Structural Analysis of Alkaline-Soluble Polysaccharide, P-1, from the Kernels of *Prunus mume* SIEB. et ZUCC.

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A polysaccharide fraction extracted with cold 0.5 M NaOH from the kernels of *Prunus mume* exhibited some biological activities. A polysaccharide, P-1, was purified from the 0.5 M NaOH extract by ion-exchange chromatography and gel-filtration. The results of the structural analysis of P-1 to determine the relationship between the activities and the structure are described in this paper. In the mild acid hydrolysis of P-1, the non-dialyzable hydrolysate (I-3) believed to be its core portion was obtained. The yield of I-3 was 26.0% and contained 59.8% uronic acid as galacturonic acid (GalA). The neutral sugars of I-3 were composed of rhamnose, xylose and galactose in a molar ratio of 1.0:3.4:0.3 following analysis by gas-liquid chromatography. The molecular weight of I-3 was estimated to be ca. 14000 by gel-filtration on Toyopearl HW55F. I-3 exhibited the mitogenic activity toward spleen cells as well as P-1. These facts appeared to confirm that I-3 was the core part of P-1 and important for its biological activity. I-3 was successfully reduced by the Taylor and Conrad method to avoid so much repetition. Methylation analysis of the reduced hydrolysate by gas-liquid chromatography and gas chromatography-mass spectroscopy showed that the ratio of 1,4-linked galactopyranosyl and 1,3,4-linked galactopyranosyl residues were significantly increased in comparison with native I-3. These results suggested that I-3 was composed of 1,4- and 1,3,4-linked galacturonic acid residues in the main chain.

Key words *Prunus mume* kernel; polysaccharide; controlled hydrolysis; uronic acid; methylation analysis; mitogenic activity

We have been studying the biologically-active components of *Prunus mume* SIEB. et ZUCC. (Japanese name, ume), and earlier described the biological activities of some of the extracts. The hexane extracts obtained from the fruit extract and kernels *P. mume* exhibited the antimutagenic activities.¹⁾ Some of the polysaccharide fractions extracted with either water or alkali showed various biological activities, and we also reported the biological activities and the characterization of polysaccharide (P-1) obtained from cold 0.5 M NaOH extract.²⁾

In this paper, we studied the chemical structure of P-1 by a mild hydrolysis with 0.01, 0.05 and 0.1 M trifluoroacetic acid (TFA) to elucidate the relationship between this structure and biological activities.

MATERIALS AND METHODS

Preparation of Alkaline-Soluble Polysaccharide (P-1)²⁾

The preparation methods of the polysaccharide fractions from the kernels of *Prunus mume*, a cultivar name of Nanko produced in Mie prefecture, were described previously, and P-1 was isolated from the dialyzable fraction extracted with 0.5 M NaOH using Toyopearl 650 M ion-exchange chromatography followed by Toyopearl HW65 gel-filtration.

Controlled Hydrolysis P-1 (500 mg) was hydrolyzed with 0.01 M TFA at 100 °C for 3 h, and dialyzed against distilled water overnight. The dialysis was repeated 2 times. The resulting dialyzable (E-1) and non-dialyzable (I-1) fractions were concentrated and lyophilized. Fraction I-1 was hydrolyzed with 0.05 M TFA, 100 °C for 3 h and dialyzed to prepare dialyzable (E-2) and non-dialyzable

(I-2) fractions. Fraction I-2 was again hydrolyzed with 0.1 M TFA, 100 °C for 3 h and dialyzed to prepare dialyzable (E-3) and non-dialyzable fractions. The non-dialyzable fraction of the hydrolysate with 0.1 M TFA was designated I-3. A diagram of the controlled hydrolysis is shown in Fig. 1.

Fig. 1. Diagram of Controlled Hydrolysis Procedure of P-1

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Physicochemical Analysis The neutral sugar content of I-3 was calculated from the standard sugars, L-rhamnose (L-Rha), D-xylose (D-Xyl), L-arabinose (L-Ara) and D-galactose (D-Gal), mixed according to the molar ratio of I-3 obtained following gas-liquid chromatography (GLC). The uronic acid content of I-3 was calculated from the D-galacturonic acid (D-GalA) standard. Detection of the neutral sugars³⁾ and uronic acid⁴⁾ was carried out using methods described previously.

Analysis of component sugars of I-3 was carried out as follows: I-3 (5 mg) was hydrolyzed with 1 ml 1 M TFA at 100 °C for 3 h. The hydrolysate was evaporated to remove TFA and dried under reduced pressure. The residue was dissolved in 0.5 ml of water and divided into two portions. One of them was derivatized to alditol acetates⁵⁾ in the usual way, and analyzed by GLC using a Shimadzu GC-8A instrument fitted with a glass column (0.26 × 200 cm) packed with 3% ECNSS-M on Gaschrom Q under conditions of 170 °C column temperature and 60 ml (N₂ gas)/min flow rate. The other portion of hydrolysate was submitted to thin-layer chromatography (TLC) on an Avicel SF plate (10 × 10 cm) and high performance thin-layer chromatography (HPTLC) plate (10 × 10 cm). The solvent system used was ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v). Spots were detected by spraying with *p*-anisidine–HCl reagent.⁶⁾

The carbon-13 nuclear magnetic resonance (¹³C-NMR) spectral analysis of P-1 and I-3 was carried out using a Bruker AM 400. The spectra were recorded at 27 °C for solutions in deuterium oxide (D₂O), and were obtained in the pulsed Fourier-transform mode with complete proton decoupling.⁷⁾

The molecular weight of I-3 was measured by gel-filtration using a column of Toyopearl HW 55F and eluted with 0.1 M NaOH.

Reduction of Acidic Moieties Uronic acid moieties in I-3 were reduced with NaBH₄ by the modified Taylor and Conrad method.⁸⁾ Briefly, the polysaccharide (10 mg) was dissolved in 10 ml of water containing 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC, 50 mg). As the reaction proceeded, the pH value of the reaction mixture was maintained at pH 4.75 with 0.1 M hydrochloric acid. The whole reaction was allowed to proceed for 2 h. After hydrogen ion uptake had ceased, sodium borohydride solution (800 mg in 10 ml of water) was added gradually to the reaction mixture using a hypodermic syringe; pH value of the mixture was maintained at 7.0 with 4.0 M hydrochloric acid. The reduction product was isolated by dialysis, then lyophilized and designated I-3R.

Methylation Analysis The reduced product was methylated three times by the method of Hakomori.⁹⁾ Then, the methylated product was formalized with 90% formic acid (1 ml) at 100 °C for 3 h in a sealed tube. After removal of the formic acid by evaporation, the residue was hydrolyzed with 1 M TFA at 100 °C for 6 h followed by evaporation to dryness. The resulting partially *O*-methylated sugars were reduced with sodium borohydride at room temperature for 8 h to the corresponding alditol, and then derivatized to alditol acetates followed by acetylation.⁵⁾ The resulting products were analyzed by

GLC and gas chromatography–mass spectroscopy (GC-MS).

GLC analysis of partially *O*-methylated alditol acetates was performed with a glass column (0.26 × 200 cm) packed with 3% ECNSS-M on Gaschrom Q (80–100 mesh). Flow rate of the carrier gas (N₂) was 60 ml/min, and the column temperature was 165 °C. The corresponding alditol acetates of *O*-methylated sugars were identified by retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as 1.0.¹⁰⁾

GC-MS analysis of partially *O*-methylated alditol acetates was performed under the following conditions. The gas chromatograph used was a Varian 3400 instrument fitted with a capillary column (0.025 mm × 30 m) of DB-5, the column oven temperature was 150–200 °C and carrier gas was 4.0 ml (He)/min of the flow rate. The electron impact mass spectra were recorded by a Finnigan MAT INCOS 50 instrument. The ion source temperature was 180 °C and EI was 70 eV.

Biological Activity Mitogen assay was performed to investigate the biological effects on the controlled hydrolysates of P-1 using spleen cells from C3H/HeN male mice, followed by assessment using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]¹¹⁾ and alkaline phosphatase (ALP)¹²⁾ methods as described previously.

RESULTS

Controlled Hydrolysis of P-1 To gain information about its architecture, P-1 was degraded with mild acid hydrolysis using TFA in the order of 0.01, 0.05 and 0.1 M. After each hydrolysate was dialyzed against distilled water, the dialyzable and non-dialyzable fractions were lyophilized respectively. The dialyzable fractions were designated E-1, E-2 and E-3, and then the non-dialyzable fractions were designated I-1, I-2 and I-3. The yield of each fraction is shown in Table I: the yields for dialyzable fractions were 18.8% (E-1), 39.8% (E-2) and 15.4% (E-3), and the yield for the non-dialyzable fraction (I-3) obtained finally by the partial hydrolysis was 26.0%.

The molar ratio of mono-saccharides in each dialyzable fraction is also shown in Table I. Almost all of the Ara residue contained in P-1 was hydrolyzed under milder conditions, 0.01 or 0.05 M TFA, though Xyl, Gal and uronic acid were gradually hydrolyzed in any concentration of TFA, and existed in the dialyzable fractions; most of the uronic acid remained in the non-dialyzable fraction even under 0.1 M TFA and was contained in I-3 at the rate

TABLE I. Component Sugars of Controlled Hydrolysates of P-1

Fraction	Yield (%)	Molar ratio of sugars				GalA (%)
		Rha	Ara	Xyl	Gal	
E-1 ^{a)}	18.8	nd ^{d)}	7.4	1.0	0.2	30.6
E-2 ^{b)}	39.8	nd ^{d)}	8.3	1.0	0.5	9.2
E-3 ^{c)}	15.4	nd ^{d)}	nd ^{d)}	1.0	0.4	26.9
I-3	26.0	0.3	nd ^{d)}	1.0	0.1	59.8

a, b, c) E-1, E-2 and E-3 are the molar ratios of mono-saccharides contained in dialyzable fractions of controlled hydrolysates. d) nd: not detected.

of ca. 60%.

Physicochemical Properties of I-3 I-3 contained 59.8% of uronic acid (as GalA), and neutral sugars. The component neutral sugars were identified by GLC and TLC as Rha, Xyl and Gal in the molar ratio of approximately 1.0:3.4:0.3. The results suggested that the core portion of P-1 which had molecular weight of over 2000000 was constituted of Rha, Xyl, Gal and GalA in the molar ratio of 1.0:3.4:0.3:7.0, respectively.

Gel-filtration of I-3 was carried out using Toyopearl

Fig. 2. Chromatogram of I-3 by Gel-Filtration

Ten mg of I-3 dissolved in 0.5 ml of water was applied to a Toyopearl HW55F column (2.2×90 cm) and then eluted with 0.1 M NaOH. Eluted fractions were detected individually by the phenol-H₂SO₄ method.

Fig. 3. Determination of Molecular Weight of I-3 by Gel-Filtration on Toyopearl HW55F Eluted with 0.1 M NaOH

A, Dextran T-110; B, Dextran T-70; C, Dextran T-40; D, I-3; E, Dextran T-10.

HW 55F column and the chromatogram obtained is shown in Fig. 2. The molecular weight of I-3 was found to be approximately about 14000 by gel-filtration (Fig. 3).

The ¹³C-NMR spectra of P-1 and I-3 are shown in Fig. 4. As shown above, I-3 showed a strong carbonyl signal at 174 ppm suggesting a high content of uronic acid, and this was also confirmed from the signal for galactopyranosyluronic acid¹³⁾ which appeared at about 100 ppm. Comparison of the patterns of P-1 and I-3 showed that most of the signals in I-3 were attributable to minor signals in P-1. Sharp signals in P-1 came from areas around the I-3 core.

Reduction Product of I-3 with NaBH₄ The component sugars in the reduction product of I-3, I-3R, were identified as Rha, Xyl and Gal by GLC in the molar ratio of approximately 1.0:1.5:4.0. In comparison with the molar ratio of I-3, Xyl of I-3R was decreased to less than half. Thus, an irregular degradation of Xyl occurred under a condition of severe reduction by NaBH₄ and HCl.

Mitogenic Activity of I-3 and I-3R The mitogenic activity against spleen cells of C3H/HeN mice was examined to confirm whether or not the controlled hydrolysates of P-1 exhibited biological activity. As shown

TABLE II. Mitogenic Activity of Controlled Hydrolysates towards C3H/HeN Spleen Cells^{a)}

Sample	μg/culture	MTT mean ± S.D. ^{b)} (S.I.)	ALP mean ± S.D. ^{b)} (S.I.)
Control		0.068 ± 0.0014 (1.0)	0.533 ± 0.0573 (1.0)
P-1	250	0.127 ± 0.0045 (1.9)	1.429 ± 0.1689 (2.7)
	50	0.131 ± 0.0134 (1.9)	0.785 ± 0.0813 (1.5)
	10	0.106 ± 0.0088 (1.6)	0.563 ± 0.0702 (1.1)
I-1	250	0.111 ± 0.0091 (1.6)	0.999 ± 0.0952 (1.9)
	50	0.103 ± 0.0052 (1.5)	0.948 ± 0.0360 (1.8)
	10	0.086 ± 0.0061 (1.3)	0.689 ± 0.0281 (1.3)
I-2	250	0.138 ± 0.0016 (2.0)	1.100 ± 0.0579 (2.1)
	50	0.102 ± 0.0080 (1.5)	0.769 ± 0.0401 (1.4)
	10	0.089 ± 0.0117 (1.3)	0.723 ± 0.0130 (1.4)
I-3	250	0.133 ± 0.0013 (2.0)	1.141 ± 0.1528 (2.1)
	50	0.120 ± 0.0069 (1.8)	0.894 ± 0.0264 (1.7)
	10	0.098 ± 0.0062 (1.4)	0.692 ± 0.0297 (1.3)
LPS	2	0.296 ± 0.0218 (4.4)	2.854 ± 0.0211 (5.4)
	0.4	0.234 ± 0.0070 (3.4)	2.786 ± 0.0415 (5.2)
	0.08	0.159 ± 0.0110 (2.3)	2.157 ± 0.0261 (4.0)

a) 5×10^5 cells/100 μl were mixed with 50 μl of each sample and 50 μl of twofold concentration of RPMI 1640 medium without fetal calf serum, and cultured for 72 h in humidified 5% CO₂. After being cultured, 25 μl of each cultured mixture was transferred to a new tissue culture plate, and MTT and ALP assay was performed. b) The results are expressed as mean O.D. ± S.D. of triplicate cultures. LPS: lipopolysaccharide.

Fig. 4. ¹³C-NMR Spectra of P-1 and I-3 in Deuterium Oxide

A, I-3; B, P-1.

TABLE III. Mitogenic Activity of I-3 and I-3R towards Spleen Cells^{a)}

Sample	$\mu\text{g/culture}$	MTT	ALP
		mean $\pm$ S.D. ^{b)} (S.I.)	mean $\pm$ S.D. ^{b)} (S.I.)
Control		0.068 $\pm$ 0.0014 (1.0)	0.533 $\pm$ 0.0573 (1.0)
I-3	250	0.133 $\pm$ 0.0013 (2.0)	1.141 $\pm$ 0.1528 (2.1)
	50	0.120 $\pm$ 0.0069 (1.8)	0.894 $\pm$ 0.0264 (1.7)
	10	0.098 $\pm$ 0.0062 (1.4)	0.692 $\pm$ 0.0297 (1.3)
I-3R	250	0.081 $\pm$ 0.0063 (1.1)	0.563 $\pm$ 0.0260 (1.1)
	50	0.076 $\pm$ 0.0024 (1.1)	0.580 $\pm$ 0.0120 (1.1)
	10	0.085 $\pm$ 0.0034 (1.2)	0.528 $\pm$ 0.0099 (1.0)
LPS	2	0.296 $\pm$ 0.0218 (4.4)	2.854 $\pm$ 0.0211 (5.4)
	0.4	0.234 $\pm$ 0.0070 (3.4)	2.786 $\pm$ 0.0415 (5.2)
	0.08	0.159 $\pm$ 0.0110 (2.3)	2.157 $\pm$ 0.0261 (4.0)

a) Spleen cells (5×10^5 /culture) from C3H/HeN mice were mixed with 50 μl P-1 (5 mg/ml) and cultured without fetal calf serum. After being cultured for 72 h, MTT and ALP assay was performed. b) The results are expressed as mean O.D. $\pm$ S.D. of triplicate cultures.

TABLE IV. Relative Molar Ratio of Alditol Acetates Derived from Methylated I-3 and I-3R

Component	Molar ratio ^{a)}	
	I-3	I-3R
2,3,4-Tri- <i>O</i> -methyl xylitol	1.2	0.6
3,4-Di- <i>O</i> -methyl rhamnitol	1.0	1.0
2,3,4,6-Tetra- <i>O</i> -methyl galactitol	0.5	0.3
2,3,6-Tri- <i>O</i> -methyl galactitol	nd ^{b)}	3.2
2,6-Di- <i>O</i> -methyl galactitol	nd ^{b)}	1.4

a) The molar ratios were calculated from mol masses and peak area of derivatives on an ECNSS-M column. b) nd: not detected.

in Table II, all of the hydrolysates, I-1, I-2 and I-3, exhibited similar mitogenic activity to P-1. However, the mitogenic activity of I-3R was not similar to that of I-3 (Table III). These results strongly suggest that I-3 is the core portion of P-1, and that the acidic moieties of I-3 are significant for the mitogenic activity.

Methylation Analysis of I-3 and I-3R The methylated alditol acetates derived from I-3 and I-3R were identified by the retention time of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol on GLC¹⁰⁾ and the fragmentation patterns on GC-MS.^{10,14)} The derivatives of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylitol, 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl rhamnitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol were analyzed in both I-3 and I-3R. However, I-3R gave the high peaks of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol and 1,3,4,5-tri-*O*-acetyl-2,6-di-*O*-methyl galactitol which were not detected in the original I-3. The molar ratios of these derivatives are shown in Table IV. These results strongly suggest that 2,3,6-tri-*O*-methyl galactitol and 2,6-di-*O*-methyl galactitol increased in methylated I-3R are originated in acidic moieties of I-3, galacturonic acid residues.

DISCUSSION

It is generally accepted that the mitogenic activity of certain polysaccharides has some correlation with their effectiveness, e.g., prophylactic action against certain etiological agents for human gastro-intestinal diseases.

Therefore, to determine the specific substance(s) effective in promoting human health, mitogenic activity as one of biological activities was tested for the compounds obtained from the kernels of *Prunus mume*.

Of the six fractions extracted with various solvents from the kernels of *P. mume*, the alkaline-soluble polysaccharide fraction extracted with 0.5 M NaOH had the highest sugar content. P-1 purified from this polysaccharide fraction was therefore used in further experiments such as the identification and quantification of individual sugar components. Analysis of P-1 fraction by TLC and GLC revealed that the component sugars were mainly Ara and GalA, and included Rha, Xyl and Gal as minor component sugars. In the weak hydrolysis condition with 0.01 and 0.05 M TFA, primarily Ara was isolated as a mono- and/or oligo-saccharide from the core part. This suggests that Ara is present on the terminal or the tip portion of P-1, and that the architecture of P-1 is an acidic, highly branched heteroglycan-like pectin. The results of the ¹³C-NMR spectra of P-1 and I-3 showed most of the signals in I-3 to be minor signals in P-1. I-3 was thus assumed to be the core of P-1.

The data shown in this and a previous paper²⁾ suggested strongly that the mitogen found in *P. mume* has different properties than bacterial lipopolysaccharide, this is shown especially in its reactivity to C3H/HeJ mice. A core part given by the partial hydrolysis of P-1 and designated I-3, contained a higher ratio of galacturonic acid. The fact that the activity between P-1 and I-3 resembled each other suggests strongly that I-3 core part is the essential domain showing mitogenic activity by P-1. That the reduction of GalA in I-3 significantly reduced the activity strongly supported that the GalA part is important for the mitogenic activity of I-3 and P-1. However, commercially available pectin and pectic acid showed almost no mitogenic activity (data not shown). Methylation analysis of I-3R suggested that the major linkage of GalA was (1 $\rightarrow$ 4) and that it was sensitive to pectinase (data not shown). These facts suggested that even though the outline of the linkages of I-3 resembled those of "pectic acid", a certain specific linkage in I-3 is essential to show mitogenic activity.

In comparison with the molar ratio shown in the component sugar analysis and methylation analysis of I-3, one of the component sugars, Xyl, gave a reduced ratio in methylation analysis. Thus, we analyzed the molar ratio of component sugars in I-3 under different hydrolysis conditions: 0.5, 1.0 and 2.0 M TFA for 5 and 10 h at 100 °C. Under these conditions, the content of Xyl was decreased in accordance with increase in the concentration of TFA and the period of hydrolysis (data not shown). Previously, Shimizu *et al.*¹⁵⁾ prepared oligosaccharides from a plant polysaccharide by hydrolysis using 2.0 M TFA at 100 °C for 3 h. The conditions we used in this report are not significantly stronger than those used in preparing oligosaccharide. However, since I-3 contains Rha residue which is sensitive to acid hydrolysis, we could not choose more severe conditions. I-3 thus appears to contain both acid-sensitive and -resistant glycosidic linkages, so that the molar ratio would contain some inaccuracy. We also used Taylor's reduction, but the reaction sometimes lost a part

of the component sugars.¹⁶⁾ This would explain the contradiction in the molar ratio in the component sugar analysis and the methylation analysis. Although reduction of the activity after Taylor's reduction strongly supported the significance of GalA residue, we could not completely rule out the contribution of another component sugar on the mitogenic activity because the drastic reaction to Taylor's reduction sometimes causes loss of a component, though not a specific component.

Further structural analysis to identify active site of I-3 is worthy investigating.

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