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Plant Mucilages. VI.[1] Three Disaccharides obtained from Plantasan by Partial Acid Hydrolysis

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Plantasan, the seed mucilage of *Plantago major* L. var. *asiatica* Decaisne, has been subjected to partial acid hydrolysis, and three disaccharides were obtained. Analysis of components, periodate oxidation, methylation and enzymic degradation studies provided the evidences that they are O-β-D-xylopyranosyl-(1→2)-D-xylopyranose, O-β-D-xylopyranosyl-(1→4)-D-xylopyranose, and O-β-D-glucopyranosyl-uronic acid-(1→5)-L-arabinofuranose.

The previous structural investigation[3] of plantasan, the seed mucilage of *Plantago major* L. var. *asiatica* Decaisne,[4] provided the evidence that the acidic polysaccharide has a high branched structure and D-xylose forms its backbone chain. In this paper, the isolations and characterizations of three disaccharides from partial acid hydrolysates of plantasan are described, and informations on the main part of the acidic polysaccharide having a complicated structure are discussed.

According to a variety of glycosidic linkages which are cleaved on acid hydrolysis at remarkably different rates, the partial hydrolysis was carried out in two conditions. At each condition, the products were fractionated by active charcoal column chromatography, then oligosaccharides were isolated by the means of paper partition chromatography (PPC). Two neutral disaccharides (I and II) and an acidic disaccharide (III) were obtained from the hydrolysate with 0.1 N sulfuric acid at 90° for 2 hr. The acidic disaccharide III was also isolated in better yield from the hydrolysate with 1 N sulfuric acid at 90° for 3 hr.

The homogeneity of the each disaccharide was proved by cellulose thin-layer chromatography (TLC) and by gas-liquid chromatography (GLC) of its trimethylsilyl derivative. Table I shows *Rf* values on TLC and retention times on GLC in several conditions. The trimethylsilyl derivative of III gave anomeric two peaks on GLC, but the trimethylsilyl derivative of the reduction product of III showed a single sharp peak.

Table I. *Rf* Values of Disaccharides and Retention Times of their Trimethylsilyl Derivatives

	Cellulose TLC (*Rf*)			GLC (t_R)	
	Solvent A	Solvent B	Solvent C	Condition A	Condition B
Disaccharide I	0.59	0.44	0.40	36.1	34.0
Disaccharide II	0.43	0.35	0.27	39.5	36.8
Disaccharide III	0.14	0.15	0.09	38.7, 40.2	36.0, 37.7
Reduction Product of III	—	—	—	40.4	36.7

See "Experimental" on the solvents and conditions.

1) Part V: M. Tomoda and S. Nakatsuka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2491 (1972).
2) Location: *1–5–30, Shibakôen, Minato-ku, Tokyo, 105, Japan.*
3) M. Tomoda and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **20**, 778 (1972).
4) M. Tomoda and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 1214 (1971).

Neutral disaccharides I and II showed negative specific rotations, and contrarily, acidic disaccharide III showed a high positive specific rotation as follows: I, $[\alpha]_D^{20}$ −28.9° (c=0.7, H_2O); II, $[\alpha]_D^{20}$ −5.2° (c=0.7, H_2O); III, $[\alpha]_D^{20}$ +148.8° (c=1.0, H_2O).

TLC of the hydrolysates and GLC of trimethylsilyl derivatives of the methanolysates of disaccharides showed that I and II are composed of D-xylose, and III is composed of L-arabinose and D-glucuronic acid. As the result of reexamination on the component sugars of plantasan by TLC of hydrolysate and by GLC of trimethylsilyl derivative of the methanolysate, it was confirmed that hexuronic acid in plantasan is not D-galacturonic acid, but D-glucuronic acid.

After reduction of the disaccharides with sodium borohydride, the products were methanolyzed, then determinations of the methanolysates were carried out by GLC after trimethylsilylation. The results revealed that I and II are D-xylosyl D-xylose, and III is an aldobiouronic acid, that is, D-glucuronosyl L-arabinose.

As the results of periodate oxidation, mole values of consumed periodate, liberated formic acid and formaldehyde per one mole of the each disaccharide are shown in Table II. These data suggest the presences of 1→2 or 1→3 glycosidic linkage for I, 1→4 glycosidic linkage for II, and 1→5 glycosidic linkage for III.

Table II. Results[a] of Periodate Oxidation

	Consumed IO_4^-	Liberated HCOOH	Liberated HCHO
Disaccharide I	3.98	1.66	0.90
Disaccharide II	3.89	2.60	0
Disaccharide III	4.70	3.91	0

a) mole values per one mole of substrate

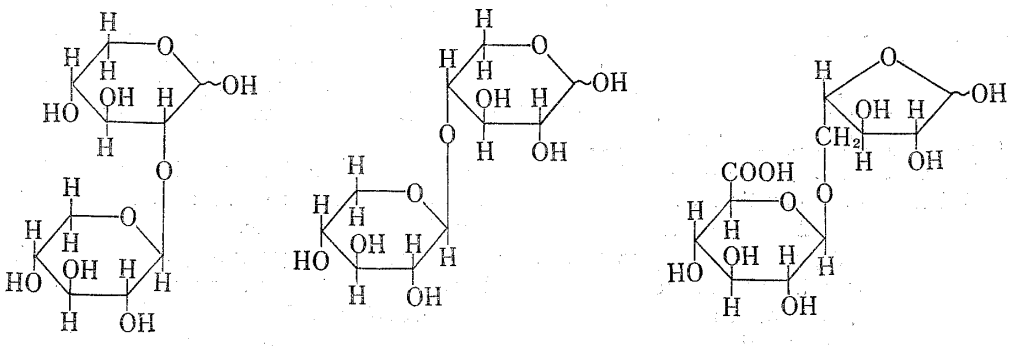
On the other hand, the disaccharides were subjected to oxidation with bromine followed by mild periodate oxidation and Smith degradation.[5] Trimethylsilyl derivatives of the products were analyzed by GLC, and the results showed that I produced glyceric acid and ethylene glycol, and II produced glycolic acid, glycerol and ethylene glycol. These results show the presences of glycosidic linkages of 1→2 type for I and 1→4 type for II. By the same treatment, III produced glycolic acid, glycerol and glyceric acid. But the analysis of Smith degradation product of fully periodate-oxidized III revealed the presence of ethylene glycol instead of glycerol, so the appearance of glycerol in the product of the former mild periodate oxidation followed by Smith degradation is probably due to incomplete oxidation. This result supports the presence of 1→5 glycosidic linkage for III.

Methylations of the disaccharides were performed with sodium hydride and methyl iodide in dimethyl sulfoxide.[6] The fully methylated products were methanolyzed, then analyzed by GLC. Methyl glycosides of 2,3,4-tri-O-methyl D-xylose and 3,4-di-O-methyl D-xylose were identified from I. Methyl glycosides of 2,3,4-tri-O-methyl D-xylose and 2,3-di-O-methyl D-xylose were identified from II. And methyl glycosides of 2,3,4-tri-O-methyl D-glucuronic acid methyl ester and 2,3-di-O-methyl L-arabinose were identified from III. Thus in addition to the data on periodate oxidation studies, the results of methylation study proved finally that I is D-xylopyranosyl-(1→2)-D-xylopyranose, II is D-xylopyranosyl-(1→4)-D-xylopyranose, and III is D-glucuronopyranosyl-(1→5)-L-arabinofuranose.

To confirm the configurations of glycosidic linkages, I was digested with β-xylan xylanohydrolase and 13% of xylose was liberated after incubation for one day. II was digested with β-1,4-xylan xylanohydrolase and 97% of xylose was liberated after incubation for one

5) H. Yamaguchi and M. Kuriyama, *J. Biochem.*, **71**, 239 (1972).
6) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

day. And III was treated with β-D-glucuronide glucuronohydrolase and 99% of glucuronic acid was liberated after incubation for one day. These results of enzymic action gave the evidence that the three disaccharides have β-glycosidic linkages, and the values of specific ratations also support this conclusion. The structures of the three disaccharides are shown in Chart 1. Disaccharides I and III are new compounds, but II is a known one and it was identified on TLC and GLC by comparison with β-1,4-D-xylobiose prepared from corncob xylan.[7]



"Disaccharide I"
O-β-D-xylopyranosyl-(1→2)-D-xylopyranose

"Disaccharide II"
O-β-D-xylopyranosyl-(1→4)-D-xylopyranose

"Disaccharide III"
O-β-D-glucopyranosyluronic acid-(1→5)-L-arabinofuranose

Chart 1. Structures of Disaccharides

The fact that D-xylobioses having 1→2 and 1→4 glycosidic linkages were obtained from plantasan as its partial hydrolysates supports the result[3] of study on the backbone structure of the acidic polysaccharide by Smith degradation, and the present investigation added the evidence that the configuration of linkages among all D-xylose residues is β-type. The isolation and the structural determination of an acidic disaccharide elucidated the fact that D-glucuronic acid in plantasan links with L-arabinofuranose by β-1→5 glycosidic linkage. As described in the previous report,[3] we have already known that L-arabinofuranose links with D-xylopyranose residues in the backbone chain. Any oligosaccharides composed of two or more hexuronic acid residues were not found in the partial acid hydrolysates, so it is conceivable that D-glucuronic acid exists as terminal residues.

Experimental

Solutions were concentrated at 40° or below with rotary evaporators under reduced pressure. Specific rotations were measured by the use of JASCO model DIP-SL automatic polarimeter. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ion detector.

Isolations of Disaccharides by Hydrolysis with 0.1N H_2SO_4——Plantasan (1.58 g) was dispersed into 0.1N H_2SO_4 (160 ml) and heated under reflux at 90° for 2 hr. After cooling to room temperature and removal of insoluble part by filtration, the filtrate was neutralized with barium carbonate, then the filtrate was passed through a column (2×5 cm) of Dowex 50 W-X8 (H^+). The eluate and washings were combined and concentrated to 10 ml, then applied to a column (2×13 cm) of active charcoal (for chromatographic use, Wako-Junyaku Co.). The charcoal was treated before use with hot 15% AcOH followed by washing with water. The column was eluted successively with water (700 ml), 5% ethanol (550 ml) and 15% ethanol (500 ml). Fractions were collected at 50 ml and carbohydrates in eluates were measured by phenol-sulfuric acid method.[8] The eluates obtained from the column were divided into five groups: Frac. 1, tubes 1 to 4; Frac. 2, tubes 5 to 14; Frac. 3, tubes 15 to 21; Frac. 4, tubes 22 to 25; Frac. 5, tubes 26 to 35. The yields were 69.1 mg in Frac. 1, 117.0 mg in Frac. 2, 94.8 mg in Frac. 3, 87.9 mg in Frac. 4 and 96.1 mg in Frac. 5.

7) R.L. Whistler and C.-C. Tu, *J. Am. Chem. Soc.*, **74**, 3609 (1952).
8) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

Fractions 2, 3, 4, and 5 were respectively applied to PPC. PPC was carried out by ascending method using Tôyô-Roshi No. 50 and solvent C in Table I, BuOH: pyridine: H_2O (6: 4: 3). Disaccharide I was obtained from a part showing *Rf* value of 0.39 by means of the extraction with water. Similarly, II was isolated from a part showing *Rf* value of 0.29, and III was obtained from a part showing *Rf* value of 0.10. Yields of I were 10.8 mg from Frac. 3 and 13.7 mg from Frac. 4, and yields of II were 11.3 mg from Frac. 2, 21.8 mg lrom Frac. 3 and 14.3 mg from Frac. 4. And moreover, 46.7 mg of III was obtained from Frac. 5.

Isolation of III by Hydrolysis with 1N H_2SO_4——Plantasan (1 g) was heated with 1N H_2SO_4 (100 ml) at 90° for 3 hr under reflux. After neutralization with barium carbonate and treatment with Dowex 50 W-X8 (H^+), the eluate and washings were combined and concentrated, then applied similarly to a column (2×13 cm) of active charcoal. The column was eluted successively with water (300 ml), 5% ethanol (400 ml) and 15% ethanol (350 ml). Fractions were collected at 50 ml and the eluates obtained from the column were divided into three groups: Frac. 1, tubes 1 to 6; Frac. 2, tubes 7 to 14; Frac. 3, tubes 15 to 21. The yields were 559.1 mg in Frac. 1, 103.3 mg in Frac. 2 and 115.4 mg in Frac. 3. Frac. 3 gave one spot in PPC having *Rf* value of 0.10, and this is identified as III by TLC and GLC. It (21.3 mg) was also obtained from a part showing the same *Rf* value by PPC of Frac. 2.

TLC and GLC of Disaccharides——TLC was carried out using Avicel SF cellulose and following solvent systems in addition to solvent C: A, AcOEt: pyridine: AcOH: H_2O (5:5:1:3); B, C_6H_5OH: 1% NH_4OH (2: 1). Samples were revealed with benzidine[9] and silver nitrate[10] reagents.

For analysis by GLC, each of samples (0.3 mg) was dissolved in pyridine (0.1 ml), then hexamethyldisilazane (0.02 ml) and trimethylchlorosilane (0.01 ml) were added for the preparation of trimethylsilyl derivatives. Following two conditions were used for GLC: A, column, 3% SE 52 on Chromosorb W (80 to 100 mesh) (0.3 cm×2 m long stainless steel); programmed column temperature, increase in 3° per min from 130 to 280°; carrier gas, N_2 (20 ml per min); B, column, 2% OV 17 on Chromosorb W (80 to 100 mesh) (0.3 cm×2 m long stainless steel); programmed column temperature and carrier gas are the same as condition A.

Qualitative Analyses of Components——Disaccharides were hydrolyzed with 2N H_2SO_4 at 100° for 6 hr, then neutralized with barium carbonate. The filtrates were passed through a small column of Dowex 50 W-X8 (H^+). The hydrolysates were analyzed by the same TLC as described above.

On the other hand, disaccharides were methanolyzed with 4% methanolic HCl in a sealed tube at 75° for 16 hr, then HCl was removed by the repeated addition and evaporation of methanol. The methanolysates were trimethylsilylated and applied to GLC in the same manner as described above. The results are shown in Table III.

Table III. *Rf* Values of Hydrolysates and Retention Times of Trimethylsilyl Derivatives of Methanolysates

	Cellulose TLC (*Rf*)			GLC (t_R)	
	Solvent A	Solvent B	Solvent C	Condition A	Condition B
Hydrolysate of I	0.58	0.38	0.43		
Hydrolysate of II	0.58	0.38	0.43		
Hydrolysate of III	0.49, 0.18	0.46, 0.13	0.36, 0.07		
Xylose	0.58	0.38	0.43		
Arabinose	0.49	0.46	0.36		
Glucuronic acid	0.18	0.13	0.07		
Methanolysate of I				12.0	10.3
Methanolysate of II				12.0	10.3
Methanolysate of III				9.1, 15.1, 19.9	7.7, 16.4, 18.3, 19.2
Methyl xyloside				12.0	10.3
Methyl arabinoside				9.1	7.7
Methyl glucuronide methyl ester				15.1, 19.9	16.4, 18.3, 19.2

Reduction of Disaccharides——The sample (10 mg) was dissolved in water (1 ml) and added sodium borohydride (5 mg). After standing for 30 min at room temperature, the pH of the reaction mixture was adjusted to 5 by addition of Dowex 50 W-X8 (H^+). The resins were filtered off, and after washing with water and methanol, the filtrate and washings were conbined and evaporated. Methanol was added to the

9) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).
10) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).

residue and evaporated again. The treatment with methanol was repeated three times, then the final residue was dissolved in small amount of water and lyophilized.

Determination of Components——Determinations of pentitol and pentose as the components of reduced disaccharides were carried out by GLC of the methanolysates. Condition B was used for the determination and mannitol was used as internal standard (t_R of trimethylsilyl derivative of it, 15.1). Glucuronic acid was estimated by carbazole method.[11] The results revealed that molar ratios of xylitol: xylose are 1.0: 1.03 in reduced I and 1.0: 1.05 in reduced II, and of arabinitol: glucuronic acid is 1.0: 0.96 in reduced III.

Qualitative Analyses of Components of Reduction Products——Hydrolysis, methanolysis and trimethylsilylation were carried out as described above. TLC was done using Wakô-gel B5 and following solvent systems: D, MeCOEt: AcOH: MeOH (3: 1: 1); E, BuOH: AcOH: ether: H_2O (9: 6: 3: 1). 2N H_2SO_4 was used for detection at 150°. For GLC, conditions A and B were used. Table IV shows *Rf* values of components of reduction products of disaccharides in TLC and retention times of their trimethylsilyl derivatives in GLC.

Table IV. *Rf* Values of Components of Reduced Disaccharides and Retention Times of Trimethyisilyl Derivatives of Methanolysates

	Si-gel TLC (*Rf*)		GLC (t_R)	
	Solvent D	Solvent E	Condition A	Condition B
Hydrolysate of reduced I	0.81, 0.68	0.66, 0.50		
Hydrolysate of reduced II	0.81, 0.68	0.66, 0.50		
Hydrolysate of reduced III	0.55, 0.48	0.54, 0.37		
Xylose	0.81	0.66		
Xylitol	0.68	0.50	13.3	9.3
Arabinose	0.64	0.44		
Arabinitol	0.55	0.54	13.8	9.5
Glucuronic acid	0.48	0.37		
Methanolysate of reduced I			12.0, 13.3	9.3, 10.3
Methanolysate of redused II			12.0, 13.3	9.3, 10.3
Methanolysate of reduced III			13.8, 15.1, 19.9	9.5, 16.4, 18.3, 19.2

Periodate Oxidation——This was performed in a dark place. Disaccharide I (3 mg) was oxidized with 0.05M sodium metaperiodate (10 ml) at 5°, and II (3 mg) was oxidized with 0.05M sodium metaperiodate (10 ml) at room temperature. And in case of III, the sample (3 mg) was oxidized with 0.01M sodium metaperiodate (25 ml) at 5°. The periodate consumption was measured by a spectrophotometric method.[12] The oxidations were completed after two days for I, nine days for II, and six days for III. Then, formic acid liberation was measured by a titration with 0.01N NaOH and formaldehyde was estimated by chromotropic acid method.[13]

Bromine Oxidation followed by Smith Degradation——The sample (0.5 mg) was dissolved in water (10 ml) and added bromine (0.1 ml). The solution was left at room temperature for one day, then excess of bromine was removed by aeration with N_2. After neutralization with silver carbonate, the solution was filtered and evaporated to dryness. The residue was oxidized with 0.015M sodium meta-periodate (3 ml) at 0° for 30 min. The reaction mixture was subsequently reduced with sodium borohydride (30 mg) at 0° overnight. After addition of acetic acid up to pH 5, the solution was treated with saturated aqueous lead acetate (0.5 ml) at 0° and the precipitate was filtered off. The filtrate was passed through a column (0.8×7 cm) of Dowex 50 W-X8 (H^+) and the effluent was evaporated to dryness. The residue was treated with repeating dissolution in ethanol followed by evaporation. The residue was hydrolyzed with 0.5N HCl (1 ml) at 100° for 5 hr, then evaporated to dryness. The hydrolysate was dissolved in pyridine (0.1 ml) containing trimethylolpropane (0.1 mg) as an internal standard, and subjected to trimethylsilylation with hexamethyldisilazane (0.02 ml) and trimethylchlorosilane (0.01 ml). The products were analyzed by GLC.

GLC: column, 5% SE 30 on Chromosorb G (80 to 100 mesh) (0.3 cm×2 m long stainless steel); programmed column temperature, increase in 5° per min from 70 to 200°; carreir gas, N_2 (30 ml per min); t_R, ethylene glycol 8.4; glycolic acid 10.6; glycerol 17.4; glyceric acid 19.9; trimethylolpropane 21.0.

11) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).
12) *a*) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.*, **1957**, 1216.
13) M. Lambert and A.C. Neish, *Can. J. Research*, **28B**, 83 (1950).

Methylation and Methanolysis——Sodium hydride (20 mg) was mixed with dimethyl sulfoxide (2 ml) and the mixture was stirred at 70° for 1 hr. The sample (10 mg) was dissolved in dimethyl sulfoxide (2 ml) and the solution was added into this mixture. After 20 min stirring at 70°, methyl iodide (1 ml) was added and the reaction mixture was stirred overnight at room temperature. All procedures were carried out in nitrogen atmosphere. After dilution with water (30 ml), the mixture was extracted with chloroform (30 ml) four times. The extract was dried with Na_2SO_4 and the filtrate was evaporated. The residue was methylated again under the same condition. The infrared spectra of the final product had no absorption near 3400 cm^{-1}. The fully methylated products of I and II were respectively heated with 1% methanolic HCl (1 ml) in a sealed tube at 75° for 4 hr. After cooling, the solution was evaporated and HCl was removed by the repeated addition and evaporation of methanol. In the case of the fully methylated product of III, the methanolysis was performed with 1% methanolic HCl in a sealed tube at 75° for 10 hr.

Analysis of Methanolysate——Chloroform solution of the methanolysate was applied to a gas chromatograph. The following two conditions were used: C, a column (0.3 cm × 2 m long stainless steel) packed with 15% Poly-butane 1,4-diol succinate on Chromosorb W (80 to 100 mesh) at 175° with a flow of 20 ml per min of N_2; D, a column (0.3 cm × 2 m long stainless steel) packed with 5% Neopentylglycol succinate on Chromosorb G (60 to 80 mesh) at 150° with a flow of 20 ml per min of N_2. Table V shows relative retention times of the products obtained by methanolysis to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside in the two conditions.

Table V. Relative Retention Times[a] of Methylation Products

	Condition C (15% BDS)	Condition D (5% NPGS)
Methanolysate of methylated I	0.46, 0.52, 1.32, 1.81	0.34, 0.67, 1.47, 3.33
Methanolysate of methylated II	0.47, 0.53, 1.84	0.34, 0.66, 3.37
Methanolysate of methylated III	1.64, 1.88, 3.26	1.44, 1.74, 2.97
Methyl 2,3,4-tri-O-methyl-D-xyloside	0.46, 0.53	0.34, 0.66
Methyl 3,4-di-O-methyl-D-xyloside	1.33, 1.81	1.47, 3.34
Methyl 2,3-di-O-methyl-D-xyloside	1.84	3.37
Methyl 2,3-di-O-methyl-L-arabinoside	1.62, 1.87	1.42, 1.73
Methyl 2,3,4-tri-O-methyl-D-glucuronide methyl ester	3.28	2.95

a) methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside=1.00

Enzymic Degradation——Disaccharide I (0.5 mg) was dissolved in 0.2 ml of 0.01M acetate buffer (pH 5.7) containing 0.04% of β-xylan xylanohydrolase (from the culture fluid of *Streptomyces* sp. E86[14]), and the solution was incubated at 40° for one day. II (1 mg) was dissolved in 0.4 ml of 0.01M phosphate buffer (pH 6.5) containing 0.02% of β-1,4-xylan xylanohydrolase (from the culture fluid of *Malbranchea* sp.[15]), and the solution was incubated at 45° for one day. And III (1 mg) was dissolved in 0.4 ml of 0.01M acetate buffer (pH 5.0) containing 0.02% of β-D-glucuronide glucuronohydrolase (from marine mollusc, Miles-Seravac Co.), and the solution was incubated at 40° for one day. In each case, the rise of reducing activity was measured by the method of Park and Johnson.[16]

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14) I. Kusakabe, T. Yasui, and T. Kobayashi, *Nippon Nôgeikagaku Kaishi*, **43**, 145 (1969); T. Kobayashi, T. Yasui, and I. Kusakabe, Report of the Annual Meeting of the Agricultural Chemical Society of Japan in 1972, p. 242.
15) M. Matsuo, T. Yasui, and T. Kobayashi, Report of the Annual Meeting of the Agricultural Chemical Society of Japan in 1971, p. 220.
16) J.T. Park and M.J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).