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Studies on an Antitumor Polysaccharide RBS Derived from Rice Bran. II.[1] Preparation and General Properties of RON, an Active Fraction of RBS

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An antitumor polysaccharide RON was obtained by fractionating RBS (a saccharide derived from rice bran) as the non-adsorbed fraction on diethylaminoethyl-Sepharose CL-6B. RON is a dextran-like α-glucan composed mainly of α-1,6-glucosidic linkages with a small amount of C-3 branches. Methylation analysis showed that the molar ratio of non reducing terminal : 1,6-linkage : 1,3,6-linkage was 1 : 25 : 1.2. Its molecular weight is over 1000 kilodaltons (kDa), the specific rotation is $[\alpha]_D^{20}$ +205°, it contains almost no protein and no starch, and it contains a small amount of inorganic substances.

RON has potent antitumor activities against syngeneic tumors, Meth-A fibrosarcoma and Lewis lung carcinoma not only by intraperitoneal administration but also by oral administration, having optimum doses around 30 mg/kg. It is rare that an α-glucan such as RON has potent antitumor activities. Therefore, RON could be an interesting material to elucidate the relationship between the structure and antitumor activities of polysaccharides.

Keywords——rice bran; polysaccharide; rice bran saccharide (RBS); RON; antitumor substance; immunomodulator; dextran; α-1,6-glucan; Meth-A fibrosarcoma; Lewis lung carcinoma

Introduction

Many antitumor polysaccharides from natural products have been reported and some of them are in clinical use as anticancer agents based on their immunomodulating activities. Lentinan[2] from *Lentinus*, schizophyllan[3] from *Schizophyllum*, grifolan[4] from *Grifola*, scleroglucan[5] from *Sclerotinia*, and so on have been studied extensively. The above polysaccharides are all β-glucans composed mainly of β-1,3-glucosidic linkages.

On the other hand, we have reported an antitumor glucan (RBS)[1] (rice bran saccharide) that had a potent antitumor activity against allogeneic mouse tumor, sarcoma-180, not only by intraperitoneal administration but also by oral administration. RBS is an α-glucan composed mainly of α-1,6-glucosidic linkages.

Two fractions having antitumor activities were obtained by further purification of RBS by anion exchange chromatography. The main component, RON, was an α-glucan with almost no protein. RON exhibited potent antitumor activities against syngeneic mouse tumors, Meth-A fibrosarcoma and Lewis lung carcinoma, by oral administration.[6]

This paper deals with the physical and chemical properties, structure, and antitumor activities of RON.

Materials and Methods

Preparation of RON——Procedures for the preparation of RBS, the mother compound of RON, have been

described previously.[1] $RBS_{30}F_1$ was applied to a diethylaminoethyl (DEAE)-Sepharose CL-6B column, and eluted with water; fractions not adsorbed on the column were collected, dialyzed against water, and lyophilized to get a pure white powder, RON. The adsorbed portion of $RBS_{30}F_1$ was eluted stepwise with 0.1 and 0.5 N NaCl solutions, dialyzed against water, and lyophilized to give RIN and R5N, respectively.

Analytical Procedures——Carbohydrate content was determined by the phenol–sulfuric acid method using glucose as a standard. Protein content was determined by the Lowry–Folin method[7] using bovine serum albumin as a standard. Ultraviolet (UV) absorption was taken on a Hitachi 200-20 spectrophotometer. The infrared (IR) spectrum was measured with a Hitachi 270-30 infrared spectrophotometer. Optical rotation was determined on a JASCO DIP-4 digital polarimeter in formamide. The nuclear magnetic resonance (NMR) spectrum was recorded on a Varian VXR-400 spectrometer in D_2O. Chemical shifts (δ) were expressed in ppm downfield from tetramethylsilane (TMS) as an external standard.

Methylation Analysis——RON was methylated with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide by the method of Hakomori.[8] The fully methylated derivative was hydrolyzed with formic acid and dilute sulfuric acid. The hydrolyzate was reduced with $NaBH_4$ and acetylated with acetic anhydride and pyridine. The resulting alditol acetates were analyzed with a Shimadzu GC-9A gas liquid chromatography.

Enzymatic Digestion——RON was digested with an endodextranase (1,6-α-D-glucan 6-glucanohydrolase, from *Penicillium* sp.,[9] Seikagaku Kogyo Co. Ltd.) and exodextranases (1,6-α-D-glucan glucohydrolase[10] and 1,6-α-D-glucan isomaltohydrolase[11] from *Arthrobacter globiformis*, Sapporo Breweries Ltd.). The liberated glucose and oligosaccharides were analyzed by high performance liquid chromatography (HPLC) or thin layer chromatography.

Measurement of Antitumor Activity——Meth-A Fibrosarcoma (Meth-A): Meth-A cells were kindly supplied by Dr. H. Satoh (Sasaki Institute), and were maintained in ascites form in female BALB/C mice. Ten BALB/C mice (7 weeks old, female, CRJ) per group were inoculated with Meth-A cells (6×10^4 cells/mouse) subcutaneously on day 0. Each sample dissolved in saline was administered orally or intraperitoneally on days 1—10. Average tumor weight was determined about 30 d after the tumor inoculation. The inhibition ratio was calculated from the average tumor weight of the test group *vs.* the control group.

Lewis Lung Carcinoma (3LL): 3LL cells were kindly supplied by Dr. T. Tashiro (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research), and were maintained in solid form subcutaneously in female C57BL mice. Ten BDF_1 mice (7 weeks old, female, CRJ) per group were inoculated with 3LL cells (1×10^5 cells/mouse) subcutaneously on day 0. The same procedure as above was followed.

Results

Preparation and Antitumor Activities of RON

$RBS_{30}F_1$ was fractionated into 3 fractions by passing it through a DEAE-Sepharose CL-6B column as shown in Fig. 1. Antitumor activities against Meth-A and 3LL are shown in Tables I and II, respectively. The general properties of each fraction are summarized in Table III. Although RIN had antitumor activities at the same level as RON, it was a minor component and seemed to have a more complicated structure, so we selected RON for further studies.

RON was a major component of RBS and had potent antitumor activities against syngeneic tumors, Meth-A and 3LL, especially by oral administration, and both activities were comparable with those of 5-fluorouracil (5-FU), a positive control. Optimum doses for

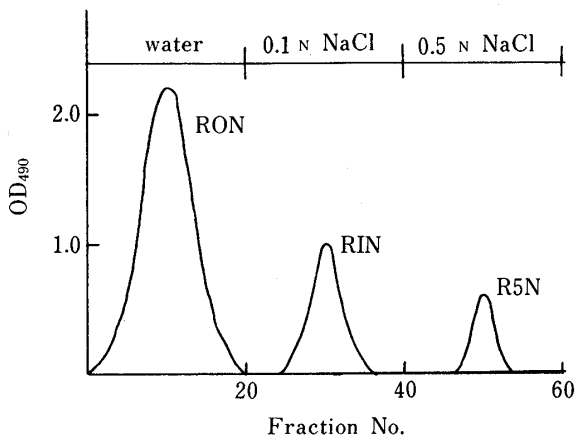

Fig. 1. Fractionation of RBS with DEAE-Sepharose CL-6B

Table I. Antitumor Activity of Each Fraction of RBS against Meth-A Fibrosarcoma

Sample[a]	Dose (mg/kg)	Route	Tumor weight (g±S.D.)[b]	Inhibition ratio (%)[d]	Complete regression
Control	—	—	5.34±1.98	—	0/10
5-FU[c]	20	*p.o.*	2.91±2.36	45.5[e]	2/10
RBS	30	*p.o.*	2.77±2.27	48.1[e]	2/10
RON	10	*p.o.*	4.22±2.82	21.0[f]	1/10
	30	*p.o.*	2.93±2.43	45.1[e]	3/10
	100	*p.o.*	3.94±2.63	26.2[f]	1/10
	30	i.p.	2.67±1.78	50.0[e]	2/10
RIN	30	*p.o.*	3.19±2.40	40.3[e]	2/10
R5N	30	*p.o.*	5.05±2.17	5.4	0/10

Meth-A cells (6×10^4 cells/mouse) were inoculated subcutaneously on day 0. Ten mice were used in each group. *a*) Each sample was administered orally or intraperitoneally on days 1—10. *b*) Tumor weight was measured at day 30. *c*) A positive control. *d*) Statistical significance was evaluated by applying Student's *t*-test. Significant difference from the control, *e*) $p<0.01$, *f*) $p<0.05$.

Table II. Antitumor Activity of Each Fraction of RBS against Lewis Lung Carcinoma

Sample[a]	Dose (mg/kg)	Route	Tumor weight (g±S.D.)[b]	Inhibition ratio (%)[d]	Complete regression
Control	—	—	2.65±0.75	—	0/12
5-FU[c]	20	*p.o.*	1.44±0.46	45.7[e]	1/10
RBS	30	*p.o.*	1.41±0.45	46.8[e]	1/10
RON	10	*p.o.*	1.87±0.55	29.4[g]	0/10
	30	*p.o.*	1.49±0.44	43.8[e]	1/10
	100	*p.o.*	1.92±0.57	27.5[g]	0/10
	30	i.p.	1.38±0.40	47.9[e]	1/10
RIN	30	*p.o.*	1.51±0.70	43.0[f]	1/10
R5N	30	*p.o.*	2.89±0.77	−9.1	0/10

Lewis lung carcinoma cells (1×10^5 cells/mouse) were inoculated subcutaneously on day 0. Ten mice were used in each group. *a*) Each sample was administered orally or intraperitoneally on days 1—10. *b*) Tumor weight was measured at day 30. *c*) A positive control. *d*) Statistical significance was evaluated by applying Student's *t*-test. Significant difference from the control, *e*) $p<0.001$, *f*) $p<0.01$, *g*) $p<0.05$.

Table III. Summary of General Properties of Each Fraction Derived from RBS

Property	RON	RIN	R5N
Ratio (%)	80	15	5
Molecular weight[a]	>1000 kDa	>1000 kDa	>1000 kDa
Sugar component[b]	Glucose	Glucose	Uncertain
Sugar linkage	Almost linear	Branched	Uncertain
Protein[c]	Almost none	+	+
Antitumor activity[d]	+, +	+, +	−, −

a) Estimated by gel filtration on Sepharose CL-6B (appeared in the void volume). *b*) Confirmed by HPLC of total hydrolyzate of each fraction. *c*) Confirmed by the Lowry–Folin method. *d*) Against Meth-A fibrosarcoma (left) or Lewis lung carcinoma (right) by oral administration (see Tables I and II).

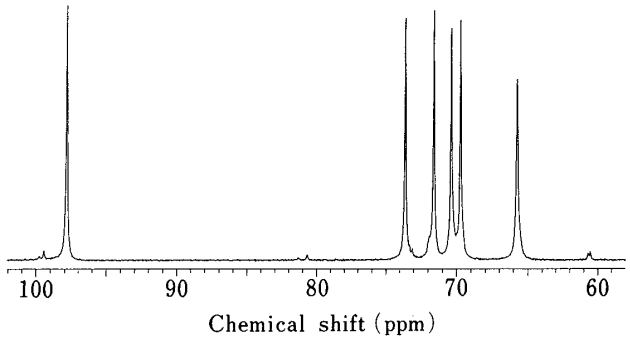

Fig. 2. ^{13}C-NMR Spectrum of RON

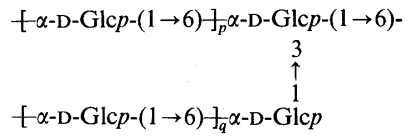

Fig. 3. Probable Structure of the Sugar Part of RON

Glc*p*: glucopyranose. $p+q=24$ (p, q: 0—24).

both tumors seemed to be around 30 mg/kg.

Physical and Chemical Properties of RON

RON was found to be a glucan composed of glucose as a sole sugar component; it contained almost no protein, showed no characteristic UV absorption, and contained a small amount of inorganic substances (less than 2%). Elemental analyses were as follows: Calcd for $C_6H_{10}O_5$: C, 44.45; H, 6.22. Found: C, 44.00; H, 6.15; ash, 1.00. IR absorptions were observed at 763, 840, and 918 cm^{-1}, which are characteristic of an α-linked glucose. Specific rotation was $[\alpha]_D^{20}$ +205° ($c=0.5$, formamide). The ^{1}H-NMR spectrum of RON showed a singlet at δ 5.0 ppm characteristic of H-1 of α-D-glucopyranosyl residue. In the ^{13}C-NMR spectrum (Fig. 2), a signal observed at 97.85 ppm with a $^1J_{CH}$ of 172.1 Hz was assigned to the anomeric carbon of the α-D-glucopyranosyl residue. The signal for C-6 of the α-D-glucopyranosyl residue in RON was shifted to δ 65.74 ppm, downfield from δ 63.55 ppm for C-6 in unsubstituted α-D-glucopyranose. Signal assignments were as follows: δ 60.55, 65.74 (C-6), 69.74 (C-5), 70.38 (C-2), 71.60 (C-4), 73.59, 80.66 (C-3), and 97.85, 99.44 ppm (C-1). These data indicated that RON had α-1,6 bonds as the main linkage. Binding positions of glucose were further confirmed by methylation analysis. 2,3,4,6-Tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, and 2,4-di-*O*-methyl-D-glucose were identified in the molar ratio of 1 : 25 : 1.2.

Enzymatic Digestion of RON

Complete enzymatic digestion of RON with an endodextranase led to the disappearance of high-molecular glucan and the generation of glucose, isomaltose, and a small amount of branched oligosaccharides. In the case of exodextranase (common name; glucodextranase or isomaltodextranase), only glucose or isomaltose was accumulated and a high-molecular glucan still remained.

Probable Structure of RON

Based on the results described above, it can be concluded that RON is mainly composed of α-1,6-linked D-glucopyranose units and has a small amount of branches linked through the C-3 position. A probable structure of RON is presented in Fig. 3.

Discussion

An antitumor polysaccharide RON was obtained by fractionating RBS derived from rice bran. RON is an α-glucan consisting of α-1,6-glucosidic linkages with a small amount of branching at C-3. Its molecular weight is over 1000 kilodaltons (kDa), and it contains almost no protein. Contamination with starch can be ruled out because it showed no starch–iodine reaction.

The structure of RON bears a close resemblance to that of dextran. However, dextran T-2000 ($\overline{MW}$: 2000 kDa, a product of Pharmacia Fine Chemicals AB, Uppsala, Sweden) and native dextran (prepared from a culture broth of *Leuconostoc mesenteroides* ATCC 10830, a producer of NRRL B-512 dextran[12]) showed almost no antitumor activities in our assay system. The relationship between the structure of this glucan and the antitumor activity is very interesting and is now being studied.

There are a few reports that some higher plants contain a dextran-like α-glucan such as RON. Tomoda *et al.* reported that a hypoglycemic substance, panaxan A,[13] isolated from the roots of *Panax ginseng*, was an α-1,6-glucan having branching at C-3. This substance has a molecular weight of 14 kDa with a small peptide moiety (1.7%). They also reported another hypoglycemic substance, aconitan A,[14] isolated from the roots of *Aconitum carmichaeli* Debeaux (Ranunculacea). This substance was also an α-1,6-glucan having branching at C-3. It has a molecular weight of 8.7 kDa and contains no protein.

A size-reduced RON obtained by acid hydrolysis (molecular weight of 10—20 kDa) still retained the antitumor activity.[15]

RON contains a small amount of inorganic substances (less than 2% as Na, K, Mg, Ca, P, Cl, *etc.*), although dextran T-2000 and native dextran contain almost no such substances. Gel filtration on Sepharose CL-6B or ion exchange chromatography on DEAE-Sepharose CL-6B did not reduce the content of such inorganic substances in RON.

On the other hand, antitumor activities against Meth-A and 3LL become weaker when the inoculum size of these cells is increased, for example, to 1×10^6 cells/mouse. It seems to be a characteristic of RON that the antitumor activities against both tumors are about the same on intraperitoneal and oral administrations. This might suggest that RON or its digested fragments are absorbed through the intestine, retaining the biological activities.

Since allogeneic tumors have a higher antigenicity to the host than syngeneic ones, antitumor polysaccharides would be more effective against allogeneic tumors, in general. However, in the case of RON, stronger activities could not be found against allogeneic tumors, sarcoma-180 and Ehrlich carcinoma.

Studies on the mechanism of antitumor activities of RON, especially on the influence of RON upon the activation of the macrophage system, are in progress, the results will be reported elsewhere.

References

1) Part I: E. Ito, S. Takeo, H. Kado, H. Yamamoto, N. Watanabe, M. Kamimura, E. Soma, K. Uchida, Y. Mori, and T. Morinaga, *Yakugaku Zasshi*, **105**, 188 (1985).
2) H. Saito, T. Ohki, N. Takasuka, and T. Sasaki, *Carbohydr. Res.*, **58**, 293 (1977).
3) T. Norisuye, T. Yanaki, and H. Fujita, *J. Polym. Sci. Polym. Phys. Ed.*, **18**, 547 (1980).
4) N. Ohno, I. Suzuki, S. Oikawa, K. Sato, Y. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **32**, 1142 (1984).
5) N. Ohno, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 1362 (1986).
6) N. Watanabe, S. Takeo, and M. Kamimura, Proceedings of the Japanese Cancer Association, The 46th Annual Meeting, Tokyo, Sep. 1987, p. 476; S. Takeo, N. Watanabe, and M. Kamimura, *ibid*, The 46th Annual Meeting, Tokyo, Sep. 1987, p. 333.
7) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
9) J. Fukumoto, H. Tsuji, and D. Tsuru, *J. Biochem.* (Tokyo), **69**, 1113 (1971).
10) T. Sawai, T. Yamaki, and T. Ohya, *Agric. Biol. Chem.*, **40**, 1293 (1976).
11) T. Sawai, K. Toriyama, and K. Yano, *J. Biochem.* (Tokyo), **75**, 105 (1974).
12) T. Kobayashi and Y. Tsukano, *Nippon Nogeikagaku Kaishi*, **25**, 421 (1951).
13) M. Tomoda, K. Shimada, C. Konno, K. Sugiyama, and H. Hikino, *Planta Medica*, **50**, 436 (1984).
14) M. Tomoda, K. Shimada, C. Konno, M. Murakami, and H. Hikino, *Carbohydr. Res.*, **147**, 160 (1986).
15) Y. Tanigami, S. Kusumoto, T. Shiba, and S. Kotani, Abstracts of Papers, The 9th Japanese Carbohydrate Symposium, Tokyo, July 1986, p. 17.