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Polysaccharides in Fungi. IV.[1] Acidic Oligosaccharides from Acidic Heteroglycans of *Tremella fuciformis* Berk and Detailed Structures of the Polysaccharides[2]

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The acidic heteroglycans (AC and BC) prepared from the fruit bodies of *Tremella fuciformis* Berk have been partially hydrolyzed with 1 N sulfuric acid to isolate three kinds of acidic oligosaccharides (H-1, H-2 and H-3). On the basis of methylation analysis and PMR spectroscopy, the structures of these compounds were established to be O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl-(1→3)-D-mannopyranose for H-1, O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-D-mannopyranose for H-2, and 2-O-(β-D-glucopyranosyl uronic acid)-D-mannopyranose for H-3. The chemical and physical properties of H-3 were identical with those reported for the aldobiouronic acid prepared from the extracellular polysaccharides of *T. mesenterica* and other sources. These results indicate that the anomeric configuration of the non-reduced terminal D-glucopyranosyluronic acid residues of the acidic polysaccharides, AC and BC, is of β-type, and that the configuration of internal D-mannopyranose residues is of α-type. The present study also confirms that β-D-glucopyranosyluronic acid residues are linked to the position 2 of α-1→3 D-mannopyranose units in both AC and BC.

Keywords——*Tremella fuciformis*; acidic polysaccharide; acidic oligosaccharide; aldobiouronic acid; aldotriouronic acid; aldotetraouronic acid; 2-O-(β-D-glucopyranosyluronic acid)-D-mannopyranose; O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-D-mannopyranose; O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl-(1→3)-D-mannopyranose; PMR spectra

In the previous papers, we reported the isolation,[4a] antitumor activity[4b] and structural investigation[4c] of acidic polysaccharides (AC and BC) prepared from the fruit bodies of *Tremella fuciformis* Berk (edible mushroom). The results of methylation analysis and controlled Smith degradation[4c] indicated that both AC and BC were constructed by a backbone of α-1→3 linked D-mannopyranose residues with some of branches at the position 2. The branches were terminated by D-glucopyranosyluronic acid and β-D-xylopyranose residues, and by β-1→2 or β-1→3 linked D-xylopyranosyl side chains.

During the course of further structural elucidation of the acidic polysaccharides, we have now obtained three kinds of acidic oligosaccharides (H-1, H-2 and H-3) by partial acid hydrolysis of both AC and BC. The present paper describes the isolation and characterization of these oligosaccharides, and informations on the detailed structures of the polysaccharides (AC and BC).

The partial hydrolyzates of AC and BC with 1 N sulfuric acid were fractionated by a column of Amberlite CG-400 (acetate form) with water, followed by a gradient elution with acetic acid (0 to 1 M). The acidic fractions were further purified by paper partition chromato-

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[1] Part III: S. Ukai, T. Kiho, C. Hara, and K. Hirose, *Chem. Pharm. Bull.* (Tokyo), **26**, 1707 (1978).

[2] Presented partly at the 98th Annual Meeting of Pharmaceutical Society of Japan, Okayama, April, 1978.

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[4] *a*) S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1102 (1974); *b*) S. Ukai, K. Hirose, T. Kiho, C. Hara, T. Irikura, T. Kanechika, and Y. Hasegawa, *ibid.*, **20**, 2293 (1972); *c*) S. Ukai, K. Hirose, T. Kiho, and C. Hara, *ibid.*, **25**, 338 (1977).

graphy (PPC). Thus three acidic oligosaccharides (H-1, H-2 and H-3) were isolated from AC and BC, respectively.

Each oligosaccharide was homogeneous on PPC and paper electrophoresis (PE). The R_{GlcA} values in PPC, M_{GlcA} in PE and specific rotations are shown in Table I. Hydorlysis of the oligosaccharides gave mannose and glucuronic acid as component sugars. The molar ratios of these sugars were determined by a colormetric method[5] and the results are shown in Table I. Reduction of the oligosaccharides with sodium borohydride gave the oligosaccharide glycitols (HR-1, HR-2 and HR-3). The component sugars of the resulting oligomers were analyzed by PPC of their hydrolyzates, and mannitol, mannose and glucuronic acid were detected in HR-1 and HR-2, and mannitol and glucuronic acid were detected in HR-3. The formation of mannitol in these cases suggested the presence of a mannose residue as reducing end in each oligosaccharide.

Table I. Characterizations of Oligosaccharides

Oligosaccharides	Specific rotations in water	PPC R_{GlcA}	PE M_{GlcA}	Molar ratios GlcA : Man
H-1	$[\alpha]_D^{18}$ +21°(c=0.42)	0.36	0.69	1 : 3
H-2	$[\alpha]_D^{18}$ −2°(c=0.68)	0.49	0.76	1 : 2
H-3	$[\alpha]_D^{18}$ −44°(c=0.80)	0.68	0.86	1 : 1

Abbreviations: GlcA=D-glucuronic acid; Man=D-mannose.

The oligosaccharides and oligosaccharide glycitols were methylated by the method of Hakomori,[6] and methanolyzed. The methanolyzates were analyzed by gas-liquid chromatography (GLC) and GLC–mass spectrometry (GLC–MS). The results in GLC are shown in Table II. The O-methylated sugars were identified by comparing the retention times in GLC and mass spectra with those of authentic samples or the values in the literature.[7]

Table II. Relative Retention Times and Compositions of Methylated Products

Methylated sugars (Compd. No.)	Relative retention times[a]	Approximate ratios of peak area				
		HM-1	HM-2	HM-3	HRM-1	HRM-2
1,2,4,5,6-Penta-O-methyl mannitol (IV)	2.53[b]	—	—	—	2.5	2.4
Methyl 2,3,4-tri-O-methyl-D-glucuronide methyl ester (I)	2.47,[b] 3.28	1.0	1.0	1.0		
Methyl 3,4,6-tri-O-methyl-D-mannoside (II)	2.90	0.8	0.9	0.9	0.9	0.9
Methyl 2,4,6-tri-O-methyl-D-mannoside (III)	3.72	1.9	1.1	—	1.1	—

[a] Methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside=1.00.

[b] Overlapping peak. The mass spectrum of the beginning part of the overlapping peak was identical with that of the first peak (t_R: 2.47) of I listed in Table V. Also, the mass spectrum (m/e(%): 89(100), 45(86), 102(46), 59(45), 71(35), 131(28), 101(22), 87(21), 58(20), 75(20), 133(14)) taken at the maximum part of the overlapping peak revealed the pressenec of IV.[7b]

Methanolysis of permethylated H-1 (HM-1) gave methyl 2,3,4-tri-O-methyl D-glucuronide methyl ester (I), methyl 3,4,6-tri-O-methyl D-mannoside (II) and methyl 2,4,6-tri-O-methyl

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5) *a*) Z. Dishe, *J. Biol. Chem.*, **181**, 379 (1949); Z. Dishe, L.B. Shettles, and M. Osnos, *Arch. Biochem.*, **22**, 169 (1949); *b*) J.T. Galambos, *Anal. Chem.*, **19**, 119 (1967).

6) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

7) *a*) L.D. Melton, L. Mindt, D.A. Rees, and G.R. Sanderson, *Carbohyd. Res.*, **46**, 245 (1976); *b*) J. Lönngren and S. Svensson, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 29, ed. by S. Tipson and D. Horton, Academic Press, Inc., New York and London, 1974, p. 56.

D-mannoside (III) in the approximate molar ratio of 1:1:2. The same methylated sugars (I, II and III) were obtained from the methanolyzate of permethylated H-2 (HM-2), but the approximate molar ratio (1:1:1) of them was different from the ratio observed for HM-1. In the case of permethylated H-3 (HM-3), I and II were obtained in the approximate molar ratio of 1:1. On the other hand, the methanolyzate of permethylated HR-1 (HRM-1) contained 1,2,4,5,6-penta-O-methyl mannitol (IV) in addition to I, II and III. Methanolysis of permethylated HR-2 (HRM-2) did not afford III, but I, II and IV.

The detection of I in all cases revealed the presence of D-glucopyranosyluronic acid residue as non-reducing end in each oligosaccharide. The reducing end of H-1 was ascertained to be 1→3 linked D-mannopyranose residue by detection of IV in HRM-1, and by the decrease of III in GLC intensity to one half of that of III in HM-1. Similarly, the reducing end of H-2 was evident to be 1→3 linked D-mannopyranose residue due to the disappearance of III in HRM-2 and the appearance of IV in HRM-2. In the case of H-3, 1→2 linked D-mannopyranose residue was identified as reducing end because methanolysis of HM-3 gave I and II in the equal molar ratio; hydrolysis of HR-3 afforded mannitol and glucuronic acid, but no mannose. Consequently, H-3 should be an aldobiouronic acid. Besides these methylation analyses, the hydrolyzates prepared by partial acid hydrolysis or H-1 and H-2 were analyzed by PPC detecting the aldobiouronic acid (H-3) in addition to their component sugars.

The above data indicate that H-1 and H-2 are aldotetraouronic acid and aldotriouronic acid, which are substituted with D-glucopyranosyluronic acid residue at the position 2 of non-reducing end of 1→3 linked D-mannopyranotriose and 1→3 linked D-mannopyranobiose, respectively.

The anomeric configuration of each glycosidic linkage in the oligosaccharides (H-1, H-2 and H-3) was assigned by proton magnetic resonance (PMR) spectroscopy,[8] as shown in Table III. Furthermore, a treatment of each oligosaccharide with β-D-glucuronide glucuronohydrolase released D-glucuronic acid. These results indicate that the glucuronic acid residue possesses β-glycosidic linkage, and that the mannose residues as internal units possess α-glycosidic linkage. The specific optical rotation of each oligomer was compatible with these assignments.

TABLE III. PMR Spectral Data of Anomeric Protons in Oligosaccharides

	δ-Value[a] ($J_{1,2}$, Hz)		Proton assignment
H-1	4.57 (7.4)	1H	β-GlcA(1→
	5.27 (1.5)	1H	→2)-α-Man(1→
	5.11[b] (2.0)	2H	→3)-α-Man(1→
	5.11[b] (2.0)		→3)-α-Man
	4.88[c]		→3)-β-Man
H-2	4.59 (7.3)	1H	β-GlcA(1→
	5.27 (1.7)	1H	→2)-α-Man(1→
	5.16 (1.7)	1H	→3)-α-Man
	4.90 (0.9)		→3)-β-Man
H-3	4.56 (7.3)	1H	β-GlcA(1→
	5.27 (1.8)	1H	→2)-α-Man
	4.87 (0.8)		→2)-β-Man

a) All signals are observed as doublets except for c), which is broad signal.
b) Overlapping signals.

From these data, it can be concluded that H-1, H-2 and H-3 are O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl-(1→3)-D-man-

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8) G.M. Bebault, Y.M. Choy, G.G.S. Dutton, N. Funnel, A.M. Stephen, and M.T. Yang, *J. Bacteriol.*, **113**, 1345 (1973); G.G.S. Dutton and M.T. Yang, *Carbohyd. Res.*, **59**, 179 (1977).

nopyranose, O-(β-D-glucopyranosyluronic acid)-(1→2)-O-α-D-mannopyranosyl-(1→3)-D-mannopyranose, and 2-O-(β-D-glucopyranosyluronic acid)-D-mannopyranose. The structures of the oligomers are shown in Chart 1.

H-1: β-D-GlcA*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-D-Man*p*
H-2: β-D-GlcA*p*-(1→2)-α-D-Man*p*-(1→3)-D-Man*p*
H-3: β-D-GlcA*p*-(1→2)-D-Man*p*

Chart 1

The molar ratios of the component sugars of H-1 and H-2 were in fair agreement with those of the aldotetrao- and aldotrio-uronic acid isolated by Slodki[9] from the hydrolyzate of the extracellular polysaccharide of *T. mesenterica*, and also the similarities between ours and his oligosaccharides were observed in the values of specific rotations. However, the structures of his oligomers had not been clarified.

On the other hand, H-3 was identical with the aldobiouronic acid prepared from the extracellular polysaccharides of *T. mesenterica*[9,10] and the related strain, *Cryptococcus laurentii*[11] by comparison with its chemical and physical properties.

The characterization of these oligosaccharides (H-1, H-2 and H-3) confirms that the anomeric configuration of the non-reduced terminal D-glucopyranosyluronic acid residues of the acidic polysaccharides (AC and BC) is of β-type, of which feature had not been reported in the previous paper,[4] and that the anomeric linkage of the internal D-mannopyranose residues of both AC and BC is of α-type, which is consistent with our suggestion in the previous report.[4c]

The present study also demonstrates that β-D-glucopyranosyluronic acid residues are linked to the position 2 of α-1→3 linked D-mannopyranose units in AC and BC. This result is consistent with the result of the methylation analysis and controlled Smith degradation in our previous work.[4c]

Possible basic structural units in AC and BC are shown in Chart 2 on the basis of the structural feature previously proposed[4c] and the result obtained in this work with regard to the oligosaccharides.

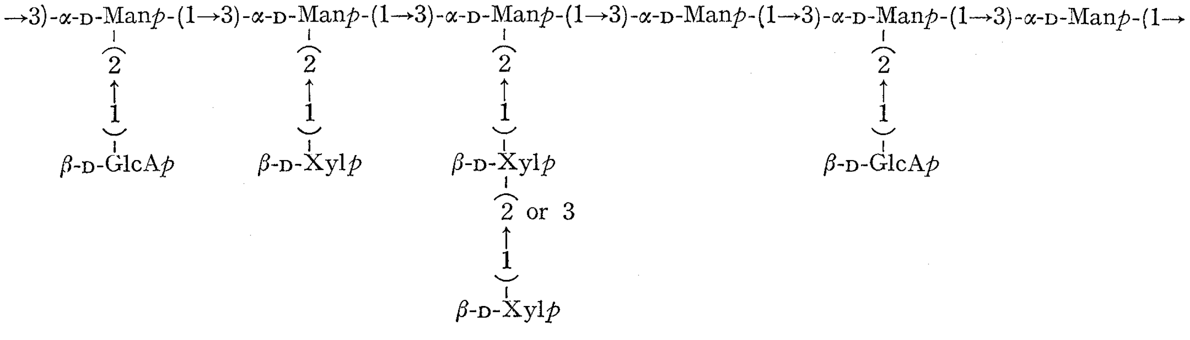

Chart 2. A Possible Structure of AC and BC

Each yield of the oligosaccharides (H-1, H-2 and H-3) was nearly equivalent between AC and BC, as given in experimental section. The observaiton supports the fact that the structures of the two acidic heteroglycans (AC and BC) are essentially similar to each other, though somewhat different in minor respects.[4] The main differences between AC and BC are in a molecular weight, in O-acetyl content and in antitumor activity.[4]

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9) M.E. Slodki, *Can. J. Microbiol.*, **12**, 495 (1966).
10) C.G. Fraser, H.J. Jennings, and P. Moyna, *Can. J. Biochem.*, **51**, 219 (1973).
11) A. Jeanes, J.E. Pittsley, and P.R. Watson, *J. Appl. Polymer Scie.*, **8**, 2775 (1964).

Studies on carbon-13 nuclear magnetic resonance spectra of these acidic oligosaccharides will be reported elsewhere.

Experimental

Specific rotations were measured by the use of a JEOL Model DIP-4 automatic polarimeter. Solutions were concentrated below or at 35° with rotary evaporators under reduced pressure.

Isolation of Oligosaccharides——To a solution of each acidic polysaccharide (AC and BC) (1 g) in H_2O (100 ml) 2 N H_2SO_4 (100 ml) was added, and the solution was allowed to stand 37° for 24 hr, and heated at 100° for 4 hr. The solution was neutralized with $Ba(OH)_2$ and $BaCO_3$, and filtered. The filtrate was passed through a column (1×10 cm) of Amberlite CG-120 (H^+) and concentrated to a small volume. The solution was applied to a column (1.2×33 cm) of Amberlite CG-400 (CH_3COO^-). The column was eluted with H_2O (200 ml), followed by a gradient elution (0—1 M) of AcOH (1 l). Fractions were collected at 10 g and examined with phenol–sulfuric acid method.[12] The acidic fractions were divided into four groups: Frac. 1, tubes 52 to 60; Frac. 2, tubes 62 to 68; Frac. 3, tubes 71 to 81; Frac. 4, tubes 89 to 98. Each fraction was concentrated to a syrup, which was dried over NaOH pellets. Fractions 1, 2 and 3 were purified by PPC. PPC was carried out by double ascending method using Whatman 3 MM paper and with solvent A, *n*-BuOH: AcOH: H_2O (2: 1: 1). Each oligosaccharide was eluted from the filter paper with H_2O. The above procedure was carried out again. Each eluate was concentrated to a small volume, and treated with a column of Amberlite CG-120 (H^+) and lyophilized to give the acidic oligosaccharides as amorphous solid: H-1 (27 mg and 35 mg) from Frac. 1, H-2 (52 mg and 55 mg) from Frac. 2, H-3 (64 mg and 68 mg) from Frac. 3, respectively. Frac. 4 contained glucuronic acid.

PPC and PE of Oligosaccharides——PPC was carried out using Toyo-Roshi No. 51 by double ascending method with solvent A. Sugars were detected by spraying a solution of alkaline $AgNO_3$.[13] PE was carried out on Whatman 3 MM paper (10×38 cm) in solvent B, pyridine: AcOH: H_2O (8: 18: 474) at the condition of 320 volts for 4 hr. Sugars were located with benzidine-TCA reagent,[14] which moved to the anode. The mobilities of H-1, H-2, H-3 and glucuronic acid were 6.3, 6.9, 7.8 and 9.1 cm, respectively.

Reduction of Oligosaccharides——Each sample (5 mg) was dissolved in H_2O (1 ml) and added $NaBH_4$ (5 mg). After standing at 5° overnight, Amberlite CG-120 (H^+) resin was added up to pH 5. The resin was removed by filtration, and the filtrate was concentrated a few times with MeOH to give the oligosaccharide glycitol (HR-1, HR-2 and HR-3).

Determination of Component Sugars——Each sample (3 mg) was hydrolyzed with 2 N H_2SO_4 (2 ml) at 100° for 6 hr. The solution was neutralized with $BaCO_3$, filtered, treated with Amberlite CG-120 (H^+), and concentrated to a syrup. The hydrolyzates were analyzed by PPC. PPC was carried out by ascending method using Toyo-Roshi No. 51 and with solvent C, *n*-BuOH: pyridine: H_2O (6: 4: 3); solvent D, *n*-BuOH: iso-ProOH: H_2O (14: 2: 4). Sugars were detected with *p*-anisidine hydrochloride[15] and alkaline $AgNO_3$[13] reagents. Table IV shows *Rf* values of components of oligosaccharides and oligosaccharide glycitols.

The component sugars were estimated on each of oligosaccharide solutions by the method of Dishe[5a] for mannose, and Galambos[5b] for glucuronic acid.

TABLE IV. *Rf* Values of Component Sugars on PPC

	Solvent C						Solvent D					
	H-1	HR-1	H-2	HR-2	H-3	HR-3	H-1	HR-1	H-2	HR-2	H-3	HR-3
Mannose	0.44	0.44	0.44	0.45	0.44	—	0.19	0.20	0.19	0.20	0.20	—
Mannitol	—	0.39	—	0.39	—	0.39	—	0.16	—	0.16	—	0.16
Glucuronic acid	0.10	0.10	0.11	0.11	0.11	0.11	0.02	0.02	0.02	0.02	0.02	0.02
(Glucuronolactone)	0.60	0.60	0.60	0.61	0.61	0.61	0.34	0.34	0.34	0.34	0.34	0.34

Methylation Analysis——The sample (2 mg) was dissolved in Me_2SO (2 ml), and the solution of methylsulfinyl carbanion (1 ml) was added. The latter reagent was prepared by mixing with NaH (10 mg) and Me_2SO (1 ml) at 65° for 1 hr. The mixture was stirred at room temperature for 1 hr, then CH_3I (1 ml) was added. The reaction mixture was stirred overnight at room temperature. All procedure were carried out in nitrogen atmosphere. The mixture was then diluted with H_2O (10 ml) and extracted with $CHCl_3$ (10 ml)

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five times. The combined $CHCl_3$ extracts were washed with H_2O (10 ml) five times and dried over Na_2SO_4, and the filtrate was evaporated to dryness.

The methylated products were heated with 5% methanolic HCl (2 ml) in a sealed tube for 6 hr in a boiling water bath. After neutralization with Ag_2CO_3 and filtration, the mixture was analyzed by GLC and GLC–MS. GLC was performed on a JEOL Model JGC-1100 gas chromatograph equipped with hydrogen flame ionization detector, using a glass column (0.3 cm × 2 m) packed with 5% neopentylglycol succinate polyester (NPGS) on Chromosorb G (AW) (60 to 80 mesh) at 150° with a flow rate of 43 ml per min of N_2.

For GLC–MS spectrometry, the products were dissolved in $CHCl_3$, and injected into a Schimadzu Model LKB-9000 gas-mass spectrometer using a glass column (0.4 × 1 m) packed with 5% NPGS at 130° with a flow of 30 ml per min helium. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 60 μA and a temperature of ion source of 160°. Table V shows the mass spectra of partially methylated methyl glycosides.

TABLE V. Mass Spectra of partially Methylated Methyl Glycosides by GLC–MS

Methyl 2,3,4-tri-O-methyl-D-glucuronide methyl ester				Methyl 3,4,6-tri-O-methyl-D-mannoside		Methyl 2,4,6-tri-O-methyl-D-mannoside	
1st peak		2nd peak					
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%
101	100	101	100	75	100	71	100
88	67	88	69	71	96	74	69
75	32	75	30	45	83	101	60
45	22	45	19	88	74	45	60
73	20	73	15	74	64	88	40
89	11	74	9	101	39	102	27
102	6	89	8	89	31	75	25
59	6	85	8	87	30	87	18
85	6	102	6	59	22	89	17
		87	6	73	16	59	15
				102	14		
				58	13		
				161	13		

Partial Acid Hydrolysis of Oligosaccharides——Each of H-1 and H-2 (each 3 mg) was dissolved in 0.5 N H_2SO_4 (2 ml), and heated in a sealed tube at 100° for 1 hr. After neutralization with $BaCO_3$ followed by a treatment with Amberlite CG-120 (H^+), the hydrolyzate was analyzed by PPC with solvent A as described above.

PMR Spectroscopy of Oligosaccharides——PMR spectra in D_2O were run at 90°, using a JEOL-FX 100 spectrometer in the pulsed, Fourier transform mode. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate was used as the external standard.

Enzymatic Degradation——A solution of each oligosaccharide (1 mg) in 1 ml of 0.01 M phosphate buffer (pH 7.0) was treated with β-D-glucuronide glucuronohydrolase (10 mg) (Sigma Co.) at 37° for 24 hr with $CHCl_3$ (1 drop). The reaction mixture was filtered, deionized with Amberlite CG-120 (H^+) and Amberlite CG-400 (CH_3COO^-), and the concentrated solution was examined by PE described above.

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