CHAPTER III

RESULTS

1. PURIFICATION OF RICE BRAN LECTIN

1.1 Yield of defatted rice bran and total protein in crude PBS extract

Rice bran was defatted by soaking in acetone in 13 lots of about 1,000 g each. Table 1 showed the average yield of defatted rice bran as 83 ± 4.4 g % (w/w). Each lot of defatted rice bran was then extracted in PBS for total protein and it was found that the average yield of total protein in crude PBS extract was 3.5±0.5 g % according to the rice bran weight.

1.2 Agglutination test with various erythrocytes

The crude PBS extract of rice bran containing total protein was screened for its lectin activity by the agglutination test with different types of trypsinized erythrocytes, which were human A, B, AB, O and rabbit erythrocytes as described in "Methods". The hemagglutination activity of lectin was observed in crude PBS extract of rice bran as shown in Table 2, because crude PBS extract agglutinated all types of human erythrocytes and showing the highest specific agglutination activity (titer=8, titer per mg protin=0.77) with trypsinized rabbit erythrocytes indicating higher affinity for N-acetyl-D-glucosamine (25). Therefore trypsinized rabbit erythrocytes were used to estimate the specific agglutination activity of rice bran lectin in all the further steps of purification.

Table 1. Yield of defatted rice bran and total protein in crude PBS extract

Sample No. (n)	Rice bran (g)	Defatted rice bran (g%)	Total protein g% (g/100 g rice bran)
13	1,000-1,180	83±4.4	3.5± 0.5

Table 2. Agglutination test of crude PBS extract with various types of trypsinized erythrocytes

Type of trypsinized erythrocyte	Titer * (per 10.40 mg protein)	Specific titer (titer/mg protein)
Human A	2	0.19
B	2	0.19
AB	1	0.09
O	1	0.09
Rabbit	8	0.77

* The assay was performed 6 times with reproducible results.

1.3 Ammonium sulphate fractionation

Ammonium sulphate was gradually added into the crude PBS extract. It was previously observed that at 20 % and 40 % ammonium sulphate saturation, no precipitate was obtained after centrifugation. Until the concentration of ammonium sulphate was brought up to 60% saturation and centrifuged, the precipitate was obtained. After precipitation, the supernatant fraction was further saturated with ammonium sulphate in the same manner and followed by the collection of 60-80% and 80-100% ammonium sulphate precipitates. The 0-60%, 60-80% and 80-100% ammoniun sulphate precipitates were dialyzed against PBS to elimimate ammonium sulphate and tested for agglutination activity with trypsinized rabbit erythrocytes as described in "Methods". It was found that only the dialysate of 0-60% ammonium sulphate precipitate showed positive specific agglutination activity with increasing specific titer of 3.76.

1.4 Column chromatography

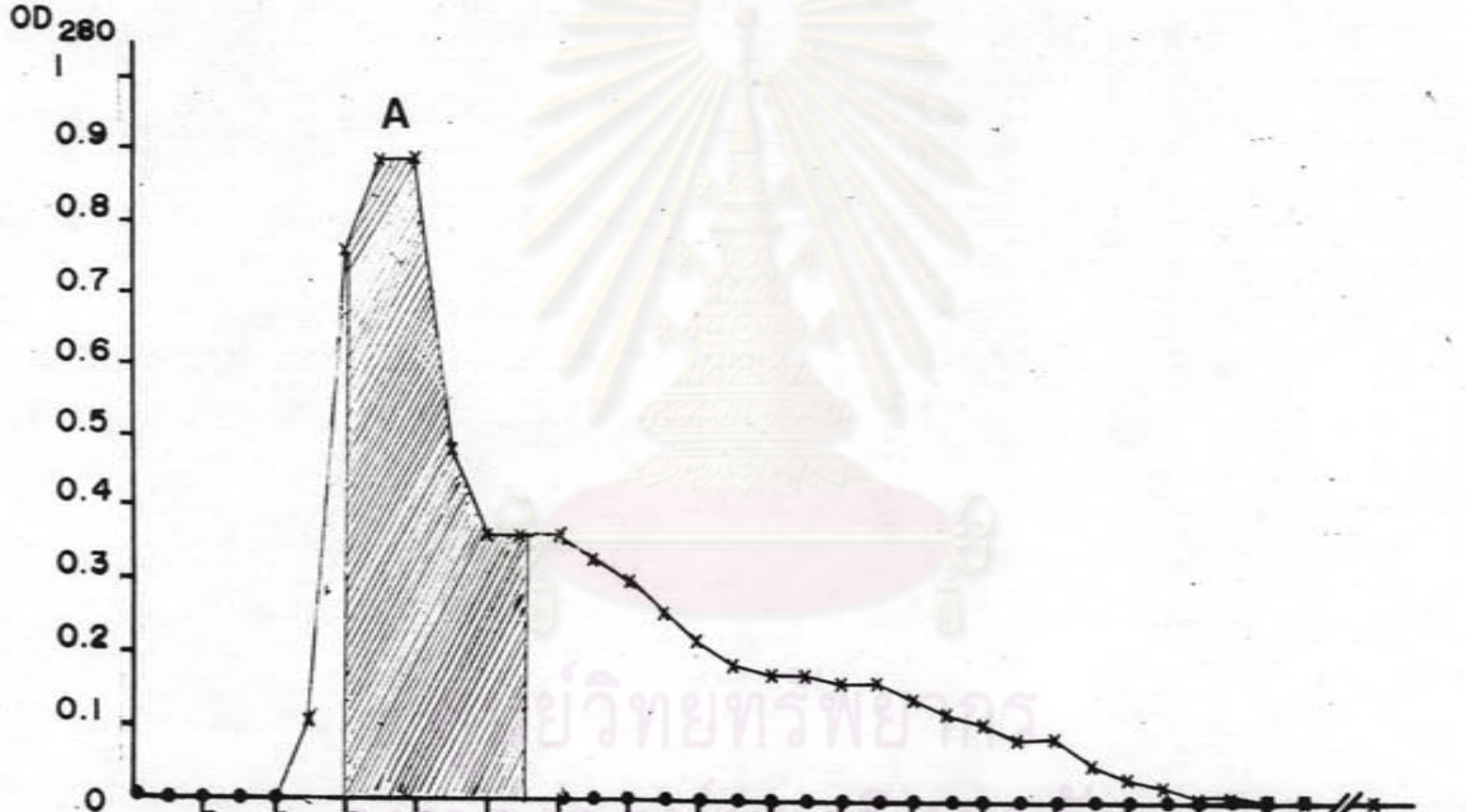
1.4.1 Purification by ion exchange chromatography

a. Sephadex G-25 gel filtration

The dialysate was applied onto a sephedex G-25 column in order to change buffer from PBS to 0.1 M Tris-HCl buffer, pH 7.4 Figure 1 shows that the protein fractions were eluted as broad single peak. When the agglutination activity of each fraction was assayed with trypsinized rabbit erythrocytes, only fraction 6-11 (A) showed positive agglutination activity. These fractions were therefore pooled, and redetermined the specific titer of the pooled fractions resulting in the specific titer of 3.76.

Figure 1. Elution profile of PBS dialysate on Sephadex G-25 column

Dialysate(17 mg protein/ml PBS) was applied on a Sephadex G-25 column and eluted with 0.1 M Tris-HCl buffer, pH 7.4 at the flow rate of 0.5 ml/min. Fractions of 3 ml were collected, measured the absorbance of protein profile at 280 nm(×—×—×—×), and determined the agglutination activity with 2 % trypsinized rabbit erythrocytes. Fractions with positive specific titer are indicated as (▨).


OD 280
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
50
FRACTION NUMBER (3 ml. / FRACTION.)

b. DEAE-Sephadex A-50 column

Pooled fraction(A) from Sephadex G-25 column was applied onto DEAE-Sephadex A-50 column to separate lectin from other protein on the basis of their difference in charge. Figure 2. shows the chromatographic profile of 4 protein peaks numbering B_1, B_2, B_3, and B_4 in the order of increasing net negative charge. They were eluted from the column in the range of 0.1-0.2 M, 0.25-0.3 M, 0.35-0.4 M and 0.43-0.46 M of linear NaCl concentration gradient respectively. When the agglutination activity of each fraction was assayed with trypsinized rabbit erythrocytes, only fractions 61-68 (B_2) showed positive specific titer. When these fractions were pooled, the specific titer of pooled B_2 was found to be increasing to 18.6 titer/mg protein.

c. Sephadex G-100 column

The B_2 obtained from DEAE-Sephadex A-50 column was applied to Sephadex G-100 column to separate lectin from other proteins on the basis of their difference in M.W. Figure. 3 shows an elution diagram of 3 protein peaks numbering C_1, C_2, and C_3 in the order of decreasing molecular weights. When the agglutination activity of each fractions 37-47 (C_3) showed positive agglutination activity. These fractions were therefore pooled and redetermined the specific titer of pooled fractions (C_3) resulting in the titer/mg protein of 20. From the position of C_3 peak in this elution diagram, it can be estimated that the M.W. of lectin was in the range of 40,000-60,000 dalton.

Figure 2. Elution profile of pooled protein fraction A on DEAE-Sephadex A-50 column

Pooled protein fraction A from Sephadex G-25 column (about 150 mg protein in 180 ml of 0.1 M Tris-HCl buffer, pH 7.4) was applied to a DEAE-Sephadex A-50 column(2.2x40 cm). The column was eluted with increasing salt gradient to 1.0 M NaCl. Fractions of 3 ml were collected, and determined the specific agglutination activity with 2 % trypsinized rabbit erytrocytes suspension. Positive agglutination activity is shown as (▨), where(- - -) stands for NaCl gradient.

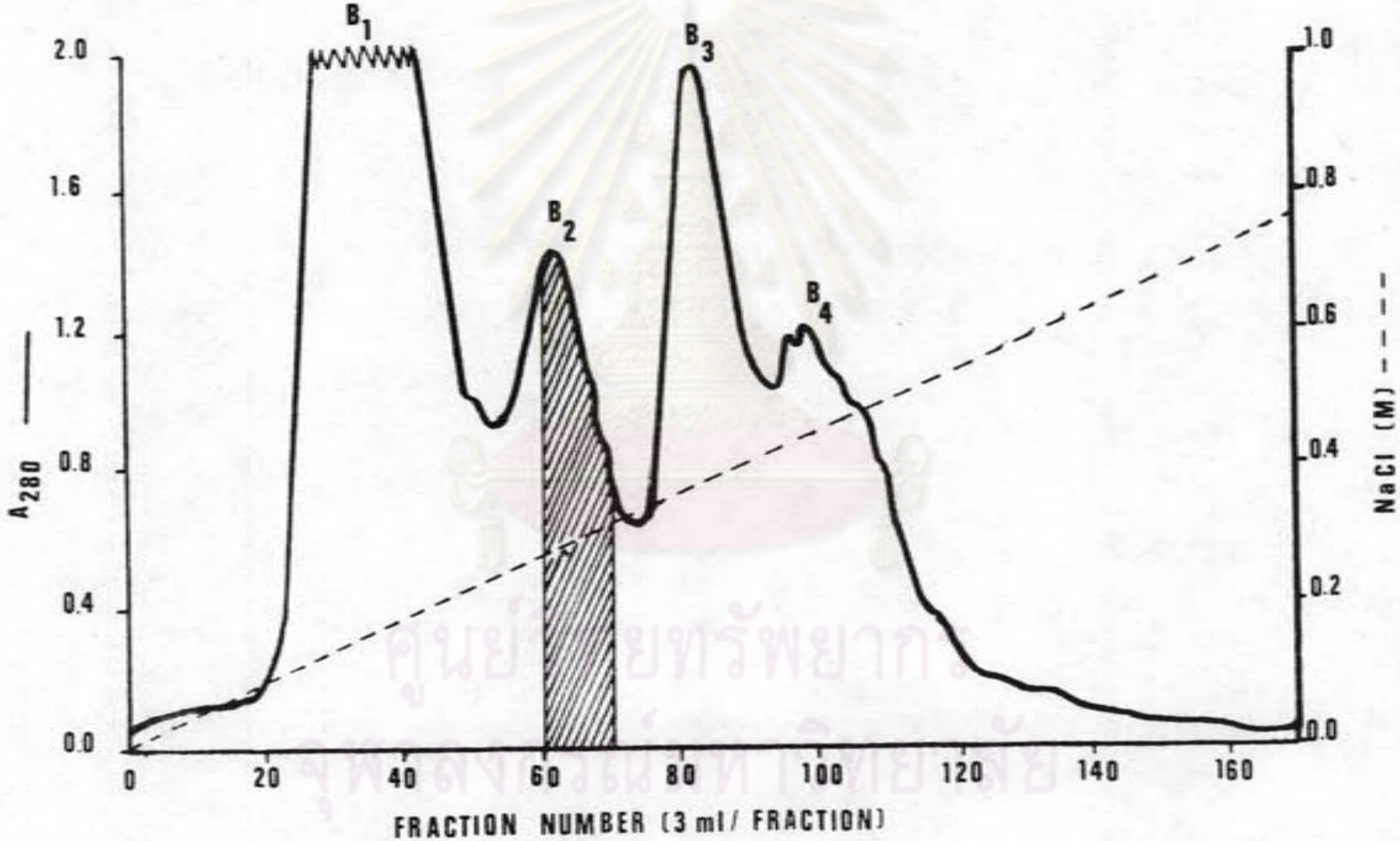
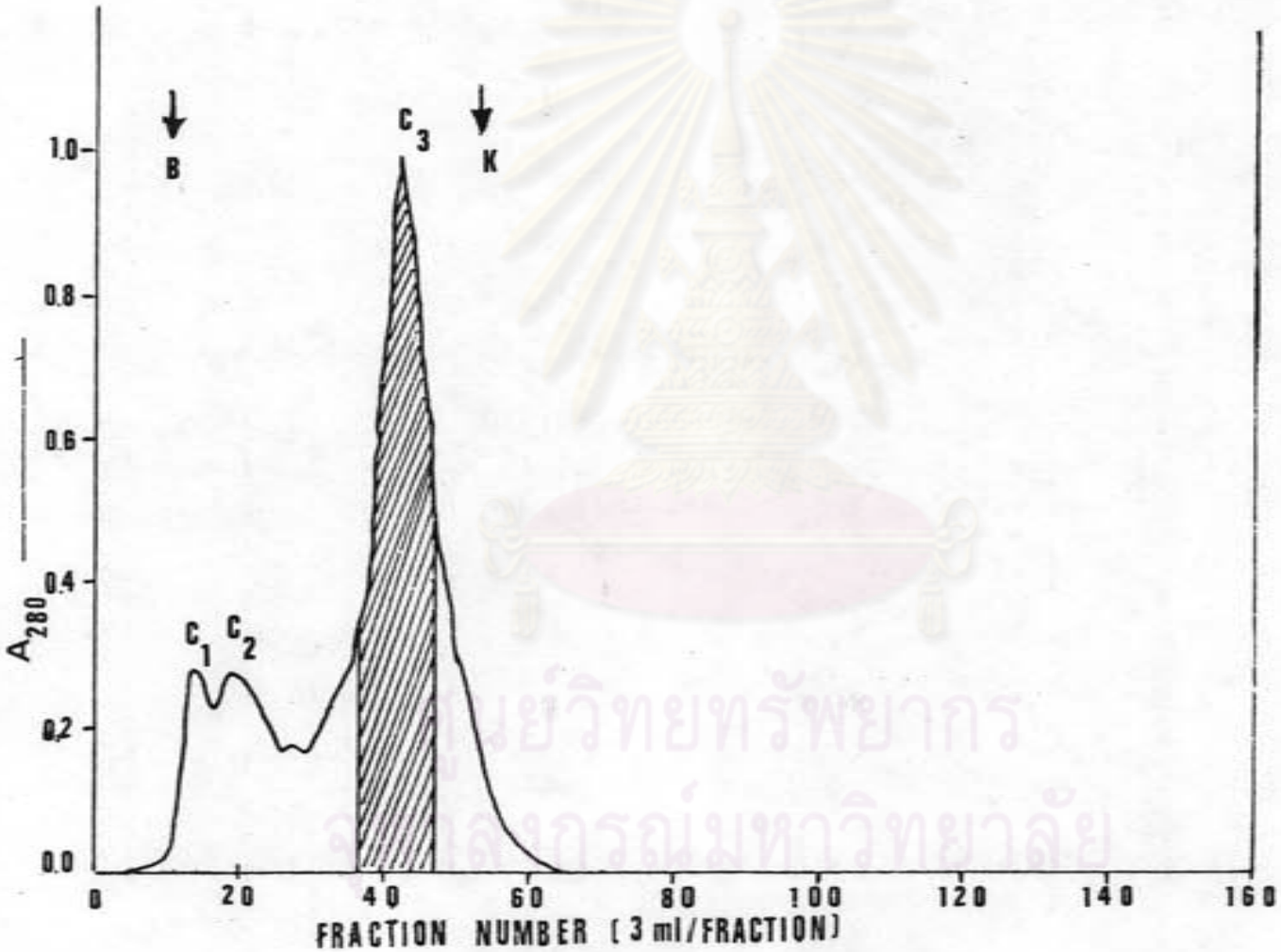

B1
B2
B3
B4
2.0
1.6
1.2
0.8
0.4
0.0
A280
1.0
0.8
0.6
0.4
0.2
0.0
NaCl (M)
0
20
40
60
80
100
120
140
160
FRACTION NUMBER (3 ml/ FRACTION)

Figure 3. Elution profile of pooled fraction B_2 on Sephadex G-100 column

Pooled protein fraction B_2 (61-68) from DEAE-Sephadex A-50 column(6.62 mg protein in 7 ml of 0.1 M Tris-HCl buffer, pH7.4) was applied to a Sephadex G-100 column (2.2x40 cm) and eluted with 0.1 M Tris-HCl buffer, pH 7.4 at the flow rate of 0.5 ml/min. Fractions of 3 ml were collected, measured the protein profile at 280 nm (———), and determined for specific agglutination activity with 2 % trypsinized rabbit erytrocytes suspension suspension, in which (▨) indicated for fractions of specific titer.

(B and K stand for blue dextran and potassium chromate markers)



1.4.2 Purification by affinity chromatography

a. N-acetyl-D-glucosamine Sepharose 4B column

From the first scheme of chromatographic method (1.4.1), the purification of 26 fold was achieved by many steps. In this experiment affinity chromatography was tried to shorten the purification steps of rice bran lectin, using its ability to bind specificly to N-acetyl-D-glucosamine covalently bound to Sepharose 4 B column matrix. Starting with the protein dialysate from step 1.3, 42.5 mg protein in 2.5 ml PBS was applied to an N-acctyl-D-glucosamine Sepharose 4B previously prepared as described in "Methods". The column was firstly eluted with PBS to wash out all the protein which has no affinity for N-acetyl-D-glucosamine as shown by the peak D_1 in Fig.4. When the specific agglutination activity of each fraction in D_1 was assayed with trypsinized rabbit erythrocytes, none of them showed specific agglutination activity. After that, the column was loaded with 0.1 M N-acetyl-D-glucosamine in PBS as displacer, D_2 the protein peak eluted by displacement of excess N-acetyl-D-glucosamine was pooled and extensively dialyzed against PBS before assay of specific agglutination activity. The titer/mg protein of 20 was also observed with D_2 peak (▨).

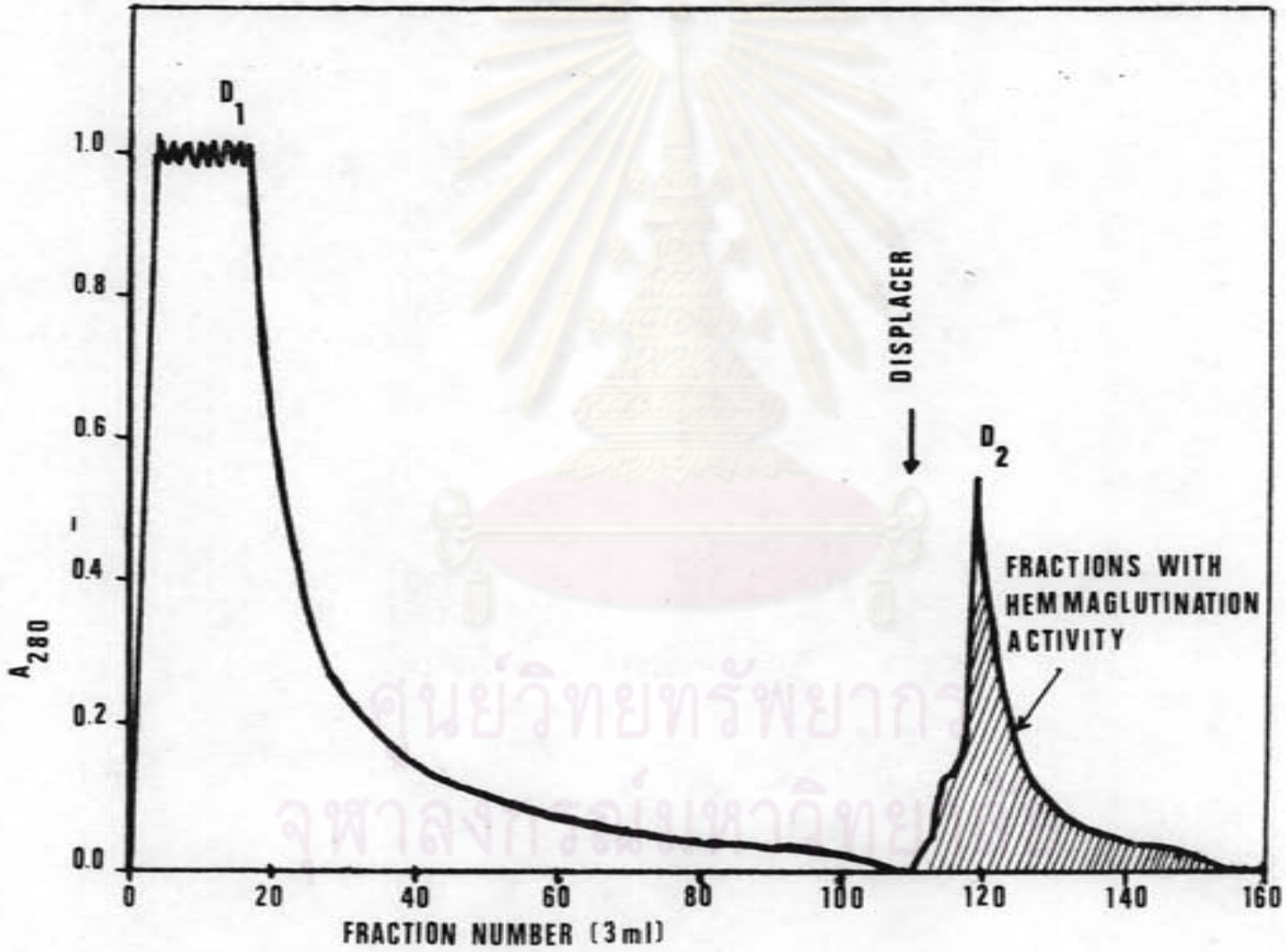
The purification of lectin from rice bran was summarized in table 3. The results indicate that single step purification by N-acetyl-D-glucosamine-Sepharose 4B is equivalent to the first scheme of purification by anion exchanger and gel filtration columns showing similar specific titer of 20, and 26 fold purification.

2. Agglutination activitey of rice bran lectin

The agglutination activity (titer) of lectin in each step tested

Figure 4. Elution profile of PBS dialysate on N-acetyl-D-glucosamine Sepharose 4B column

Dialysate (42.5 mg protein in 2.5 ml PBS) was applied on an N-acetyl-D-glucosamine Sepharose 4B column (2.2x25 cm). After loading, PBS was applied at a flow rate of 0.5 ml/min until no absorbance at 280 nm was detected in the effluent of 3ml/fraction. The column was then eluted with 0.1 M N-acetyl-D-glucosamine in PBS as displacer. Fractions of 3 ml were collected. Absorbance at 280 nm of every fraction was determined and the fractions corresponding to the protein peak were pooled, and extensively dialyzed against large volume of PBS. The specific agglutination activity was determined after no trace of N-acetyl-D-glucosamine was detected in the dialysable fraction. (———) represents the absorbance of protein at 280 nm, (▨) represents fractions of positive agglutination activity.



with trypsinized rabbit erythrocytes are demonstrated in Fig. 5. The last dilution of lectin in which erythrocytes formed a fuzzy mat on the bottom of the well is the agglutination end point. The values of specific titer of lectin shown in table 3 were calculated from the titer observed in this plate divided by their concentration of protein in mg/ml. Trypsinized rabbit erythrocyte suspensions and PBS buffer without lectin served as negative control in the last well of each row, and trypsinized erythrocytes suspensions to which wheat germ agglutinin (0.1 mg/ml) served as positive controls. (row f.)

3. Homogeneity of purified rice bran lectin

3.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to separate proteins and to test the homogeneity of a purified protein on the basis of the rate of movement of proteins in an electric field depended on their charge rather than on their mass. In this experiment, crude PBS extract, dialysate from 0-60% ammonium sulphate precipitate, B_2 from DEAE-Sephadex A-50 column , C_3 from Sephadex G-100 column and D_2 from N-acetyl-D-glucosamine-Sepharose 4B column were subjected to polyacrylamide gel electrophoresis as described it "Methods" Figure. 6 shows that number of protein bands decreased in the order of purification steps. The homogeneity of purified rice bran lectin obtained from 2 different schemes of purification was evident by the same position of a single band in gel d. and e. which were C_3 from Sephadex G-100 column and D_2 from N-acetyl-D-glucosamine Sephrose 4B column respectively.

Figure 5. Agglutination activity (titer) of rice bran lectin during purification

Crude PBS extract, dialysate of 0-60 % $(NH_4)_2SO_4$ precipitate, B_2 pooled fraction from DEAE-Sephadex A-50 column, C_3 pooled fraction from Sephadex G-100 column and D_2 pooled fraction from N-acetyl-D-glucosamine-Sepharose 4B column were assayed for agglutination activity as described in "Methods".

A. Crude PBS extract, titer = 8

B. Dialysate of 0-60 % $(NH_4)_2SO_4$ precipitate, titer = 64

C. B_2 pooled fraction from DEAE-Sephadex A-50 column, titer = 8

D. C_3 pooled fraction from Sephadex G-100 column, titer = 4

E. D_2 pooled fraction from N-acetyl-D-glucosamine Sepharose 4B column, titer = 2

F. Standard wheat germ agglutinin(0.1 mg/ml), titer = 2

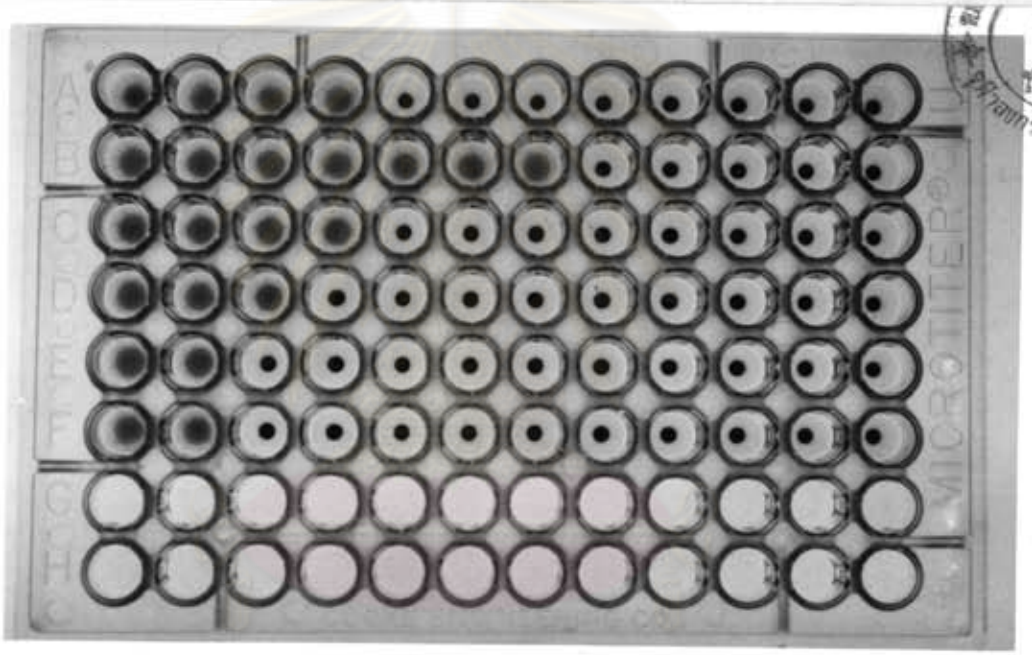


Table 3. Summary of purification of rice bran lectin

	Protein concentration (mg/ml)	Total protein (mg)	Agglutination activity		Purification fold
			Titer	Specific titer (titer/mg protein)	
Crude PBS extract	10.40	100.00	8	0.77	1
Dialysate of 0-60% $(NH_4)_2$ SO_4 precipitate	17.00	47.79	64	3.76	5
First shceme					
a. DEAE-Sephadex A-50 column	0.43	2.94	8	18.60	24
b. Sephadex G-100 column	0.20	2.04	4	20.00	26
Second shceme					
N-acetyl-D-glucosamine-Sepharose 4 B column	0.10	1.69	2	20.00	26

Figure 6. Polyacrylamide gel electrophoresis pattern

Crude PBS extract, dialysate of 0-60 % $(NH_4)_2SO_4$ precipitate, B_2 peak from DEAE-Sephadex A-50 column, C_3 peak from Sephadex G-100 column and D_2 peak from N-acetyl-D-glucosamine Sepharose 4B column electrophoresed on polyacrylamide gel electrophoresis as described in the "Methods".

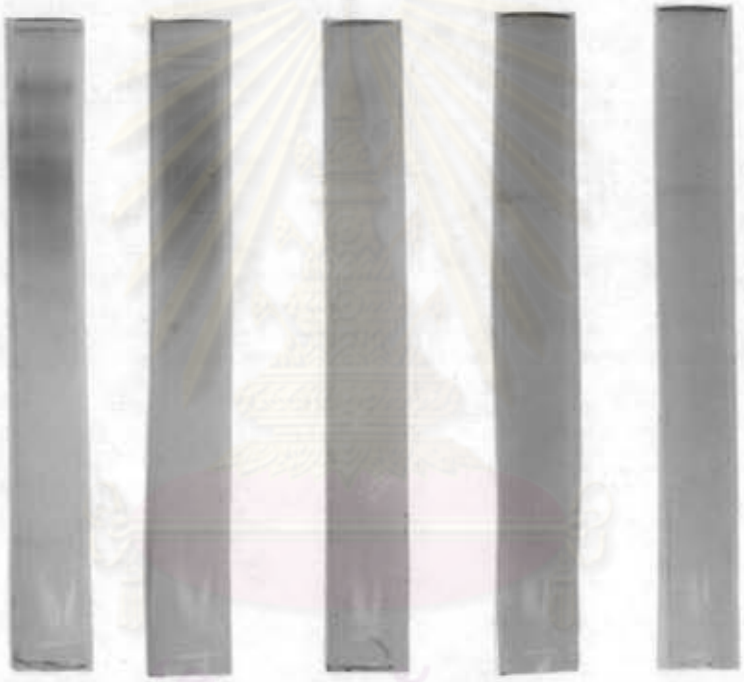
a. Crude PBS extract

b. Dialysate of 0-60 % $(NH_4)_2SO_4$ precipitate

c. B_2 peak from DEAE-Sephadex A-50 column

d. C_3 peak from Sephadex G-100 column

e. D_2 peak from N-acetyl-D-glucosamine Sepharose 4B column



3.2 Gel filtration on Sephadex G-75 column

Gel filtration was used to determine the M.W. of proteins on lectin from both schemes of separation methods were eluted on Sephadex G-75 column previously calibrated with standard M.W. markers as described in "Methods". Figure 7 shows that purified rice bran lectin from both methods were eluted as a single peak, at the same position in fraction number 32. The molecular weight of purified rice bran lectin estimated from the plot of log M.W. of several standard marker proteins versus their Kav values was 40,000 dalton.

4. Biological properties of purified rice bran lectin

4.1 Inhibition study of hemagglutination activity with various sugars

Different types of sugars, which are N-acetyl-D-glucosamine, D-glucose, D-galactose, D-arabinose and lactose were tested for inhibition of the hemagglutination activity of purified rice bran lectin with trypsinized rabbit erythrocytes as described in "Methods". At 1,000 fold concentration of purified rice bran lectin, every sugars tested could not inhibit the hemagglutination activity, except N-acetyl-D-glucosamine. It indicated that the carbohydrate binding sites on purified rice bran lectin specific for N-acetyl-D-glucosamine. To determine the minimal concentration of N-acetyl-D-glucosamine that inhibit the hemagglutination activity of purified rice bran lectin, 2-fold serial dilution of N-acetyl-D-glucosamine starting from 50 mg/ml was performed as shown in Fig. 8. At the constant amount of purified rice bran lectin (0.1 mg/ml), the minimal inhibitory concentration of N-acetyl-D-glucosamine is 12.5 mg/ml or 125 fold.

Figure 7. Estimation of the molecular weight of purified rice bran lectin on Sephadex G-75 column

Purified lectin from Sephadex G-100 and N-acetyl-D-glucosamine Sepharose 4B columns(5 mg protein in 1 ml PBS each) and standard protein markers, which were 1. BSA(M.W. 67,000), 2. α-chymotrypsinogen A(M.W. 25,000) and 3.cytochrome C(M.W. 12,270) (5 mg protein in 1 ml each) were applied on a Sephadex G-75 column (2.2x51 cm) and eluted with PBS at the flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured for the protein profile at 280 nm.

A. (●——●) represents the purified lectin from Sephadex G-100 column

(✕——✕) represents the purified lectin from N-acetyl-D-glucosamine Sepharose 4B column

↓ ↓ ↓ ↓ ↓

(B, BSA, α-chy, cyt and K stand for blue dextran, bovine serum albumin, α-chymotrypsinogen A, cytochrome C and potassium chromate markers respectively)

B. The logarithms of the molecular weights of protein markers were plotted against their Kav values

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

V_e = elution volumn for the protein

V_o = elution volumn for the blue dextran

V_t = total bed volumn

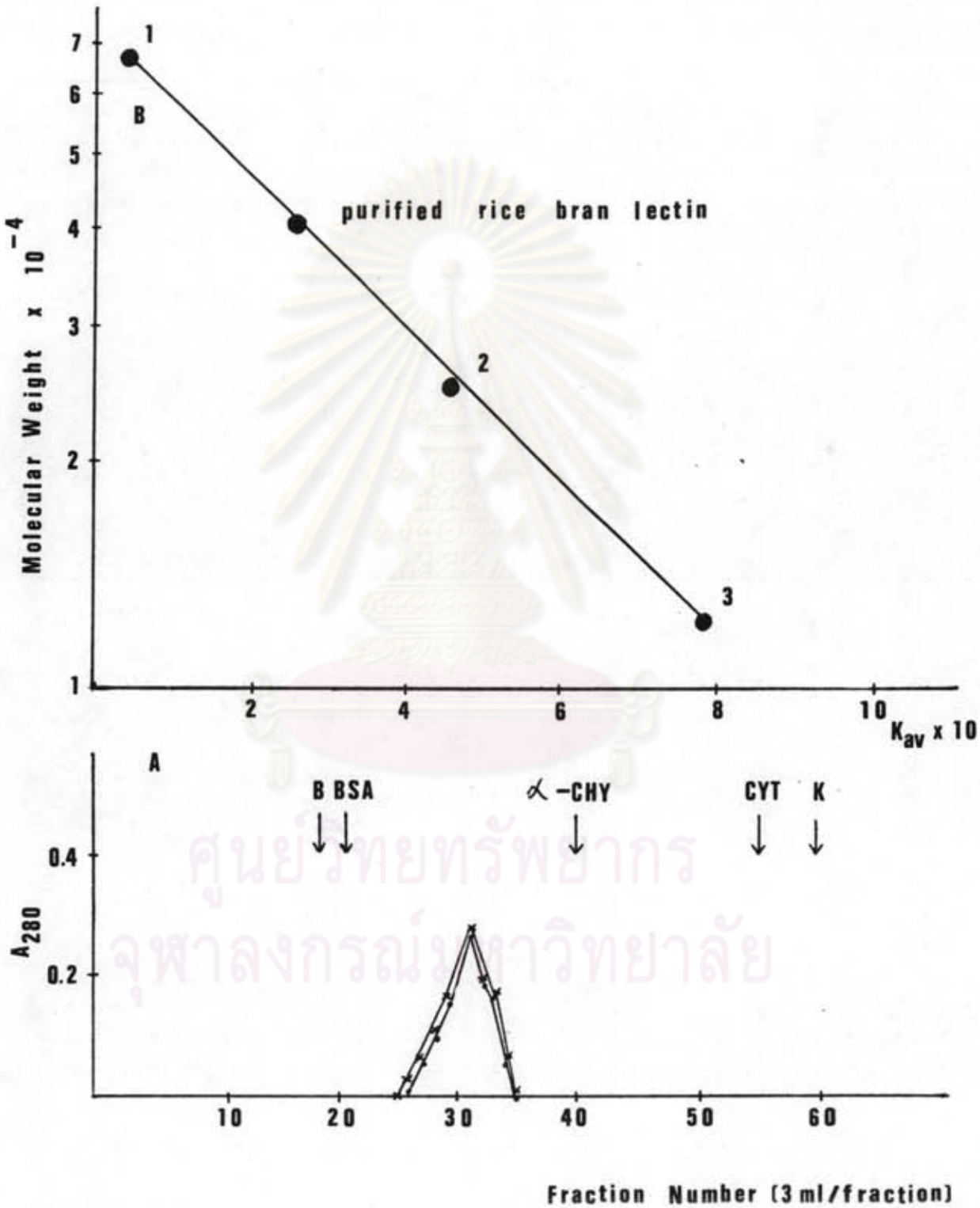
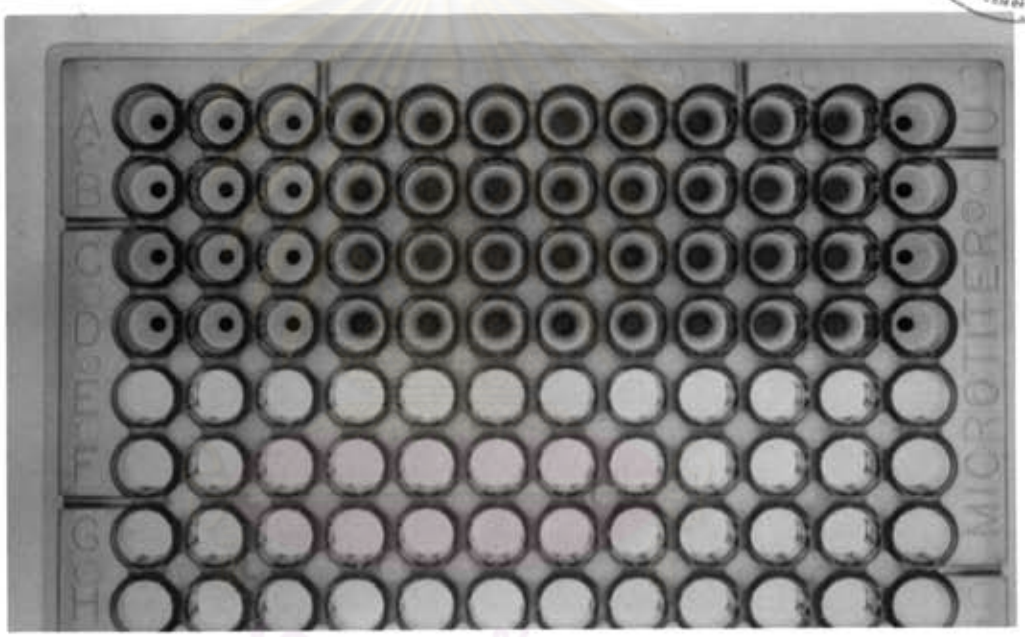


Figure 8. Inhibition study of hemagglutination activity

Twenty five microliters of purified rice bran lectin (0.1 mg/ml) from both schemes of separation were mixed with N-acetyl-D-glucosamine solution (50 mg/ml or serially diluted) allowed to stand for 30 min before addition of 25 µl trypsinized rabbit erythrocytes suspension (2% v/v).

A. and B. hemagglutination of trypsinized rabbit erythrocytes by purified lectin from Sephadex G-100 column can be inhibited by the minimal concentration of N-acetyl-D-glucosamine at well 3 (12.5 mg/ml).

C. and D. hemagglutination of trypsinized rabbit erythrocytes by purified lectin from N-acetyl-D-glucosamine-Sepharose 4B column can be inhibited by the minimal concentration of N-acetyl-D-glucosamine at well 3 (12.5 mg/ml).



4.2 Agglutination of purified rice bran lectin with nitrogen-fixing rhizospheric bacteria

Purified rice bran lectin from the two separation schemes were tested for the specific agglutination activity with some nitrogen-fixing rhizospheric bacteria (No 5, 6, 15, 17 and 18) isolated from rice rhizosphere during 1979-1981 (14). These N_2 fixers are gram negative rods and show acetylene reduction activity ranging from 50-75 nmole/OD/hr (14). These bacterial suspension (1% V/V) were used instead of trysinized rabbit erythrocytes as shown in Fig. 9a. and b. Escherichia coli another gram negative bacterium (row F.) served as comparative species. Figure. 9a. and b. show that purified rice bran lectin from Sephadex G-100 column (0.2 mg/ml), and purified rice bran lectin from N-acetyl-D-glucosamine Sepharose 4B column (0.1 mg/ml) agglutinate all these N_2-fixing bacteria with similar titer of 4 and 2 respectivety resulting in the specific titer of 20. Purified rice bran lectin cannot agglutinate Escherichia coli cells, although they are gram negative, suggesting that the purified rice bran lectin is rather specific to diazothrophs.

4.3 Inhibition study of agglutination activity with nitrogen fixing rhizospheric bacteria

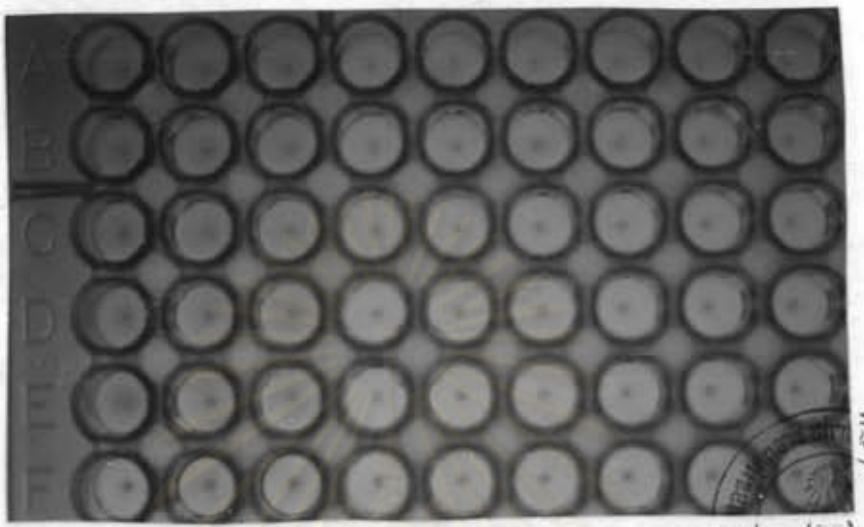
Inhibitory ability of different sugars, previously used in 4.1 were also tested with the agglutination activity of lectin with nitrogen-fixing rhizospheric bacteria as described in "Methods". Figure 10. a. and b. show that agglutination of bacterial cells rendered by rice bran lectin from column Sephadex G-100 (a) and N-acetyl-D-glucosamine-Sepharose 4B (b) can be inhibited by N-acetyl-D-glucosamine with the minimal concentration of 6.25 mg/ml. No other sugars tested exhibits

Figure 9. Agglutination of purified rice bran lectin with nitrogen-fixing rhizospheric bacteria

The bacterial culture No. 5, 6, 15, 17 and 18(1 % v/v) were agglutinated with purified rice bran lectin from Sephadex G-100 column (0.2 mg/ml) and from N-acetyl-D-glucosamine Sepharose 4B column (0.1 mg/ml)

a. Agglutination of purified rice bran lectin from Sephadex G-100 column (0.2 mg/ml)with nitrogen fixing rhizospheric bacteria A. No. 5, B. No. 6, C. No. 15, D. No. 17, E. No. 18 showing similar titer of 4. F. Escherichia coli showing negative titer.

b. Agglutination of purified rice bran lectin from N-acetyl-D-glucosamine Sepharose 4B column (0.1 mg/ml) with nitrogen-fixing rhizospheric bacteria A. No.5, B. No. 6, C. No. 15, D. No. 17 and E. No. 18 showing similar titer of 2. F. Escherichia coli showing negative titer.



a

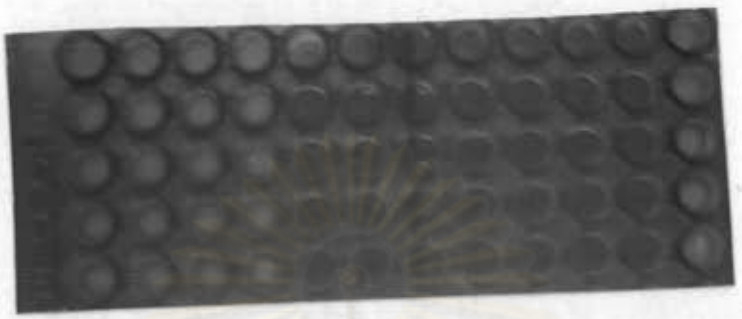
b

Figure 10. Inhibition study of agglutination activity with nitrogen-fixing rhizospheric bacteria

Twenty five microliters of purified rice bran lectin (0.1 mg/ml) from both schemes of separation were mixed with N-acetyl-D-glucosamine solution (50 mg/ml or serially diluted) allowed to stand for 30 min and then nitrogen-fixing bacterial suspension (1% v/v) was added.

a. Agglutination of bacterial cells; A. No 5, B. No 6, C. No 15, D. No 17 and E. No 18 rendered by purified lectin from Sephadex G-100 column can be inhibited by the minimal concentration of N-acetyl-D-glucosamine at well 4 (6.25 mg/ml).

b. Same as a. except the purified lectin was from N-actyl-D-glucosamine Sepharose 4B column.



a

b

the inhibitory activity, indicating that agglutination activity is specific to N-acetyl-D-glucosamine. It is noticable that the minimal inhibitory concentration of N-acetyl-D-glucosamine is only 6.25 mg/ml which is about half of the minimal concentration effective for inhibiting hemagglutination of rabbit erythrocytes.

5. Effect of boiling on the agglutination activity of purified rice bran lectin

When purified lectin from 2 schemes separation were boiled at 100°C for 15 min and assayed the hemagglutination activity with trypsinized rabbit erythrocytes, negative titer was observed which indicated that the agglutination activity of purified lectin is heat labile.

6. Sugar content

Anthrone reaction is the reaction of furfural or furfural derivative formed by dehydration of pentose or hexose with concentrate H_2SO_4. Condensation with anthrone gives characteristically colored products, which have absorbance at 620 nm with the sensitivity 20 µg. In our experiment, no trace of sugar was found in the purified lectin either from Sephadex G-100 column or N-acetyl-D-glucosamine Sepharose 4B column.

7. Subunit structure

SDS-polyacrylamide gel electrophoresis was used to separate protein subunits and to determine their M.W. on the basis of the rate of movement of proteins in an electric field depended on their mass. In this experiment, purified lectin and standard proteins(Sigma) were

subjected to SDS-polyacrylamide gel electrophoresis as described in "Methods"; Figure 11. shows that purified lectins either from ion exchange column or affinity column dissociate into 4 subunits numbering E_1, E_2, E_3 and E_4 with the approximate molecular weights of 24,000, 18,000, 14,000 and E_4 somewhat larger than 6,000 respectively.

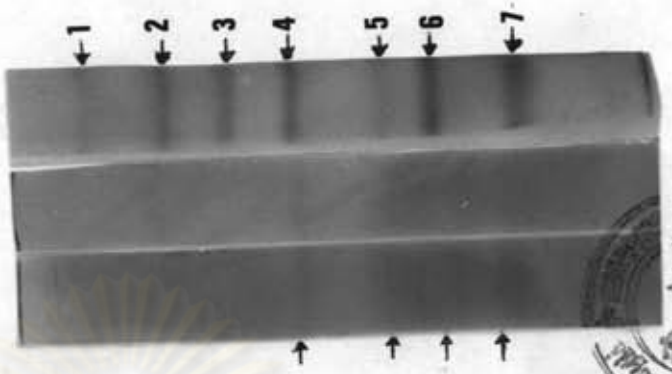
Figure 11. Estimation of the molecular weight of the subunits of purified rice bran lectin on SDS-polyacrylamide gel electrophoresis

Purified lectin from Sephadex G-100 and N-acetyl-D-glucosamine Sepharose 4B columns (50µg protein each) and standard protein markers, which were 1. BSA (M.W. 66,000), 2. ovalbumin (M.W. 45,000), 3. pepsin (M.W.34,700), 4. trypsinogen (M.W.24,000), 5. β-lactoglobulin (M.W.18,400),6. lysozyme (M.W. 14,300) and 7. insulin (M.W. 5,734) electrophoresed on SDS-polyacrylamide gel electrophoresis as described in "Methods".

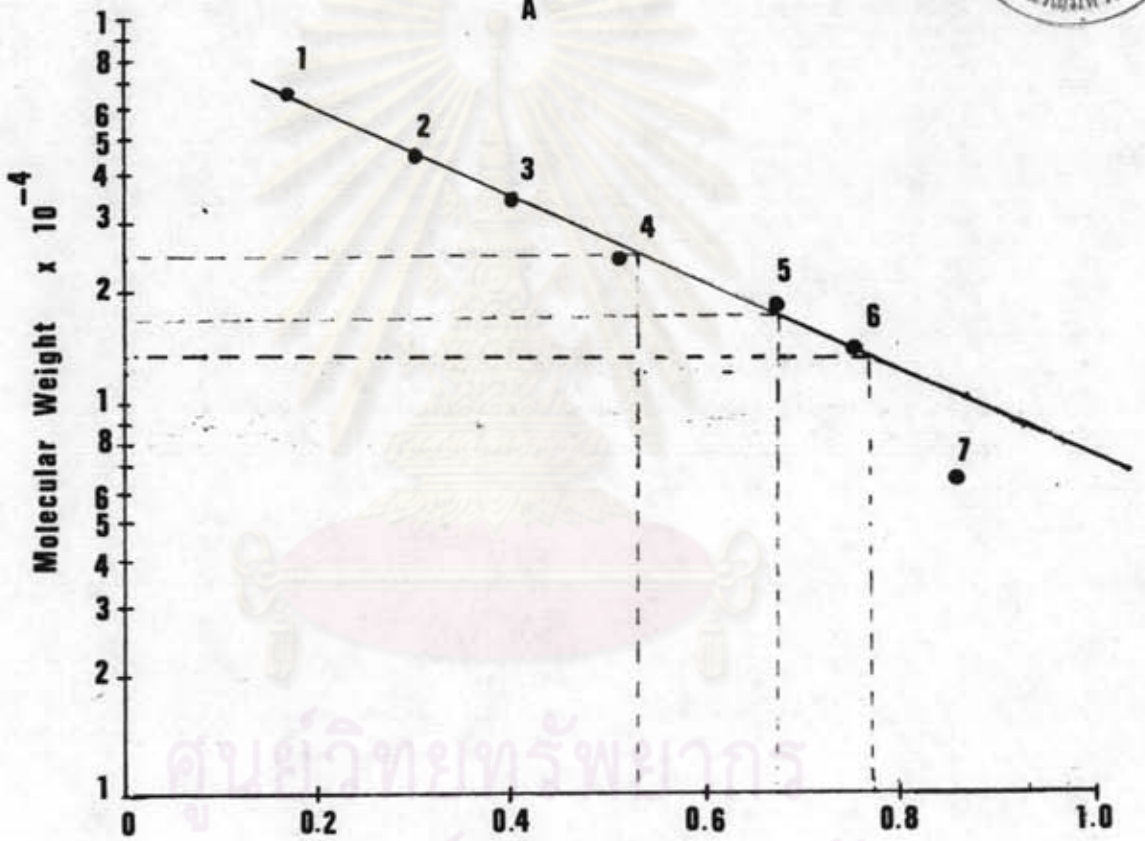
A.←represents the position of standard marker proteins and dissociation of rice bran lectin.

B. The logarithms of the molecular weights of marker proteins were plotted against their relative mobility values.

$$\text{The relative mobility} = \frac{\text{The distance of the band}}{\text{The distance of tracking dye}}$$



A



B