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Sugar composition of apple pulp polysaccharides and their enzymatic hydrolysis

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Abstract

Apple pomace was fractionated into the water-soluble and -insoluble materials. The farmer was subfractionated into the mono- and oligo-saccharide (M-OL), and the water-soluble polysaccharide (WSP) fractions. The latter (apple pulp) was subfractionated into pectic substances (PS), hemicellulose (HC), and cellulose (CL) fractions. The approximate ratio of carbohydrate in the M-OL, WSP, PS, HC and CL was 56:1:17:15:11. The M-OL fraction consisted of solbitol, glucose, fructose and sucrose in the ratio of 3:10:57:31. The action of commercial crude pectinases and cellulases on apple pulp was examined to increase the efficiency of enzymatic saccharification. About 31% of apple pulp was hydrolyzed to be soluble by PECTINASE HL from *Aspergillus* sp., and about 9% was hydrolyzed to be soluble by CELLULASE "ONOZUKA" 3S from *Tricoderma viride*. The carbohydrates content in the water-soluble fraction, and the materials solubilized by PECTINASE HL, and CELLULASE "ONOZUKA" 3S was estimated to be about 97% of the potential apple pomace carbohydrates.
Key words: Apple pomace, Biomass, Cell-wall

1. Introduction

Many studies have been conducted to develop new manufacturing techniques for use of biomass, resources. Bio-plastic, poly-lactic acid production was one of the major targets for these biomass projects.[1)] There is two approaches to produce glucose, a precursor of lactic acid, i.e., use of amylase for the raw-starch materials and use of cellulases for cellulosic biomass. In Aomori prefecture apple pomace which is rich in carbohydrates are produced more than 17,600t per year as a by-product on apple juice production.

Efficiency of bioconversion of apple pomace to poly-lactic acid is largely dependent on the use of the residue of apple fruits extract (apple pulp). Currently, apple pomace is mainly used for cattle feeds and compost. Therefore, the effective hydrolysis of apple pomace polysaccharides to monosaccharide components may lead to the increase in efficiency and yield of bio-plastic production.

Although numerous studies have been done with apple pomace polysaccharides, characterization of the component polysaccharides was not clear enough. Information on the sugar components and contents of apple pomace is useful for enzymic saccharification. Commercial enzymes for the effective saccharification of apple pulp polysaccharides were screened for.

2. Material and Method

Materials

The apple pomace was kindly provided by NKA Nihon Kajitu Kakou Kabusikikaisha, Hirosaki, Aomori.

General Method [2)]

Total sugar and acidic sugar in each fraction were determined by the phenol-sulfuric acid method and the carbazole-surfuric acid method, respectively. Analysis of the constituent neutral sugars in oligo- or poly-saccharide fraction was carried out Dionex ion chromatography system DX-300 after hydrolysis of each fraction with acid. The retention times of peaks of each sample were compared with those of standard sugars [fucose (Fuc.), arabinose (Ara.), rhamnose (Rha.), galactose (Gal.), glucose (Glc.), xylose (Xyl.) and mannose (Man.).

Analyses of the constituent mono- and oligo-saccharides in each fraction were done by Dionex ion chromatography system DX-300 as reported previously. The retention times of peaks of each sample were compared with those of standard sugars [solbitol (Sol.), glucose (Glc.), fructose (Fru.) and sucrose (Suc.).

Fractionation of carbohydrates in apple pomace

Figure 1 shows a flow-chart of the fractionation of apple pomace carbohydrates. Apple pomace (wet weight: 101.1g) was mashed and mixed for 5min with 2 volumes of distilled water, and centrifuged to separate into the water-soluble and -insoluble materials. This procedure was repeated 5 times.

A portion of the water-soluble materials (total sugar content: 7.3g) was applied to a column (2.5 × 42cm) of Bio-Gel P-2 followed by filtration through the column with water; 3-ml fractions were collected and assayed for carbohydrates. Tubes 22 to 29, and 44 to 58 were separately combined and concentrated to give a water-soluble polysaccharides (WSP) fraction (yield: 664mg as Glc. equiv.), and the mono- and oligo-saccharides (M-OL) fraction (yield: 6548mg as Glc. equiv.).

The water-insoluble materials (apple pulp) were extracted three times with 0.25% ammonium oxalate for 1.5hr at 100℃. The 0.25% ammonium oxalate extract was desalted by Bio-Gel P-2 column to give the pectic-substances (PS) fraction (yield: 2174mg as Glc.

equiv.). The 0.25% ammonium oxalate-insoluble materials were extracted three times with 24% KOH at room temperature. The 24% KOH extract was neutralized with acetic acid desalted by Bio-Gel P-2 to give the hemicellulose (HC, 1993.3mg as Glc. equiv.) fraction. The 24% KOH-insoluble materials were neutralized with acetic acid and washed with distilled water. The washed insoluble materials were freeze-dried to give the cellulose (CL, 1411.4mg as Glc. equiv.) fraction.

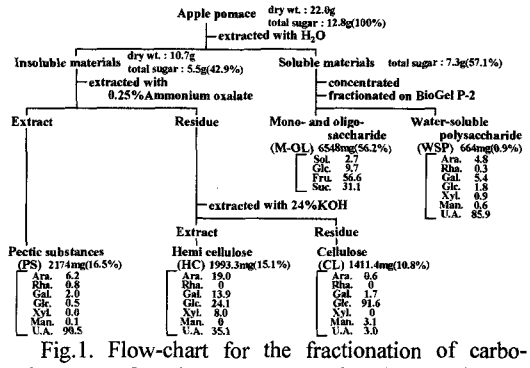

Fig.1. Flow-chart for the fractionation of carbohydrates of apple pomace, and the yields and sugar composition of the subfractions obtained.

Saccharification of apple pomace by commercial crude pectinases

Apple pomace was mashed and mixed for 5min with 2 volumes distilled water and autoclaved for 30min at 121℃. The autoclaved apple pomaces were individually incubated at 40℃ with 6 kinds of commercial crude pectinases (Table I). The enzymatic reaction was monitored after 27hr- and 51hr-incubation using both the calcium pectate method and the acid-base titration method.

	Enzymes name		Enzymes name
P-A	No enzymes	P-D	PECTINASE HL (from *Aspergillus sp.*)
P-B	PECTINASE SS (Yakult PHARMACEUTICAL IND.)	P-E	SUCRASE N(Shin Nihon Chemical)
P-C	PECTINASE 3S (Yakult PHARMACEUTICAL IND.)	P-F	SUCRASE S(Shin Nihon Chemical)
		P-G	SOLUBLE PECTINASE T(HBI Enzymes Inc.)

Table I. Commercial crude pectinases.

Influence of the concentration of substrate and enzyme on hydrolysis of apple pomace with PECTINASE HL

Apple pomace were mashed and mixed 5min with 2 or 3 volumes distilled water and autoclaved for 30min at 121℃. The autoclaved apple pomace in 2 and 3 volumes of distilled water were divided equally into 3 parts, respectively. The divided samples were individually hydrolyzed with 0.5, 1, or 1.5% PECTINASE HL for 48hr at 40℃. The hydrolysis ratios were determined by the calcium pectate method and the acid-base titration method.

Saccharification of apple pulp by commercial crude cellulases

Case 1: Apple pulp was mixed for 5min with 3 volumes of distilled water and autoclaved for 30min at 121℃. The autoclaved apple pulp were individually treated with 15 kinds of commercial crude cellulases (Table II) and incubated at 40℃. After 24hr-digestions the samples were centrifuged and separated into soluble- and insoluble-materials.

Case 2: Apple pomace was treated with PECTINASE HL for 51hr and centrifuged to obtain de-pectinated apple pomace. The de-pectinated apple pomaces suspended in 3 volumes of distilled water were individually incubated with 15 kinds of commercial crude cellulases at 40℃. After 24hr, the incubated mixtures were centrifuged and separated into soluble- and insoluble-materials.

	Enzymes name		Enzymes name
C-A	DRISERASE(Seikagaku Ind.)	C-H	CELLULASE "AMANO"90(Amano Enzyme)
C-B	MEISERASE(Meiji)	C-I	CELLULOSINE HC(HBI Enzymes Inc.)
C-C	MEISERASE P(meiji)	C-J	CELLULASE AP-3(HBI Enzymes Inc.)
C-D	TOYOSERASE(toyo jozo)	C-K	CELLULASE T"AMANO"4(Amano Enzyme)
C-E	CELLULASE "ONOZUK" 3S (from Tricoderma virde.)(Yakult PHARMACEUTICAL IND.)	C-L	CELLULOSINE HC100(HBI Enzymes Inc.)
		C-M	CELLULOSINE TP25(HBI Enzymes Inc.)
C-F	CELLULASE Y-NC (Yakult PHARMACEUTICAL IND.)	C-N	CELLULOSINE AP(HBI Enzymes Inc.)
		C-O	CELLULOSINE AC40(HBI Enzymes Inc.)
C-G	CELLULASE A "AMANO" 3(Amano Enzyme)		

Table II. Commercial crude cellulases.

In both case, the soluble- and insoluble-materials were subjected to analyses of total sugar and acidic sugar contents, and constituent monosaccharide. Degree of hydrolysis of apple pulp or de-pectinated apple pomace was calculated as follows. Degree of hydrolysis = (total sugar in the soluble materials) / (total sugar in the soluble and insoluble materials) × 100(%).

Influence of the concentration of substrate and enzyme on hydrolysis of apple pomace with CELLULASE "ONOZUKA" 3S

Apple pomace in equal volume of distilled water was hydrolyzed with PECTINASE HL for 48hr. The hydrolyzate was centrifuged to separate soluble- and insoluble-materials. The insoluble-materials (the de-pectinated apple pomace) were mixed for 5min with 0.5, 1, or 1 volumes of distilled water. Each mixture was then divided equally into 3 portions. The divided samples were hydrolyzed with 0.25, 0.5, or 1% CELLULASE "ONOZUKA" 3S for 24hr at 40℃, respectively. The degrees of hydrolysis were determined as described above.

Sequential hydrolysis of apple pulp with PECTINASE HL and CELLULASE "ONOZUKA" 3S and analyses of the hydrolyzates

Apple pulp was hydrolyzed with PECTINASE HL for 48hr and centrifuged to separate into the soluble- and insoluble-materials. The soluble-materials in 20mM Na-acetate buffer (pH5.5) were applied to column (2.5× 15cm) of DEAE Sephadex A-25 equilibrated with 20mM Na-acetate buffer (pH5.5) and eluted stepwise with 200ml of the same buffer, and 200ml of 1M NaCl in the same buffer. Fractions of 8ml each were collected and assayed for carbohydrates. The fractions eluted with Na-acetate buffer and 1M NaCl with the same buffer were desalted by Bio-Gel P-2 chromatography to give neutral fraction and acidic fraction of PECTINASE HL hydrolyzates. The both fractions were subjected to Bio-Gel P-2 chromatography to investigate the molecular weight distribution.

The insoluble-materials were hydrolyzed with CELLULASE "ONOZUKA" 3S for 48hr and separated into the soluble- and insoluble-materials by centrifugation. The soluble-materials were subjected to Bio-Gel P-2 chromatography to investigate the molecular weight distribution.

3. Result and discussion

Fractionation of carbohydrates in apple pomace and their sugar composition

As shown in the Fig. 1, carbohydrates in apple pomace were fractionated into the mono- and oligo-saccharides (M-OL), water-soluble polysaccharides (WSP) and pectic substances (PS), hemicellulose (HC) and cellulose (CL) fractions. The approximate ratio of carbohydrate in the M-OL, WSP, PS, HC and CL was 56:1:17:15:11. The M-OL fraction consisted of Sol., Glc., Fru. and Suc. in the ratio of 3:10:57:31. The content of these mono- and oligo-saccharides is very important to increase the yield of lactic acid.

The sugar compositions of WSP, PS, HC and CL fractions are shown in Fig. 1. The WSP and PS fractions contained large amounts of uronic acid. The uronic acid is derived from polygalacturonic acid (apple pectin). Conversion of these polygalacturonic acids in apple pulp to oligogalacturonic acids may be possible by an appropriate treatment such as hydrolysis with pectinase. Xylose was found in HC fraction. This may be derived from xyloglucan polysaccharides. The major component of CL fraction was glucose. Conversion of these cellulosic polysaccharides to glucose by hydrolysis with appropriate cellulases may increase the yield of lactic acid.

Saccharification of apple pomace by commercial crude pectinases

Pectinase that hydrolyzed apple pomace well was screened for. Degrees of hydrolysis of apple pomace with commercial enzyme preparations are shown in Fig. 2. PECTINASE HL (P-D in Fig. 2) caused more hydrolysis than the five other enzymes. From this result, PECTINASE HL was selected.

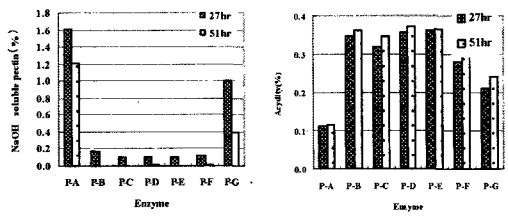

Fig.2. Hydrolysis of apple pomace with commercial crude pectinases. Details are given in the text. The degrees of hydrolysis were determined by the calcium pectate method (left) and the acid-base titration method (right). See Table 1 for P-A to P-G.

Next the influence of the concentration of substrate and enzyme on hydrolysis of apple pomace with PECTINASE HL was investigated. The results are summarized in Fig.3. The ratio of substrate to water (substrate concentration), 1:2 and 1:3 showed no significant differences in enzyme activity, and enzyme activity did not significantly differ at 0.5 and 1% PECTINASE HL concentrations. Taking the cost of the pectinase into consideration, we decided the hydrolysis condition as follows: the mixture of apple pomace (100g wet weight), PECTINASE HL (0.5g) and water (200ml) is incubated for 48h at 40℃.

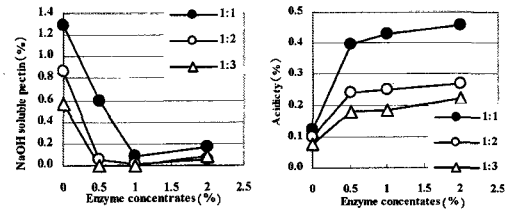

Fig.3. Influence of the concentration of substrate and enzyme on hydrolysis of apple pomace with PECTINASE HL. Details are given in the text. The ratios of 1:1, 1:2, and 1:3 mean the ratios of substrate (apple pomace) to water. The degrees of hydrolysis were determined by calcium pectate method (left) and the acid-base titration method (right). Enzyme concentration: (enzyme dry wt. / substrate wet wt.) × 100(%)

Saccharification of apple pulp by commercial crude cellulases

Cellulase that hydrolyzed cellulose in apple pomace or apple pulp well was screened for. Degrees of hydrolysis of apple pulp with commercial enzyme preparations are shown in Fig. 4. Figure 5 shows the degrees of hydrolysis of the de-pectinated apple pomace with the same enzymes. All enzymes hydrolyzed the de-pectinated apple pomace better than apple pulp. The de-pectinated apple pomace was more susceptible to the enzymes. About 85% of the de-pectinated apple pomace was hydrolyzed by TOYOSERASE (C-D), CELLULASE "ONOZUKA" 3S (C-E), and CELLULOSINE HC100 (C-L). On basis of this result and the enzyme cost, CELLULASE "ONOZUKA" 3S was selected.

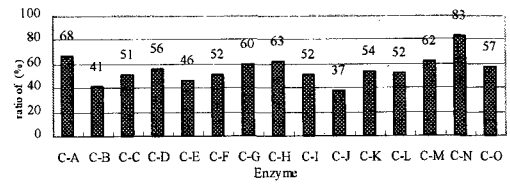

Fig.4. Hydrolysis of apple pulp with commercial crude cellulases. Details are given in the text. See Table 2 for C-A to C-O.

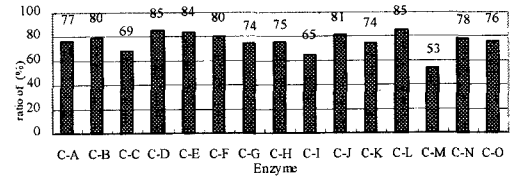

Fig.5. Hydrolysis of de-pectinated apple pomace with commercial crude cellulases. Details are given in the text. See Table 2 for C-A to C-O.

Next the influence of the concentration of substrate and enzyme on hydrolysis of de-pectinated apple pomace with CELLULASE "ONOZUKA" 3S was investigated. The results are summarized in Fig. 6. The ratio of substrate to water (substrate concentration), 1:1 and 1:1.5 showed no significant differences in enzyme

activity. There was little difference in enzyme activity among 0.25, 0.5 and 1% cellulase consideration. We decided the hydrolysis condition as follows: the mixture of de-pectinated apple pomace (100g wet weight), CELLULASE "ONOZUKA" 3S (0.5g) and water (100ml) in incubated for 24h at 45℃

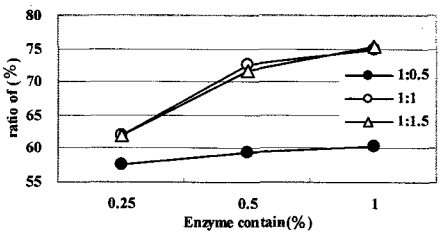

Fig.6. Influence of the concentration of substrate and enzyme on hydrolysis of the de-pectinated apple pomace with CELLULASE "ONOZUKA" 3S. Details are given in the text. The ratios of 1:0.5, 1:1, and 1:1.5 mean the ratios of substrate to water

Sequential hydrolysis of apple pulp with PECTINASE HL and CELLULASE "ONOZUKA" 3S and analyses of the hydrolyzates

First, apple pulp was hydrolyzed with PECTINASE HL for 48h. The soluble materials of the hydrolyzate were separated into neutral (PHL-N) and acidic (PHL-A) fractions by DEAE-Sephadex A-25 chromatography (Fig. 7). The PHL-N and PHL-A fractions were individually subjected to Bio-Gel P-2 chromatography (Fig. 8) to give fractions PHL-N-1, -2, and -3, and PHL-A-1, -2, and -3, respectively. Yields and sugar composition of the obtained fractions are shown in Table 3. The results show that PECTINASE HL hydrolyzes apple pulp mostly into the oligo-saccharides, the molecular weight of which are below 2000. In addition, there is a possibility that PECTINASE HL contains various kinds of carbohydrates.

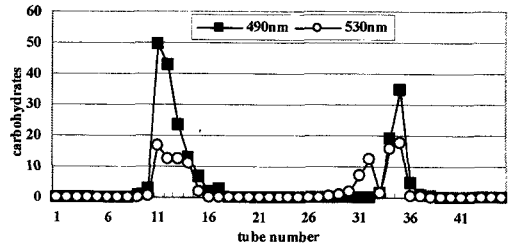

Fig.7. Separation of the PECTINASE HL hydrolyzate of the apple pulp by DEAE-Sephadex A-25 chromatography. Tubes 10 to 17, and 29 to 38 were separately combined to give the neutral (PHL-N) and acidic (PHL-A) fractions. Details are given in the text.

Next, the pectinase-treated apple pulp (insoluble-materials after hydrolyzed with PECTINASE HL) were hydrolyzed with CELLULASE "ONOZUKA" 3S for 48hr. and separated into the soluble- and insoluble-materials by centrifugation. The soluble-materials were subjected to Bio-Gel P-2 chromatography (Fig. 9) to give CLO-1, -2, and -3. Yields and sugar composition are shown in Table III. The major fraction, CLO-3 consisted of 66% glucose. The fraction CLO-3 contained other mono-saccharides in addition to glucose and glucobiose. It was found that 97% of apple pomace carbohydrates were solubilized into mono- and oligo-saccharides by sequential treatment with PECTINASE HL and CELLULASE "ONOZUKA" 3S.

Further studies on the cellulase-saccharification of apple pulp will be necessary.

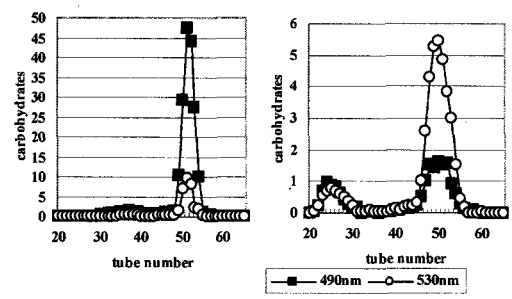

Fig.8. Separation of the PHL-N and PHL-A fractions by Bio-Gel P-2 chromatography. PHL-N (left): Tubes 31 to 41, 42 to 48, and 49 to 61 were separately combined to give fractions PHL-N-1,-2, and -3. PHL-A (right): Tubes 22 to 30, 40 to 45, and 46 to 64 were separately combined to give fractions PHL-A-1, -2, and -3.

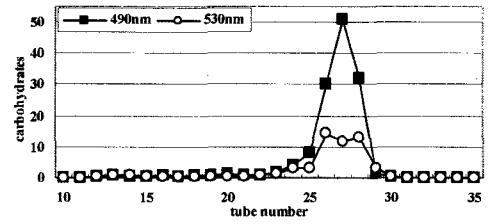

Fig.9. Separation of the CELLULASE "ONOZUKA" 3S hydrolyzate of the de-pectinated apple pulp by Bio-Gel P-2 chromatography. Tubes 12 to 16, 17 to 24, and 25 to 30 were combined to give fractions CLO-1, -2, and -3.

Fraction		amount (%)	Sugar Composition (%)							
			Fuc.	Ara.	Rha.	Gal.	Glc.	Xyl.	Man.	U.A.
PHL -N-	1	2.28	trace.	24.8	45.6	25.3	0.9	trace.	trace.	3.4
	2	1.34	trace.	35.7	trace.	10.5	13.7	15.0	5.3	20.0
	3	5.52	trace.	30.9	9.4	5.8	6.2	33.3	trace.	14.4
PHL -A-	1	3.58	trace.	5.6	1.8	17.4	35.0	5.7	2.1	32.6
	2	0.45	trace.	3.1	trace.	1.5	2.0	0.3	trace.	93.1
	3	17.62	trace.	12.2	trace.	6.6	6.2	1.7	trace.	73.4
CLO -	1	0.32	trace.	5.1	6.8	6.3	1.6	13.3	2.6	64.4
	2	0.44	trace.	2.9	0.7	7.9	20.8	13.4	1.1	53.2
	3	8.08	trace.	6.3	trace.	7.4	66.2	3.8	16.2	trace.

% apple pulp carbohydrate

Table III. Yield and sugar composition of fractions PHL-N-1 to -3, PHL-A-1, to -3, and CLO-1, to -3.

4. References

[1] Nakasaki K, Adachi T.: Effects of intermittent addition of cellulase for production of L-lactic acid from wastewater sludge by simultaneous saccharification and fermentation. *Biotechnol Bioeng.* **82(3)**:263-70. 2003

[2] S. Ito, Y. Mitsuishi, T. Okuno, and Y. Kato: Changes in the structure of xyloglucan of apple fruit during development. *J. Japan. Soc. Hort. Sci.* **73(1)**: 51-56 2004.

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