Chemical Structure of the component Involved in Immunoregulation

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Background and Objective

BioBran/MGN-3 derived from rice bran is known to have various physiological functions including NK cell and macrophage activation and its immunomodulatory function is attracting attention. In addition, BioBran/MGN-3 has been reported to have antitumor activity and its clinical application as an antitumor agent is anticipated. Application of BioBran/MGN-3 requires analyses of the mechanisms of various physiological actions and the active ingredients. Although many studies on BioBran/MGN-3 have been reported, there are few detailed studies on the active component of BioBran/MGN-3 and the immunomodulatory ingredient is unknown.

This study analyzed the component of BioBran/MGN-3 involved in immunomodulatory activity using macrophage stimulation as the indicator as well as the chemical structure of the active ingredient.

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Materials and Methods

1. Fractions of BioBran/MGN-3

1) BioBran/MGN-3 was dissolved in purified water and the soluble fraction was further separated into 50% (50 ppt), 66% (66 ppt), and 80% (80 ppt) methanol precipitation fractions and 80% methanol supernatant fraction (80 sup) by fractional alcohol precipitation.

2) The 80% methanol insoluble fraction was applied to a DEAE-Sephadex A-25 column equilibrated with 20 mM Na-acetate buffer (pH 5.0) and eluted stepwise with NaCl to obtain 5 fractions I to V. Fraction II, eluted with 0.2 M NaCl, was applied to a DEAE-Sephadex A-25 column again and subfractions II-1 to II-7 were obtained by elution using a linear concentration gradient of 0-0.5 M NaCl.

2. Evaluation of macrophage activation

Female C57BL/6 mice aged 6-7 weeks were injected intraperitoneally with 4.05% thioglycolate medium (Difco) , the abdominal skin was removed after 4 days, and 5 ml of cold PBS was introduced into the peritoneal cavity to collect peritoneal exudate cells (PEC). Mac-1^+ cells (peritoneal macrophage) were separated using a magnetic bead cell sorter (Miltenyl Biotec) and cultured in the presence of BioBran/MGN-3 or each BioBran/MGN-3 fraction for 24 hours, and the culture supernatant was isolated. NO^{2-} in the culture supernatant was determined using the Griess method and tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) were determined using ELISA (Biosource).

3. Sugar analysis for each fraction

Analyses of sugar composition and linkages (methylation) and analysis of fragments with an enzyme were performed.

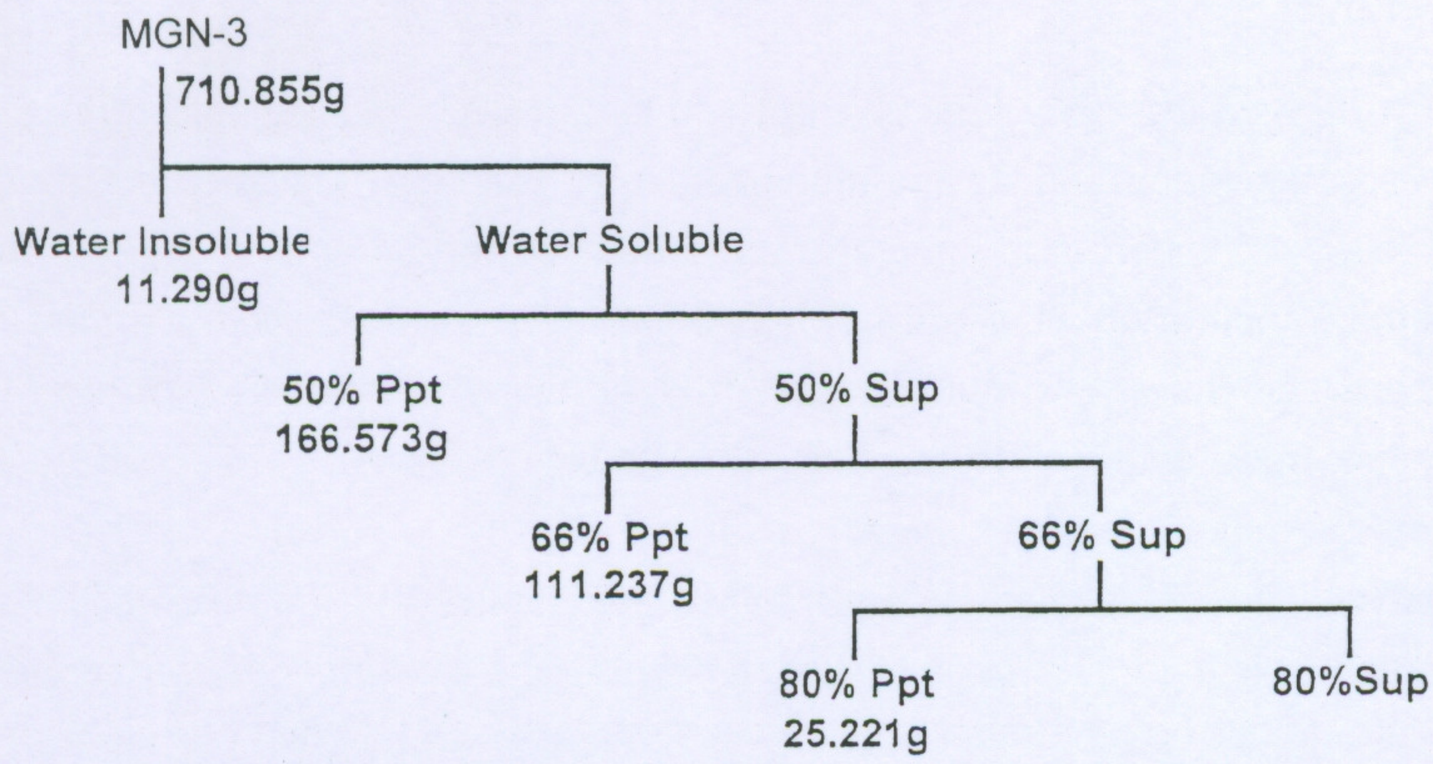

Fig. 1 Fractionation of MGN-3 by the graded methanol precipitation method

Results and Discussion

1. Search for an immunomodulatory component by alcohol fractional precipitation

BioBran/MGN-3 solution in purified water was subjected to stepwise alcohol fractional precipitation. As a result, 167 g of the 50% methanol precipitation fraction, 111 g of the 66% methanol precipitation fraction,

and 25 g of the 80% methanol precipitation fraction were obtained from about 710 g of BioBran/MGN-3 (**Fig. 1**). The yield by methanol fractional precipitation was 23.4% for 50 ppt, 15.6% for 66 ppt, and 3.5% for 80 ppt.

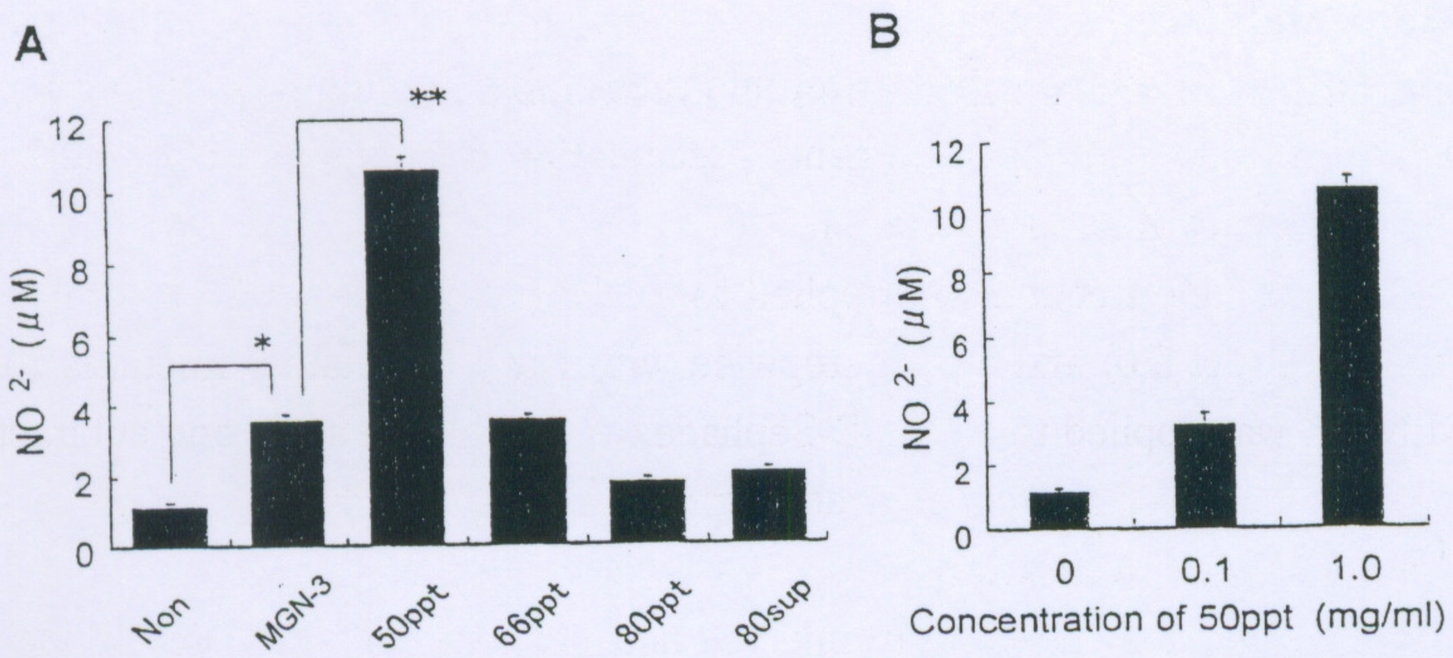

Fig. 2 **Effect on NO^{2-} production of MGN-3 subfractions. A, comparison of the NO production in respectively stimulating the macrophage in MGN-3, 50%, 66%, 80% methanol precipitation fractions or, 80% methanol soluble fraction. B, dose dependency of 50%, 66%, 80% methanol precipitation fractions on NO^{2-} production. Each bar value represents the mean ± standard deviation based on four wells.**

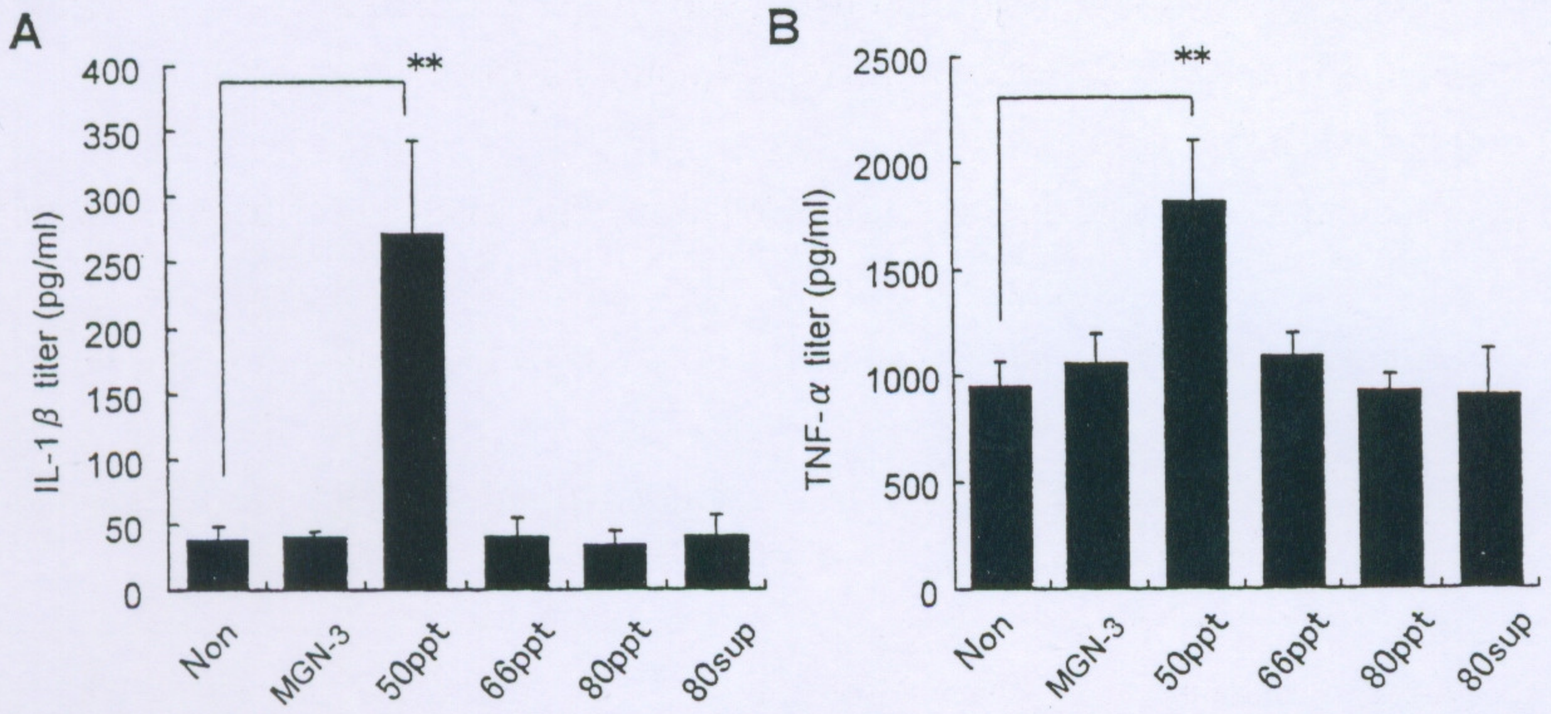

Fig. 3 **Effect on cytokine production of MGN-3 subfractions, 50ppt, 60ppt, 80ppt, and 80sup. TNF-α (A) and IL-1β (B) production in culture supernatants after 24 h of incubation were estimated. Each bar value represents the mean ± standard deviation based on four wells.**

Macrophages were stimulated *in vitro* with each precipitation fraction to compare their abilities to activate macrophages with BioBran/MGN-3. NO^{2-} production by machrophages was 3 times higher after stimulation with the 50 ppt fraction than after stimulation with unfractionated BioBran/MGN-3 (**Fig. 2A**). On the other hand, the 66% and 80% methanol precipitation fractions and 80% methanol soluble fraction induced production slightly but the NO^{2-} activities produced were equivalent to or lower than that of BioBran/MGN-3 (**Fig. 2A**). The NO^{2-} production of macrophages stimulated with the 50% methanol precipitation fraction was dose-dependent (**Fig. 2B**).

Next, the production of IL-1β and TNF-α were determined after stimulation with methanol precipitation fractions. BioBran/MGN-3 scarcely induced IL-1β, while the 50% methanol precipitation fraction induced it markedly (**Fig. 3A**). Similarly, the 50% methanol precipitation fraction markedly increased TNF-α

production (**Fig. 3B**). There are reports of the activation of immune cells with BioBran/MGN-3. Our study confirmed the activation of macrophages with BioBran/MGN-3. The ability to activate macrophages was 3-5 times higher for the 50% methanol precipitation fraction than for BioBran/MGN-3 itself and the 50% methanol precipitation fraction accounted for 23.4% of BioBran/MGN-3. From findings these, it is inferred that the immunomodulatory ingredient of BioBran/MGN-3 is present in this fraction.

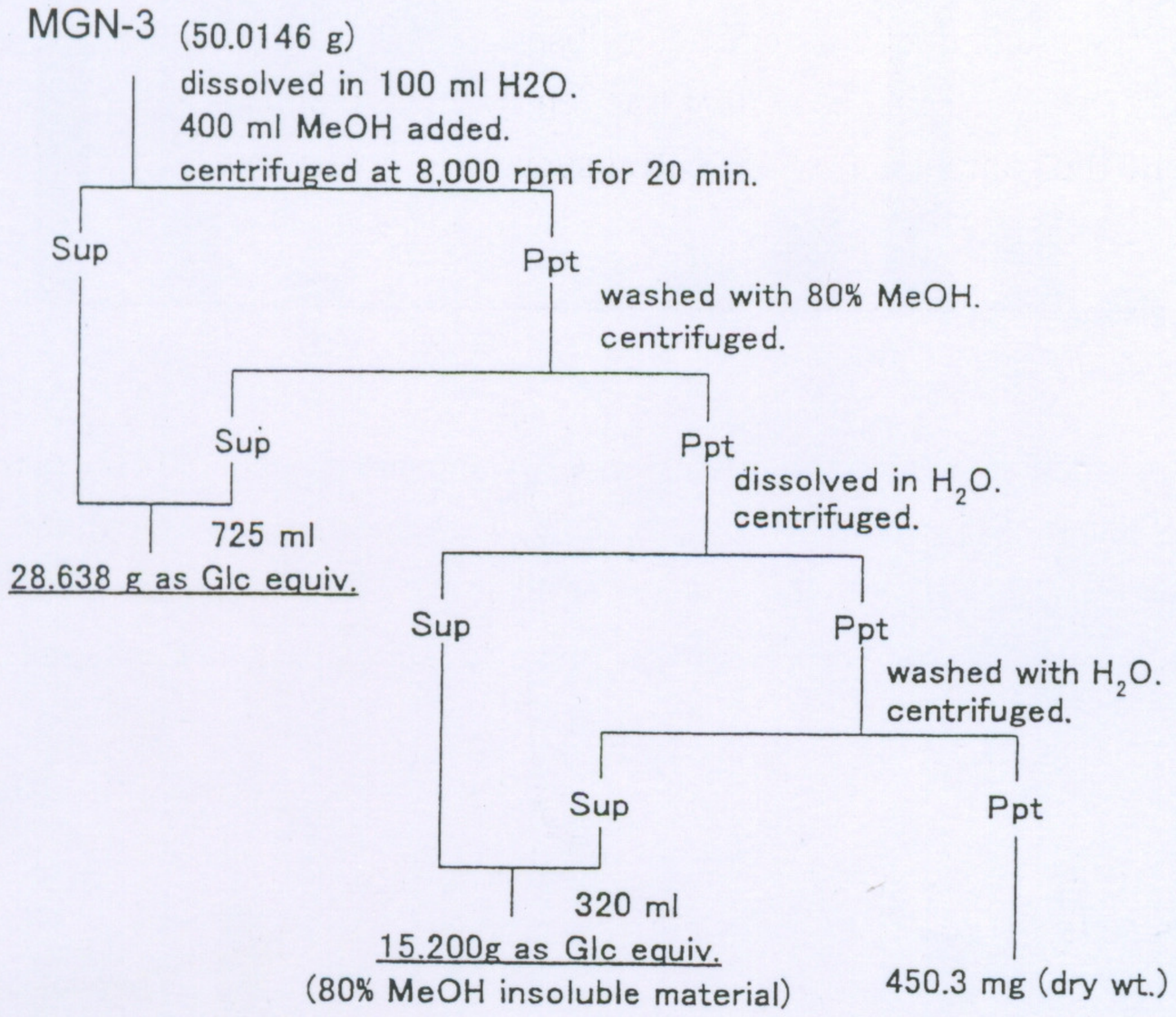

Fig. 4 A summary of preparation procedures of 80% MeOH insoluble material from MGN-3

Table 1 Neutral sugar composition of fraction I~V obtained from the 80% MeOH insoluble material by DEAE-Sephadex A-25 chromatography

Fraction	Yield (mg)	Neutral sugar composition (mol%)						
		Rha	Fuc	Ara	Xyl	Man	Glc	Gal
I	164.7							
II	115.0	1.0	0.2	23.0	15.1	2.0	48.1	10.7
III	127.2	1.9	5.9	15.7	7.5	1.3	30.1	37.8
IV	57.8	–	–	2.7	1.7	2.4	89.2	4.0
V	38.4							

80% MeOH insoluble material (14.49 g as Glc equiv./ 305ml) applied on a column (5×27cm) of DEAE-Sephadex A-25 equilibrated with 20mM Na-acetate buffer (pH 5.0) and eluted stepwise with 20mM Na-acetate buffer (fraction I), 0.2M NaCl in the same buffer (fraction II), 0.5M NaCl in the same buffer (fraction III), 1.0M NaCl in the same buffer (Fraction IV) and 0.5M NaOH (fraction V). Fractions II, III, IV and V were dialyzed against distilled water. Fraction I was treated with amylases, and then dialyzed against distilled water.

2. Search for an immunomodulatory component by ion-exchange chromatography

Methanol was added to the water soluble fraction of BioBran/MGN-3 to a final concentration of 80%, the precipitate was separated, washed with 80% methanol, and removed again, and after purification, 15 g of

the 80% methanol precipitation fraction (yield 30.4%) was obtained from 50 g of BioBran/MGN-3 (**Fig. 4**). This insoluble fraction was applied to a DEAE-Sephadex A-25 column equilibrated with a 20 mM Na-acetate buffer (pH 5.0) to obtain non-absorptive fraction I, fraction II eluted with 0.2 M NaCl, fraction III eluted with 0.5 M NaCl, fraction IV eluted with 1.0 M NaCl, and fraction V eluted with 0.5 M NaOH. Fractions II to V were dialyzed against water and fraction I was digested with amylase and there dialyzed against water. **Table 1** shows the yields and neutral sugar compositions of fractions II, III, and IV eluted with NaCl. Fraction II mainly contained arabinose, xylose, glucose, and galactose. Fraction III contained less arabinose and xylose but more galactose than fraction II. For fraction IV, glucose accounted for 89.2% of the neutral sugar while the contents of other neutral sugars were very low.

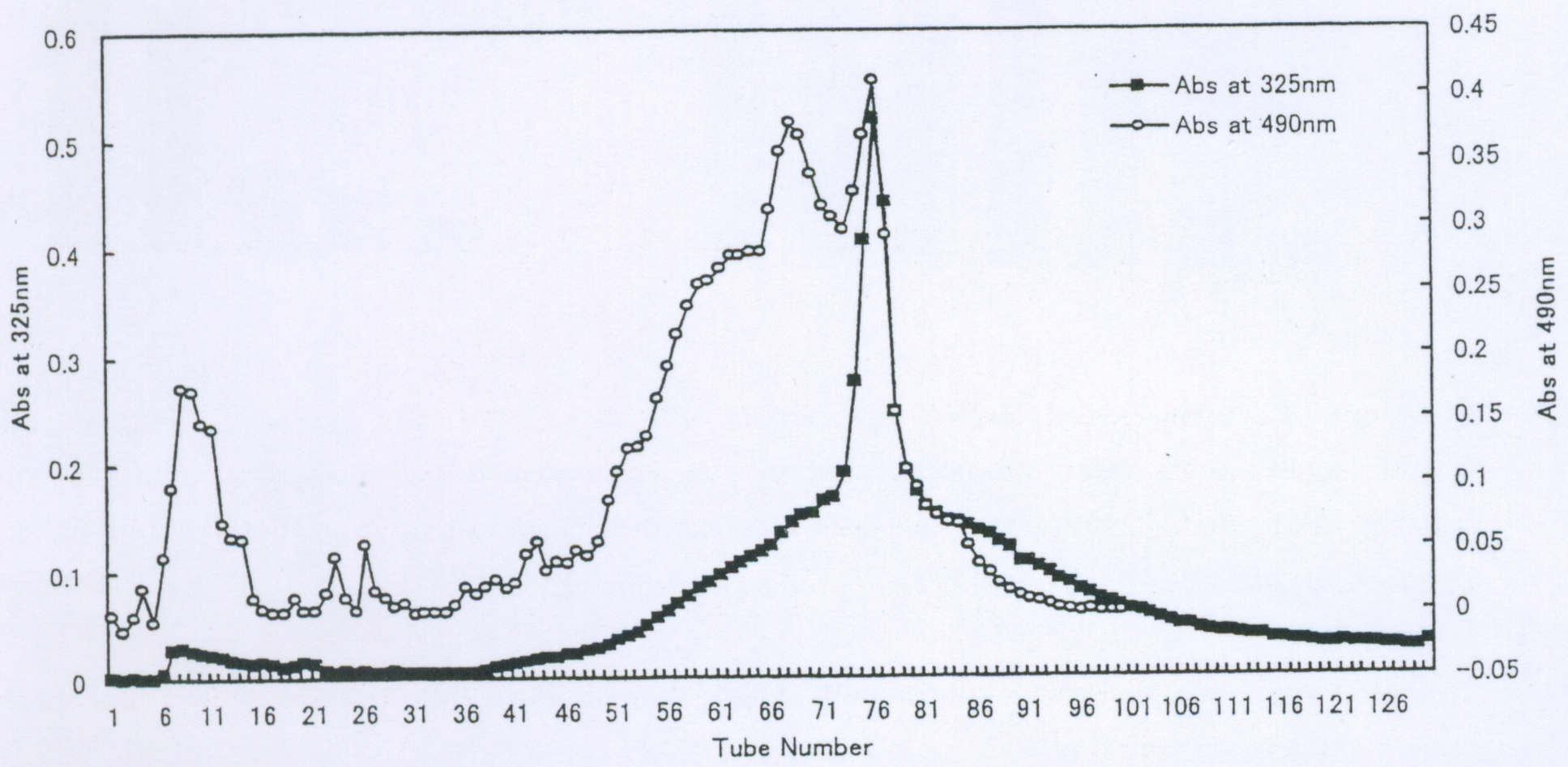

Fig. 5 DEAE-Sephadex A-25 chromatography of Fr. II. Fraction II obtained from MGN-3 was applied to a column of DEAE-Sephadex A-25 equilibrated with 20 mM Na-acetate buffer, and then the column was washed with the same buffer. The adsorbed materials were eluted with the salt gradient (0~0.5 M NaCl in the Na-acetate buffer): 9.8ml-fractions were collected. Tubes 6-15 (Fr. II-1), 36-49 (II-2), 50-53 (II-3), 54-65 (II-4), 66-73 (II-5), 74-79 (II-6) and 80-90 (II-7) were separately combined and dialyzed against distilled water.

Fraction II, with relatively high arabinose and xylose content, was applied to a DEAE-Sephadex A-25 column, for detailed fractionation by elution using a linear concentration gradient of 0-0.5 M NaCl. Based on absorption peaks of sugars, subfractions II-1 to II-7 were obtained (**Fig. 5**). In UV absorption, only subfraction II-6 showed a clear peak, suggesting that the other 6 subfractions contained almost no protein. Among the 7 subfractions, II-4, II-5, and II-6 were analyzed for macrophage activation and neutral sugar compositions. NO^{2-} production after stimulation with subfractions II-4 to II-6 was higher than that by the 50% methanol precipitation fraction and was 4-5 times higher than that with BioBran/MGN-3 (**Fig. 6A**). Lipopolysaccharide (LPS), Gram-negative outer membrane components, are known to play a priming role in the activation of machrophages. In our study, 10 μg/ml of LPS strongly stimulated macrophages. In a comparison of the subfractions with LPS, macrophages stimulated with the subfractions had NO production equivalent to or higher than those stimulated with LPS. Subfraction II-6 dose-dependently activated

machrophages and the activation inducing activity at 1/10th of the concentration was similar to that of the 50% methanol precipitation fraction (**Fig. 6B**). Among subfractions II-4 to II-6, only II-6 contained a UV absorbing component likely to be protein. Subfractions II-4 and II-5 were similar to II-6 in ability to activate macrophages, suggesting that the UV absorbing material plays no direct role in the activation of macrophages. Thus, it is inferred that polysaccharides in subfractions II-4, II-5, and II-6 have ability to activate macrophages.

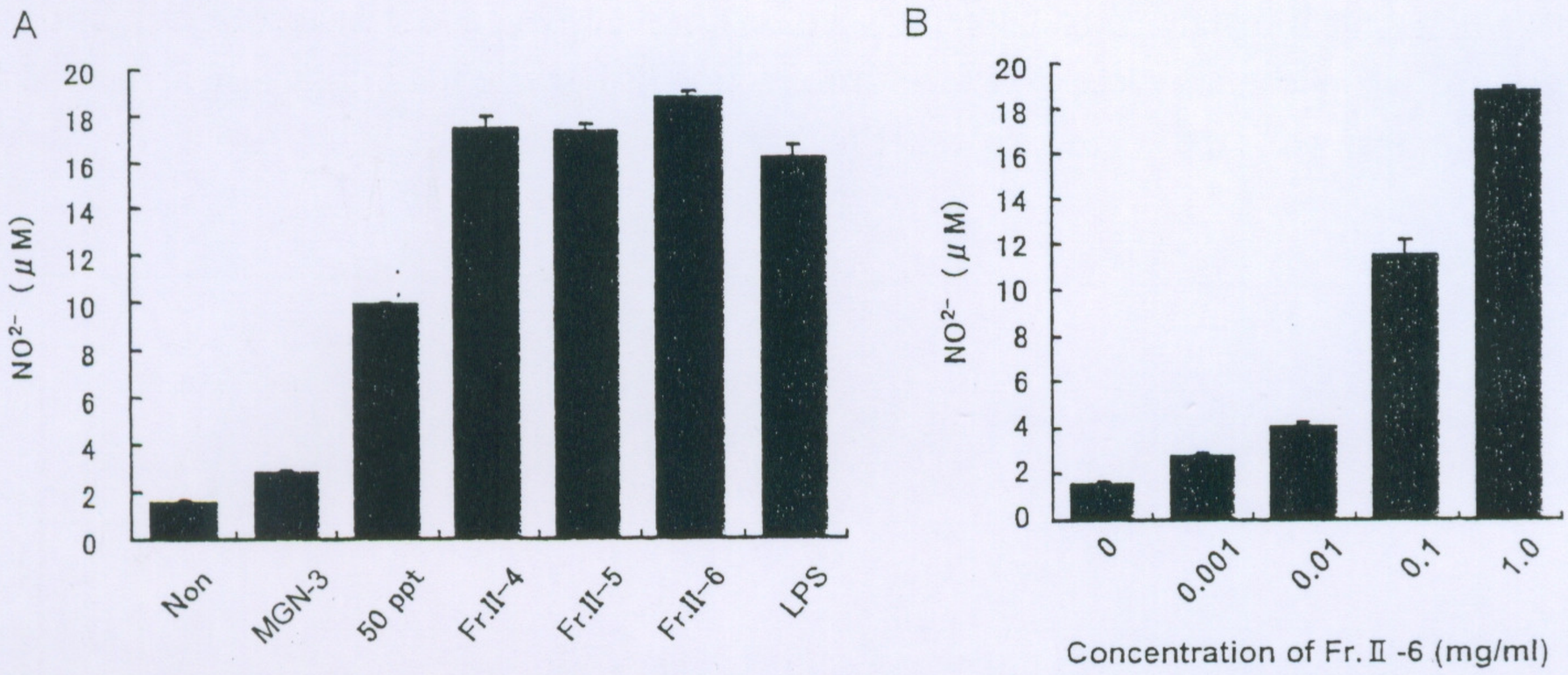

Fig. 6 **Effect on PEC activation of MGN-3 subfractions, Fr. II-4, Fr.II-5, and Fi. II-6. NO^{2-} production in culture supernatants after 24h of incubation was estimated. A, comparison of NO^{2-} production between crude MGN-3, 50% NeOH insoluble fraction (50ppt), and subfraction II eluted with 0.2M NaCl. B, dose dependency of the Fr. II-6 in NO^{2-} production from PEC.**

Table 2 **Neutral sugar composition of subfractions, II-1 ~ II-7 obtained from fraction II by DEAE-Sephadex A-25 chromatography (Fig. 5).**

Fraction (Tube Nos.)	Yield (mg)	Neutral sugar composition (mol%)						
		Rha	Fuc	Ara	Xyl	Man	Glc	Gal
II-1 (6-15)	1.6							
II-2 (36-49)	1.9							
II-3 (50-53)	1.5							
II-4 (54-65)	13.4	1.1	0.4	22.5	19.4	4.7	26.7	25.2
II-5 (66-73)	13.2	2.7	0.7	22.2	14.8	3.5	32.2	23.9
II-6 (74-79)	9.2	7.6	0.6	22.2	13.7	2.7	30.2	23.0
II-7 (80-90)	4.2							

3. Structural analysis of an immunomodulatory component

Subfraction II-6 with the highest macrophage stimulating activity was analyzed for molecular weight (MW) distribution by gel filtration using a Sepharose CL-6B (**Fig. 7**). It had a wide distribution of MW and comparison with a standard dextran suggested that the mean size was 10,000-20,000 Daltons. Methylation analysis was performed to study sugar linkages of the polysaccharides in II-6. Subfraction II-6 methylated by Hakomori's method was hydrolyzed and analyzed as alditol acetate by gas chromatography (**Fig. 8** and **Table 3**). As a result, the subfraction showed peaks of terminal non-reducing glucose, 5-linked arabinose, terminal non-reducing galactose, 4-linked xylose, 3,5-linked arabinose, 3-linked glucose, 4-linked glucose, 2,4-linked galactose, 4,6-linked glucose, and 3,6-linked galactose, showing that the main components are

arabinose, galactose, and glucose. Although it is difficult to estimate the structure of polysaccharides in II-6 from these methylated monosaccharides with various linkages, the approximate structure could be predicted based on the structures of many polysaccharides that compose the plant cell wall.

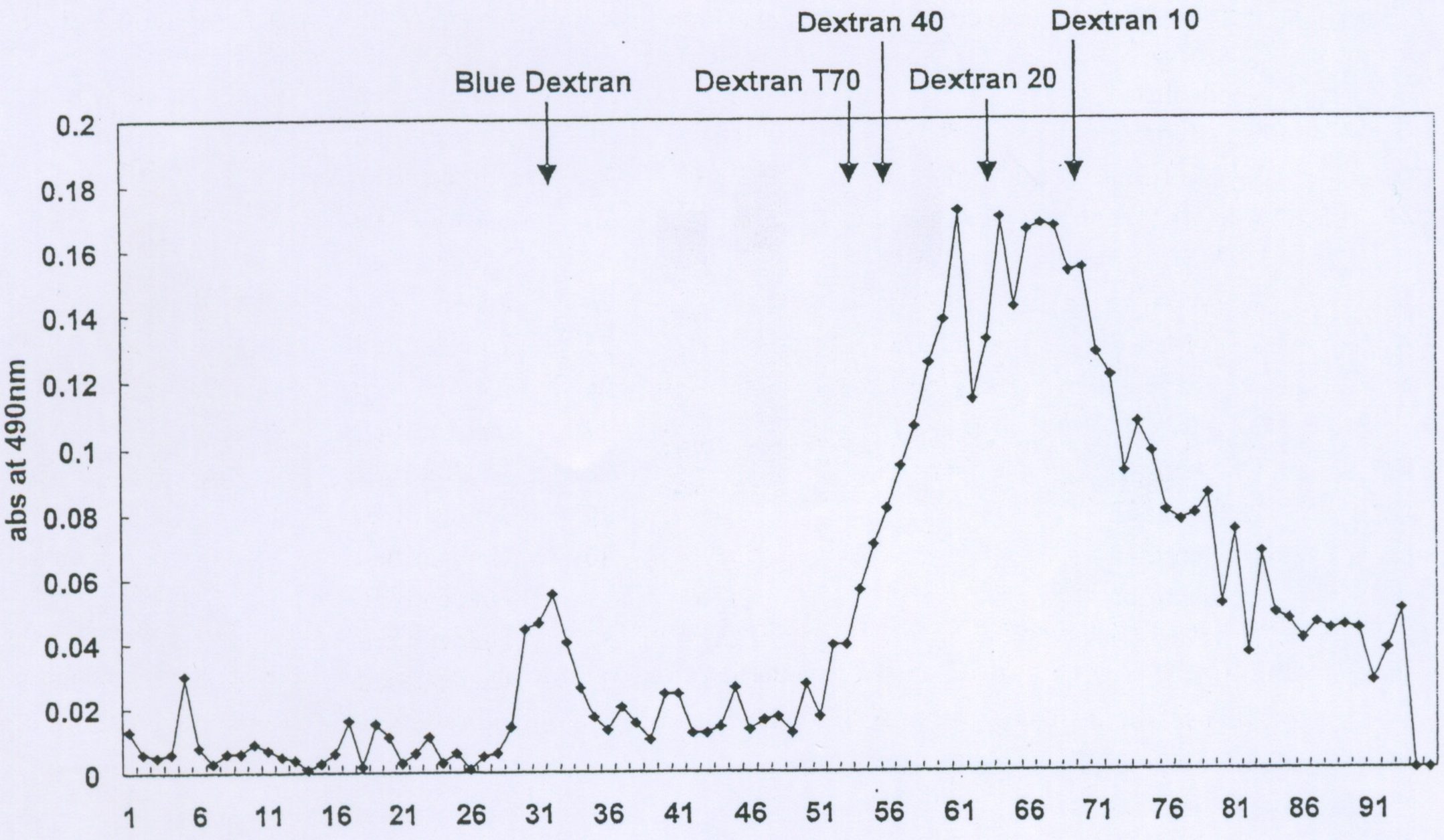

Fig. 7 Molecular weight distribution of fr.II-6

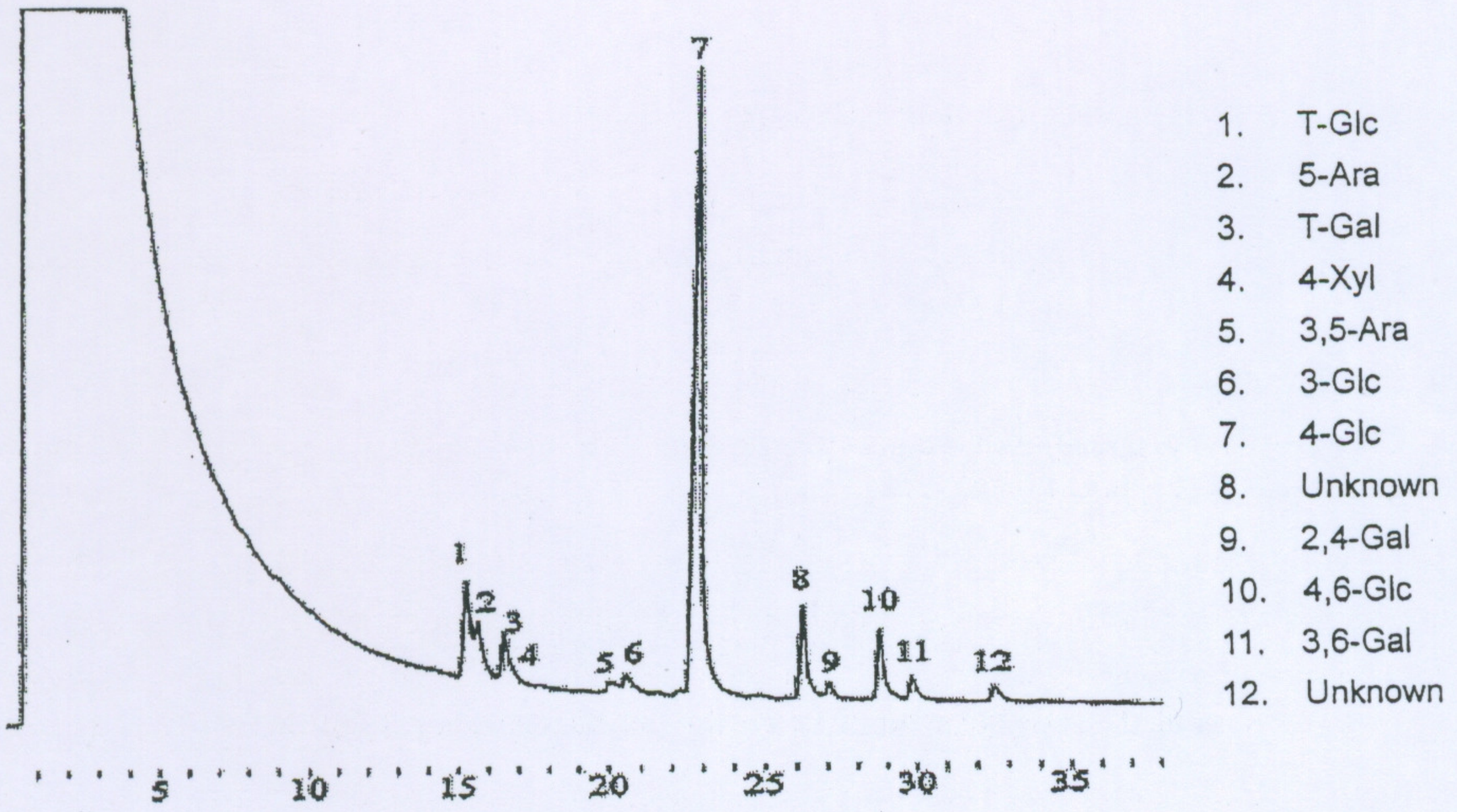

Fig. 8 Glycosidec linkage analysis of fr.II-6

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Table 3 Sugar linkage composition of fr.II-6

Peak No.	Methylated sugar linkage	Deduced glycosidic linkage*	Amount**	Peak No.	Methylated sugar linkage	Deduced glycosidic linkage*	Amount**
1	2,3,4-Me3-Ara***	T-Ara	13.9	17	2,3,6-Me3-Glc	4-Glc	12.1
2	2,3,4-Me3-Xyl	T-Xyl	0.6	18	Ara	2,3,5-Ara	3.3
3	Unidentified		0.1	19	Unidentified		0.9
4	Unidentified		0.6	20	Unidentified		0.9
5	3,4-Me2-Rha	2-Rha	1.8	21	Unidentifie		1.3
6	2,3,4,6-Me4-Glc and/or	T-Glc and/or	3.5	22	3,6-Me2-Gal	2,4-Gal	3.7
	2,3,4,6-Me4-Man	T-Man		23	Unidentified		0
7	2,3-Me2-Ara	5-Ara	13.1	24	2,3-Me2-Glc	4,6-Glc	0.3
8	2,3,4,6-Me4-Gal	T-Gal	4.6	25	Unidentified		2.9
9	2,3-Me2-Xyl and/or	4-Xyl and/or	6.2	26	2,4-Me2-Gal	3,6-Gal	6.1
	3,4-Me2-Xyl	2-Xyl		27	Unidentified		0.6
10	Unidentified		0.3	28	Unidentified		0.9
11	Unidentified		0.4	29	Unidentified		0.1
12	Unidentified		0.3	30	Unidentified		0.3
13	Unidentified		0.7	31	Unidentified		0.2
14	2,4,6-Me3-Glc		3.6	32	Unidentified		2.5
15	2,4,6-Me3-Gal and/or		2.4	33	Unidentified		2.3
	2,3,6-Me3-Man						
16	2,3,6-Me3-Gal,		9.2				
	3,4,6-Me3-Gal and/or						
	2,3,4,-Me3-Glc						

*The numerical prefixes represesnt the carbon atoms involved in glycosidic linkages in the original polysaccahrides. Prefix T indicates sugaras linked through C(O)-1 only.

**% total area.

***2,3,5-Me_3-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl-arabinitol, etc.

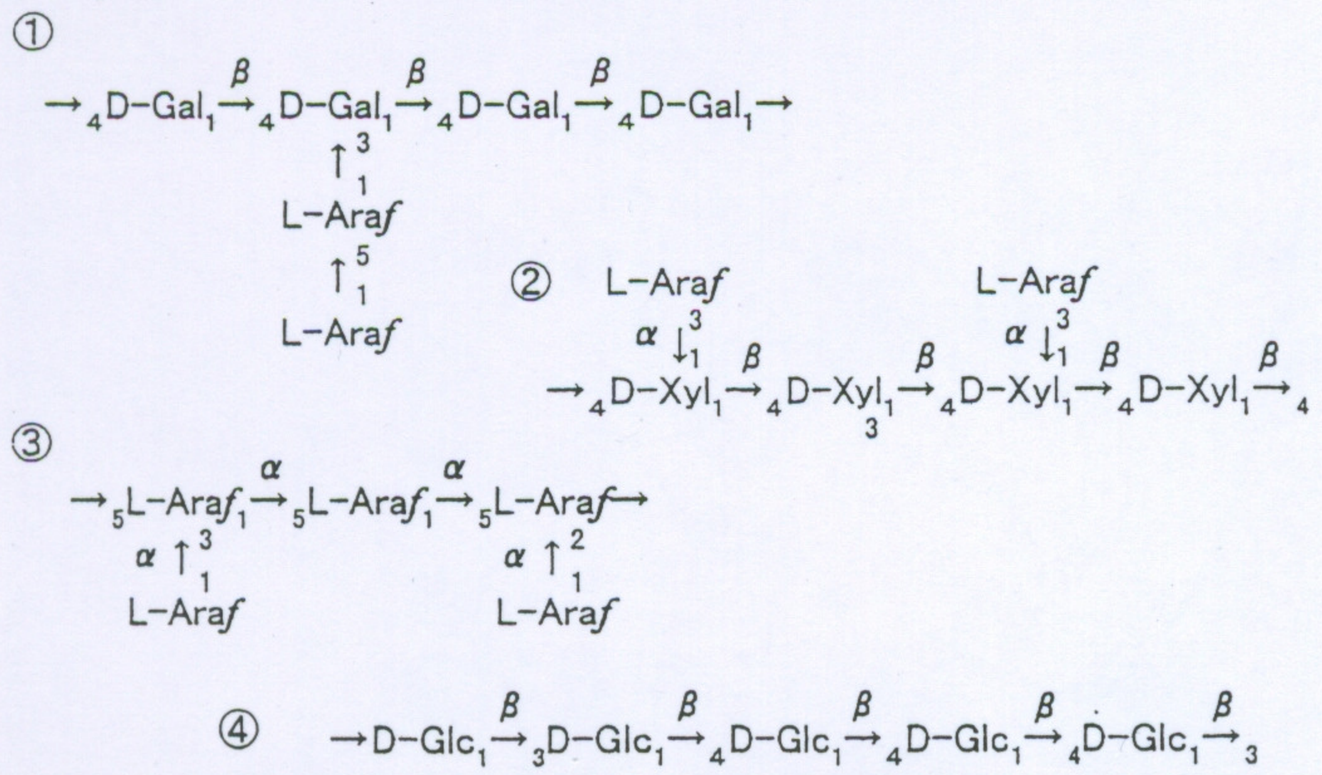

Fig. 9 Possible structures for the polysaccharide present in fr.II-6

The results of methylation analysis suggested the following polysaccharide structures (**Fig. 9**) (1)

arabinogalactan with a main chain of 1,4-β-galactan and side chains of arabinose, (2) arabinoxylan with a main chain of 1,4-β-xylan and side chains of arabinose, (3) arabinan with the main chain of 1,5-α-arabinan and side chains of arabinose, and (4) β-1,3:1,4-glucan. Further analysis is needed to determine whether these polysaccharides are mixed or present as parts of one molecule. However, the immunomodulatory ingredient of BioBran/MGN-3 is estimated to be a heteropolysaccharide of complex structure.