園学雑. (J. Japan. Soc. Hort. Sci.) 73 (6) : 574-579. 2004.

Utilization by Intestinal Bacteria and Digestibility of Arabino-oligosaccharides In Vitro

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Summary

We investigated the utilization of arabino-oligosaccharides derived from arabinan, which is abundant in side chains of apple pectin, by intestinal bacteria in vitro to clarify the mechanisms of the beneficial effects of apple ingestion on intestinal bacterial flora. Among the test strains, arabino-oligosaccharides that contain more than three units were selectively utilized by *Bifidobacterium adolescentis, Bi. longum,* and *Bacteroides vulgatus*. The digestion of arabino-oligosaccharides by artificial digestive juices was investigated in vitro. Arabino-oligosaccharides were not digested by artificial saliva, artificial gastric juice, or artificial pancreatic juice, and only slightly by small intestinal enzymes. These results suggest that arabino-oligosaccharides serve as prebiotics and that the beneficial effects of apple ingestion on intestinal bacterial flora are partly due to apple pectin.

Key Words: apple, arabino-oligosaccharides, *Bifidobacterium*, intestinal bacterial flora, prebiotics.

Introduction

Intestinal bacterial flora, which comprise a diverse collection of bacterial species, are closely related to human health. Of the 30 anaerobic genera in the human colon, *Bacteroides, Clostridium, Eubacterium, Lactobacillus* and *Bifidobacterium* are the most numerous. *Bifidobacterium* and *Lactobacillus* are beneficial intestinal bacteria; they lower blood cholesterol levels, inhibit the growth of potential pathogens, produce vitamins, etc. They are not usually thought to be pathogenic. Others, like *Escherichia coli* and *C. perfringens*, are unfavorable for human health because of their pathogenicity, production of carcinogens, etc (Hidaka et al., 1983; Mitsuoka, 1982). A predominance of *Bifidobacterium* is considered to be beneficial for human health (Okazaki et al., 1990).

Intestinal bacterial flora have remarkable stability and constancy (Bornside, 1978; Mitsuoka, 1982), but can be altered by many endogenous and exogenous factors, including diet (Mitsuoka, 1982). People on a Western diet have higher counts of *Bacteroides* than those on a Japanese diet, whereas those on a Japanese diet have higher counts of *Lactobacillus, Peptostreptococcus* and *Eubacterium* than those on a Western diet (Finegold et al., 1974). The intake of some oligosaccharides, such as fructo-oligosaccharides and xylo-oligosaccharides, which are prebiotics, increases major beneficial bacteria, *Bifidobacterium*. Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and / or activity of one or a limited number of bacterial species already residing in the colon, and thus improve the host's health (Gibson and Roberfroid, 1995). The effects of many polysaccharides from fruits and vegetables on intestinal bacteria flora, however, remain unclear. Little attention has been paid to the possible use of plant cell wall-derived oligosaccharides as prebiotic substrates. Thus, it is important to elucidate the effects of oligosaccharides and polysaccharides contained in fruits and vegetables on intestinal bacterial flora.

Daily ingestion of an apple significantly decreases *C. perfringens* (Suzuki et al., 2001). Its pectin has a strong bacteriostatic action on *Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa* and *Es. coli* (Tazawa et al., 1997). Feeding apple pectin to rats decreases levels of fecal β-glucosidase, tryptophanase, and β-glucuronidase, which are potentially important in the generation of toxins and carcinogens (Ohkami et al., 1995). Therefore, apple pectin might strongly influence the intestinal bacterial flora, although, generally, pectin does not belong to prebiotics. The

Received; November 11, 2003. Accepted; March 11, 2004.
Parts of this paper were presented at the 2000 Spring Meeting and the 2002 Autumn Meeting of the Japanese Society for Horticultural Science.

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effects of each constituent of apple, however, such as pectin, sorbitol, and oligosaccharides on intestinal bacterial flora have not been reported.

In this study, we investigated the potential of oligosaccharides derived from apple pectin as prebiotics and discussed the mechanisms underlying the beneficial effects of apple ingestion on intestinal bacterial flora.

Materials and Methods

Materials

Arabino-oligosaccharides, including arabinobiose, arabinotriose, arabinotetraose, arabinopentaose, arabinohexaose, arabinoheptaose, and arabinooctaose, and arabinan were obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland). All arabino-oligosaccharides and arabinan are α-1, 5-linked; the structure is the same as that of the complex carbohydrate backbone in apple pectin. Polygalacturonic acids and apple pectin were purchased from Sigma Chemical Co. (St. Louis, MO). Fructo-oligosaccharides and xylo-oligosaccharides were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo). Fructo-oligosaccharides are β-1, 2-linked fructose oligomers and a mixture of 1-kestose, nystose, and 1-fructofuranosyl-D-nystose. Xylo-oligosaccharides are β-1, 4-linked xylose oligomers and a mixture of xylobiose, xylotriose, etc.

Preparation of oligosaccharides from apple pectin

A solution of apple pectin (2%, w/v) was incubated with pectinase from *Aspergillus nigar* (Sigma) (0.05%, v/v) at 37℃ for 3 hr. After boiling for 10 min to inactivate the pectinase, ethanol was added to the mixture to make the final concentration of 80% (v/v). It was then centrifuged at 5000 × g for 20 min at 4℃, after which the pellet and the supernatant (80% supernatant) were dried in vacuo. The pellet was dissolved in distilled water and then made to 30% (v/v) with ethanol. The solution was centrifuged, and the supernatant and the pellet were named 30% supernatant and 30% pellet, respectively. The fractions were dried in vacuo and dissolved in distilled water for use in the in vitro experiments. Analysis by thin-layer chromatography revealed that the samples of 80% supernatant, 30% supernatant, and 30% pellet contained mainly monosaccharides, oligosaccharides, and polysaccharides, respectively (data not shown). Polygalacturonic acid as control was also treated as above.

Utilization of sugars by intestinal bacteria in vitro

Strains of *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Lactobacillus* and others (Table 2), were obtained from the culture collection of RIKEN (Institute of Physical and Chemical Research) and Laboratory of Culture Collection (Institute of Medical Science, University of Tokyo).

Utilization of sugars by intestinal bacteria in vitro was investigated according to Okazaki et al. (1990) with modifications. The bacterial strains for the study were anaerobically incubated on a BL agar plate at 37℃ with Anaero Pack (Mitsubishi Gas Chemical Company, Inc., Tokyo) and then inoculated on PYF medium (Holdeman et al., 1977) containing 0.5% (w/v) glucose or modified GAM broth and kept anaerobic overnight. A test medium was prepared by adding each sugar to PYF broth in a concentration of 0.5% (w/v). A culture fluid (5 μl) was added to 250 μl of the test medium and incubated anaerobically at 37℃ for 4 days. The utilization of each sugar was determined, based on the decrease in the medium pH. Control experiments were performed with sugar-free medium.

In vitro digestibility of sugars

In vitro digestibility of sugars, including arabino-oligosaccharides and other oligosaccharides, was investigated according to Okada et al. (1990) with modifications. All hydrolytic products were analyzed by high-performance size-exchange chromatography (HPSEC) or high-performance anion-exchange chromatography (HPAEC).

1) Hydrolysis with artificial human saliva was performed by using α-amylase from human saliva (type IX-A, Sigma). Forty microliters of artificial human saliva (40 U / ml α-amylase, 1 mM $CaCl_2$, and 20 mM ACES buffer (pH 6.0)) was added to 200 μl of sample solution (10% (w/v) sugar, 1 mM $CaCl_2$, and 50 mM Tris-maleate buffer (pH 6.0)), incubated at 37℃ for 30 min and heated at 100℃ for 5 min to stop the reaction. One unit of α-amylase liberates 1.0 μmol glucose from 0.1% (w/v) starch in 1 min at pH 6.9 at 37℃.

2) Hydrolysis with artificial gastric juice was performed with an HCl-KCl buffer (pH 2.0). HCl-KCl buffer (50 mM, 100 μl, pH 2.0) was added to 200 μl of sample solution (2.2% (w/v) sugar), incubated at 37℃ for 100 min, and neutralized with 10 mM NaOH to stop the reaction.

3) Hydrolysis with artificial pancreatic juice was performed with α-amylase from porcine pancreas (type I-A, Sigma). Artificial pancreatic juice (20 μl; 20 U・ml^{-1} α-amylase) was added to 200 μl of sample solution (10% (w/v) sugar, 1 mM $CaCl_2$, 50 mM Tris-maleate buffer (pH 6.6)), incubated at 37℃ for 6 hr, and heated at 100℃ for 5 min to stop the reaction. The enzyme activity was defined as α-amylase from human saliva.

4) Hydrolysis with small intestinal enzymes was performed with intestinal acetone powders from a rat (Sigma). Intestinal enzyme solution (20 μl; 4.3 U・ml^{-1}) was added to 200 μl of sample solution (1% (w/v) sugar, 50 mM Tris-maleate buffer (pH 6.6)), incubated at 37℃ for 3 hr, and heated to 100℃ for 5 min to stop the reaction. One unit of intestinal enzymes liberates 1.0 μmol glucose from 1.0% (w/v) maltose in 1 min at pH 6.0 at 37℃.

Analytical methods

After each reaction, arabinobiose, arabinotriose, raffinose, 1-kestose, isomaltose, and sucrose were analyzed with HPSEC; arabinotetraose, arabinopentaose, arabinohexaose, and maltopentaose were analyzed with HPAEC. HPSEC was performed on an LC-10A vp system (Shimadzu, Kyoto) equipped with Shodex SUGAR KS-801 (300 × 8.0 mm) that was eluted at 60℃ with Milli Q water at 0.5 ml· min^{-1}. The eluate was monitored with an RID-10A refractive index detector (Shimadzu). Taurine was measured as an internal control. HPAEC was performed on a DX-300 Carbohydrate system (Dionex, Sunnyvale, CA) equipped with a Dionex CarboPac PA-1 (250 × 4.0 mm) in combination with a Carbopac PA-1 guard column (50 × 4.0 mm); the column eluted at 25℃ with 100 mM NaOH at 1.0 ml· min^{-1} The sugars were analyzed by using a gradient of sodium acetate in 100 mM NaOH as follows: 0-5 min, 0 mM; 5-25 min, 0-600 mM. The eluate was monitored using DX-300 pulsed amperometry (Dionex). Ethylene glycol was used as an internal control. The hydrolysis rate was calculated from the following formula: Hydrolysis rate (%) = 100-100 (Sa / Sb), in which Sa represents samples after treatment and Sb represents samples before treatment.

Table 1. Utilization of sugars derived from apple pectin by *Bifidobacterium adolescentis* in vitro.

	Before pectinase treatment	After pectinase treatment			
		Before fractionation	Monosaccharide-rich fraction	Oligosaccharide-rich fraction	Polysaccharide-rich fraction
Sugars derived from polygaracturonic acid	-	-	±	-	-
Sugars derived from apple pectin	-	±	±	+	-

Judgment of bacterial growth: ++, same level of growth compared to glucose; +, weaker growth compared to glucose; ±, a little growth; -, no growth.

Table 2. Utilization of arabino-oligosaccharides by intestinal bacteria in vitro.

Bacteria		Origin	A1	A2	A3	A4	A5	A6	A7	A8	AN	FOS	XOS
Bifidobacterium	*adolescentis*	JCM 7046	++	±	+	++	++	++	++	++	-	++	++
	bifidum	JCM 1255	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	infantis	JCM 1210	-	-	-	-	-	-	n.d.	n.d.	-	++	±
	longum	JCM 1217	++	++	++	++	++	++	++	++	++	++	-
	breve	JCM 1192	-	-	-	-	-	-	n.d.	n.d.	-	+	±
Lactobacillus	*acidophilus*	JCM 1132	-	-	-	-	-	-	n.d.	n.d.	-	+	-
	casei	JCM 1109	-	-	-	-	-	-	n.d.	n.d.	-	++	-
	salivarius	JCM 1040	-	-	-	-	-	-	n.d.	n.d.	-	+	-
Eubacterium	*lentum*	JCM 9979	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	limosum	JCM 6421	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	aerofaciens	JCM 10188	-	-	-	-	-	-	n.d.	n.d.	-	±	-
Propionibacterium	*acnes*	JCM 6425	-	-	-	-	-	-	n.d.	n.d.	-	-	-
Bacteroides	*distasonis*	JCM 5825	-	-	-	-	-	-	n.d.	n.d.	-	±	±
	fragilis	IID 1643	-	-	-	-	-	-	n.d.	n.d.	-	+	-
	thetaiotaomicron	JCM 5827	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	vulgatus	JCM 5826	++	++	++	++	++	++	++	++	++	++	+
Clostridium	*difficile*	JCM 1296	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	paraputrificum	JCM 1293	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	ramosum	JCM 1298	-	-	-	-	-	-	n.d.	n.d.	-	±	-
	perfringens	JCM 1290	-	-	-	-	-	-	n.d.	n.d.	-	-	-
Escherichia	*coli*	JCM 5491	+	-	-	-	-	-	n.d.	n.d.	-	-	-
Klebsiella	*pneumoniae*	JCM 1662	++	±	-	-	-	-	n.d.	n.d.	-	+	+
Enterococcus	*faecalis*	JCM 5803	-	-	-	-	-	-	n.d.	n.d.	-	-	-
Peptostreptococcus	*prevotti*	JCM 6490	-	-	-	-	-	-	n.d.	n.d.	-	-	-
Veillonella	*alcalescens*	IID 5225	-	-	-	-	-	-	n.d.	n.d.	-	-	-
Mitsuokella	*multacida*	JCM 2054	+	-	-	-	-	-	n.d.	n.d.	-	-	-

A1, arabinose; A2, arabinobiose; A3, arabinotriose; A4, arabinotetraose; A5, arabinopentaose; A6, arabinohexaose; A7, arabinoheptaose; A8, arabinooctaose; AN, arabinan; FOS, fructo-oligosaccharide; XOS, xylo-oligosaccharide.

Judgement of bacterial growth: -, ΔpH<0.5; ±, 0.5 ≦ ΔpH<1.0; +, 1.0 ≦ ΔpH<1.5; ++, 1.5 ≦ ΔpH; n.d., not determined; ΔpH=(test pH)-(control pH).

Results

Utilization of apple pectin by Bifidobacterium in vitro

Table 1 shows utilization of apple pectin and sugars derived from it by *Bifidobacterium*. *Bi. adolescentis* utilized sugars in oligosaccharide-rich fractions derived from apple pectin, but not in other fractions, including apple pectin (before pectinase treatment), pectinase-treated apple pectin (before fractionation), monosaccharide-rich fractions and polysaccharide-rich fractions. On the other hand, *Bi. adolescentis* did not utilize polygalacturonic acid (before pectinase treatment) or sugars derived from it.

Utilization of arabino-oligosaccharides by intestinal bacteria in vitro

Apple pectin is characterized by arabinose-rich side chains (Schols et al., 1990). Oligosaccharides are used as prebiotics more often than polysaccharides. The above results demonstrate that *Bifidobacterium* utilizes oligosaccharides derived from apple pectin, but not oligogalacturonic acid. Thus, we investigated the utilization of arabino-oligosaccharides and arabinan by intestinal bacteria in vitro (Table 2). The monosaccharide, arabinose, was more or less selectively utilized by *Bi. adolescentis, Bi. longum, Ba. vulgatus, Es. coli, Klebsiella pneumoniae* and *Mitsuokella multacida* among the test strains. Of these, arabinobiose was readily utilized by *Bi. longum* and *Ba. vulgatus*, but slightly by *Bi. adolescentis* and *K. pneumoniae*, where it was unaffected by *Es. coli* and *M. multacida*. Arabinotriose was strongly utilized only by *Bi. longum* and *Ba. vulgatus*. Arabinotetraose, arabinopentaose, arabinohexaose, arabinoheptaose, and arabinooctaose were strongly utilized by *Bi. longum* and *Ba. vulgatus*, as well as *Bi. adolescentis*. Fructo-oligosaccharides and xylo-oligosaccharides, which are common prebiotics, were utilized by more bacterial species than arabino-oligosaccharides. Arabinan was utilized only by *Bi. longum* and *Ba. vulgatus*, but not by *Bi. adolescentis*. *Clostridium, Es. coli*, and *K. pneumoniae*, which are harmful to humans, did not utilize arabinan as an energy source.

Digestibility of arabino-oligosaccharides in vitro

The digestion of arabino-oligosaccharides by artificial digestive juices was investigated in vitro (Table 3), since, to be prebiotics, a substance must reach the large intestine without digestion. Maltopentaose, a control sugar, was digested slightly by artificial gastric juice, but completely by artificial saliva, artificial pancreatic juice, and the small intestinal enzymes. Maltose was hardly digested by the artificial gastric juice but 86% by the small intestinal enzymes. Raffinose and 1-Kestose, non-digestible sugars, were digested a little by artificial gastric juice, but remained undigested in the presence of artificial saliva, artificial pancreatic juice, and the small intestinal enzymes. Likewise, arabino-oligosaccharides were not digested at all by artificial saliva, artificial gastric juice, or artificial pancreatic juice, and only a little by the small intestinal enzymes.

Discussion

Since pectin is degraded and fermented in the large intestine by intestinal bacteria, it is thought to affect intestinal bacterial flora. The beneficial effects of dietary fibers, including pectin, on intestinal bacterial flora remain unclear, however, and not all fibers are necessarily prebiotics. Pectin is utilized by *Bacteroides* and *Eu. eligens*, but not *Bifidobacterium* in vitro (Salyers et al., 1977a; Salyers et al., 1977b). Feeding high-methoxylated pectin to rats resulted in an increase of *Bacteroides* (Aoe et al., 1988); whereas, feeding 5% pectin to rats did not affect intestinal bacterial flora (Wise et al., 1982). Human ingestion of 35 g pectin per day for more than 2 weeks did not affect intestinal bacterial flora (Drasar and Jenkins, 1976). One reason

Table 3. Hydrolysis rate (%) of arabino-oligosaccharides with artificial digestive juices in vitro.

	Artificial saliva	Artificial gastric juice	Artificial pancreatic juice	Small intestinal enzymes	Total hydrolysis rate
Arabinobiose	0	0	0	2	2
Arabinotriose	0	0	0	2	2
Arabinotetraose	0	0	0	4	4
Arabinopentaose	0	0	0	4	4
Arabinohexaose	0	0	0	3	3
Raffinose	0	1	0	0	1
1-Kestose	0	7	0	0	7
Isomaltose	0	0	0	47	47
Maltose	n.d.	0	n.d.	86	-
Maltopentaose	100	2.8	100	100	100
Sucrose	n.d.	1	n.d.	32	-

n.d.; not determined.

why the effects of pectin on intestinal bacterial flora remain unclear is that the composition and structure of pectin vary depending on its source.

Apple pectin might beneficially affect intestinal bacterial flora, because it has strong bacteriostatic action (Tazawa et al., 1997) and suppresses the levels of enzymes related to carcinogenesis (Ohkami et al., 1995). This suggests that the beneficial effects of daily consumption of apple on intestinal bacterial flora (Suzuki et al., 2001) are principally due to its pectin. It is important to clarify the mechanism to improve fruits or to improve the efficiency of pectin. Since oligosaccharides are noted as prebiotics, we investigated the utilization by *Bifidobacterium* of oligosaccharides derived from apple pectin and polygalacturonic acid, which are the main constituents of pectin (Table 1). The findings that *Bi. adolescentis* utilizes oligosaccharides derived from apple pectin but not its polygalacturonic acid and rhamnogalacturonan backbone indicate that the pectin *per se* is not prebiotic.

The most striking feature of apple pectin is the presence of large amounts of arabinose residues in the side-chains (Schols et al., 1990). Apple pectin is composed of more than 20% (w/w) of arabinose (Aspinall and Fanous, 1984). Thus, it is possible that the beneficial effects of apple pectin on intestinal bacteria are attributed to its arabino-oligosaccharides. Arabino-oligosaccharides might be part of the apple pectin metabolized during the fruit ripening stage, since arabinan exists in it as side chains and arabinose residues are released from it then (Yoshioka et al., 1994). The ingested pectin reaches the large intestine without degradation and absorption; it is completely degraded there by intestinal bacteria (Titgemeyer et al., 1991). Arabino-oligosaccharides might be liberated during this process. It is important to investigate the utilization of arabino-oligosaccharides by intestinal bacteria.

Arabinose, a monosaccharide, is more or less selectively utilized by *Bifidobacterium* (Table 2). The combination of arabinose makes its utilization more selective. Arabino-oligosaccharides, especially those that consist of more than three units, are selectively utilized only by *Bi. adolescentis, Bi. longum* and *Ba. vulgatus* of the strains tested. They were more selectively utilized than fructo-oligosaccharides and xylo-oligosaccharides, which are common prebiotics. Furthermore, to prevent lifestyle-related disease through improvement of intestinal bacterial flora with prebiotics, it is important to increase *Bi. adolescentis* and *Bi. longum* because they are the predominant bifidobacteria in human adults (Benno and Mitsuoka, 1989). Thus, arabino-oligosaccharides are suitable as prebiotics, because they are strongly utilized by *Bi. adolescentis* and *Bi. longum*.

These results demonstrate that in humans, arabino-oligosaccharides probably reach the large intestine without being digested. They are very effective prebiotics that mainly underlie the beneficial effects of apple on intestinal bacterial flora. Further study to investigate their effects in vivo is necessary before they can be used as prebiotics.

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アラビノオリゴ糖の腸内細菌による資化性と消化性

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摘　要

リンゴの腸内フローラ改善効果のメカニズムを解明するために，リンゴペクチンの特徴であるアラビナンに由来するアラビノオリゴ糖の腸内細菌による資化性をin vitroで調べた．三糖以上のアラビノオリゴ糖は，調査した26菌種のうち*Bifidobacterium adolescentis*，*Bifidobacterium longum*，*Bacteroides vulgatus*にのみ資化され，ビフィズス菌に極めて特異的利用されることが明らかとなった．アラビノオリゴ糖のヒト消化液による分解性をin vitroで調べたところ，人工唾液，人工胃液，人工膵液には全く分解されず，人工小腸液により数％分解されただけであった．以上のことからアラビノオリゴ糖は効果の高い新規のプレバイオティックスであること，リンゴの腸内フローラ改善効果はペクチンによることが示唆された．

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