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Study on the cultivation of apios (*Apios Americana* Medikus) in the upland field converted from paddy and its carbohydrate composition

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For effective use of the upland field converted from paddy*, it is necessary to ask what new upland crops can be grown in paddy** with more value. We chose apios (*Apios americana* Medikus) which has many effects on human health as one of the upland crops grown in paddy, and proceeded to elucidate whether apios is raised in any place in Aomori Prefecture, to determine the carbohydrate composition of apios tuber, and to search for a biochemical function of the carbohydrate components in apios tuber.

Apios was considered to be a candidate of upland crops grown in paddy in Aomori Prefecture based upon the following:

1) Apios is grown in upland fields converted from paddy in Aomori Prefecture. And, when compared with a common field, a similar harvest is expected.
2) The carbohydrate composition of apios tuber is very similar to that of pulses.
3) The growth of DLD-1 human tumor cells is reduced by oligosaccharide fractions from apios tubers.

Key words : upland field converted from paddy, upland crops grown in paddy, apios, cultivation, Carbohydrate

1. INTRODUCTION

Today, Japan faces an important turning point of rice policy which has been carried out for 34 years, as upland crops , (wheat, soybean, feed crops, buckwheat, etc.) are mainly being raised in paddy fields instead of rice. However, we still have a problem in that paddy fields are not used efficiently for rice production. Some parts of the paddy fields are not only used inefficiently but also not used at all. It is very important for us to study efficient new usage of paddy fields for upland crops.

Apios (*Apios Americana* medikus) is a native North American vine of the pulse family, it has a double leaf, and produces fattened tubers on the rhizome, which resemble a rosary. It is reported that apios is effective in improving the conditions of illnesses of cancer, polonaises, high blood pressure, diabetes, etc.[1] Cultivation and sale of apios are carried out in a very limited area in Japan.

On the basis of these facts, we chose apios as one of the upland crops grown in paddy, and proceeded to elucidate whether or not apios grows in any place in Aomori Prefecture, to determine the carbohydrate composition of apios tuber, and to search for a biochemical function of carbohydrate components in apios tuber.

2. MATERIALS AND METHODS

Materials: Apios (mother tubers: about

*The term "Upland field converted from paddy" is a converted paddy field used to grow upland crops.
**The term "Upland crops grown in paddy" are crops grown in a paddy field instead of rice.

5g/tuber) were harvested from an upland field, converted from paddy in Hirosaki-shi, Aomori in 2003.

Cultivation: Apios tubers harvested in 2003 were budded indoors early in April, grown to about 10 cm, and then planted in upland fields converted from paddy in Hirosaski-shi, Nakasato-machi, and Kamikita-machi. In addition, apios tubers were planted in common fields in Hirosaki-shi and Iwaki-machi. Tubers of similar size were planted at 20cm intervals with ten stocks per row and 2 rows per experimental site. Rows were 1.2m long and 2.5 m^2. Planting rate = 400 stocks / 1 *are*. The planting and harvest were done on May 4, 2004 and November 6, 2004, respectively.

Fractionation of carbohydrate in apios tubers:

Apios tubers which were grown at the upland field converted from paddy in Hirosaki-shi, Aomori prefecture in 2003 were used for this experiment. Apios tubers were freeze-dried after harvesting. The freeze-dried apios tubers were homogenized and triturated in a blender to give apios powder. The apios powder (50.0 g dry wt.) was treated with 50 ml of 80% methanol for 2 h at 100°C, and then centrifuged for 30 min at 3,000 rpm. The precipitate was treated twice more in sequence with 80% methanol under the same conditions. The final precipitate was washed with 50 ml of acetone and dried to give the 80% methanol insoluble fraction. The supernatant and the acetone washings were combined and concentrated to dryness (the 80% methanol soluble fraction).

The 80% methanol insoluble fraction suspended in 50 ml of 50 mM Na-acetate buffer (pH 4.5) was heated in a boiling water-bath for 10 min. The resulting 80% methanol insoluble fraction was incubated with a mixture of isoamylase (118 units) and glucoamylase (7 units) under a covering of a few drops of toluene at 40°C. After 24 hr of incubation, the enzyme-treated 80% methanol insoluble fraction was centrifuged at 3,000 rpm for 30 min. The precipitate was treated once more in sequence with the mixture of enzymes under the same conditions. The final precipitate was washed with water. The water-washed precipitate was freeze-dried to give the water-insoluble polysaccharide fraction (cell-wall polysaccahrides fraction). The total combined supernatant and washings were concentrated to a small volume to give (glucose derived from starch in apios tubers).

The water-insoluble polysaccharide fraction (cell-wall polysaccharide fraction) was treated with 50 ml of 0.25% ammonium oxalate for 1 hr at 100°C, and then pelleted by centrifuging for 10 min. This extraction process was repeated twice more. Then the combined extract (pectic substance fraction, PS) was dialyzed against distilled water and freeze-dried. The residue from the extraction by ammonium oxalate was treated with 50 ml of 4% KOH under N_2 for 24 hr at room temperature. The extraction by 4% KOH was repeated once, after which the residue was extracted twice with 50 ml of 24% KOH for 24 hr under the same conditions. The combined 4% KOH extract (hemicellulose I fraction, HC-I) and the 24% KOH extract (hemicellulose II fraction, HC-II) were individually neutralized with acetic acid, dialyzed against distilled water, and finally freeze-dried. The residue from 24% KOH extraction was exhaustively washed with water and freeze-dried to give the cellulose fraction (CL).[2]

The total carbohydrate content in each fraction was determined by the phenol-sulfuric acid method with glucose as a standard.[3]

Analysis of mono- and oligo-saccharides in the 80% methanol soluble fraction by high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD): HPAEC-PAD analysis of mono- and oligosaccharides in the 80% methanol soluble fraction was done on a Dionex ion chromatography system DX-300 (Dionex gradient pump, Dionex pulsed electrochemical detector with a gold working electrode, and an Ag/AgCl reference electrode) interfaced with an AI-450 workstation. All eluents were degassed by flushing them with helium and pressurized with the Dionex eluent degas module. Separations were performed at 20°C on a column (4 x 250 mm) of Dionex CarboPac PA 1 anion exchange resin with a CarboPack guard column, using a flow rate of 1 ml/min. Oligosaccharides were eluted with the following NaOAc gradient profile in 100 mM NaOH, 0-30 min, -50 mM; 30-50 min, 50 mM; 50-60 min, 50-500 mM.[4]

Fractionation of the 80% methanol soluble fraction by Bio-Gel P-2 and the effect of the fractions on growth of DLD-1 human tumor cells:

A portion of the 80% methanol soluble

fraction was concentrated to dryness, then dissolved in distilled water, and centrifuged to separate soluble and insoluble materials. The soluble materials (755mg as glucose equivalent) was applied on a column of Bio-Gel P-2, and eluted with water. Fractions of 20ml each were collected and assayed for carbohydrates by the phenol-sulfuric acid method with glucose as a standard. Tubes 20-25, 26-36, 37-39, 40-44, 45-48 and 49-60 were separately combined and concentrated to give fractions 1 to 6. Each was hydrolyzed with 2 M trifluoroacetic acid for 4 hr at 100°C. The hydrolyzate was evaporated to dryness. Neutral sugar composition of the hydrolyzate was determined by HPAEC-PAD analysis.

Fractions 1 to 6 were tested for effects on the growth of DLD-1 human tumor cells and the multiplication control effect was able to be checked. DLD-1 cells were grown at 37°C in RPMI-1640 medium with 10% FBS in a 5% CO_2 humidified incubator. The cells in RPMI-1640 medium with 10% FBS were planted at a cell density of 2 x 10^3 cells/well in a 96-well micro-plate. Plants were incubated with or without each fraction.[5)]

3. RESULT AND DISCUSSION

Cultivation of apios tubers in the upland field converted from paddy and common fields

The parent tubers of apios were grown in the upland field converted from paddy in Hirosaki-shi, Nakasato-machi and Kamikita- machi, and in the common fields in Hirosaki-shi and Iwaki-machi from May 4, 2004 to November 6, 2004. After harvesting, the weight and size of tubers obtained from the upland field converted from paddy and the common fields were determined and compared (Table I). The amount of daughter tubers per 0.05 are from the upland fields converted from paddy in Hirosaki-shi, Nakasato-machi, and Kamikita- machi were 2,465 g, 1,566 g and 593 g, respectively. On the other hand, that in the common field in Hirosaki-shi and Iwaki-machi were 1,083 g and 1,564 g, respectively. According to reports, the amount of harvest of apios is 250～600 kg/10 are. Present cultivation experiments show that the amount of daughter tubers from the upland field converted from paddy and the common fields are 117～489kg/10 are and 214～310 kg/10 are, respectively. In addition, the length and width of individual daughter tuber were measured to determine the size distribution of harvested daughter tubers in terms of by length plus width vs. weight (data not shown). Distinct differences between apios tubers from the upland field converted from paddy and those from the common field were not observed. This means that apios can be grown in converted paddy crops fields in Aomori Prefecture. Even when compared with a common field, a similar harvest is expected.

(Table I, Total amount of harvest)

Area	Mother tuber g	Rhizome g	Doughter tuber g	Daughter tuber kg/10a
Upland field converted from paddy				
①Hirosaki-shi	576.8	62.3	2,465.7	489.2
②Nakasato-machi	114.9	320.1	1,566.3	310.8
③Kamikita-machi	105.0	94.7	593.3	117.7
Common field				
④Hirosaki-shi	87.1	275.8	1,083.0	214.9
⑤Iwaki-machi	136.6	68.4	1,564.4	310.4

(Table I-I, Classification by weight)

	2L %	L %	M %	S %	2S %	3S %	Waste %
Upland field converted from paddy							
①Hirosaki-shi	3.1	4.6	23.5	20.5	23.8	12.3	12.3
②Nakasato-mach	0.0	7.7	19.7	17.7	24.7	15.0	15.2
③Kamikita-mach	0.0	2.7	14.8	30.8	24.2	11.2	16.2
Common field							
④Hirosaki-shi	0.0	14.6	23.7	13.1	21.6	14.4	12.6
⑤Iwaki-machi	3.9	18.3	29.2	25.1	12.9	5.6	5.0

2L >=30g, L 15～30g, M 8～15g, S 5～8g, 2S 3～5g
3S 2～3g, waste <2g, Rhizome <1g

Carbohydrate composition of apios tuber

The apios powder prepared by homogenaization of freeze-dried apios tubers was fractionated into the 80% methanol soluble and insoluble fractions. The 80% methanol soluble fraction corresponds to the mono- and oligo-saccharide fraction. Starch in the 80% methanol insoluble fraction was hydrolyzed into glucose by treatment with isoamylase and glucoamylase.

The water-insoluble fraction (cell wall polysaccharide fraction) obtained after the removal of starch from the 80% methanol insoluble fraction was fractionated into four fractions, PS (pectic substance), HC-I (hemi cellulose-I), HC-II (hemicellulose-II) and CL fractions, by using successive extraction with 0.25% ammonium oxalate, 4% KOH, and 24% KOH, and subsequent dialysis of the individual extracts as described in a previous paper. The water-insoluble fraction consisted of PS, HC (I plus II) and CL in the ratio of 20.4:18.3:61.3.

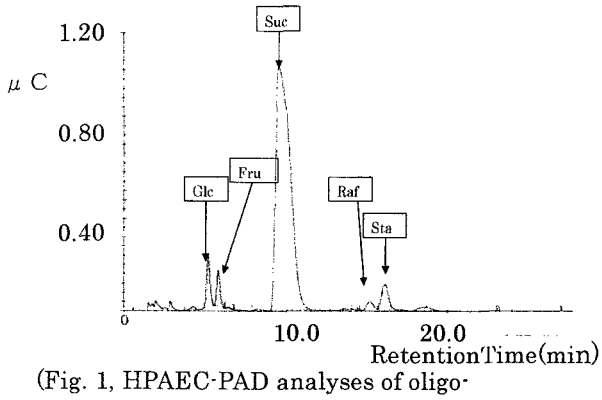
The carbohydrate content of apios tuber was 506.7 mg per 1g dry wt. The approximate ratio of mono- and oligo-saccharides, starch and

cell-wall polysaccharides was 36.1:55.6:8.3.

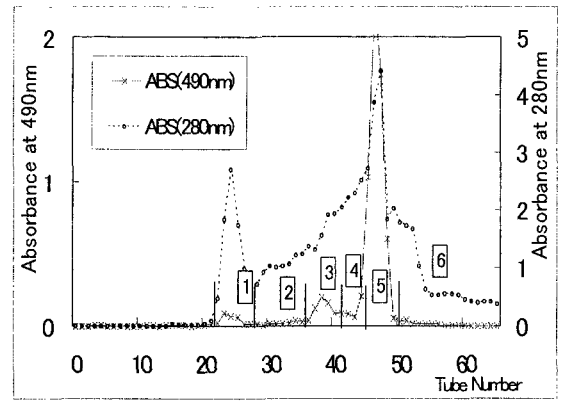
In order to obtain accurate information on the mono- and oligo-saccharide composition in the 80% methanol soluble fraction, the 80% methanol soluble fraction was subjected to HPAEC-PAD analysis. Fig. 1 shows the elution profile. As the major mono- and oligo-saccharides, glucose, fructose, sucrose, rafinose and stachyose was detected in a ratio of 4.3:3.0:84.8:1.5:4.6:1.7. The mono- and oligo-saccharide composition of apios tuber is very similar to those of pulses.

Effect on the growth of DLD-1 of the 80% methanol soluble materials obtained from apios tubers

In order to investigate the effect on the growth of human cancer cell, DLD-1 of the 80% methanol-soluble materials obtained from apios tubers, the 80% methanol-soluble fraction was subjected to fractionation by gel-filtration chromatography on Bio-Gel P-2. Fig. 2 shows the elution profile. The yields of fractions 1～6 are shown in Table II with their neutral sugar composition. Fractions 1～6 were tested for effects on the growth of DLD-1 human tumor cells. Results are shown in Fig. 3. The cell growth was reduced by fractions 1, 2 and 3. Fractions 1～6 tested did not inhibit the growth of human MRC-5-30. Fractions 1, 2 and 3 are found to have heterooligosaccharides. Present results suggest that the growth inhibition depends on the kind of heterooligosaccharides. More precise structural analysis of heteroologosaccharides in fractions 1 to 3 and detailed study on the inhibition of DLD-1 cell growth will be necessary to obtain more information about the inhibition mechanism.



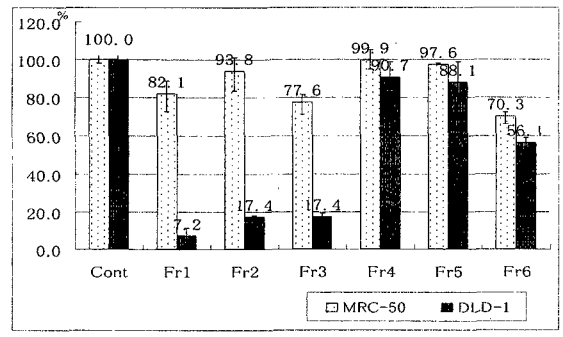
(Fig. 1, HPAEC-PAD analyses of oligo-saccharide)



(Fig. 2, Separation by Gel Filtration through Bio-Gel P-2 of the 80%methanol soluble fraction)

(Table II Yields and sugar composition from the 80% methanol soluble fraction of apios separated by Bio-Gel P2)

Fr	Total sugar (mg)	Ratio (%)	Sugar Composition (mol %) Fuc	Ara	Rha	Gal	Glc	Xyl	Man
Fr1	25.7	3.5	0.79	78.1	0	13.05	7.03	1.01	0
Fr2	22.9	3.1	2.68	18.72	5.03	16.26	42.25	1.3	13.75
Fr3	56.2	7.6	6.99	5.06	2.64	46	39.31	0	0
Fr4	52.4	7.1	0	0	0	37.11	62.89	0	0
Fr5	560.6	75.8	–	–	–	–	–	–	–
Fr6	22.2	3.0	–	–	–	–	–	–	–



(Fig. 3, Effect of each fraction obrained from the 80% methanol soluble fraction)

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