

[Original]

Studies on the Preparation of Radioactive Iodine Labelled Concanavalin-A, Lectin Extracted from Korean Native Plant "Banha", and Their Conjugation Products and the Hemagglutination Tests of These Labelled Compounds in Vitro.

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Abstract

Concanavalin-A, lectin extracted from Korean native plant "Banha" (*Pinellia Termanata*) and their conjugation products with tyrosine and 5-iodo-6-aminouracil were labelled by radioactive iodine-125, and these labelled compounds were subjected to a hemagglutination test in vitro. The labelling procedures were presented with respect to labelling yield and the trends on the conjugation reaction of lectin with various amino acids were discussed. The preliminary results on the hemagglutination tests against cancer tissues were presented and the potential clinical applications of these labelled compounds were discussed.

요 약

Concanavalin-A, 한국산 반하(半夏)로부터 추출된 Lectin 및 이들과 tyrosine 또는 5-iodo-6-aminouracil을 결합시킨 화합물들을 각각 방사성 요오드-125로 표지하였으며 표지된 화합물들을 사용하여 생체외부 시험법으로 혈액응고 시험을 실시하였다. 표지반응에 관하여 표지수율과 함께 그 실험조건을 보고하였으며 Lectin 과 여러 아미노산계통 화합물들과의 결합반응성을 논의하였다. 암세포에 대한 혈액응고 시험결과를 보고하였으며 이들 표지화합물들의 임상적 실용전망에 관하여서도 논의하였다.

Introduction

Various lectins, cell-agglutinating and sugar specific proteins have been known to provide a new tool for studying malignant cell surface such as a cancer cell¹⁾.

Especially, concanavalin-A extracted from the jack bean meal (2.5 to 3.0 percent by weight) has extensively been studied to ascertain its specific agglutination properties towards a malignant cell, the cell transformed by DNA tumor viruses or chemical carcinogens²⁾. On the other hand, a kind of glycoprotein extracted from the Korean native plant "Banha" (*Pinellia Ter-*

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nata) had clinically been observed to afford a specific hemagglutinating property on the malignant cell, which is expected to be useful to locate cancer cells or to be applied for a cancer therapy⁶⁾.

In this research the labelling procedure of concanavalin-A and "Banha" extracted lectin was at first investigated to afford the radioactive labelled compound for the cancer research with respects to the specific agglutinating property of lectins as described above. The extract from "Banha" is to be purified and the effective main component of the extract will be separated and identified. The concanavalin-A and the purified "Banha" lectin will be labelled by ¹²⁵I according to the procedures reported in the previous investigations and reaction parameters of affording a high labelling yield will further be investigated. The labelled compound would be subjected to the clinical investigation at the Cancer Hospital for the purpose of evaluating the feasibility of the labelled compound for the clinical applications.

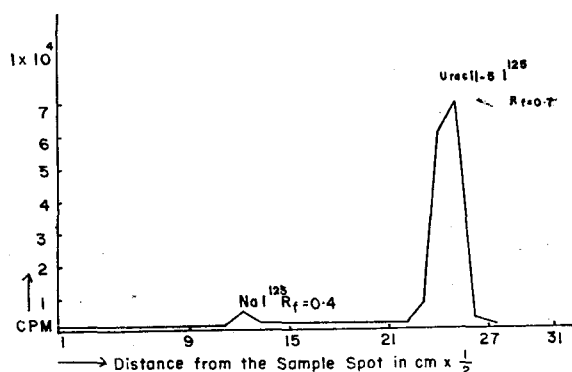
Crosslinking products of various proteins themselves have been applied for preparing an effective immuno-adsorbent³⁾, and coupling products of proteins and enzyme have also been achieved by means of glutaraldehyde and they were proved to be useful for identifying an antigen or an antibody⁴⁾. Especially, conjugation products of concanavalin-A and ferritin have been used for ascertaining the topology of cell surface⁵⁾, which may eventually be useful to understand the specific agglutinating properties of a lectin. These trends of researchs seemed to be very promising to investigate the clinical applicability of lectins for cancer therapy or diagnosis, since the binding site between lectins and the malignant cell in a

agglutinating reaction is expected to be concerned with the topology or chemical moiety of the cell surfaces. It is, therefore, considered to be worthwhile to investigate the conjugation reaction between "Banha" lectin and one of such anticancer drugs as antimetabolites, which may give a modified or specific agglutinating property towards a malignant cell and results some local uptakes of the anticancer drug itself by the malignant cell surface when the conjugation products are administered and hydrolysed in vivo on the surface of a cell. The conjugation reaction would better be studied through a radioactive tracer method, since the reaction is of a micro scale order. The relevant compounds may be labelled by radioactive iodine and the conjugated products may be subjected to a clinical investigation in vitro to evaluate the particular agglutinating behaviors for a cancer cell.

Experiments

1. Preparation of 5-iodouracil labelled by ¹²⁵I.

A catalytical amount of hydrogen peroxide



Developing Solvent: n-Butanol saturated with H₂O at 20°C by descending system

Paper: Whatmann #1

Fig 1. Paper Chromatogram of the Uracil-5 ¹²⁵I

Table 1. The Labelling of Uracil, 6-Aminouracil, and Tyrosine with radio isotopes.

Compounds	quantity of reagent(mg)	quantity of KI(mg)	quantity of Chl-T* or H ₂ O ₂ **	Labelling yield***%	Radio nucleide	Remarks
Uracil	20	1	0.1 ml	90	¹²⁵ I	in acetate Buff solution. (PH 4.8) at 100° C.
Uracil-5-Br	20	1	0.1 ml	95	"	"
6-Aminouracil	1	0.01	0.1 ml	60	"	"
D-Tyrosine	0.8	0.01	0.1 mg	80	"	in saline solution (PH8-9)
Uracil	56	59	0.1 ml	20	⁸² Br	"
Uracil-5 Br	200			4.3	⁸² Br	recoil reaction

*Chloroamine-T

**30% H₂O₂

***The labelling yield was estimated by comparing the areas of the peaks of the unreacted iodine and product on a PPC.

was added dropwise to a boiling aqueous solution of uracil or 5-bromouracil, Na ¹²⁵I, and acetate buffer (PH 4.8), and the resulting mixture was further refluxed for 1 hour. The compositions of the reaction mixture was checked by a descending PPC and the labelling yield of the product was evaluated by checking the radioactivity of the peak of the reported R_f value⁷. The reaction parameters, PPC, and the labelling yield were given in the table 1 and the Fig. 1.

2. Preparation of 5-bromouracil labelled by ⁸²Br.

The same procedures as above were applied using K⁸² Br. The K⁸² Br was prepared by neutralizing an aqueous solution of NH₄ ⁸²Br with 5% KOH solution and evaporate the solution to dryness. The results were shown in the table. 1.

3. Preparation of 6-aminouracil

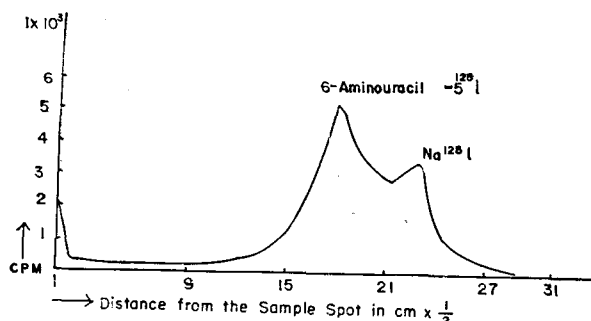
The reported procedure for the preparation of diaminouracil hydrochloride⁸ was adopted. In a three necked round bottom flask equipped with a stirrer, reflux con-

denser, and glass-col mantle, 2.0 g of metallic sodium was dissolved in 50 ml. of absolute ethanol. To the solution, 4.6 ml. of ethyl cyanoacetate and 2.6 g. of urea were added and the mixture was heated at 80°C under stirring for 4 hrs. The reaction mixture was slowly dissolved in hot water (80°C) and the solution was heated for 20 minutes. This hot solution was neutralized by glacial acetic acid to give a frothing. The frothings were separated from the mixture by centrifuge and the separated solid product was washed several times with distilled water. The dried solid product melted at 360°C. The yield was about 60%. The chemical structure of the product was identified by means of IR and UV spectrophotometry.

4. Preparation of 6-aminouracil labelled by ¹²⁵I.

The procedure described for labelling 5-iodouracil was applied. The labelled product was identified by PPC (See Fig. 2). The reaction parameters and labelling yield were shown in the table 1.

5. Preparation of tyrosine labelled by ¹²⁵I.



Developing Solvent: 75% Methanol in Water at 20°C

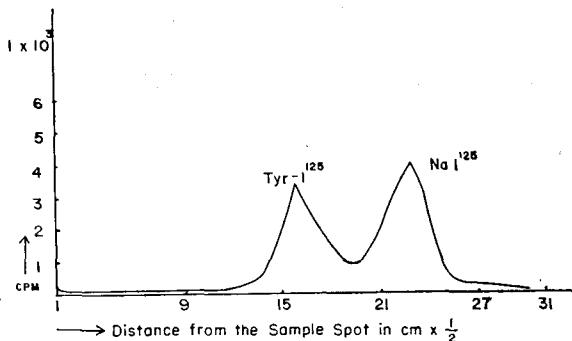
Paper: Whatmann #1

Fig. 2. Paper Chromatography of 6-Aminouracil-5 ^{125}I .

The chloramine-T procedure⁹⁾ was adopted. In the saturated aqueous solution of tyrosine an adequate amount of sodium bicarbonate solution was added to adjust the PH of the entire solution at 8-9. To this solution, 0.1 mg of potassium iodide and Na ^{125}I solution were added and the reaction mixture was stirred at room temperature for 1 hr. The reaction mixture was examined by PPC to identify the product (See Fig. 3). The reaction parameters and labelling yield were shown in the table 1.

6. Preparation of glutaraldehyde

According to the reported procedure¹⁰⁾, the mixture of acrolein (0.5 mole) and ethyl vinyl ether (0.55 mole) was reacted in a stainless bomb at 140-150°C for 12 hrs. The reaction mixture was distilled under reduced pressure. The fraction boiled at 38°C (8mm/Hg) was collected and hydrolysed with 62.5 ml of distilled water acidified by 5.2 ml of hydrochloric acid. The hydrolysed mixture was extracted with ethyl ether. The ether extract was dried under anhydrous calcium chloride and the ether was distilled off. The residue was distilled under reduced pressure to obtain the product boiled at



Developing Solvent: 75% Methanol in H₂O at 20°C
Paper: Whatmann #1

Fig. 3. Paper Chromatography of Tyrosine- ^{125}I

50°C (6 mm/Hg). The yield was averaged 38-40%.

7. Separation of lectin portion from the "Banha" extract.

a. The preparation of Banha extract.
The dried root of "Banha" plant (*Pinellia Ternata*) was pulverized in a fine powder¹¹⁾. 100 g of the powder was suspended in 500 ml of 0.15 M NaCl solution and the suspension was stirred at 4°C for 2 hrs. The resulting suspension was filtered and the filtrate was centrifuged using a refrigerated centrifuge equipped with a SS-34 Rotor. (11,000-12,000 r. p. m., 145,000-150,000g) The yellowish supernatant liquid was separated and it was saturated with 30% solution of ammonium sulfate. The saturated solution was neutralized using ammonium hydroxide solution and stirred for 4 hrs. The solution was again centrifuged and the supernatant liquid was separated. The supernatant solution separated was again saturated with 80% solution of ammonium sulfate and it was centrifuged. The precipitate was separated and dissolved in 150 ml of cold distilled water. The solution was dialysed against distilled water and it was again dialysed against 1 M solution of sodium chloride. The dialysed product was centrifuged and

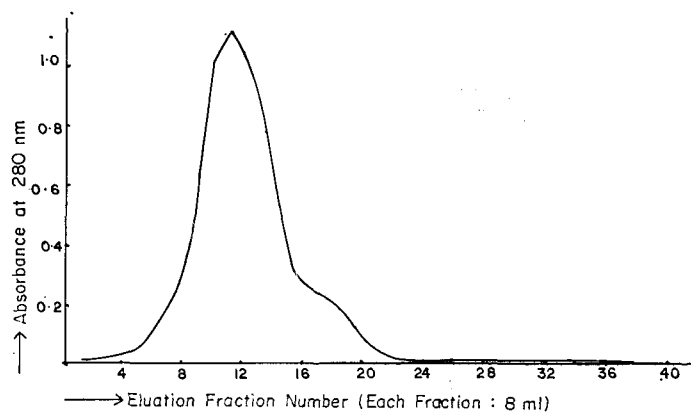
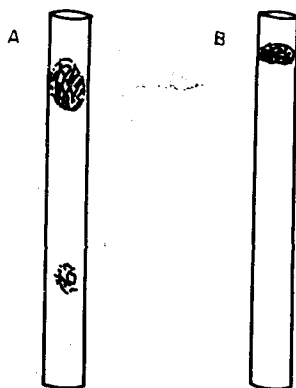


Fig. 4. Elution Curve of the purified lectin solution by Shephadex G-120 column Chromatography



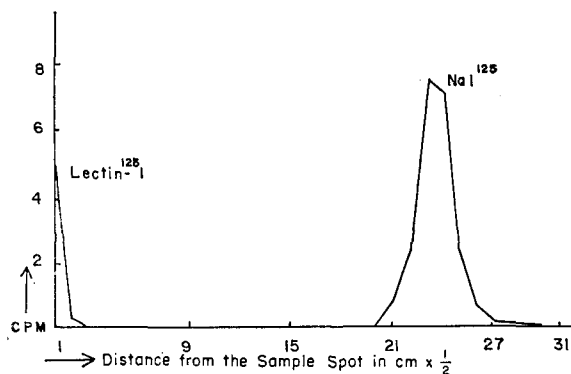
Amidoblack 10B Staining PAS Staining
Fig. 5. 5% SDS-polyacrylamide gel electrophoresis²⁰⁾ of purified lectin from Banha root stained for protein and glycoprotein.

Electrophoresis; Const. Current, 8 ma per gel.

the clear supernatant liquid was separated as the purified extract.

b. The separation of lectin portion from the extract.

The purified extract was chromatographed at the flow rate of 35-40 ml/hr. through a Shephadex G-120 column presaturated by 1 mol. solution of sodium chloride. The eluate from the column was collected 8 ml interval and each fraction was examined for its UV absorption at λ_{max} 280 nm (See Fig. 4), and the fractions showing absorp-



Developing Solvent: 75% Methanol in Water at 20°C

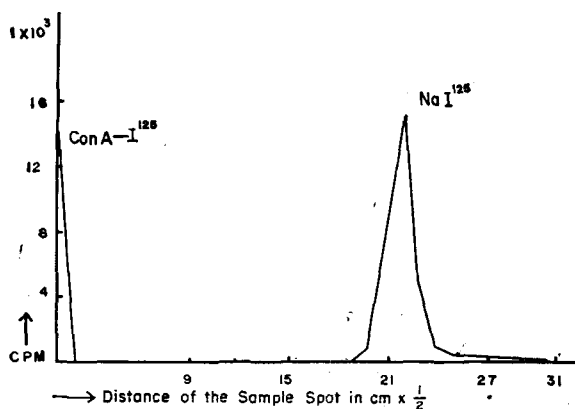
Paper: Whatmann #1

Fig. 6. Paper Chromatography of the Lectin-¹²⁵I.

tion at 280 nm were collected as the active lectin portion of the extract. The collected fractions were dialysed against distilled water. The dialysed extract was stored at 4°C as a stock solution of "Banha" lectin or lyophilized as a dried powder.

8. Determination of molecular weight of "Banha" lectin.

According to the Gel electrophoresis procedure for polyacrylamide¹²⁾ a phosphate buffer (pH 7.2) solution of "Banha" lectin lyophilized as above was subjected to a gel



Developing Solvent: 75% Methanol in Water at 20°C

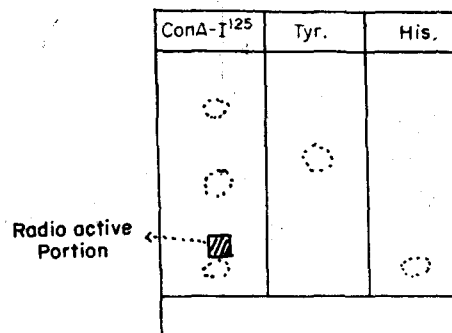
Paper: Whatmann #1

Fig. 7. Paper Chromatography of the Con-A-¹²⁵I

electrophoresis in a tube (ID 5mm, Length, 10 cm) (See. Fig. 5). It was found that the molecular weight of the lectin amounted to 150,000 when the position of the spot was compared with that of Con-A of known molecular weight²⁰.

9. Preparation of "Banha" lectin labelled by ¹²⁵I.

The reported procedure for labelling concanavalin-A¹³ was applied. The purified lectin was acetylated and the acetylated product was dialysed against distilled water. The dialysed solution (2 ml) was mixed with Na ¹²⁵I, (desired amount of radio activity) potassium iodide (0.1 mg) and the PH of the resulting solution was adjusted at 8.0 by means of a sodium bicarbonate solution. To this solution, 0.5 ml of chloramine-T (2 mg/ml) was added dropwise and the reaction mixture was reacted for 1 hr at 20°C. The reaction mixture was examined by PPC to determine the labelling yield (35%) (See. Fig. 6). The labelled lectin was separated from the reaction mixture by means of a dialysis.



Developing Solvent: Butanol: Acetic Acid: Water (4:1:5 V/V)

Paper: Whatmann #1

Solvent Front: 20 cm

Fig. 8. Paper Chromatography of the hydrolyzed lectin

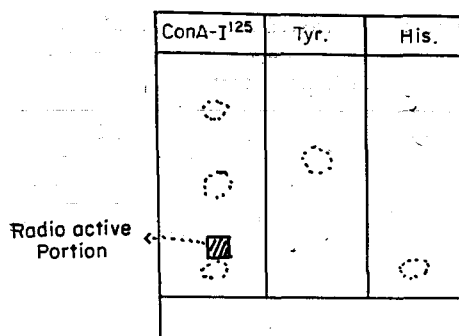
10. Preparation of concanavalin-A labelled by ¹²⁵I.

The concanavalin-A (Sigma Chem, Co. USA) was acetylated and the acetylated product was labelled by the procedure described above. The PPC of the labelled mixture was shown in the Fig. 7. The labelling yield was 50%. The labelled product was separated by dialysis.

11. Hydrolysis of labelled lectin and concanvalin-A

a. Hydrolysis of labelled lectin

2 ml. of labelled lectin and 10 ml. of 6N-HCl were mixed together in a round bottomed flask equipped with a reflux condenser and the resulting mixture was refluxed for 20hrs. The resulting solution was concentrated to a small volume and was examined for its amino acid compositions by PPC. The R_f values of each amino acid components on the PPC were studied both by radioactivity counting and coloring reagent such as ninhydrin. Separately, a standard solution of tyrosine and histidine was prepared and their R_f values on the PPC were examined by means of coloring re-



Developing Solvent; Butanol : Acetic Acid : Water
(4 : 1 : 5 V/V)

Paper: Whatmann #1

Solvent Front : 20 cm

Fig. 9. Paper Chromatography of the hydrolyzed Con-A-¹²⁵I

gent such as ninhydrin. The obtained R_f values on both PPC were compared and the compositions of the hydrolyzed product of labelled lectin were evaluated. (See Fig. 8)

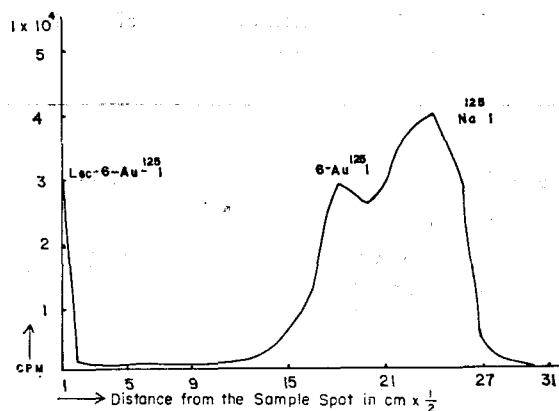
b. Hydrolysis of labelled concanavalin-A.

The same procedure described in a, was followed. The result was shown in Fig. 9.

12. Preparation of the conjugated lectins.

a. Conjugated product between lectin and labelled 6-aminouracil.

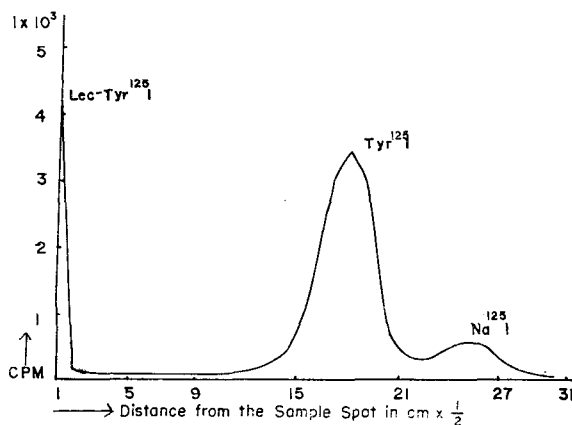
The glutaraldehyde conjugation procedure⁵⁾ was applied for the present system. 1.0 ml of the solution of 6-aminouracil labelled (0.1 M acetate buffer solution, PH= 4.8) 5 mg. of the lectin, and a small amount of NaCl were dissolved to make up a solution and 0.1 ml of 1% aqueous solution of glularaldehyde was further added to this solution. The resulting solution was kept at room temperature (20–22°C) for 2 hrs. The reaction mixture was examined for it's conjugated product by means of PPC as shown in Fig. 10. The conjugation reaction solution was dialysed against distilled water to remove the low molecular weight components and the dialyzed product was exa-



Developing Solvent: 75% Methanol

Paper: Whatmann #1

Fig. 10. Paper Chromatography of 6-Aminouracil-¹²⁵I Conjugated Lectin.



Developing Solvent: 75% Methanol in H₂O at 20°C

Paper: Whatmann #1

Fig. 11. Paper Chromatography of Tyr-¹²⁵I conjugated Lectin

mined again for its purity by PPC and the radioactivity of pure conjugated product was measured to check the overall labelling yield, which amounted to 15.0%.

b. Conjugated product between lectin and labelled tyrosine.

The same procedur described in a was followed. The overall labelling yield was 18.0%. The PPC data was given in Fig. 11.

13. Hemagglutination tests (in vitro) for labelled "Banha" lectin and conjugated lectins.

Table 2. Agglutination test of ^{125}I -labelled glycoprotein extracted from "Banha" Plant.

case	Total (cpm)			Sediment (cpm)		
	RBC	WBC		RBC	WBC	
		Lym.	Granulocyte		Lym.	Granulocyte
C3Hf	1182	2149	1880	780	1026	975
Hartley	2181	1911	2493	1464	1315	1382
Strain 2	3127	3129	2087	1941	1998	945
Case A	2505	1838	2656	731	1248	1072
Case B	1398	—	1869	505	—	1158

Table 3. Agglutination test of ^{125}I -labelled glycoprotein extracted from "Banha" Plant.

Case	Total (cpm)		Sediment (cpm)	
	RBC	WBC	RBC	WBC
Ehrlich	9909		5716	
Sarcoma-180	7992		4338	

Case	RBC	WBC	RBC	WBC
Strain 2	10346	9082	1523	5296
.. . . .	10058	8822	2852	4839
Case A	11183	9488	1231	4079
Case B	9101	9063	1354	4196

Table 4. Agglutination test of 6-Aminouracil and Tyrosine conjugated Lectin

Case	Radioactivity	Total (cpm)		Sediment (cpm)		
		RBC	WBC	RBC	WBC	
Case A	Heparin	U*	12851	14059	4229	1540
		Tyr**	17917	18225	3234	2678
	EDTA	U	15297	13958	5075	1851
		Tyr	16363	16248	2845	2367
Case B	Heparin	U	13428	14737	4821	2222
		Tyr	—	11015	—	1567
	EDTA	U	13602	13502	5469	2686
		Tyr	—	—	—	—

* 6-Aminouracil-5 ^{125}I conjugated lectin** Tyrosine- ^{125}I conjugated lectin

Standard procedure—The blood samples from Hartley, strain 2 guinea pig, C3Hf mice, and leukemia cases were separated for their RBC and WBC components, and

the WBC portion was further separated for Lym. and Granulocyte. The separated portions were mixed with a definite amount of labelled lectins or labelled conjugated

Table. 5. Agglutination test of 6-Aminouracil and Tyrosine conjugated lectin.

Case	Radioactivity		Total (cpm)		Sediment (cpm)	
Ehrlich	U.*		33265		18180	
	Tyr.**		26336		4337	
Sarcoma-180	U.		31600		26052	
	Tyr.		24485		4504	
Case	organ	R B C		W B C		
C3Hf	U.	36837	34825	21333	6460	
	Tyr.	27271	25203	6694	3067	
Strain 2	U.	31567	35877	(42620)	14422	
	Tyr.	24863	26946	5235	3569	
Hartly	U.	33295	42009	(35297)	16797	
	Tyr.	25901	25438	4247	4291	

* 6-Amino Uracil-5 ¹²⁵I Conjugated lectin

** Tyrosine-¹²⁵I Conjugated lectin

lectins and the mixture was shaken vigorously to complete the hemagglutination reaction. Each system was examined for the hemagglutination by a microscope.¹⁹⁾ After being ascertained the hemagglutination of a sample system, the system was centrifuged and the radioactivities of both supernatant liquid and precipitate were counted. The results were summarized in table. 2. In cases of Ehrlich and Sarcoma-180, the hemagglutination reaction for a system was completed at first and the resulting mixture was sedimented. The radioactivity of the sedimented fraction of each system was measured and evaluated. The results were summarized in table 3, 4, and 5, respectively.

Results and Discussion

As shown in table 1, labelling of uracil, 6-aminouracil, and D-tyrosine could be achieved with relatively high labelling yields and purities by means of chloroamine

-T⁹⁾ or H₂O₂⁹⁾ procedures. In case of labelling an uracil by the radioactive bromine-82, a rapid direct bromination of an uracil could be achieved by means of the reactor produced K²⁸Br, whereas the recoil labelling procedure could give only the low labelling yield(20%) together with many kinds of decomposed products. The prepared products are all examined by PPC, which showed the same composition of the labelled products as reported in literatures (See Figs 1-3). Generally, the hydrogen peroxide procedure could give the better labelling yields in cases of uracil derivatives than those of the chloroamine-T procedure. Probably, the hydrogen peroxide could act as the better oxidizing agent in the present reaction systems. Even though the labelled products prepared are mainly utilized as the tracer compounds for the precursor purposes in this paper, they may independently be used for the basic research of the clinical applications for detecting tumours of any kinds in this country, as

many literatures reported their potential applications in the clinical works.

Since the saline extract of a "Banha" root could clinically show a selective hemagglutination reaction towards various tumour cells,⁶⁾ it was considered that the extract would contain some sorts of lectin equivalent to concanavalin-A or the similars, which had been known to show a selective hemagglutination reaction.¹⁾ Therefore, the saline extracts of Banha are prepared and they are subjected to the separation scheme adopted for the separation of con-A from jack-meal bean as reported.¹¹⁾ As shown in Fig. 4 the eluate from a Shephadex column did not show the UV adsorption as the later parts of the elution fractions which are the fractions eluated by a D-glucose solution. The fractions showing UV adsorption at λ max 280nm belong to the fraction numbers of from 4 to 24. These parts of the fractions were considered as the inactive protein for the hemagglutination reaction in case of the separation of the concanavalin-A¹¹⁾, but the present portions could give the distinct agglutinating activity when they are subjected to an agglutination test as shown in tables 2-3. The lyophilized product from the fractions (2.0g from 100g of the dried Banha root) gave the broad bands near at the region of molecular weights 120,000-170,000 and showed some tails at those of the lower region when they are examined through a polyacrylamide gel electrophoresis. (see Fig. 5) However, they showed the distinct deep coloring of a glycoprotein by the PAS staining in the column B as shown in Fig. 5. Furthermore, the purified extract showed a distinct sugar reaction towards the chemical tests and the hydrolyzed product

of it had shown the amino acid residues on the PPC (see Fig 8. and 9). Since the concanavalin-A was proved to be a non glycoprotein which showed an average molecular weight of 80,000 and lectin separated from the lotus tetragonolohus¹⁾ showed three different molecular weights, the glycoprotein separated as above from the Banha would be a mixture of the glycoproteins, molecular weights of which are 120,000-170,000. Further quantitative identifications for the purity of the glycoprotein would be necessary to ascertain whether it is composed of a pure lectin or not, as concanavalin-A was proved to be 98% pure lectin.¹¹⁾ But the extract from the Banha could not show an affinity to the Shephadex gel as described above and hence there were no fractions showing UV absorption at the D-glucose elution stage. On basis of these facts, the glycoprotein from Banha could be considered qualitatively to be a lectin of an average molecular weight of 150,000. The hydrolyzed product of the Banha lectin labelled by ¹²⁵I was compared to those of the concanavalin-A labelled by ¹²⁵I through PPC. Both of them showed the same R_f value of their amino acid residues on PPC, which are more closed to the histidine residue. It could, therefore, be considered that the glycoprotein separated from the Banha root was labelled at the same amino acid residue as compared those of concanavalin-A. Though the presence of the subunit or impurities in the Banha lectin should, quantitatively, be proved, the lectin from the Banha would contain the amino acid residue resembled to that of the concanavalin-A.

The labellings of the concanavalin-A and the lectin separated from the Banha are

proceeded smoothly by means of a modification of the H_2O_2 -lactoperoxidase procedure¹⁸⁾ of labelling the concanavalin-A. The increase of labelling yield would further be expected when the concentration of the reaction mixture is adjusted depending on their application in vitro.

The agglutination tests by means of labelled Banha lectin thus prepared gave interesting results as listed in tables 2 and 3. Generally, the high radioactivity was observed in the agglutination mixture, but the sedimented fractions of the mixture could not retain all of the initial radioactivity. Especially, RBC portions could retain less than 50% of the initial radioactivity in their sedimented fractions, which indicated that the binding of the Banha lectin with a RBC is not substantial enough to keep their binding in the sedimented particles. In cases of the WBC portions the initial radioactivity of agglutination mixture could be retained in the sedimented fraction of the lymphocytes of WBC, whereas the retain of the radioactivity was greatly decreased in case of the granulocyte of the WBC. The agglutination tests for case A (Leukemia) could show a considerable retention of the radioactivity in the sedimented particles of the WBC fraction, which would be substantial enough to identify a high agglutination behavior of the Banha lectin for the case.

Further investigations utilizing the lectin conjugated with a biologically active amino acid such as tyrosine or uracil derivatives showed a decreased retention of the radioactivity in the sedimented fraction of a WBC, whereas it was slightly increased in case of a RBC fraction. These observations would indicate that the binding sites of the

Banha lectin towards a agglutination reaction with the RBC and the WBC was decreased, because of the conjugation between the lectin and its counterpart. Furthermore, the agglutination by Banha lectin is specifically dependent on the binding moiety of the RBC or the WBC. Various conjugation partners for the Banha lectin were searched, but the tyrosine and the 6-amino 5-iodouracil could only be able to give their conjugation products, whereas 5-iodouracil or equivalents had failed to conjugate with the lectin.

In conclusion, the Banha lectin, when it is labelled by ^{125}I , could show a quantitative profile for the agglutination test and its binding of the WBC portion was substantial enough to identify such case as a leukemia. The binding sites of the Banha lectin with the blood cell were decreased when it was conjugated with an amino acid or an uracil derivative, which would predict the Banha lectin has the specific binding potentialities towards blood cells, especially such a malignant WBC as a leukemia.

Acknowledgement

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