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In vitro fermentability of a pectin fraction rich in hairy regions

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Abstract

Pectins are widely used in the food industry as gelling agents, and are also recognised to promote human health as dietary fibre. Commercially available pectins are similar in composition, with high uronic acid contents. A pectin rich in hairy regions was extracted under alkaline conditions. This sample contained 56 g neutral sugars but only 15 g uronic acids/100 g and had a very complex molecular structure, with highly branched arabinan side chains. It was subjected to *in vitro* fermentation with fresh human faecal bacteria and compared to a commercial pectin and a larch arabinogalactan. The two pectins were metabolised completely within 6 h, whereas approximately 20% of the arabinogalactan remained un-degraded after 24 h. Higher amounts of short-chain fatty acids were produced from the pectin hairy regions than from the commercial preparation with propionate being more than doubled. Concomitantly, the pH was lowered to a larger extent. These two features are of interest from a physiological point of view, since they are considered to be beneficial to colon health. Thus, pectin fractions rich in hairy regions might be an interesting dietary fibre with slightly improved physiological properties compared to commercial pectins.

Keywords: Pectin; Hairy regions; Neutral sugars; *In vitro* fermentability; Short-chain fatty acids

1. Introduction

Pectins, mainly found in the middle lamella and primary cell wall of higher plants, are a very heterogeneous group of polymers with respect to molecular weight (MW), polydispersity and chemical structure. Their major component is α-D-1,4-galacturonic acid (GalA), whose carboxyl groups can be methyl esterified to different extents. Pectins consist of smooth and hairy regions. Smooth regions are built up by partly methoxylated linear chains of α-D-1,4-GalA units, whereas neutral sugars (NS) are either present in low amounts or completely absent (Brett & Waldron, 1990). The hairy regions consist of rhamnogalacturonan I and II. Rhamnogalacturonan I (RG I) is the second important type of polysaccharide of pectins. Its backbone consists of an alternating sequence of 1,4-linked α-D-GalA and 1,2-linked α-L-rhamnose. Branching sites are located on the rhamnose units. Side chains consisting of arabinans, galactans or two different types of arabinogalactans can be attached at position *O*-4 (Carpita & Gibeaut, 1993). Rhamnogalacturonan II (RG II) is a complex pectic polysaccharide with a low MW, whose backbone only contains GalA. The side chains consist of rhamnose and some rare sugars, such as apiose, 2-*O*-methyl-xylose, 2-*O*-methyl-fucose, 3,4-linked fucose, 2-linked glucuronic acid, aceric acid, and others (Vidal et al., 2000).

Pectins are used in food as additives due to their ability to form gels at low concentrations. Since they are not metabolised during the passage through the human digestive tract, they are considered as dietary fibre, and have gained interest in the last decades because of their postulated positive effects on health such as cholesterol-lowering (Brown, Rosner, Willet, & Sacks, 1999), cancer-preventing (Umar, Morris, Kourouma, & Sellin, 2003), and blood

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glucose-regulating effects (Jenkins et al., 1995). Nowadays, pectins are still extracted with the main purpose of being added to food as gelling agent, as thickener and stabiliser. Extraction is carried out with diluted acid followed by precipitation with an alcohol. The resulting polysaccharides are high in GalA (usually >75/100 g ash-free dry matter (May, 1990)) and low in NS contents. Thus, industrial pectins result to be rather similar in their composition and structure, independently on their source and/or modification such as partial saponification and partial hydrolysis. In a previous work (Gulfi, Arrigoni, & Amadò, 2005), industrial apple and citrus pectins were shown to have GalA and NS contents in typical ranges of 50–75 g GalA and 5–20 g NS/100 g, respectively. Large homogalacturonan (HG) and rather small RG I regions, as well as similar NS side chains (small galactans and arabinogalactans) were modelled in the pectin structures. The minor compositional and structural variations were shown not to influence the *in vitro* fermentability pattern of the pectins, in case their degree of methoxylation (DM) was similar. It was supposed that larger differences were probably necessary to affect the fermentation behaviour of these natural polymers, which are very easily metabolised by colonic bacteria. The final aim would be to identify pectins with improved physiological properties such as an enhanced short-chain fatty acid (SCFA) production or slower fermentability. To study this further, an alternative extraction method was applied to yield pectin fractions which differ from commercial pectins in NS and GalA distribution and in structure in general. Alcohol insoluble residue (AIR) of apple was extracted under alkaline conditions. The resulting pectin, rich in NS (hairy regions), was chemically characterised and investigated for its physiological behaviour *in vitro*. It was compared to a commercial pectin with high GalA content and to a branched neutral polysaccharide, arabinogalactan from larch.

2. Experimental

2.1. Materials

AIR of ripe apples (*var.* Cloche), isolated and analysed by Fischer and Amadò (1994), was used as starting material for the extraction of pectin hairy regions under alkaline conditions. The extraction procedure of Oosterveld, Beldman, Schols, and Voragen (1996) was applied with minor modifications. 570 ml NaOH 4 M containing $NaBH_4$ 0.01 M were added to 20 g of AIR and stirred at 80 °C for 2 h. The suspension was filtered through a glass filter (pore size 16–40 μm; Duran-Schott, Mainz, D). To the remaining mash NaOH 4 M 1:1 (v/v) was added again. The suspension was kept at 80 °C for 2 h, filtered, cooled down and the two filtrates were pooled. After neutralisation with HCl 37%, the solution was dialysed (MW cut-off 12–14 kDa; ServaPor 29 mm, Serva, Heidelberg, D). In order to remove co-extracted xyloglucans, the isolate was inoculated with a xyloglucan-specific *endo*-1,4-β-D-glucanase preparation (gift from Novozymes A/S, Bagsvaerd, DK) from *Aspergillus aculeatus*, which was checked previously by Lutz (2003) for side activities on different poly- and oligosaccharides. A suspension of 6 g sample in 900 ml NaOAc 0.05 M (pH 3.5) was incubated with 54 ml glucanase solution (1% (w/v)) at 37 °C for 24 h. The enzyme was inactivated for 10–15 min at 100 °C and, after cooling, the reaction mixture was dialysed as described above and freeze-dried.

A commercial apple pectin, Apple LM ULV (a low methoxyl and low viscosity preparation from Obipektin AG, Bischofszell, CH), and a larch arabinogalactan (Larex Inc., White Bear Lake, USA) were used as substrates for comparative purposes in this study.

2.2. Composition and physical properties

Uronic acids (UA) were determined spectrophotometrically according to Blumenkrantz and Asboe-Hansen (1973), and NS content was assessed by GLC-FID after hydrolysis and derivatisation of the sugar monomers to alditol acetates, as described earlier (Gulfi et al., 2005).

Before methylation analysis, reduction of UA was performed. Methylation analysis and the subsequent modelling of the structure were carried out as described by Gulfi et al. (2005).

The DM was determined by HPLC according to a method published by Voragen, Schols, and Pilnik (1986), with some modifications (Gulfi et al., 2005). The same method was applied to check pectin hairy regions for residual acetate, originating from the buffer used to enzymatically degrade xyloglucans.

For the determination of MW and intrinsic viscosity (IV), the samples were suspended in $NaNO_3$ 0.1 M, heated at 70 °C for 10 min and kept to hydrate at 4 °C over night. After filtration (0.2 μm, Titan Syringe Filters, Infochroma AG, Zug, CH), high performance size-exclusion chromatography was performed as described by Gulfi et al. (2005) with some modifications. Mobile phase was $NaNO_3$ 0.1 M. The light scattering (LS) detectors were calibrated with a pullulan standard with a MW of 212 kDa (Shodex Standards, P-82, Showa Denko K.K., Kawasaki, J). For Apple LM ULV the refractive index increment was calculated by measuring the on-line refractive index (RI) response of solutions with concentrations between 0.4 and 2 mg/ml, (a value of 0.158 ml/g was obtained). For the pectin hairy regions a refractive index increment from literature was taken (0.146 ml/g; Theisen, Johann, Deacon, & Harding, 2000). Peak molecular weights (M_p) were calculated from the LS and corresponded to the MW of the most represented polymer populations of a sample.

2.3. In vitro fermentation experiment

To test fermentability, the *in vitro* method described by Lebet, Arrigoni, and Amadò (1998) was used. Substrates

Table 1
Chemical composition and physical features of the investigated samples

	Pectin hairy regions	Apple LM ULV	Arabinogalactan
Dry matter (g/100 g)	96	91	92
Uronic acids (g/100 g)	15	68	*nd*
Neutral sugars (g/100 g)	56	9	*nd*
Arabinose	*38*	*<1*	10
Galactose	*8*	*4*	68 [a]
Glucose	*3*	*2*	*nd*
Xylose	*3*	*1*	*nd*
Rhamnose	*1*	*1*	*nd*
Fucose	*1*	*<1*	*nd*
Mannose	*3*	*<1*	*nd*
DM (%)[b]	<1	40	*nd*
M_p (kDa)[c]	34	28	*nd*
Intrinsic Viscosity (dl/g)	0.2	0.4	*nd*

nd, not determined. $n = 2$–4, SD < 7%.
[a] SD = 12%.
[b] Degree of methoxylation.
[c] Peak molecular weight.

hydrated over night were incubated at 37 °C for 24 h with fresh human faeces under continuous shaking and strictly anaerobic conditions. Sampling was done after 0, 1, 2, 3, 4, 6, 8, and 24 h, since pectins are easily fermentable substrates. Pectins and arabinogalactan were incubated and analysed in duplicates ($n = 2$). Blank (only inoculum) and lactulose samples were fermented as controls ($n = 1$). Total gas production, pH decrease, and production of SCFA were measured as described by Lebet et al. (1998). After centrifugation, supernatant and solid residues were separated and frozen or freeze-dried, respectively. Total substrate disappearance (as sum of UA and NS) of pectins, arabinogalactan and blank was assessed spectrophotometrically as described by Gulfi et al. (2005), whereas lactulose disappearance was evaluated by high performance anion exchange chromatography with pulsed amperometric detection as described by Scheiwiller, Arrigoni, Brouns, and Amadò (submitted).

3. Results and discussion

3.1. Chemical and structural characterisation

In Table 1 chemical composition and physical features of the pectin hairy regions and of the two samples which were fermented for comparison are presented. Moisture contents were low (max. 9/100 g). Apple LM ULV, which

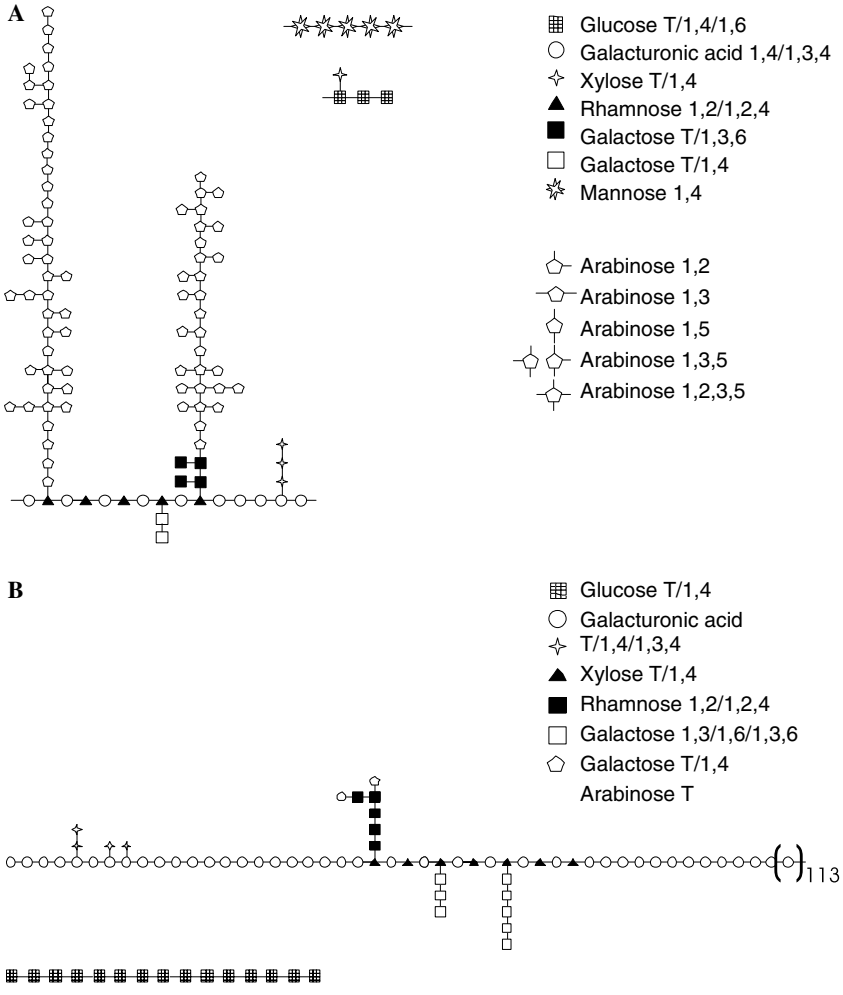

Fig. 1. Molecular model for the pectin hairy regions (A) and for Apple LM ULV (B).

had been selected because of fruit source, low DM, low viscosity, and good solubility, had the typical sugar composition of industrial pectin preparations, with high UA and low NS contents. According to the producer, commercial, non-standardised pectins contain 2–3 g ash/100 g. The mineral content of the arabinogalactan was not known but estimated to be low: according to the specifications the sample should consist of approx. 95/100 g arabinogalactan, on a moisture-free basis. Water content should range between 2 and 6/100 g; slightly more water was found, probably due to hydration during storage. The measured arabinose (Ara) and galactose (Gal) contents of the arabinogalactan were lower than expected from the producer's specifications. It is not known which method was used by the producer to determine Ara and Gal, therefore it is difficult to compare the actual yield of arabinogalactan with the specification value.

Acetate was quantified in the pectin hairy regions and less than 0.5/100 g were found, hardly any residual acetate from the buffer was left after dialysis. The DM of the pectin isolate was approx. 0%, whereas Apple LM ULV had a DM below 50%, as expected for low methoxyl pectins.

Only 15/100 g UA were present in the pectin fraction obtained by alkaline extraction, since most of the homogalacturonan backbone was degraded and its fragments were removed by dialysis. The main constituent was Ara, a NS which is sensitive to acids (Guillon & Thibault, 1990) and therefore present only in low amounts in commercial pectins. The alkaline extraction used in this experiment favoured the preservation of the arabinan side chains of pectin. In industrial pectins however, the predominant NS is usually Gal, in apple pectins possibly glucose originating from residual starch (Kravtchenko, Voragen, & Pilnik, 1992). Correspondingly, the main NS component of Apple LM ULV was Gal followed by glucose, whereas Ara was present in very low amounts. The Ara:Gal ratio in the isolated NS-rich pectin fraction was 5:1, whereas in the larch arabinogalactan it was 1:7, which corresponded well to the 1:6 found by Fitzpatrick, Roberts, and Witherly (2004) for the same commercial preparation. However, both polysaccharides were characterised by a high degree of branching (DB).

Both pectins had a comparably low IV and their M_p was also rather similar. Physical characteristics were not assessed for the arabinogalactan. The arabinogalactans investigated by Fitzpatrick et al. (2004) had a MW of approx. 20 kDa.

The molecular structures of the NS rich pectin and of Apple LM ULV were modelled after methylation analysis. An average molecule of 100 sugar residues for the pectin hairy region is shown in Fig. 1A. Because of the predominance of GalA in Apple LM ULV, 200 sugar residues were used to picture the molecular model (Fig. 1B), in order to make small RG regions and NS side chains better visible. The mol% figures for the single sugar residues are given in Table 2.

Table 2
Molar sugar distribution (mol%) for the pectin hairy regions and for Apple LM ULV

Sugar residue	Pectin hairy regions	Apple LM ULV
Galacturonic acid		
t-GalA*p*	0.08	0.84
1,3-GalAp	–	0.28
1,4-GalA*p*	7.83	73.63
1,2,4- or 1,3,4-GalA*p*	0.41	1.53
1,4,6-GalA*p*	0.06	1.57
	8.38	**78.34**
Rhamnose		
t-Rha*p*	–	0.18
1,2-Rha*p*	1.21	2.28
1,2,4-Rha*p*	2.32	1.11
1,2,3,4-Rha*p*	–	0.19
	3.53	**3.75**
Arabinose		
t-Ara*f*	24.60	0.94
t-Ara*p*	1.93	–
1,2-Ara*f*	0.37	–
1,3-Ara*f*	3.21	–
1,5-Ara*f*	20.13	–
1,3,5-Ara*f*	14.64	–
1,2,3,5-Ara*f*	5.14	–
	70.02	**0.94**
Galactose		
t-Gal*p*	3.60	0.92
1,3-Gal*p*	–	1.45
1,4-Gal*p*	1.08	2.73
1,6-Gal*p*	0.18	0.60
1,3,4- or 1,2,4-Gal*p*	0.10	–
1,3,6-Gal*p*	2.13	0.35
1,4,6-Gal*p*	0.14	0.16
	7.22	**6.20**
Xylose		
t-Xyl*p*	1.21	2.58
1,4-Xyl*p*	1.68	0.77
	2.89	**3.36**
Glucose		
t-Glc*p*	0.87	1.42
1,4-Glc*p*	1.11	5.88
1,4,6-Glc*p*	0.32	0.11
	2.31	**6.76**
Mannose		
1,4-Man*p*	**4.66**	–

Bold values correspond to the sum of each monosaccharide group.

Most of the backbone of the pectin hairy region was composed of RG (RG:HG = 7:5), whose DB was very high (0.66, whereas Apple LM ULV had a DB = 0.35). Small amounts of xyloglucan and mannan were modelled beside the pectin. Only few Gal residues were found. The arabinogalactans contained above all Ara, and the galactans were very small. A xylogalacturonan region was modelled beside the RG. Lutz (2003) also detected short 1,4-bound xylooligomers, and proposed the xylogalacturonan to be a small side chain attached to a rhamnose residue. In the model presented here, the xylooligomer was positioned on a GalA residue of the backbone. The arabinan side chains appeared to be very complex and highly branched. It is known that

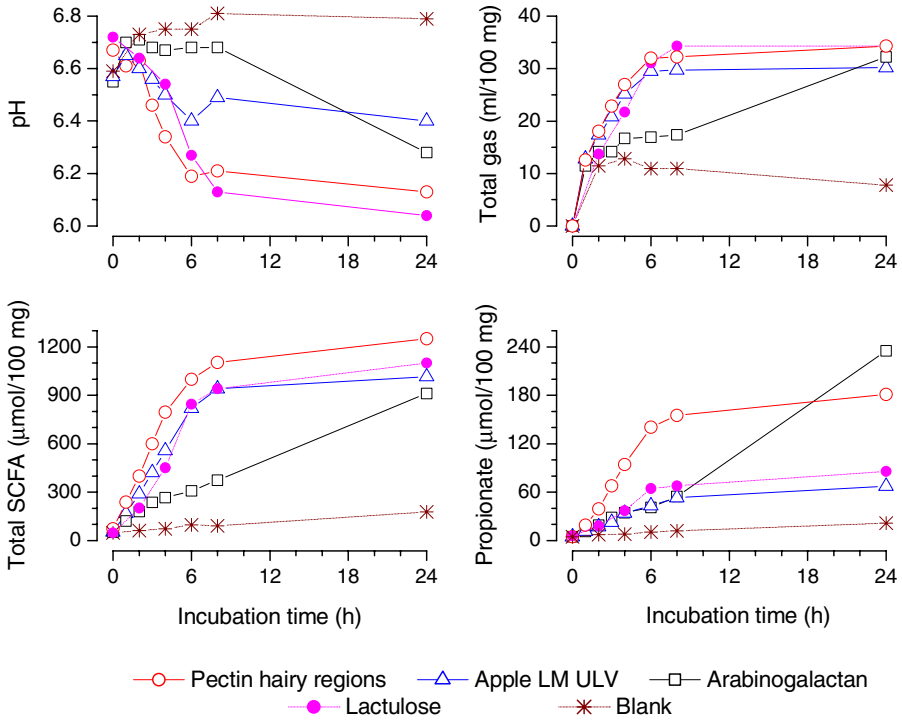

Fig. 2. pH, total gas, total SCFA and propionate production during *in vitro* fermentation of different samples.

Ara residues in hairy regions of apple pectins are present as highly branched side chains (Aspinall & Fanous, 1984). Using different extraction methods, Schols, Vierhuis, Bakx, and Voragen (1995) isolated hairy regions of apple pectin and identified high-MW fragments, where RG rich in Ara and xylogalacturonans were present. They detected 44–66 mol% Ara in the different hairy regions, which had a residual GalA content of 12–25 mol%. A similar mol% distribution for these two sugars was found in the present work: 70 mol% Ara and 8 mol% GalA (Table 2). Oosterveld, Beldman, Schols, and Voragen (2000) isolated pectin hairy regions from sugar beet pulp with NaOH 4 M and found a similar composition as the present one. Their fractions also contained highly branched arabinans. As in the hairy regions obtained here, 1,3,5-linked Ara was predominantly identified as branch point. Lutz (2003) extracted pectic fractions with NaOH 4 M from the same AIR as used here, but after sequential extraction with CDTA, Na_2CO_3 and NaOH 0.1 M. Compared to the hairy regions presently obtained, the fraction isolated by Lutz contained higher amounts of glucose and Gal and less Ara, the arabinans being highly branched as well.

The hairy region of Apple LM ULV was very small compared to the smooth region (RG:HG molar ratio = 8:70). The DB was much lower than that of the hairy regions extracted presently. Small galactans and type II arabinogalactans were modelled. The few Ara residues, which were found, were terminal and were modelled in the arabinogalactan. 1,4-Linked glucose was probably originating from starch, and the few xylose residues were assumed to be part of a small xylogalacturonan region.

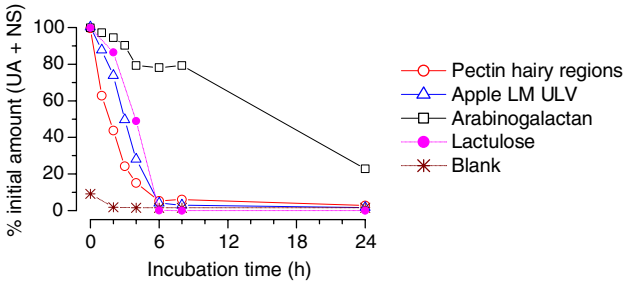

Fig. 3. Substrate disappearance during *in vitro* fermentation of different samples (values for Blank in mg).

The arabinogalactan was not subjected to methylation analysis. According to the literature, arabinogalactan in larch wood consists of a β-1,3-galactan to which β-1,3-linked arabinooligosaccharides or β-1,6-linked galactooligosaccharides are attached at position 6 as side chains (Chandrasekaran & Janaswamy, 2002; Sakamoto & Thibault, 2001).

3.2. *In vitro* fermentability

In order to investigate their physiological behaviour, the pectin hairy regions were fermented *in vitro* together with the other two substrates for comparison. As shown in Figs. 2 and 3, the pectin hairy regions were fermented more extensively than Apple LM ULV and the larch arabinogalactan in particular. pH and gas production were influenced in a stronger way by this substrate. The total amount of SCFA produced from the pectin hairy regions after 24 h was higher than from both lactulose and Apple LM ULV,

whereas the rate of production was similar for these three substrates. In contrast, SCFA were released nearly linearly and much more slowly from the arabinogalactan, but the end value at $t = 24$ h was almost as high as for Apple LM ULV. Acetate accounted for 75–78 mol% of the total SCFA for both pectin samples compared to 63 mol% for the arabinogalactan, whereas butyrate ratios of 10–13 mol% were found for all three substrates (results not shown). Most interestingly, the higher content of NS of the pectin extracted under alkaline conditions led to a clear shift in SCFA ratios. Propionate ratios of 7 and 15 mol% were determined for Apple LM ULV and for the pectin hairy regions, respectively. Additionally, the absolute amount of propionate produced from pectin hairy regions was more than doubled compared to Apple LM ULV (Fig. 2). However, the highest end value was reached by the arabinogalactan, from which propionate was formed at an almost linear rate. It has been postulated that propionate plays a role in the cholesterol-lowering effect of dietary fibre by inhibiting the utilisation of acetate for cholesterol synthesis (Wolever, Spadafora, & Eshuis, 1991). Propionate also appears to influence glucose and insulin levels in blood (Jenkins et al., 1995). This SCFA was furthermore shown to induce apoptosis in colorectal tumour cell lines, albeit being less effective than butyrate but more effective than acetate (Hague, Elder, Hicks, & Paraskeva, 1995).

Total substrate disappearance was fast for both pectin samples as well as for lactulose (Fig. 3). The pectin hairy regions turned out to be the most efficiently degraded substrate, particularly in the initial phase: after 2 h of fermentation more than 50% of the pectin hairy regions had been already metabolised. The two pectins and lactulose were completely fermented within 6 h. In contrast, about 20% of the initial amount of larch arabinogalactan was still present after 24 h of fermentation. The arabinogalactan was characterised by a slow fermentability which could be well approximated by a linear relationship with a slope of $-3\,h^{-1}$ (linear relationships for Apple LM ULV and the NS-rich pectin fraction were much steeper, with slopes of -17 and $-15\,h^{-1}$, respectively). Larch arabinogalactan has a helical structure consisting of a β-D-galactan main chain with arabinose/galactose side chains which possibly protect the helix core from external influences such as microbial attack (Chandrasekaran & Janaswamy, 2002). It has previously been shown that highly branched arabinogalactan fractions are digested less efficiently by colonic bacteria (Salyers, Arthur, & Kuritza, 1981).

4. Conclusions

Taking all fermentation parameters into account, the pectin hairy regions revealed to be a very readily fermentable substrate for human colonic bacteria. It was degraded at a similar rate as Apple LM ULV but showed a more substantial impact on pH and SCFA production. Moreover, the SCFA pattern obtained by fermenting pectin hairy regions was characterised by a clearly higher amount of propionate. These features are of physiological interest, since a low pH and high concentrations of butyrate and propionate are known to be beneficial to colon health. A shift to higher propionate values can therefore be considered to improve the physiological properties of pectins, which are above all acetate producers. Compared to industrial pectins high in GalA, pectin hairy regions would be a better candidate for fibre enrichment. Whether the latter would exhibit similar physiological characteristics *in vivo*, cannot be predicted from the present study. Moreover, at present it is difficult to estimate whether an alkaline extraction would be industrially feasible as far as costs and yields are concerned.

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