condition increased about 3-5 fold compared to free-living *Klebsiella* R15 on D7-D8 after inoculation. As confirm by Western blot analysis in Figure 3.8, bacteria fraction either in free living(lane 1,2) or associative condition (lane 3) revealed GS protein with higher intensity in associative condition, where total protein loaded were equal. GS protein of rice only (RD7) was zero, because the anti-GS of *Klebsiella* R15 did not cross-react with rice root cytosolic GS. Western blot analysis of rice root extracts from both cytosolic GS fraction and membrane-bound fraction on D7, 8 did not cross-react with GS antibody of bacteria(Figure 3.8, lane 7, 8 and 5), although the amount of total protein loaded were equal. GS protein could be detected in endorhizospheric *Klebsiella* R15 plus rice root pellet fraction (Lane

Table 3.5 Changes in the GS protein of free-living *Klebsiella* R15 and associative *Klebsiella* R15

Source of GS	Day	GS protein ug·(mg protein)$^{-1}$ (**)	GS Ratio	Total cytosolic protein(mg)	Protein Ratio
R15 rhizossheric fraction					
R15	0	26.6$\pm$7.0 (5)		1.91$\pm$0.75	
R15	7	13.7$\pm$1.7 (3)	1.0	0.58$\pm$0.26	1.0
R15+RD7	7	70.5$\pm$22.9 (3)	5.1	1.18$\pm$0.15	2.0
R15	8	13.1$\pm$1.8 (2)	1.0	1.03$\pm$0.36	1.0
R15+RD7	8	42.7$\pm$2.8 (2)	3.2	2.15$\pm$0.29	2.1
R15 endorhizospheric/rice root pelle fraction (150 plants)					
RD7	7	0	1.0	1.12$\pm$0.73	1.0
R15+RD7	7	2.42$\pm$0.49 (2)	2.4	1.18$\pm$0.65	1.1
RD7	8	0	1.0	0.93	1.0
R15+RD7	8	1.73 (1)	1.7	1.20	1.3

(**) : Values in brackets are number of experiments.

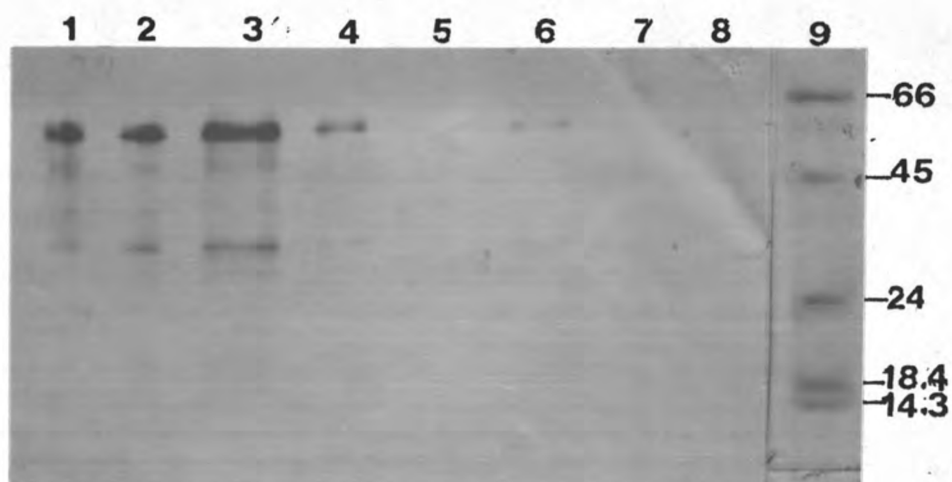


Figure 3.8 Comparison of GS protein in free-living and associative *Klebsiella* R15 and rice root extracts by Western blot analysis.

Lane 1 Crude extract of in free living *Klebsiella* R15 on D0, 1.4 μg

Lane 2 Crude extract of in free living *Klebsiella* R15 on D7(*Klebsiella* R15 + Rd7), 1.4 μg

Lane 3 Crude extract of rhizospheric *Klebsiella* R15 fraction on D7, 1.4 μg

Lane 4 Crude extract of endorhizospheric *Klebsiella* R15 plus rice root pellet fraction on D7 (*Klebsiella* R15 + RD7), 1.4 μg

Lane 5 Rice root pellet fraction(RD7 only), 1.4 μg

Lane 6 Crude extract of endorhizospheric *Klebsiella* R15 plus rice root pellet fraction(*Klebsiella* R15 + RD7), 0.7 μg

Lane 7 Rice root cytosol fraction (RD7 only), 1.4 μg

Lane 8 Rice root cytosol faction on D7 after *Klebsiella* R15 inoculation, 1.4 μg

Lane 9 The mixture of standard molecular weight markers.

4, 6) but not in rice root pellet fraction only (lane 5), demonstrated that the enzyme protein must derive from *Klebsiella* R15.

3.9 <u>Change in GS specific activity of free-living *Klebsiella* R15 after inoculation into rice seedlings cv RD7.</u>

The specific activity of GS in *Klebsiella* R15 associated with rhizosphere of rice root increased about 7-9 fold compared to free-living *Klebsiella* R15 on D7, D8(Table 3.6). In addition, the GS specific activity of *Klebsiella* R15 in association with rhizosphere was higher than free-living *Klebsiella* R15 on D0 in which those free-living *Klebsiella* R15 were grown in LB then changed to NF medium only 6 h. However specific activity of *Klebsiella* R15 associated with rhizosphere of rice(D7,8) were higher exactly than free-living *Klebsiella* R15(D0).

In the supernatant fraction of rice root, plant GS specific activity slightly decreased in rice root that had been inoculated with *Klebsiella* R15 (Table 3.7). These values represented cytosolic Gsr which differed from the rice root pellet fraction after inoculation of *Klebsiella* R15 where GS specificity activity was increased in comparison to rice root pellet due to bacterial infection, so that increasing activity belonging to *Klebsiella* R15 (endorhizospheric) was observed. These results correspond with spread plate method(Table 3.4) and immunoblotting of GS protein band (Figure 3.8) and increasing quantity of GS protein by ELISA(Table 3.5)

Table 3.6 Changes in the GS specific activity of free-living *Klebsiella* R15 and associative *Klebsiella* R15 versus change in total proteins.

Source of GS	Day	Specific activity* (**)	GS Ratio	Total cytosolic protein(mg)	Protein Ratio
R15 rhizospheric fraction					
R15	0	0.37±0.16 (5)		1.91±0.75	
R15	7	0.10±0.05 (3)	1.0	0.58±0.26	1.0
R15+RD7	7	0.92±0.48 (3)	9.2	1.18±0.15	2.0
R15	8	0.08±0.01 (2)	1.0	1.03±0.36	1.0
R15+RD7	8	0.56±0.01 (2)	7.0	2.15±0.29	2.1
R15 endorhizospheric/Rice root pelleted fraction (150 plants)					
RD7	7	0.32±0.21 (2)	1.0	1.12±0.73	1.0
RD7+R15	7	0.59±0.48 (2)	1.8	1.18±0.65	1.1
RD7	8	0.19 (1)	1.0	0.93	1.0
RD7+R15	8	0.31 (1)	1.6	1.20	1.3

* : (μmol γ-Glutamyl hydroxamate) $\cdot$(mg protein)$^{-1}\cdot$min^{-1}

(**) : Values in brackets are number of experiments.

Table 3.7 Changes in the GS specific activity of rice root supernatant fraction(150 plants)

Source of GS	Day	Specific activity* (**)	GS Ratio	Total cytosolic protein(mg)	Protein Ratio
RD7	7	1.82±0.92 (3)	1.0	4.24±0.85	1.0
RD7+R15	7	1.39±0.74 (3)	0.8	3.46±0.79	0.8
RD7	8	0.75±0.21 (2)	1.0	3.99±1.66	1.0
RD7+R15	8	0.65±0.27 (2)	0.9	3.52±1.45	0.9

* : µmol γ-Glutamyl hydroxamate ·(mg protein)$^{-1}$·min^{-1}

(**) : Values in brackets are number of experiments.

3.10 Concurrent changes in nitrogenase activity and GS activity in associative *Klebsiella* R15.

The increasing in nitrogenase activity in associative *Klebsiella* R15 with rhizosphere of rice was evident by comparison of ARA profiles between free-living *Klebsiella* R15 and RD7 during D2-D6 after inoculation with *Klebsiella* R15 (Figure 3.9). From Table 3.8, nitrogenase activity of *Klebsiella* R15 associated with rice (*Klebsiella* R 15 + Rd7) was 535-fold higher than in the free-living *Klebsiella* R15 (*Klebsiella* R15, 2.9 $nmol \cdot tube^{-1} \cdot day^{-1}$) and 443-fold higher than that of free-living rice seedlings(RD7 only, 3.5 $nmol \cdot tube^{-1} \cdot day^{-1}$) on D6. The specific activity of GS, as determined after on D7 and D8 determined after nitrogenase activity increased to stationary phase, increased merely 7-9-fold from free-living *Klebsiella* R15 (Table 3.6). The small increase in GS specific activity was incomparable to that of nitrogenase activity (400-500 fold).

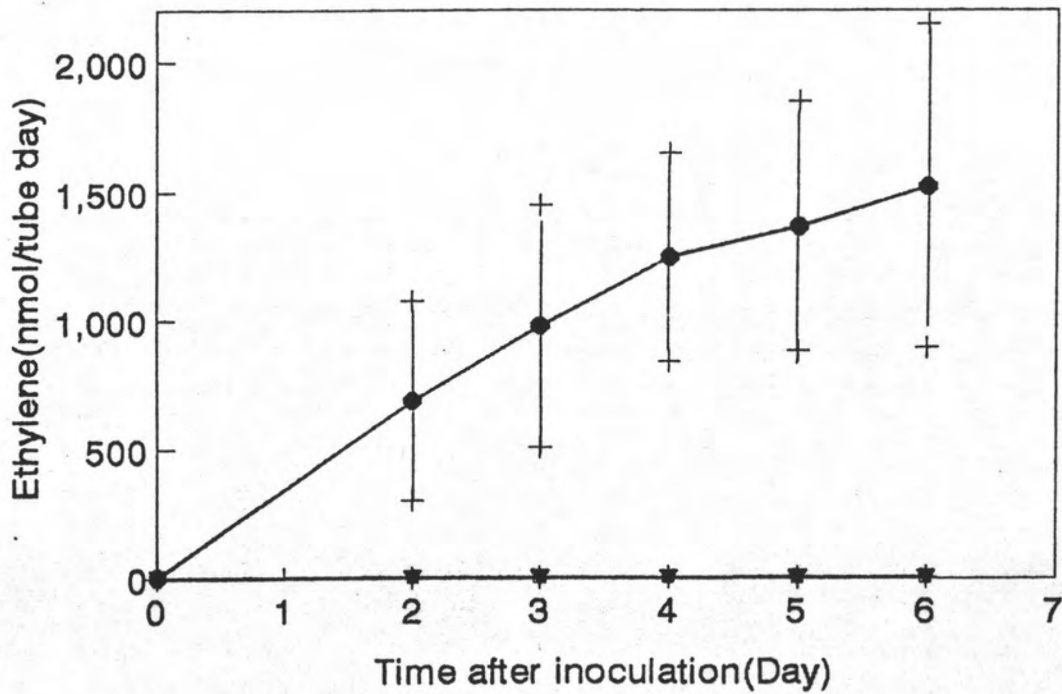


Figure 3.9 Comparison of nitrogenase activity in *Klebsiella* R15, RD7 and *Klebsiella* R15 +RD7 after *Klebsiella* R15 was inoculated in rice seedlings. Nitrogenase activity expressed as nmol C_2H_4 $tube^{-1}$ day^{-1}; mean of five experiments are shown. Standard deviations are indicated by bars.

▼ *Klebsiella* R15

■ RD7

● *Klebsiella* R15 +RD7

Day after inoculation	Bacteria/Rice Growth condition	Nitrogenase fixing activity (nmol C_2H_4·tube·day)
0	R15 (Organic N——N_2)	1.9 ± 1.3
6	R15 (N_2-grown)	2.9 ± 1.0
6	RD7 (N_2-grown)	3.5 ± 1.9
6	R15/RD7(N_2-grown)	1552.1 ± 626.4

Table 3.8 Rate of N_2 fixation in *Klebsiella* R15, RD7 and *Klebsiella* R15 + RD7 after inoculation with *Klebsiella* R15.