CHAPTER III

RESULTS

3.1 The stability of GS in crude enzyme preparation

From preliminary studies of the GS in crude preparation, the activity was not consistent, being varied from experiment to experiment. This probably due to the unstability of the enzyme or the unappropriate buffer utilized. The first attempt made was to select the most suitable conditions to store the enzyme, with the emphasis on the choice of buffer, temperature, and time of storage.

The buffers TME, pH 7.4 and imidazole-HCl, pH 7.5 previously described for the preparation of GS from *Bacillus subtilis* (50) and for the preparation of GOGAT from *Azospirillum brasilense* (45) were considered. After preparation of the GS from *Klebsiella* spp.R15, the crude enzyme was divided in 2 parts and seperately kept in the buffer mentioned at $4^{\circ}C$ and $-20^{\circ}C$ for a certain time before assay for the GS activity (both biosynthetic and transferase activities) and protein concentration.

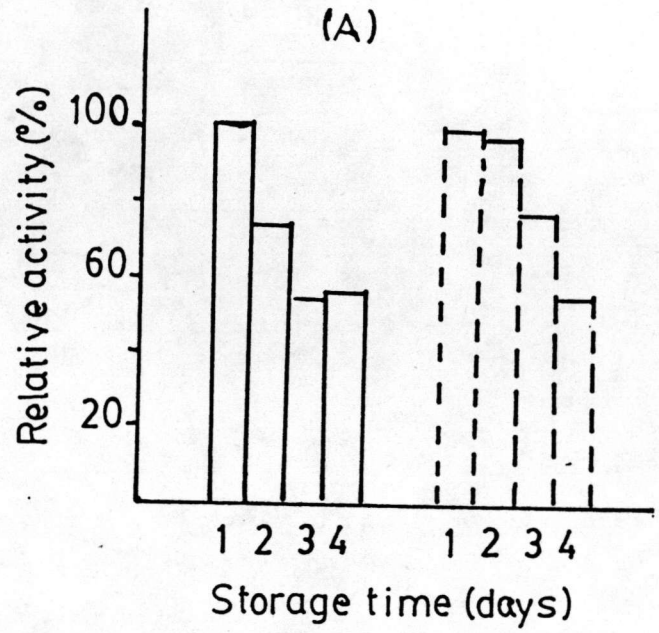
The results on the stability of crude enzyme in different buffers were shown in fig.3.1 and fig.3.2. The results from fig.3.2 clearly show that the GS was unstable in imidazole-HCl buffer when stored either at $4^{\circ}C$ or

-20°C. Both biosynthetic and transferase activities could not be detected in the third day, unless the volume of the enzyme in the assay was increased to about 5 folds. Fig.3.1 shows that both biosynthetic and transferase activities were retained over 85% on the third day when stored at -20°C. However, the slow decrease in activities was observed when stored at 4°C. These results indicate that TME buffer is more suitable than imidazole-HCl buffer in stabilizing Klebsiella spp.R15 GS.

3.2 The stability of GS in frozen cells

The stability of GS in frozen Klebsiella cells (-20°C) is shown in fig.4. TME buffer, pH 7.4 and imidazole-HCl buffer, pH 7.5 were used as washing and suspending buffers. Packed cells were kept frozen and crude preparations were prepared from each fraction daily. Activity before freezing was considered as day 0.

Result from figure 4 again shows that TME buffer (A) seeems to be more appropriated than imidazole-HCl buffer (B) for enzyme preparation. The specific activities, both biosynthetic and transferase, were comparably high and constant in the former buffer upon storage. TME was thus used as buffer for enzyme preparation throughout this thesis and crude extract was always prepared from overnight frozen cells.



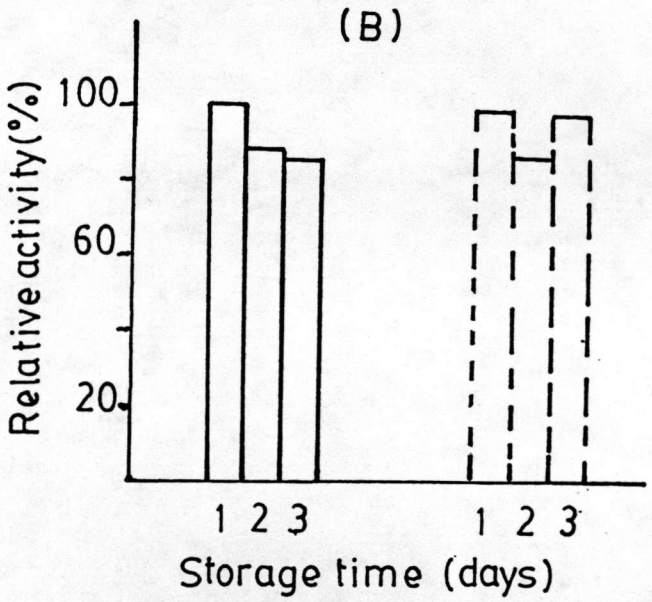


Fig.3.1 **Stability of** GS in crude enzyme fraction in TME buffer, pH7.4. 0.65 mg/ml of crude enzyme solution was seperately stored at 4°C (A) and -20°C (B) for the time indicated before assaying for GS activity. ☐ and ⬚ represent the biosynthetic and transferase activities, respectively.

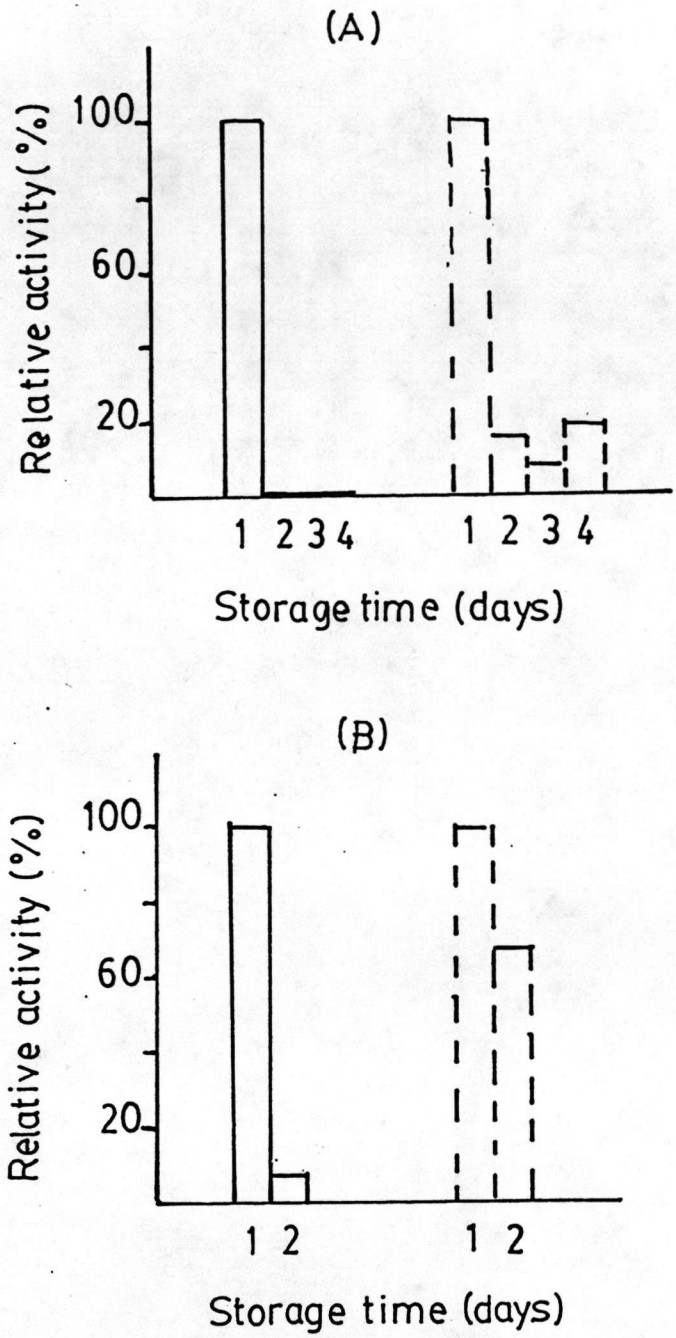


Fig.3.2 Stability of GS in crude enzyme fraction stored in imidazole-HCl buffer, pH 7.5. 0.60 mg/ml of crude enzyme solution was seperately stored at 4°C (A) and -20°C (B) for the time indicated before assaying for GS activity. ☐ and ⬚ represent the biosynthetic and transferase activities, respectively.

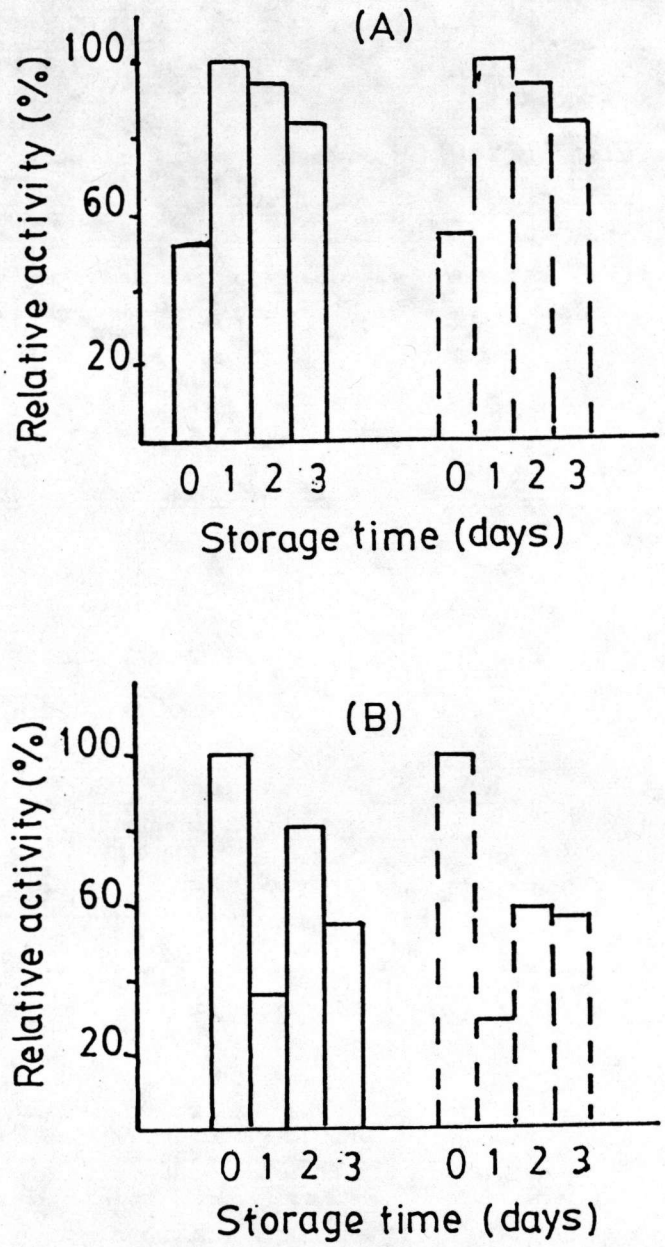


Fig.4 Stability of GS in frozen Klebsiella cells. Packed cells were kept frozen (-20°C) and used for crude preparations in (A) TME buffer and (B) imidazole-HCl buffer. ▯ and ⋯ represent the biosynthetic and transferase activities, assayed at the indicated times.

3.3 Production of GS in NF medium

In ammonium limited medium, such as in low concentration of ammonium or when using nitrate or molecular nitrogen as nitrogen sources, most microorganisms derepress GS (2). In order to obtain high activity of GS from Klebsiella spp.R15, the bacteria were cultured in NF medium. The growth of Klebsiella spp.R15 in NF medium, and specific activity of GS are shown in fig.5. It was observed that both biosynthetic and transferase activities were highest in the late log phase of growth when OD_{420} was 0.7.

However the biosynthetic activity which measured the active form of GS was always lower than the transferase activity, the indicator of the total activity of GS (both adenylylated and deadenylylated forms)(43).

3.4 Comparative studies of GS, GOGAT and GDH activities in crude enzyme

The possible significance of GS in the ammonium assimilating pathway in Klebsiella spp.R15 was investigated by comparing the specific activity of GS, GDH and GOGAT. Crude enzyme fractions were prepared from cultures grown in NH_4^+-limited (NF) and NH_4^+-excess (NFA100) media as described in the materials and methods.

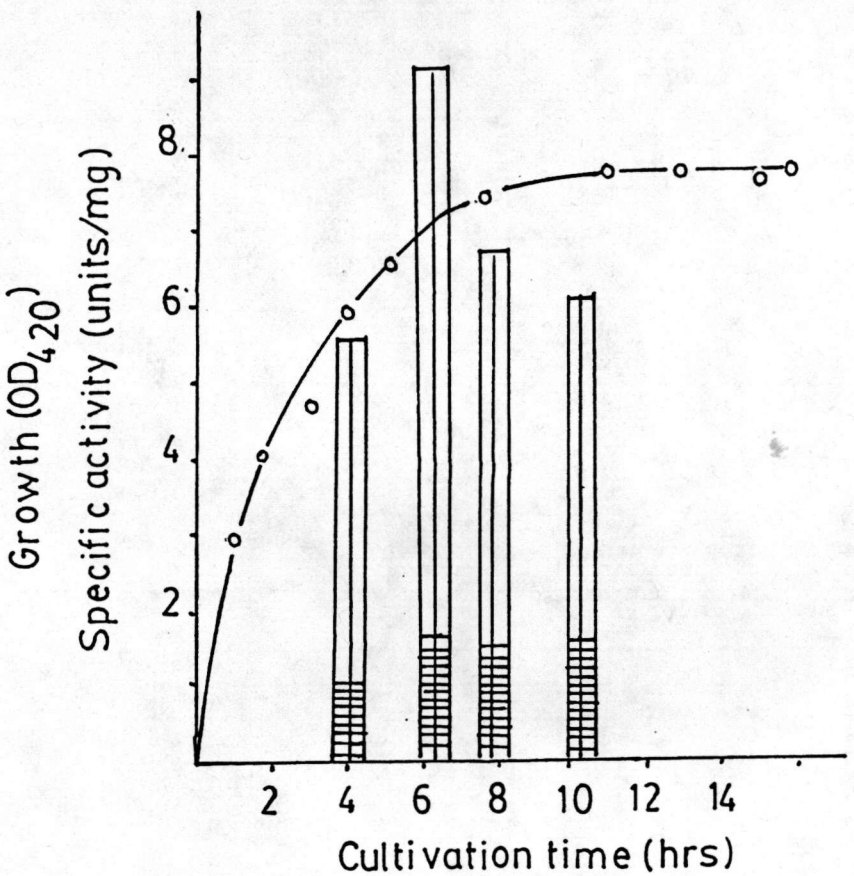


Fig.5 Production of GS in NF medium

Klebsiella spp.R15 was grown in NF medium, and growth was measured by OD_{420}. (o——o). The cells were harvested at the indicated times and used as sources for crude enzyme preparations. GS activities were determined by both biosynthetic (▯) and transferase (▦) assays

The result shown in fig.6 suggests that both biosynthetic and transferase activities of GS were repressed in NH_4^+-excess culture (NFA100) and derepressed in NH_4^+-limited culture (NF). In contrast to GS, GOGAT activity was higher in NH_4^+-excess than in NH_4^+-limited condition.

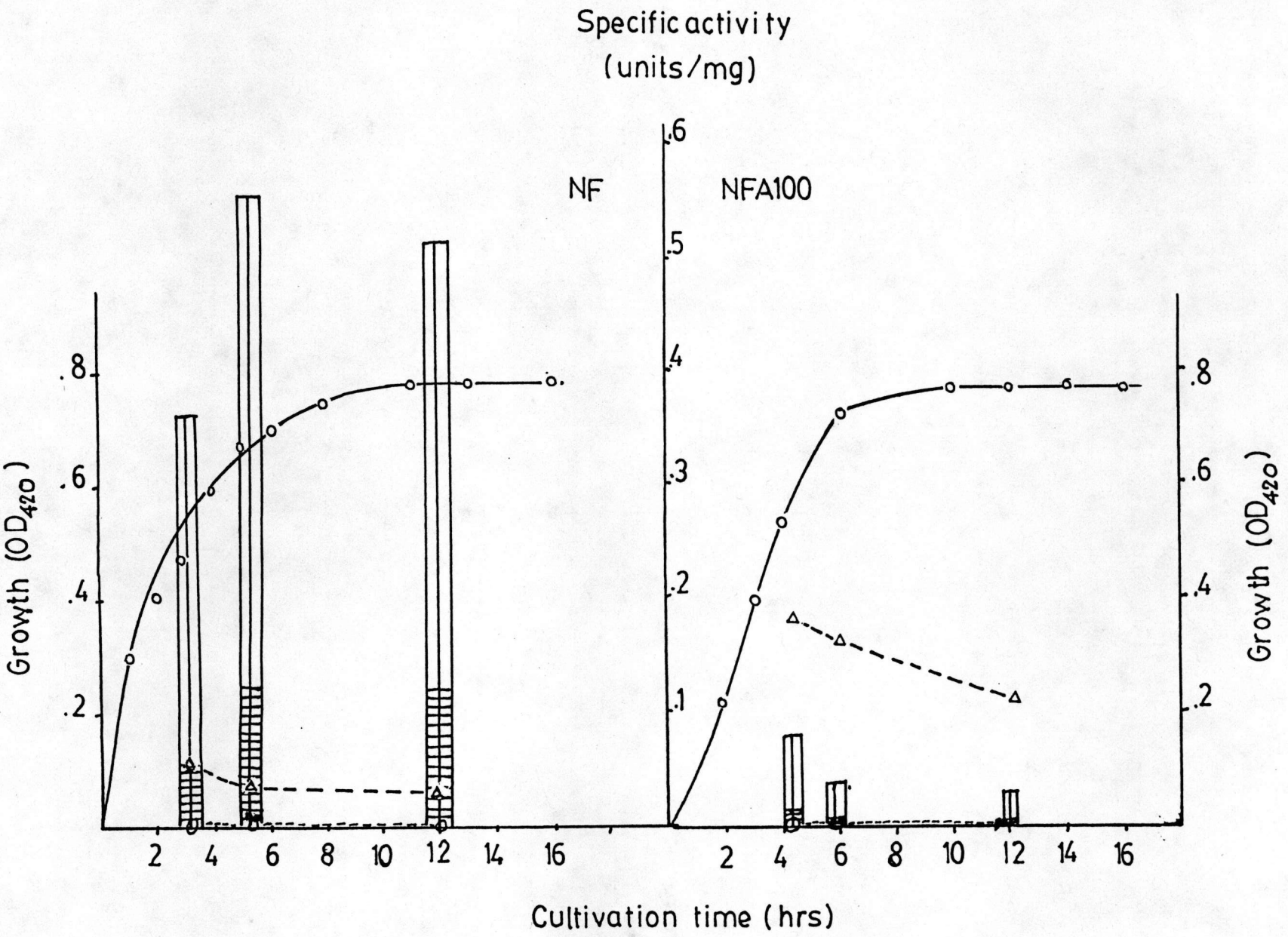
GDH activity in crude enzyme fraction from both cultures were too low to be detected, by measuring the rate of oxidation of NADH. The activity could only be detected from NFA100 but not from NF culture, if the enzyme solutions were 5 fold concentrated (data not shown), indicating very low GDH activity in Klebsiella spp.R15, especially when grown in NF medium.

These results strongly indicate that in Klebsiella spp.R15, GS is the key enzyme in the assimilation of NH_4^+ in both NH_4^+-limited and NH_4^+-excess conditions. The bacteria does not use GDH as the key enzyme for this process. NH_4^+-substrate may increase the rate of glutamine formation via GS reaction which consumes large amount of ATP. Klebsiella spp.R15 may decreased ATP utilization by decreasing the GS synthesis to a certain extent. The remaining GS is still operating since the presence of very low activity of GDH may not be capable of assimilating all ammonium available.

For the next purification of GS, the enzyme was

Fig.6 Specific activity of GS, GOGAT and GDH during growth in NH_4^+-limited and NH_4^+-excess media.

Klebsiella spp.R15 was grown in NF and NFA100 as described in the materials and methods. The growth was measured by OD_{420} (●—○). The cells were harvested and assayed for GS (biosynthetic ▓, transferase ▒), GDH (o----o), and GOGAT (▲----▲) activities at the indicated times.


Specific activity
(units/mg)
NF
NFA100
Growth (OD420)
Growth (OD420)
Cultivation time (hrs)
.2
.4
.6
.8
.1
.3
.5
2
4
6
8
10
12
14
16

always from cells grown in NF medium.

3.5 Purification and properties of GS

3.5.1 Purification of GS from NF culture.

Procedure of GS purification in this Klebsiella spp.R15 was adapted from that described for Nitrobacter agilis (46).

GS of Klebsiella spp.R15 was purified about 35 fold by affinity chromatography on Blue Sepharose CL-6B (fig.7) and gel filtration on Sepharose-4B (fig.8). Results of purification was summarized in table 3. Details of each purification step are the followings.

Heat treatment

Although the result from table 3 shows that this step did not increase the purity of GS, it was performed because some contaminating proteins were eliminated as demonstrated by polyacrylamide gel electrophoresis in fig.9.

Blue Sepharose CL-6B

Profile of affinity column is shown in fig.7. The affinity ligand shows specific binding to the enzyme, large amount of the protein without GS activity was washed off by the equilibrating buffer and GS activity was specifically eluted with the substrate, ADP. Sixty-one fold of purification and 291% yield were obtained from

this column. Protein determination was done by dye-binding (49) instead of A_{280} measurement since ADP also absorbs at this uv wavelength.

Sepharose-4B

Although disc gel electrophoresis at pH 8.3 shows that the enzyme from Blue Sepharose CL-6B column migrated as a single protein band (fig.9), SDS-polyacrylamide gel electrophoresis (fig.10) demonstrated that the enzyme still be contaminated with some small molecular weight proteins. The enzyme was thus further purified with Sepharose-4B gel filtration column.

Sepharose-4B profile of the enzyme is shown in fig.8. The result shows that other smaller molecular weight proteins could be removed by this gel filtration. The purification fold and percent recovery were greatly decreased (table 3). The enzyme may be unstable in very diluted state, and the protein concentration of the enzyme peak is only 0.07 mg/ml.

When equal amount of proteins from the Blue Sepharose CL-6B and Sepharose-4B column were loaded onto polyacrylamide gel electrophoresis, more intense activity band was observed on the later.

3.5.2 Polyacrylamide gel electrophoresis

The enzyme from each step of purification moved as a single activity band in polyacrylamide gel

Fig.7 Blue Sepharose CL-6B chromatography of Klebsiella spp.R15 GS

24 ml "crude enzyme fraction" of NH_4^+-limited culture (2 L) was loaded on Blue Sepharose CL-6B column (7.0X1.8 cm) equilibrated with 10 mM Tris-HCl, 1 mM $MnCl_2$ buffer pH 7.5. The column was then washed with about 250 ml of the same buffer while maintaining the flow rate at 10.9 ml/hr. The 3 ml fractions were collected and assayed for GS transferase activity (o——o). Protein concentration (Δ——Δ) was assayed by coomassie staining method. GS elution was performed with 2 mM ADP in the previous buffer indicated by the arrow (↓) when protein concentration in the eluants was negligible. 1 ml fractions were collected and assayed for transferase activity (o——o) and protein concentration (Δ——Δ). ⟷ indicates the fractions with high GS activity which were pooled for further purification.

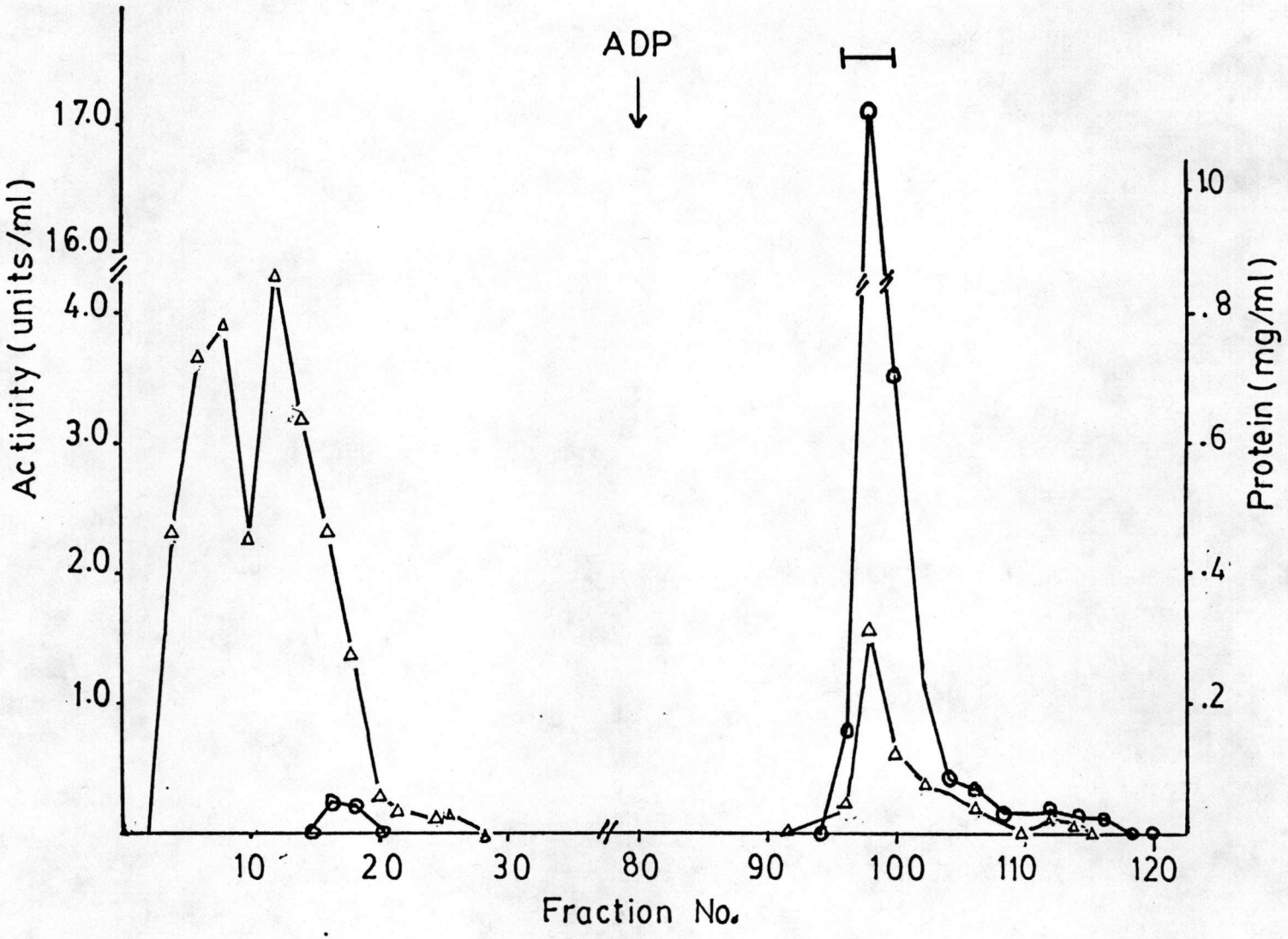

ADP
17.0
16.0
4.0
3.0
2.0
1.0
Activity (units/ml)
10
.8
.6
.4
.2
Protein (mg/ml)
10
20
30
80
90
100
110
120
Fraction No.

Fig.8 Sepharose 4B chromatography of Klebsiella spp.R15 GS

Active fractions from Blue Sepharose CL-6B were pooled and dialysed overnight against 50 mM Tris-HCl buffer pH 7.5. The dialyzed fraction was concentrated on an amicon-10 microconcentrater to 2 ml and loaded onto a Sepharose 4B column (56X1.8 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.5. The enzyme was eluted with the same buffer (flow rate 13.6 ml/hr). 2ml fractions were collected and assayed for transferase activity (o——o) and protein concentration (Δ—Δ). ⊢—⊣ indicates the fractions which posses high GS activity and were pooled for polyacrylamide gel electrophoresis and kinetic studies. To determine molecular weight of the enzyme, the column was calibrated with A-Thyroglobulin (669,000), B-Ferritin (440,000) and C-Alcohol dehydrogenase (150,000)

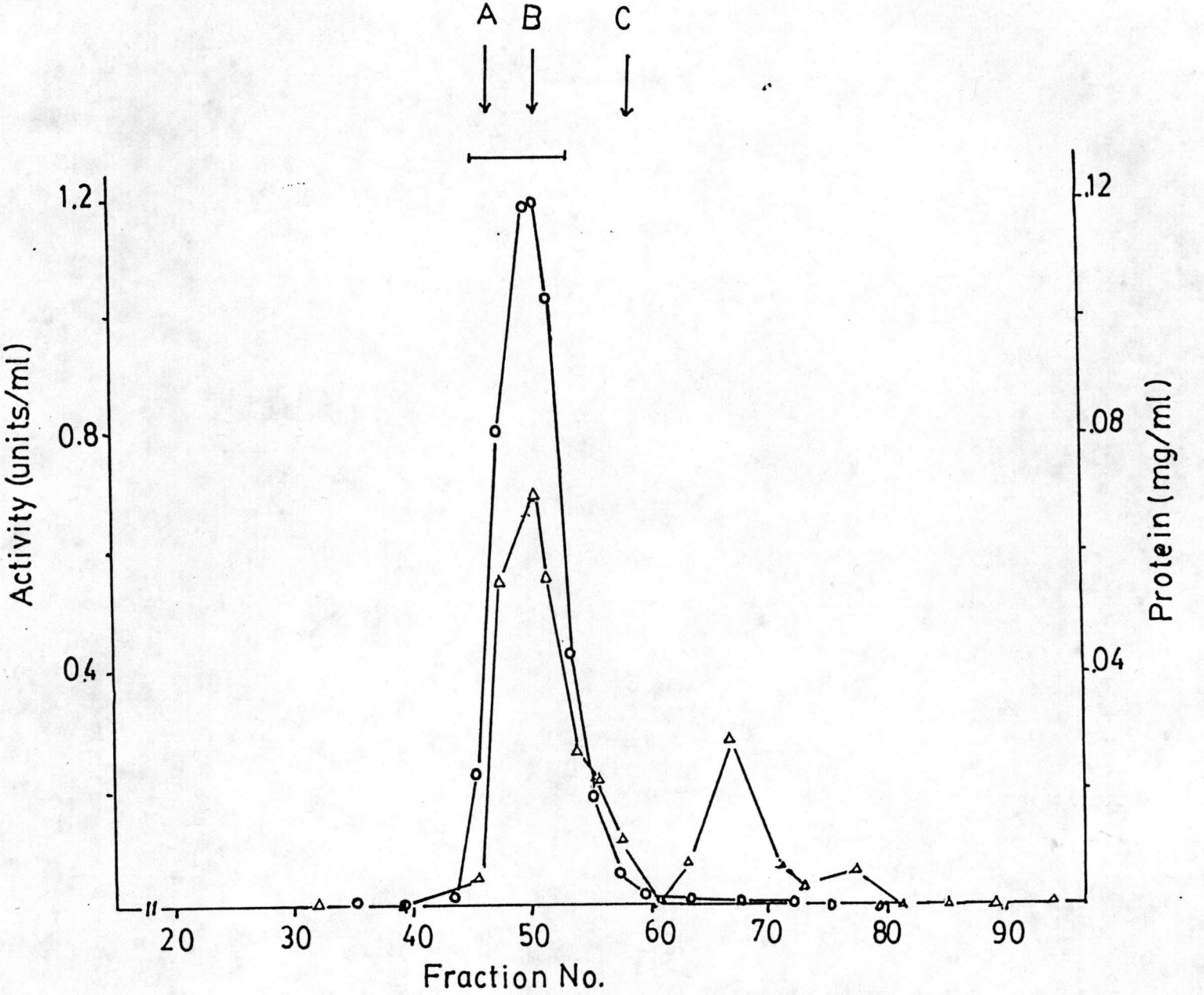

A
B
C
1.2
0.8
0.4
Activity (units/ml)
.12
.08
.04
Protein (mg/ml)
20
30
40
50
60
70
80
90
Fraction No.

Table.3 Purification of GS

Enzyme purification and enzyme assay were as described in the materials and methods. All purification steps (except heat treatment) were performed at 4°C. One enzyme unit is defined as μmole γ-glutamyl hydroxamate produced min^{-1} and specific activity as number of units (mg protein)$^{-1}$

fractions	activity	protein	sp.act.	vol.	total act.	total prot.	fold	%yield
	μmol/min/ml	mg/ml	μmol/min/mg	ml	μmol/min	mg		
Crude enzyme	1.2	1.6	0.7	25	29.3	40.1	1	100
Heat treated	1.1	1.5	0.7	25	27.7	38.3	1.0	94.6
Blue Seph.CL-6B	17.0	0.4	44.8	5	85.1	1.9	61.4	291.1
Sepharose-4B	0.6	0.03	25.4	18	11.4	0.1	34.7	39.0

electrophoresis (fig.9). Protein staining of Blue Sepharose CL-6B fraction and Sepharose-4B fraction showed a single band. This result indicates that GS from both columns was very pure, but the result from SDS-polyacrylamide gel electrophoresis (fig.10) shows a single protein band only in Sepharose-4B fraction. It was thus clearly proved that the purified enzyme moved as a single band in polyacrylamide gel electrophoresis under both non-denaturing and denaturing conditions.

3.5.3 Molecular weight determination

The molecular weight of the purified enzyme determined by gel filtration (Sepharose-4B,fig.8) was calculated to be 430 kDa (fig.11). The enzyme appeared as one band in SDS-polyacrylamide gel (fig.12), and its molecular weight was calculated as 54 kDa (fig.13). Thus the enzyme is very likely to compose of 8 homologous subunits.

3.5.4 pH optima of SVP-treated and nontreated purified GS

Study on GS in E.coli by Stadtman, et al.(51) demonstrated that the GS is controlled by adenylylation-deadenylylation modification. Since the adenylyl groups of GS can be removed by snake venom phosphodiesterase(SVP), various adenylylated states of GS can be prepared for studying enzyme properties.

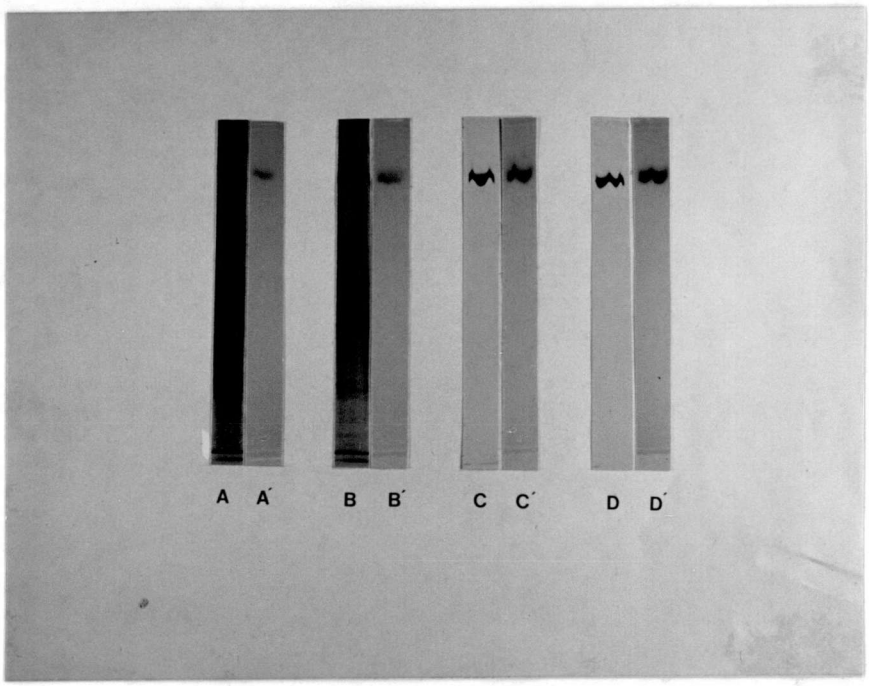


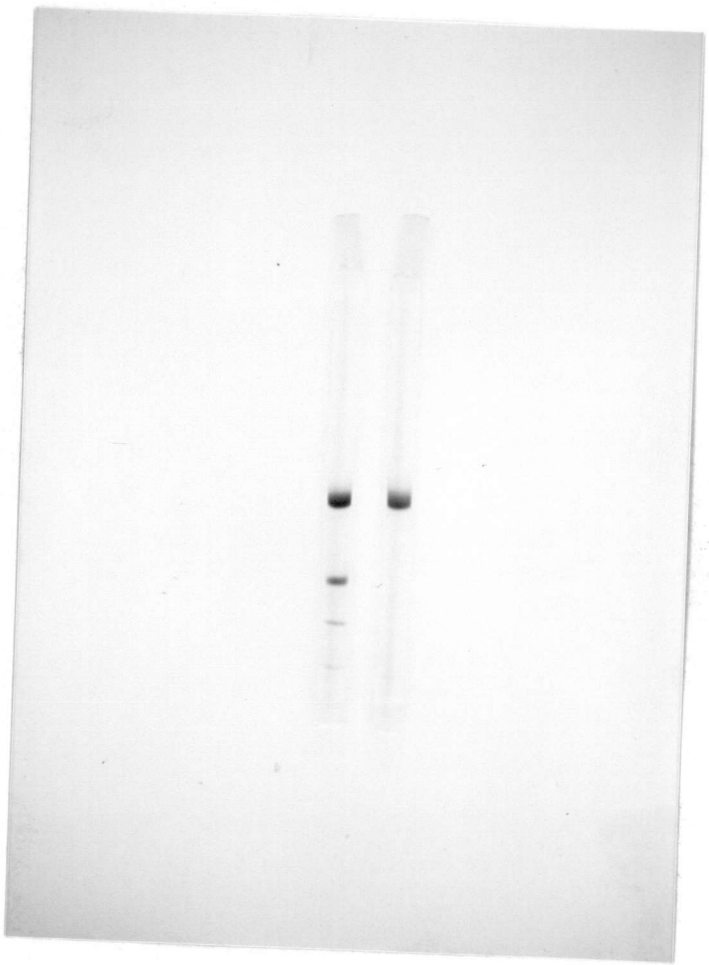
Fig.9 Polyacrylamide gel electrophoresis of GS. Protein stain (A, B, C, D). Activity stain (A', B', C', D')

A,A' : 200(.06), 400(.12) μg(umol/min) of crude enzyme fraction

B,B' : 200(.05), 400(.11) μg(umol/min) of heat-treated fraction

C,C' : 40(.438), 25(.523) μg(umol/min) of Blue Sepharose CL-6B fraction

D,D' : 40(.486), 25(1.018) μg(umol/min) of Sepharose 4B fraction



(a) (b)

Fig.10 SDS-Polyacrylamide gel electrophoresis of GS. Bands were stain for protein.

(a) 40 μg of Blue Sepharose CL-6B fraction

(b) 20 μg of Sepharose 4B fraction

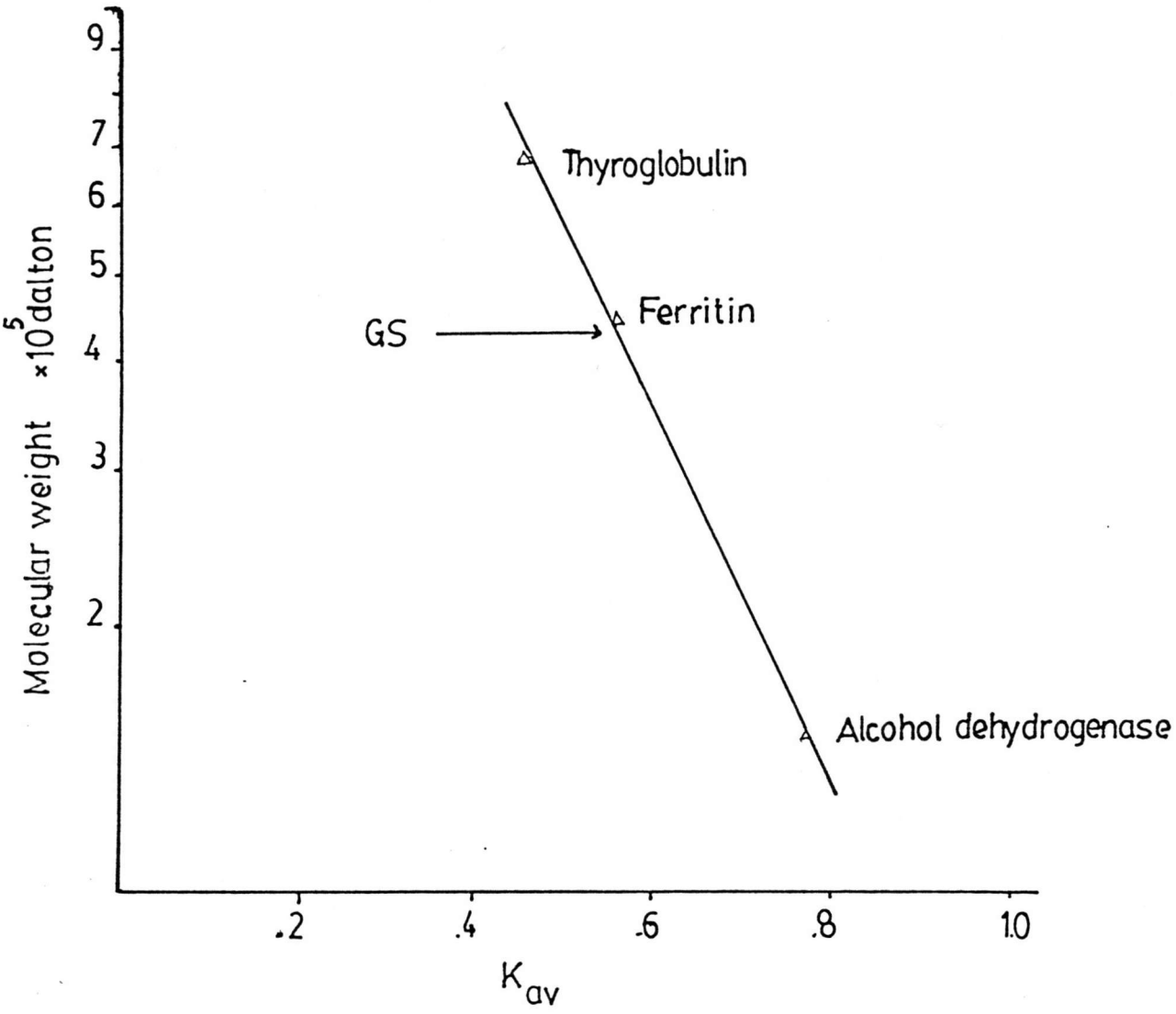


Fig.11 Determination for the molecular weight of GS. Standard proteins were: thyroglobulin (669,000), ferritin (440,000) and alcohol dehydrogenase (150,000).

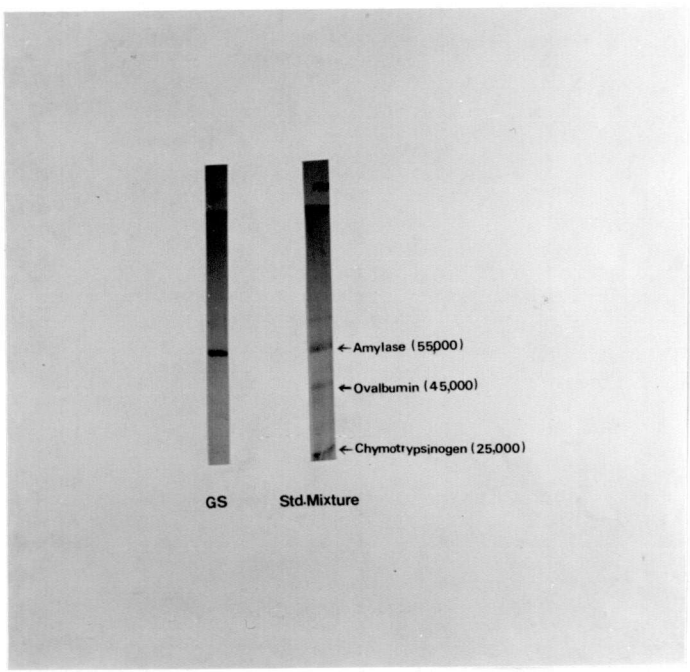


Fig.12 SDS-polyacrylamide gel electrophoresis of purified GS compared with standard proteins: amylase (55,000), ovalbumin (45,000), chymotrypsinogen (25,000).

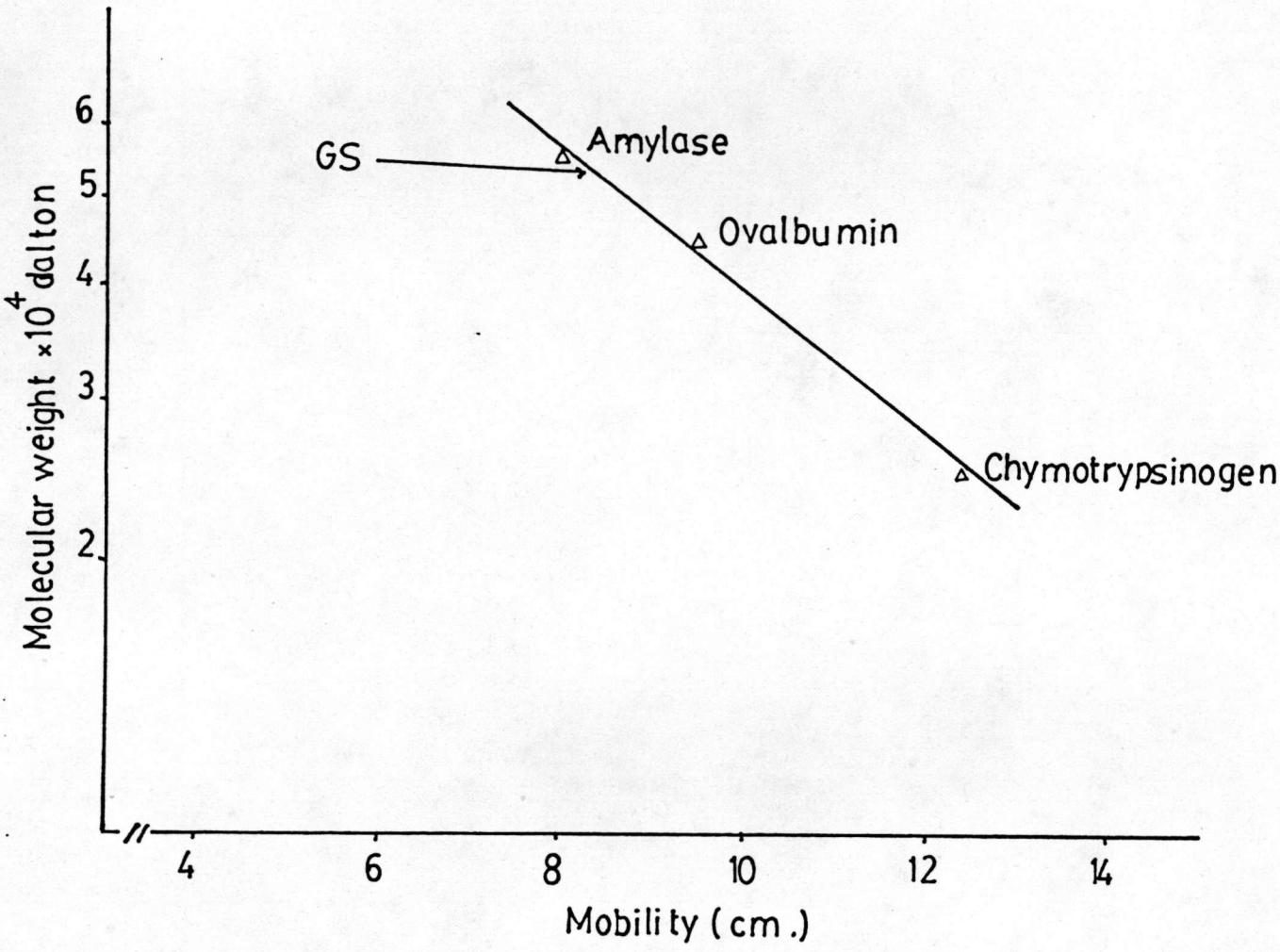


Fig.13 Determination for the molecular weight of the GS subunit. Standard proteins were amylase (55,000), ovalbumin(45,000), and chymotrypsinogen(25,000).

To study the pH optima of various adenylylated states of GS, purified enzyme from NH_4^+-limited cells treated or untreated with SVP were prepared for the assay.

Treating the purified enzyme with SVP caused the decrease in GS activity. But pH optima remained unchanged at 6.6-6.8 and 8.0. Fig.14 also shows that SVP treatment significantly decreased GS activity at the optimum pH (6.6-6.8) but slightly increased the activity at pH 8.0. Isoactivity pH was observed at pH 7.6.

3.5.5 Purification of highly adenylylated GS

Previous report indicated that the addition of ammonium to the cultures of enterobacteriaceae after induction of the GS resulted in the covalent modification of the enzyme by adenylylation (6). To obtain adenylylated GS of Klebsiella spp.R15 in large quantity, the NH_4^+-limited grown cells were shocked with 20 mM ammonium chloride for 15 minutes prior to the harvesting. The cells were extensively washed with 50mM Tris-HCl, pH 7.5 to remove ammonium before being broken by sonication.

Crude enzyme fraction from these ammonium-shocked cells had an increase in the specific transferase activity (48.4%) compared to that from non-shocked cells as shown in table 4. The increase in activity may be due to the adenylylation on the GS molecule. This was

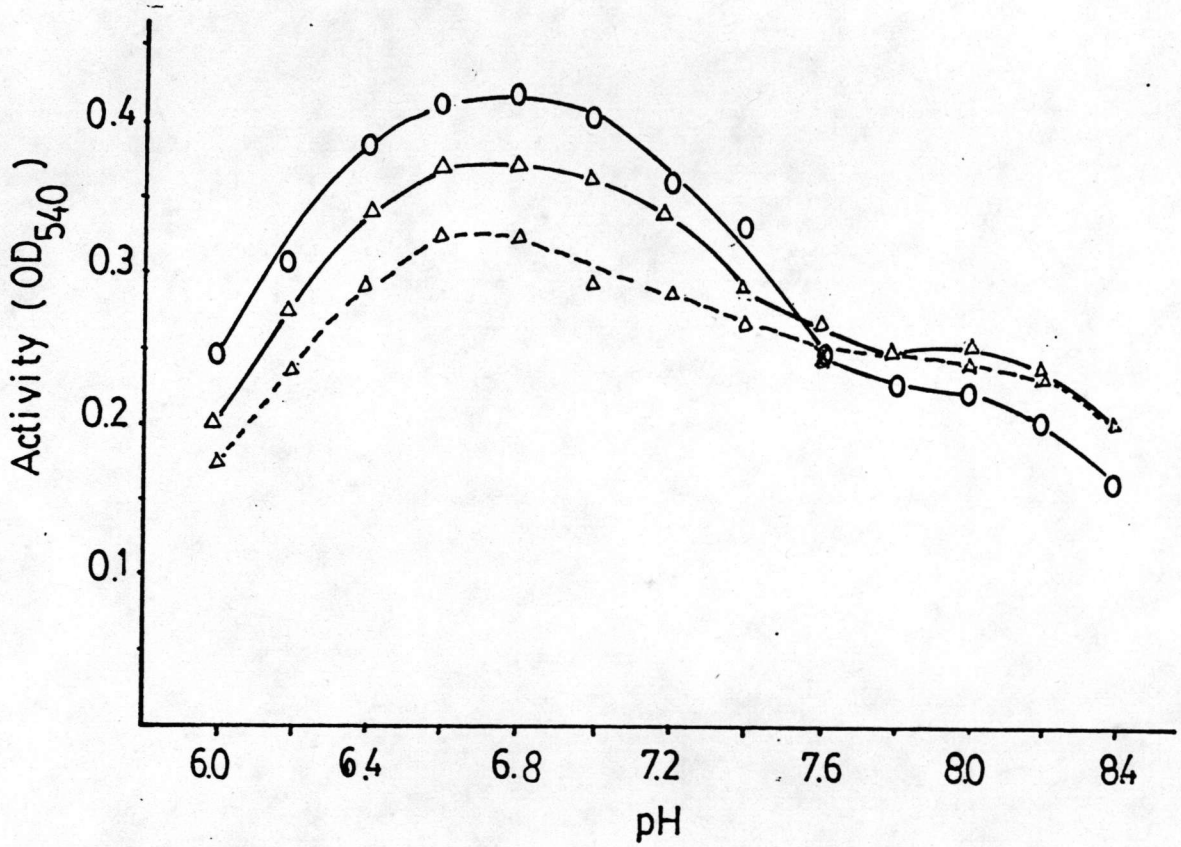


Fig.14 pH optima of purified GS

The purified enzyme was incubated at 37°C without (o——o) or with (Δ——Δ), (Δ----Δ) snake venom phosphordiesterase for 15 mins (Δ——Δ) or 30 mins (Δ---Δ). After incubation, aliqout samples were withdrawn for assay of transferase activity.

supported by the result in fig 14. which shows that the higher the adenylylated GS, the higher the transferase activity.

Table 4. Effect of ammonium on Klebsiella spp.R15 GS transferase activity

Addition of NH_4^+ prior to harvest cells	Activity. (units)	Prot.conc. (mg/ml)	Activity. (units/mg)
-	1.2	1.6	0.7
+	1.7	1.6	1.1

The enzyme obtained from ammonium-shocked cells was further purified by heat treatment, and Blue Sepharose Cl-6B affinity chromatography (fig.15) in the same conditions previously described for non-shocked cells enzyme (fig.7). The affinity chromatographic profiles of these two enzymes were different but Sepharose-4B profiles were the same. Table of purification was shown in table 5.

About 50% (25 units) of activity was eluted slightly after the wash off fraction by low concentration buffer. The remainder was retained in the column and

Fig.15 Blue Sepharose CL-6B chromatography of Klebsiella spp.R15 GS from NH_4^+-shocked cell culture. Culture volume, condition and procedure of GS purification were as described in the materials and methods except 20mM ammonium chloride was added to the culture 15 minutes before harvesting. o——o, transferase activity; △——△, protein concentration; $\overset{GS_B}{\longleftarrow\!\!\longrightarrow}$ indicates the fractions with high GS activity which were pooled for further purification.

$\overset{GS_A}{\longleftarrow\!\!\longrightarrow}$ indicates the fraction with high GS activity which were pooled for characterization.

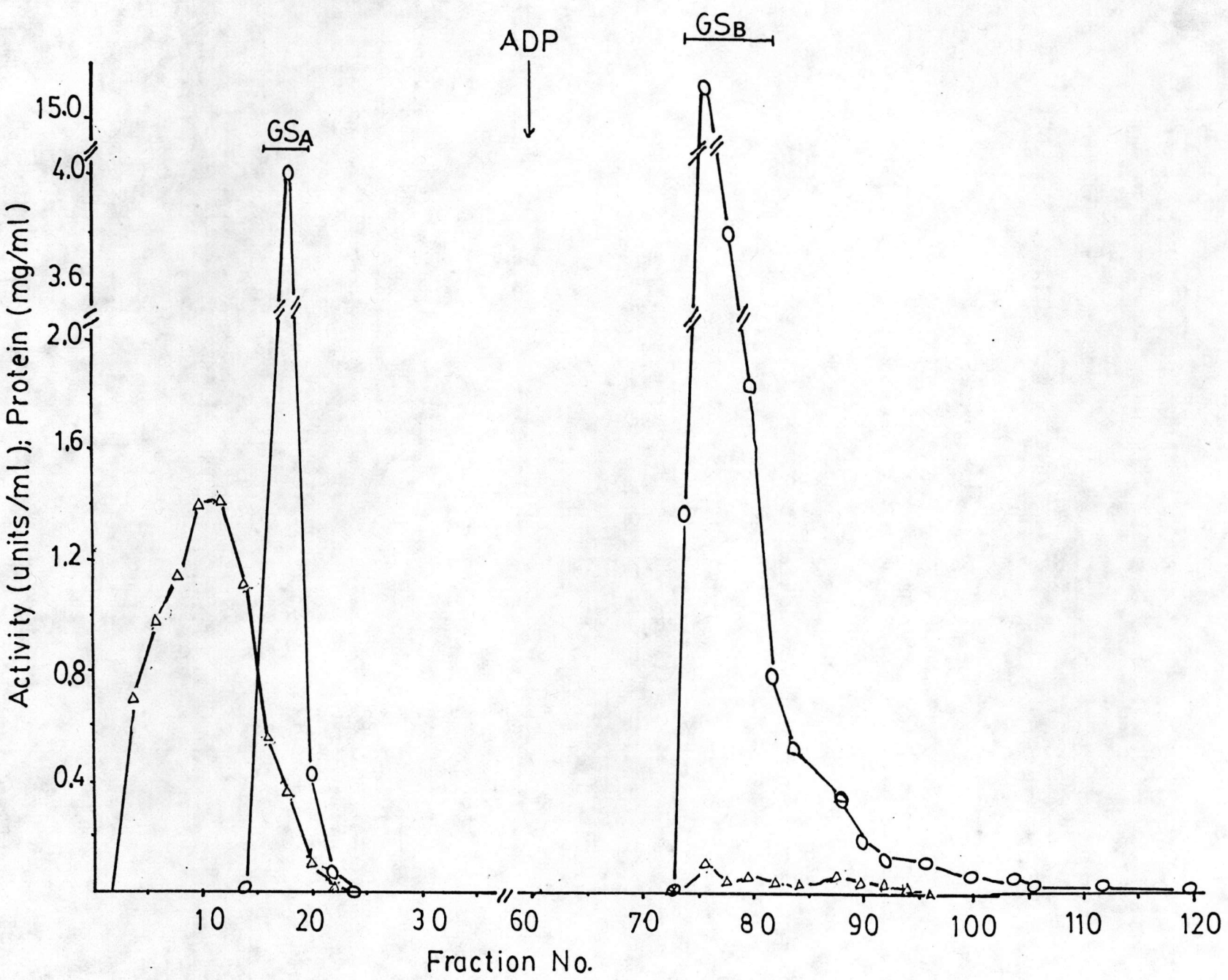

ADP
GSB
GSA
15.0
4.0
3.6
2.0
1.6
1.2
0.8
0.4
Activity (units/ml); Protein (mg/ml)
10
20
30
60
70
80
90
100
110
120
Fraction No.

Table.5 Purification of GS from ammonium-shocked cells.

Enzyme purification and enzyme assay were as described in materials and methods, except 20mM NH_4Cl was added in NF culture 15 mins before harvesting. All purification steps (except heat treatment) were performed at 4°C. One enzyme unit is defined as μmole γ-glutamyl hydroxamate produced min^{-1} and specific activity as number of units $(mg\ protein)^{-1}$

fractions	activity	protein	sp.act.	vol.	total act.	total prot.	fold	%yield
	μmol/min/ml	mg/ml	μmol/min/mg	ml	μmol/min	mg		
Crude enzyme	1.7	1.6	1.1	31	52.2	48.2	1	100
Heat treated	1.6	1.4	1.1	31	50.0	44.5	1.0	95.7
Blue Seph CL-6B	5.0	0.1	56.3	8.4	42.1	0.8	52.0	80.6
Sepharose-4B	1.7	0.03	62.2	22	36.9	0.6	57.4	70.0

after therein, eluted specifically with 2mM ADP. The enzyme in the first peak was considered to be the highly adenylylated form with several reasons discussed later.

3.5.6 Stability of purified GS

The stability of the purified enzyme obtained from NH_4^+-limited cells and NH_4^+-shocked cells were studied and the results are shown in fig.16. Activity of the purified enzyme from NH_4^+-limited cells (fig.16 (a)) was stable within 25 days but gradually decreased to about 70% if storage time was prolonged for another 45 days. The same results were observed in both 4°C and -20°C conditions. Fig.16 (b) shows that the stability of purified GS from ammonium-shocked cells was slightly different from the former. The enzyme was less stable, its activity decreased to about 60% within 35 days.

3.5.7 Heat stability

In an attempt to distinguish the two forms of GS induced in NFA100 medium and separated by Blue Sepharose CL-6B column (fig.15), pool GS_A and GS_B were tested for heat stability. The enzymes were incubated at various temperatures (37°C, 50°C, and 57°C) and aliqouts were withdrawn at time intervals (fig.17) and allowed to stand at 4°C before assaying for the GS activity.

The results (fig.17) show that both highly and slightly adenylylated forms of the GS Klebsiella spp.R15

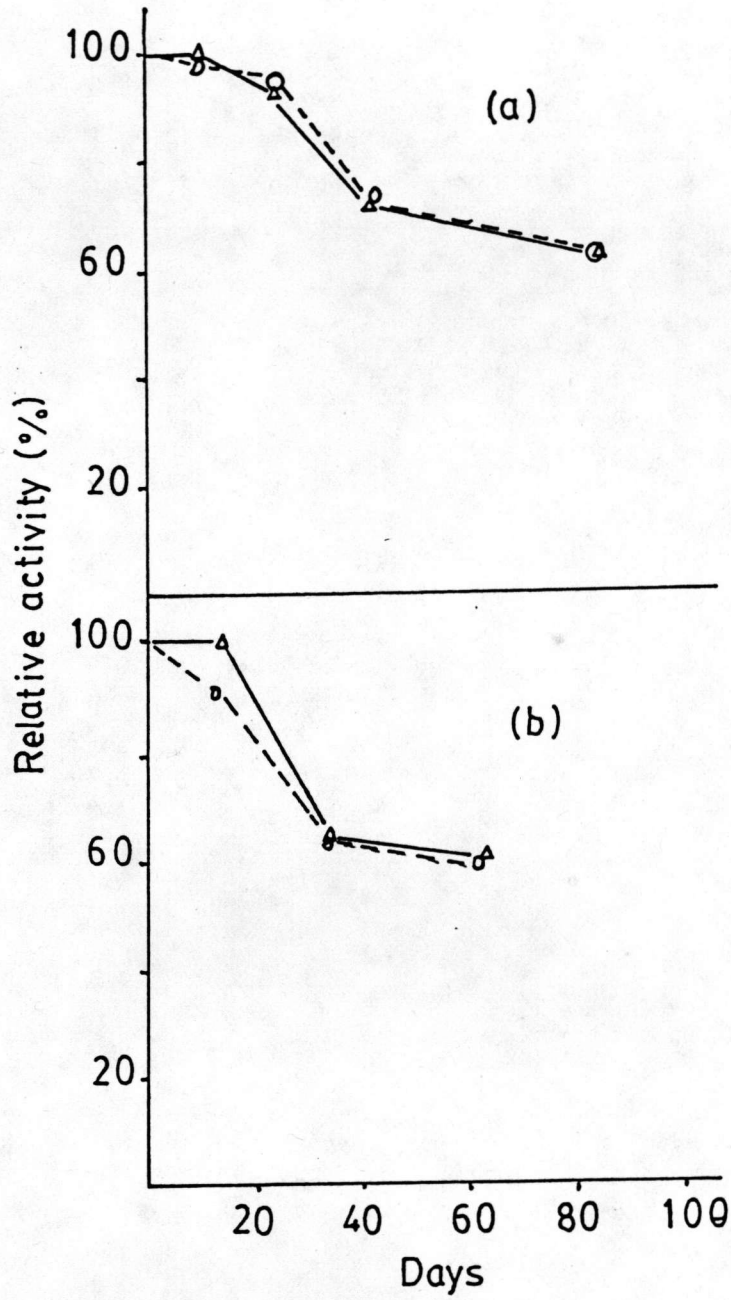


Fig.16 Stability of the purified GS in 50mM Tris-HCl pH7.5

(a) 0.058 mg/ml enzyme obtained from the NF culture and (b) 0.023 mg/ml enzyme obtained from ammonium-shocked cells, were kept at -20°C (o---o) and 4°C (Δ——Δ) at the indicated time before assaying the enzyme activity.

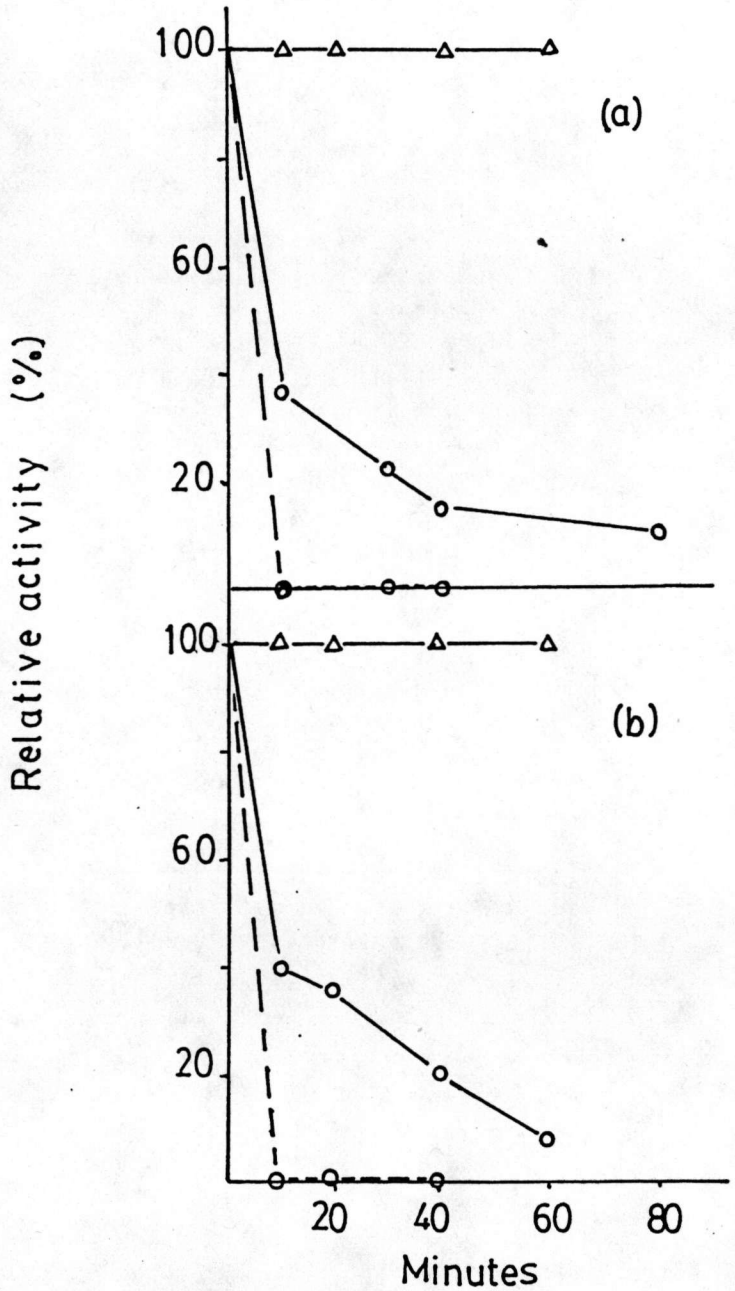


Fig.17 Heat stability of γ-glutamyl transferase activity of the nontreated GS_B (a) and GS_A (b). The enzymes in 50 mM Tris-HCl pH 7.5 were incubated at 37°C (Δ——Δ), 50°C (o___o) and 57°C (o---o) for the indicated time before being transfered to 4°C until assay.

were not different in heat stability pattern. The enzyme were completely stable at 37°C for more than 60 min but significantly labile at 50°C and 57°C.

3.6 Kinetic studies of the purified GS

The following fractions of the purified enzymes obtained from ammonium-shocked cells were used for kinetic studies:-

1.The GS_B, the purified enzyme eluted from the affinity column (fig.15) with ADP and further purified by Sepharose-4B. This was refered to as "nontreated GS_B"

2.The GS_B which was treated with SVP at 37°C for 1 hr.(SVP-treated GS_B)

3.The GS_A, the enzyme in the wash off fractions of the affinity column (fig.15).

These three enzymes differer by their degrees of adenylylation.

3.6.1 NH_2OH concentration dependent, K_m

Fig.18 shows the NH_2OH requirements of nontreated GS_B, SVP treated GS_B , and GS_A enzymes. Enzyme activities were measured at various NH_2OH concentrations with 80 mM glutamine. K_m for NH_2OH of these three enzymes were calculated from Lineweaver-Burk plot (fig.19). The values are shown in table 6.

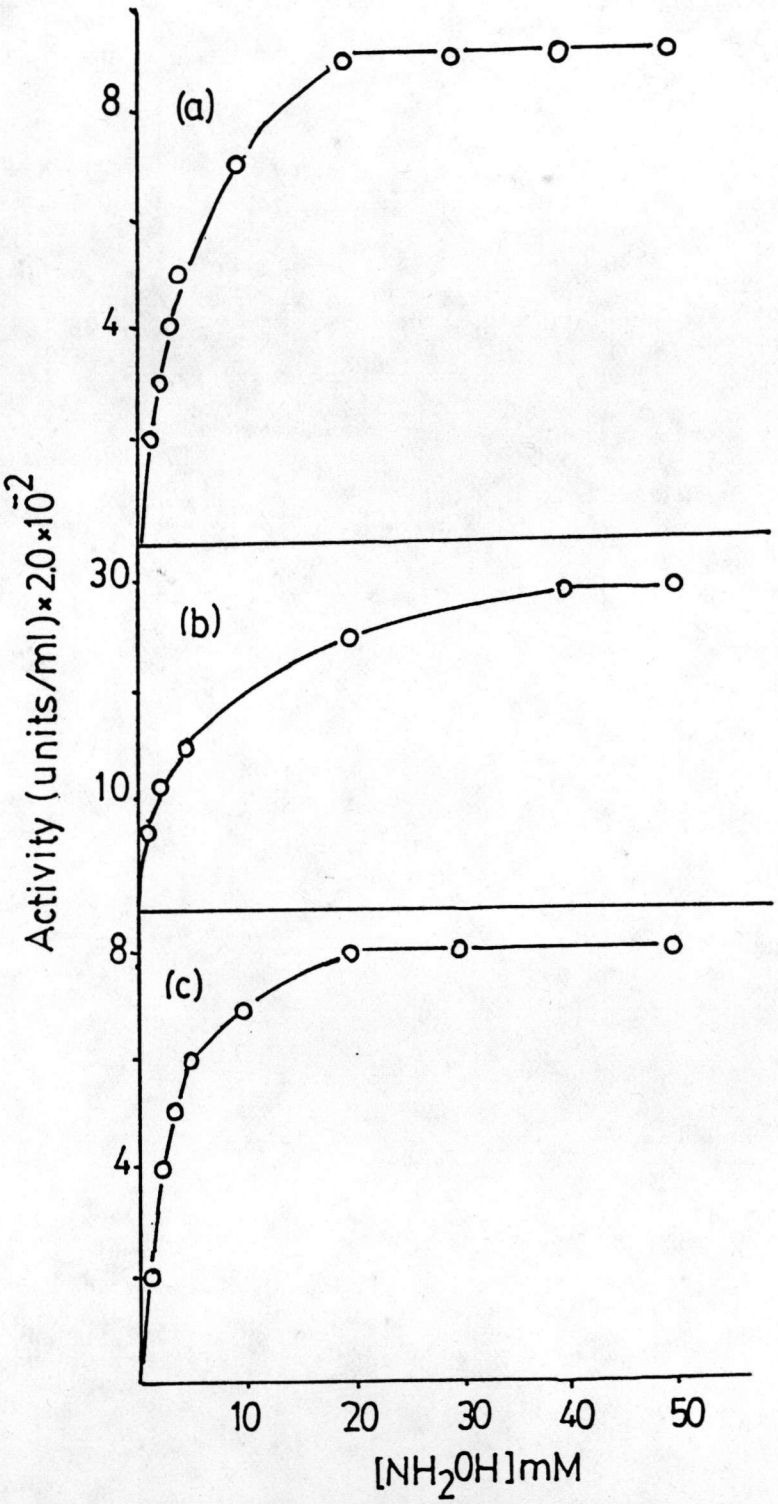


Fig.18 Effect of NH_2OH concentration on transferase activity of GS.

The nontreated GS_B (a), SVP-treated GS_B (b), and GS_A (c) were assayed with the indicated concentrations of NH_2OH. Reactions were carried out as described in the methods.

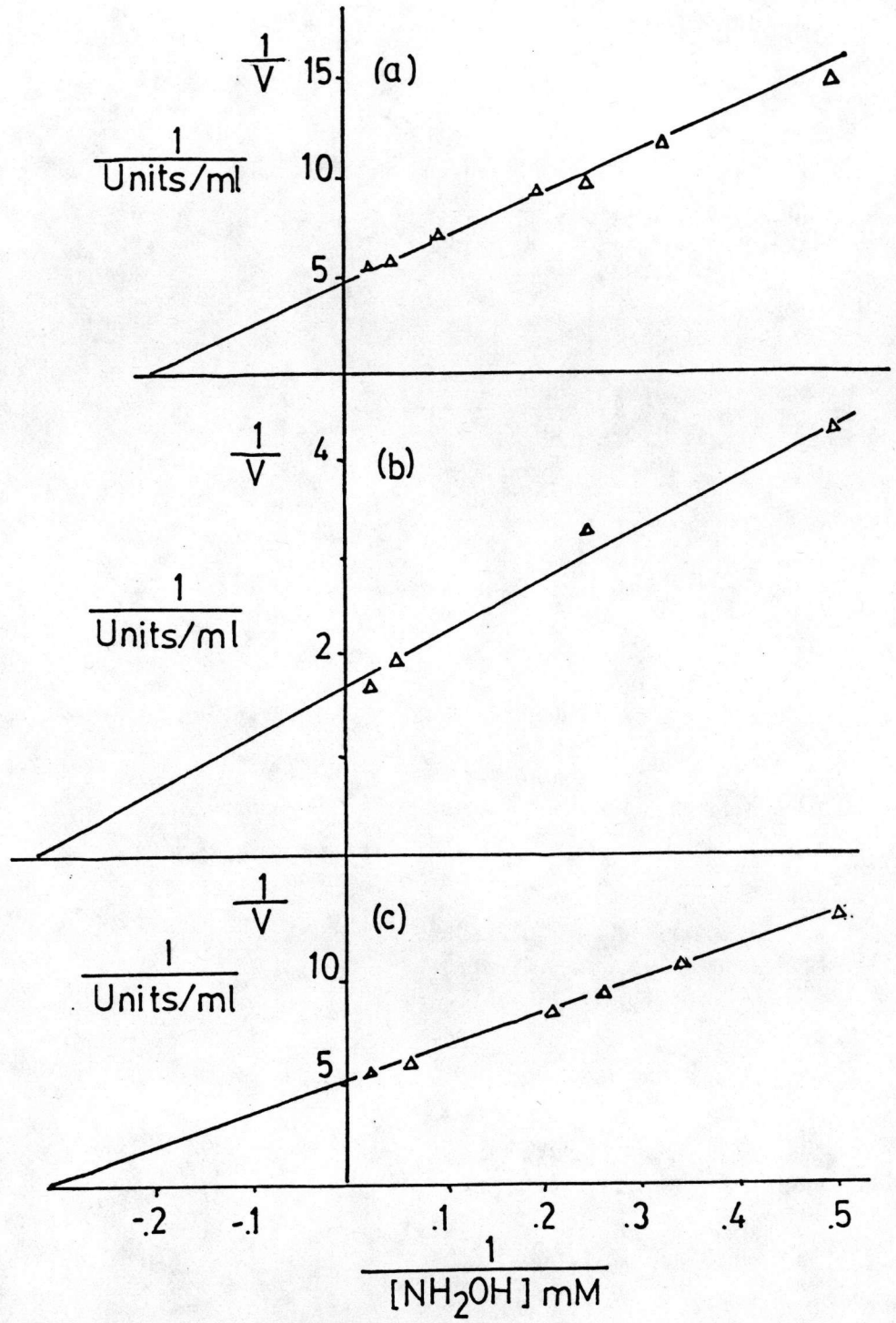


Fig.19 Double-reciprocal plot of the effect of varying the concentration of NH_2OH on transferase activity of glutamine synthetase.

The enzymes were nontreated GS_B (a), SVP-treated GS_B (b), and GS_A (c).

3.6.2 Glutamine concentration dependent, K_m

Fig.20 shows the glutamine requirement of nontreated GS_B, SVP-treated GS_B and GS_A enzyme. Enzyme activities were measured at various glutamine concentrations with saturated (50 mM) concentration of NH_2OH. K_m for glutamine of these three enzymes were calculated from Lineweaver-Burk plot (fig.21). The values are shown in table 6.

Table 6. The K_m values for NH_2OH and glutamine of the nontreated GS_B, SVP-treated GS_B and GS_A enzyme

enzymes	K_m (mM)	
	Gln	NH_2OH
nontreated GS_B	15.4	4.9
SVP-treated GS_B	2.0	3.0
GS_A	18.2	3.3

Table 6 shows the different K_m values for glutamine and hydroxylamine in three adenylylated states of the enzyme. Affinity of the enzyme for glutamine is greatly decreased with adenylylation. Different degree of adenylylation slightly changes the affinity of the enzyme for hydroxylamine.

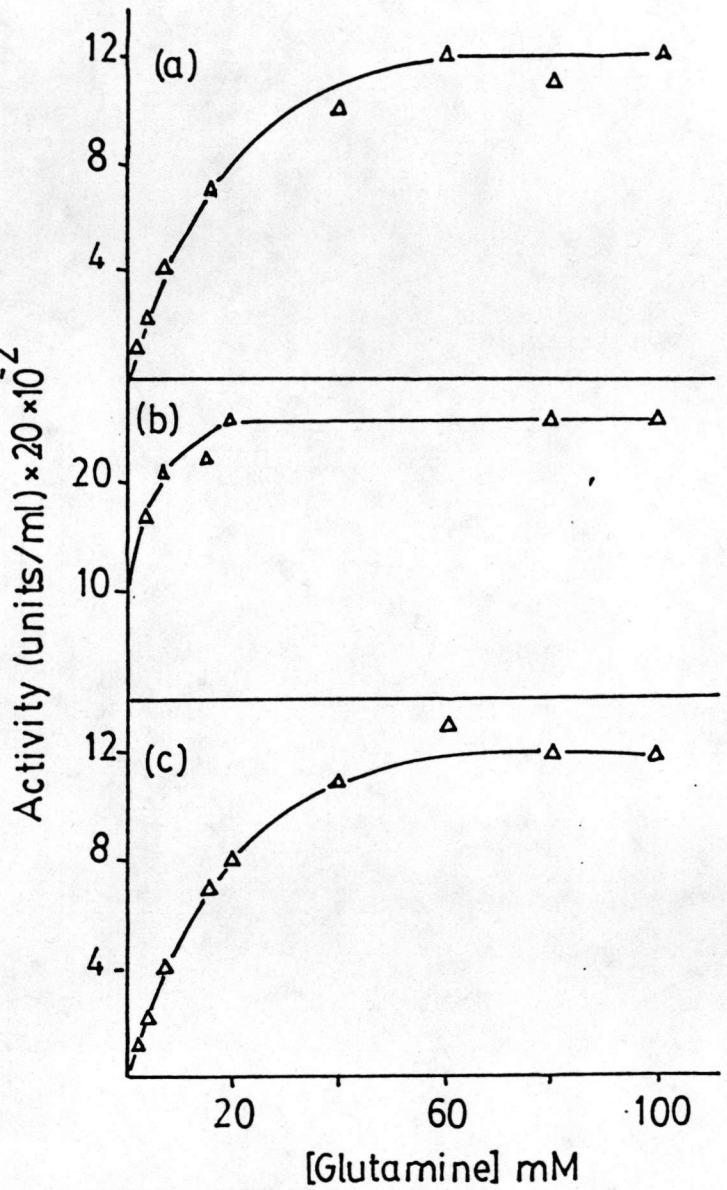


Fig.20 Effect of glutamine concentration on transferase activity of GS.

The nontreated GS_B (a), SVP-treated GS_B (b), and $GS_{A/}^{(c)}$ were assayed with the indicated concentrations of glutamine. Reactions were carried out as described in the methods.

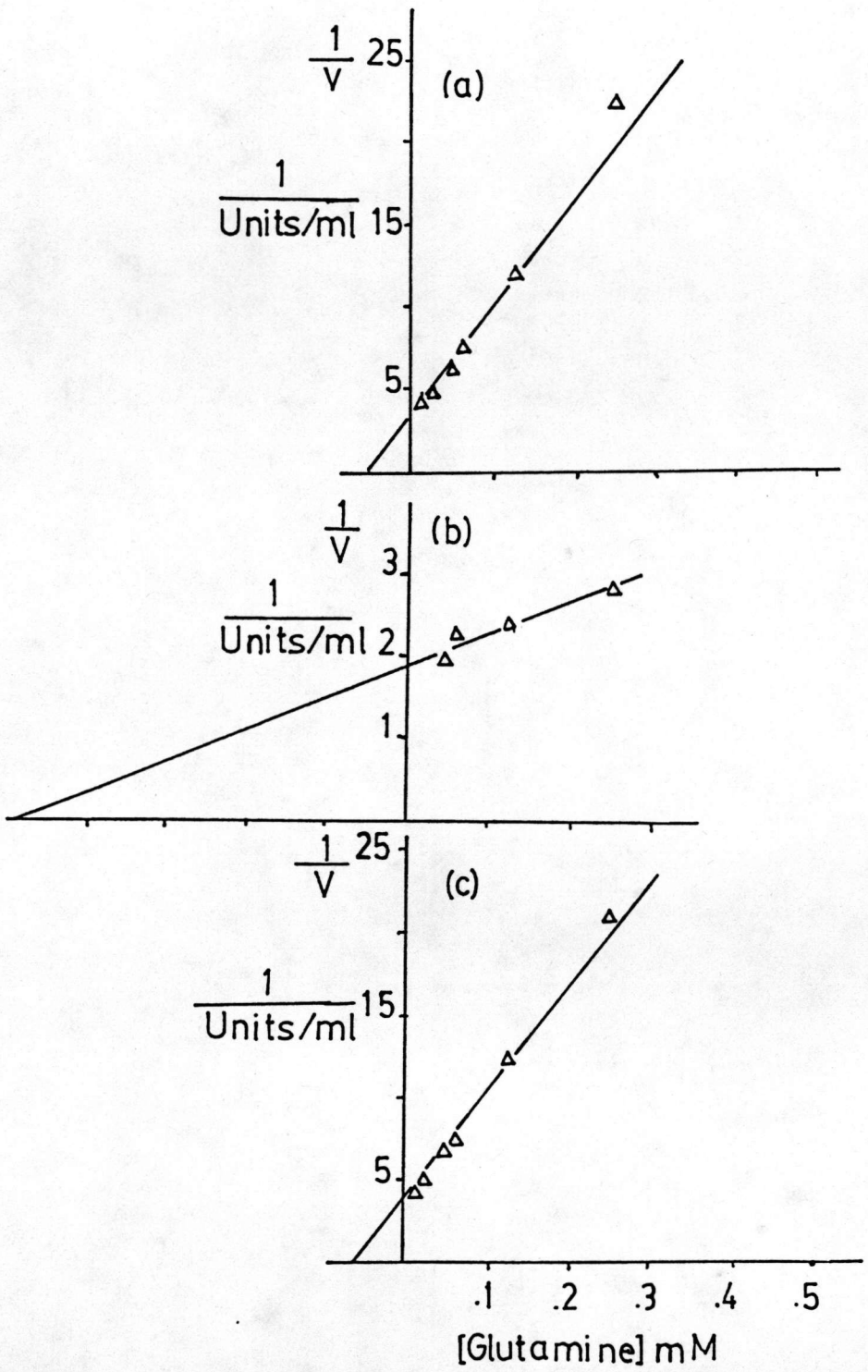


Fig.21 Double-reciprocal plot of the effect of varying the concentration of glutamine on transferase activity of GS.

The enzymes were : nontreated GS_B (a), SVP-treated GS_B (b), and GS_A (c).

3.6.3 Feedback inhibition

Bender, et al.(44) have reported on the feedback inhibition of Klebsiella aerogene GS. Screening for amino acids and nucleotides with inhibitory effect on the transferase activity of the Klebsiella spp.R15 enzyme with different degree of adenylylation was thus performed. The results are shown in table 7.

5mM of isoleucine, leucine, histidine and phenylalanine had no or slightly inhibitory effect on these three states of Klebsiella GS. 10mM of these amino acids increased the inhibition about 1-2 folds. The most pronounced inhibitory effect was with alanine which inhibited all three adenylylated states of GS. Unlike alanine, AMP showed significant higher inhibition on GS_A, and nontreated GS_B (37%, and35% inhibition) than SVP-treated GS_B (4.2% inhibition). The result suggests that while alanine serves as coarse negative effector by decreasing all the GS activity, AMP may be more specific by exerts a fine-tuning control only over the adenylylated enzyme.

When feedback inhibition with combined effectors was tested on GS_A and nontreated GS_B (table 8), the result from calculation (described in the appendix) suggests that the inhibition on both enzymes are of cumulative type (not additive). This characteristic emphasizes the regulatory

Table 7. Effect of L-amino acids and 5'-AMP on γ-glutamyl transferase activity of the nontreated GS_B, SVP-treated GS_B and GS_A.

Enzyme activity was determined as described in materials and methods with the addition of 5 or 10 mM amino acids or AMP in the reaction mixture. Each assay was performed in triplicate.

Compound added	% inhibition				
	GS_A		Nontreated GS_B		SVP-treated GS_B
	5mM	10mM	5mM	10mM	5mM
Gly	19.1	35.9	11.9	24.1	26.7
Ala	61.7	77.0	57.8	70.8	72.7
His	6.7	11.5	1.9	4.9	3.6
Trp	13.0	27.8	7.8	31.6	3.0
Phe	3.3	7.8	11.9	16.2	4.9
Ile	3.9	5.0	0.3	0	1.8
Leu	0	6.1	5.9	1.1	0.6
AMP	36.7	54.6	34.9	55.1	4.2

Table.8 Effect of combined effectors on γ-glutamyl transferase activity of the GS_A and nontreated GS_B.

Enzyme activity was determined as described in materials and methods with the addition of combined effectors (each at 5mM) in the reaction mixture. Each assay was performed in duplicate.

Combined effectors added	% inhibition					
	GS_A			Nontreated GS_B		
	observed	calculated		observed	calculated	
		cumulative	additive		cumulative	additive
Gly +Ala	60.3	69.0	80.8	63.4	66.3	69.7
Gly +AMP	43.2	48.8	55.8	49.8	42.6	46.8
Ala +AMP	65.8	75.8	98.4	63.9	72.5	92.7

role of GS in this bacteria.

3.6.4 Effect of NH_4Cl on the transferase activity

When varied NH_2OH at the saturated concentration of glutamine, the transferase activity in the presence of various concentrations of NH_4Cl was assayed. Fig.22 shows Lineweaver-Burk plots of the effect of NH_4Cl on transferase activity of nontreated GS_B (A), and SVP-treated GS_B (B). The results demonstrated that NH_4Cl decreased the affinity of NH_2OH to both enzyme forms.

3.7 Isoelectric focusing (IEF) of nontreated GS_B and SVP-treated GS_B

Fig.23 confirms the presence of covalently bound adenylyl group on the GS molecule. SVP-treated (lane B) and nontreated (lane A) GS_B enzyme were applied onto isoelectric focusing at pH 5-7. The nontreated GS_B had three major bands (A1, A2, A3) which implies the three different adenylylated states of GS. Treating the enzyme with SVP for 1 hr, only one major band was remained (B2 at the position identical to A2). Many minor bands appeared under B2 position (B3). The disappearance of the more acidic band on the IEF gel provides evidence for the removal of adenylylate moieties.

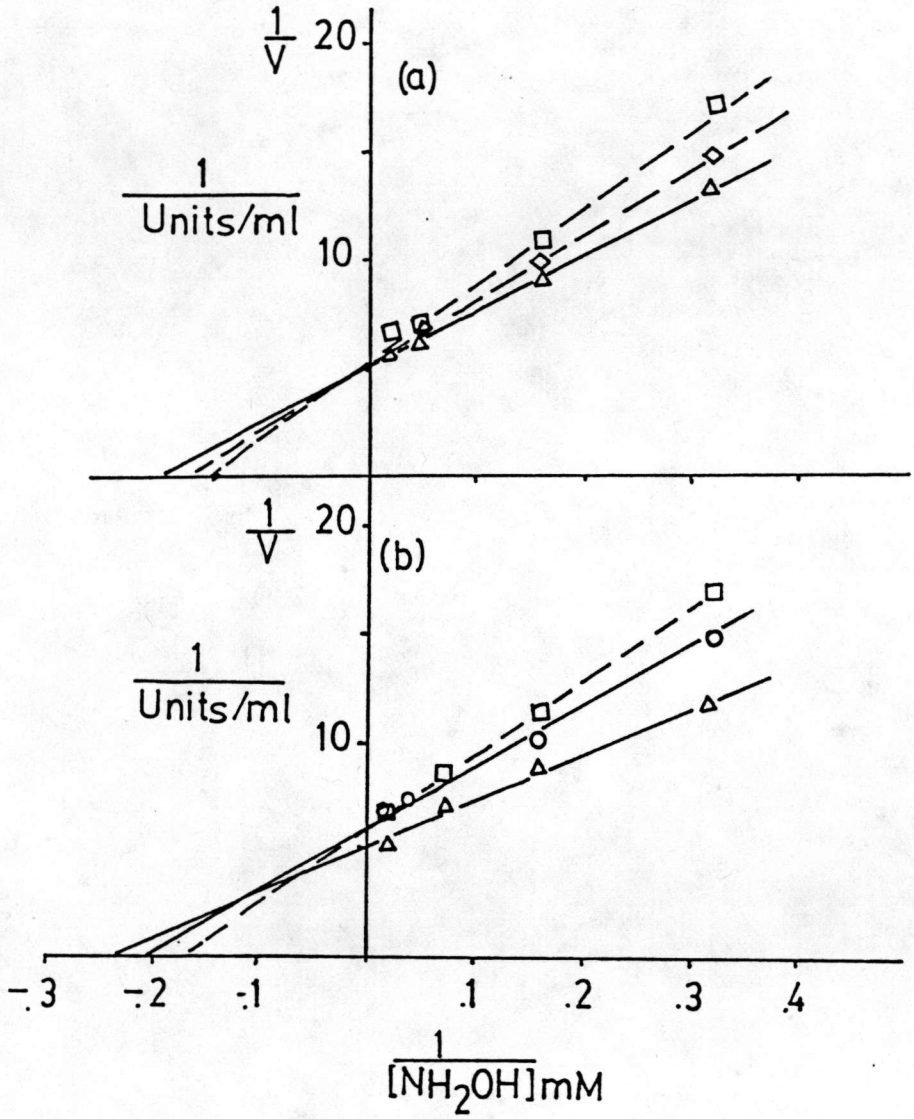


Fig.22 Lineweaver-Burk plots of the effect of NH_4Cl on transferase activity of purified glutamine synthetase obtained from nontreated GS_B (a); and SVP-treated GS_B (b) at various concentrations of NH_2OH. The concentrations of NH_4Cl are 0mM (△___△), 5mM(◇---◇), 20mM(o____o), and 30mM (□---□) and the enzyme activity was assayed as described in materials and methods.

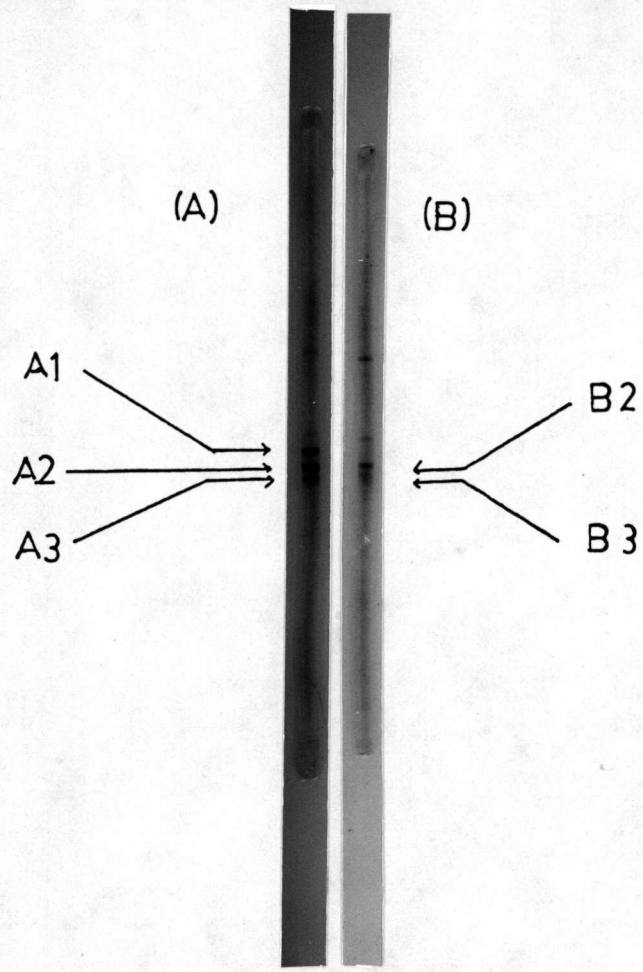


Fig.23 Isoelectric focusing (IEF) of nontreated GS_B and SVP-treated GS_B

10 µg of nontreated GS_B (A) and SVP-treated GS_B (B) were applied on IEF as described in the methods. The enzymes were prepared by incubating with or without SVP for 1 hr, 37°C. The pH of the upper side and the lower side of the IEF gel tube was 5 and 7, respectively.