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Production of Short Chain Arabinooligosaccharides by Hydrolysis of Arabinan Using a Commercial Mixed Glycanase Preparations

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Abstract

Oligosaccharides are bioactive carbohydrates that have properties beyond their content of calories; however, structure and degree of polymerisation were believed to play a major role in such bioactivity in particular the ones with degree of polymerisation 2-10. Oligosaccharides of specific degree of polymerisation were prepared by hydrolysis of arabinan using a commercial mixed glycanase preparation (VISCOZYME™ L) in a membrane reactor. A lab scale ultrafiltration unit was operated at a pressure of 1 bar, a stirrer speed of 250 rpm and a working volume of 200 ml. The temperature was controlled at 35°C and pH at 4.5.

Enzymatic hydrolysis of arabinan produced oligosaccharides with a wide range of degree of polymerisation values but favoured degree of polymerization > 10. In batch reaction arabinan concentration 5% WV^{-1} was higher in terms of quantities of oligosaccharides than 2.5% though the production delayed (5 h vs. 3 h for 5% and 2.5% respectively). Oligosaccharides of degree of polymerisation 2-10 were produced after 2 hrs of reaction time when arabinan concentration was 5% WV^{-1} using fed-batch hydrolysis. Diafiltration using buffer as substitute for hydrolysate has failed to produce similar results in comparison to fed-batch. Small amount of AOS of dp 2-10 were produced only at 3 h.

We showed that production of arabino-oligosaccharides with specific degree of polymerisation was achievable within short time (2 h). Overall results suggested that hydrolysis of arabinan in a controlled reactor to produce short chain AOS was mainly affected by; substrate concentration and time of reaction.

Keywords: Prebiotics; Arabino-oligosaccharides; AOS; Ultrafiltration; Enzymatic hydrolysis

Introduction

There has been increased interest in recent years in the application of oligosaccharides as prebiotic food ingredients [1]. Polysaccharides of the cell wall are not hydrolysed by human intestinal brush border enzymes, therefore they have potential to use for production of a wide range of oligosaccharides that serve as prebiotics [1,2]. An example of such a polysaccharide is arabinan. Arabinan is a branched pectic polysaccharide linked by 1,3 and 1,5 α-L-arabinofuranosyl residues [3] extractable from sugar beet pulp by hot alkali [4]. Sugar beet pulp is a co-product of the beet sugar industry in Europe where around 100 million tons of beets are processed annually to produce around 15 million tons of white sugar while the remains contain 5.5 million tons of dried pulp [5].

The efficacy of prebiotics toward promoting human health is strongly related to their chemical structure [6,7]. It was found that Arabino-oligosaccharides (AOS) have the ability to support growth of some species of Bifidobacteriumin pure culture [8] and may have potential as prebiotics. In addition, AOS have been studied in mixed faecal culture [9,10] and they resulted in a stimulation of bifidobacteria to varying extent, depending on molecular weight. Accordingly, lower molecular weight fractions of AOS have led to maximum growth in bifidobacteria [9]. More recently, Onumpai et al. [11] have investigated fermentation of pectic substrates in a human gut model including arabinan and oligoarabinosides (average dp 6), and found that the structure of oligosaccharides fractionated from pectins had a great impact on their fermentation by human faecal bacteria with greatest bifidogenic activity seen with low-molecular weight of arabinooligosaccharides and oligogalactosides.

Most of the commercially available oligosaccharides are manufactured by the use of enzymatic methods including depolymerisation of macromolecules [12,13] and synthesis of oligosaccharides using glycosyltransferases or glycosidases [14]. A range of endo- or exo-glycosidases can be used for the depolymerisation of polysaccharides to produce oligosaccharide mixtures with various degrees of polymerization (dp). Such processes, if accompanied by an ultrafiltration (UF) separation step could be used to produce oligosaccharides of particular molecular weights [15,16]. Enzymatic manufacturing of oligosaccharides has been reported by many researchers [17,18], however, manufacturing of novel oligosaccharides and / or optimizing production is still a challenge for the food industry.

The aim of this study was to investigate the effect of different concentrations of arabinan and different ratios of enzyme-arabinan in production of AOS (in particular bioactive range 2-10 dp) by using enzyme membrane reactors.

Materials and Methods

Substrate preparation

Arabinan was kindly provided by British Sugar (Peterborough, UK) and was reconstituted in water and ultra-filtered to remove carbohydrates of ≤ 5 KDa molecular weight. Ultrafiltration was carried out using a polyethersulfone membrane with 5 KDa MWCO, 40 cm^2 effective area (Millipore, Billerica Massachusetts, USA). Subsequently 0.2 M sodium succinate buffer, pH 4.5, equivalent to the removed permeate volume was added each hour. The applied pressure for preparative ultrafiltration was 2 bars and temperature was maintained at 10°C.

Enzyme preparation

Viscozyme™ L was obtained from Novo Nordisk (Novozyme, Bagsvaerd, Denmark). Viscozyme was diluted byone part to one part with 0.2 M sodium succinate buffer, pH 4.5, then ultrafiltered as described above. Buffer equivalent to the removed permeate volume was added each hour. The ultrafiltered enzyme was diluted againwith buffer and theprotein concentration was determined using bicinchoninic acid (BCA) method [19] which was 54 mg ml^{-1}.

Enzymatic assay

All enzymatic reactions were carried out at pH 4.5 and 35°C. To determine the ability of viscozyme tohydrolyse arabinan, a reaction mixture (200 ml) was prepared by mixing 166 ml of arabinan solution (5% arabinan, WV^{-1}) with 34 ml of viscozyme solution. Sodium azide, 0.1% WV^{-1} (Sigma-Aldrich, Dorset, UK) was added to inhibit any microbial growth. The mixture was incubated at 35°C for 24 h. Hydrolysis of arabinan was monitored and 3 ml of the solution were pipetted at different time intervals (0, 1, 2, 4, 6, 10, and 24 h of incubation) and liberated arabinose was quantified with reference to L-(+)-Arabinose (Sigma-Aldrich, Dorset, UK) as standard. Perchloric acid (BDH, Poole, UK) 200 µl of 70% was added to each sample in order to stop the enzymatic reaction. The samples were mixed thoroughly and left to settle for 5 min. The solutions were centrifuged at 10000x g for 5 min, the supernatant collected and adjusted to 3 ml using buffer. Arabinose as reducing sugar was measured according to Somogyi [20].

Ultrafiltration unit

A laboratory scale ultrafiltration unit (GYROSEP™ 300 stirred cell, Techmate Ltd, Milton Keynes, UK) and a polyethersulfone membrane with 5 KDa MWCO, 40 cm^2 effective area (Millipore, Billerica Massachusetts, USA) were used. The unit was operated at apressure of 1 bar, a stirrer speed of 250 rpm and a working volume of 200 ml. The temperature was controlled in a water bath at 35°C and maintained for 24 h. All solutionsused in this experiment were buffered at pH 4.5 with 0.2 M sodium succinate buffer.

After each run of the experiment the ultrafiltration unit was washed with 300 ml deionised water without pressure for 10 min followed by 200 ml 0.2 M NaOH for 30 min withoutpressure and a further 15 min under a pressure of 1 bar. The unit then was re-washed again for 10 min under 1 bar pressure with 300 ml deionized water. The membrane was kept in deionised water at 4°C for further use. Between runs, the membrane was validated against a water flux of 12 ml $min^{-1}$and permeated under a pressure of 1 bar.

Production of AOS

To understand the enzyme-substrate behaviour in producing different sizes of AOS two concentrations of arabinan were chosen 2.5% and 5% WV^{-1}. Production of AOS was performed under variable ratios of enzyme-substrate (Table 1). Depending on the best mixing ratio of enzyme to substrate two sets of conditions were conducted: the first involved substrate (5%, WV^{-1}, 100 ml) mixed with enzyme solution.

Substrate		Mixing Ratios	
		Arabinan	Viscozyme (54 mg protein ml^{-1})
Arabinan solution (5% WV^{-1})	Reaction 1	83	17
	Reaction 2	67	33
Arabinan solution (2.5% WV^{-1})	Reaction 1	83	17
	Reaction 2	67	33

Table 1: Arabinan - viscozyme ratios.

Permeate was substituted by arabinan (5% WV^{-1}) in hourly basis in a fed-batch manner for 6 h in total. This was conducted to keep the substrate concentration almost constant. Feed-batch enzymatic hydrolysis system is recommended over batch system in terms of quantities of produced sugars [21] and possibility of wider range of AOS with variable degree of polymerisation.

The second involved mixing ofsubstrate (5%, WV^{-1}, 100 ml) with enzyme preparation of similar volume. Buffer (pH 4.5), equivalent to permeate, was added every hour for 6 hrs in total; this diafiltration approach resulted in prolonged enzymatic digestion of the partially hydrolysed arabinan remaining in the reactor. Moreover, Rezaei et al. [22] found that diafiltration improved the activity of xylanase and cellulase as commercial preparations of enzymes contain chemical additives to enhance enzyme recovery. Such chemicals may interfere negatively with enzyme activity and can be removed by diafiltration.

The membrane of diafiltration was cleaned as mentioned above after running the experiment to insure a good flow rate.

Permeate analysis

To monitor arabinan hydrolysis and the liberation of arabinose, determination of reducing sugar and total carbohydrate in permeate was conducted according to Somogyi [20] and Dubois et al. [23] respectively.

To determine the different dp of hydrolysate the permeate was collected, freeze dried and applied to the Bio-Gel P2 column(Bio-Rad Laboratories Ltd. Hemel Hemp stead Hertfordshire, UK) at a concentration of total carbohydrate of 50 mg ml^{-1}. A volume (10 ml) was applied to the column using deionized water at a flow rate of 2.2 ml min-1 and elution monitored by refractive index. Bio-gel P2 column was calibrated using glucose and dextrans of different molecular weight (500, 1000, 2000 and 5000 Da MW) (Sigma-Aldrich, Dorset, UK).

Results and Discussion

Enzymatic hydrolysis of arabinan

Viscozyme was able to release 0.001 mmol arabinose min^{-1} per mg protein. After 6 h, the liberated arabinose reached a maximum quantity of 3.5 g out of 5.0 g (WW^{-1}) of the initial arabinan or 70% (Figure 1). The remaining 30% of arabinan may represent unhydrolyzable backbone of original polysaccharide.

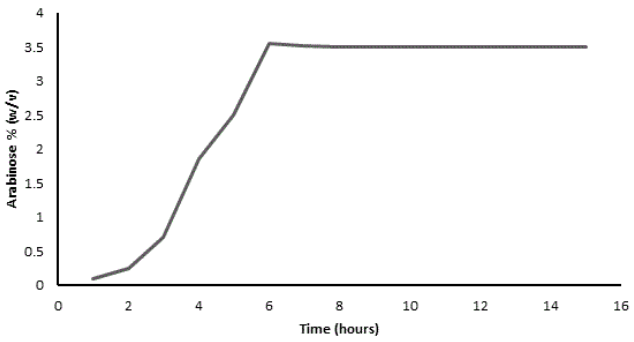

Figure 1: Arabinose release by viscozyme-catalysed arabinan hydrolysis in a batch reaction. Arabinan (5% WV^{-1}) was mixed with viscozyme (54 mg protein ml^{-1}) in ratios of 83 % (VV^{-1}) arabinan and 17% (VV^{-1}) viscozyme. The mixture was incubated at 35°C for 24 h and samples were taken to quantify arabinose amount released in the medium using arabinose calibration curve.

In batch hydrolysis, two concentrations of arabinan were initially used 5% and 2.5% WV^{-1} with different mixing ratios. Total sugar (TS) concentration in the permeate was less when 2.5% was used consequently, resulting in a higher reducing sugar (RS) to total sugar (TS) ratio. This indicated that arabinose was the major product of hydrolysis in the permeate. Moreover, as reaction progressed and as arabinan has been depleted, viscozyme has more opportunity to react with unhydrolysed part of arabinan. Such a situation has resulted in more reducing sugar in the permeate after 5 h of reaction time (27 and 20.4 for reactions involved 2.5% arabinan vs. 6.1 and 19 for reactions involved 5% arabinan) (Table 2).

This result is supported by a study of Spagnuolo et al. [24] who used enzymatic hydrolysis of arabinan followed by ultrafiltration. They found a progressive increase in reducing sugar (arabinose) by increasing enzyme concentration and / or reducing substrate concentration. As the aim of this study was to produce variable oligosaccharides rather than arabinose, 5% arabinan with 83:17 mixing ratio was used thereafter.

Gel Permeation (Bio-Gel P2)

Batch reaction: Arabinan 5% solution was prepared and was found to be 96.5% (WW^{-1}) free of arabinose after ultrafiltration as detected by Bio-gel P2 column. The ultrafiltered arabinan contained some oligosaccharides of a dp > 10 and a polysaccharide of a molecular weight ≥5 KDa.

Production of AOS (dp 2-10) occurred at 5 h of reaction time with 5% arabinan (Table 3A). Production of AOS (dp 2-10) in lesser amounts occurred from 2 h - 3 h of reaction time with 2.5% arabinan (Table 3B). Relative area under curves indicated that arabinan at 5% resulted in more AOS (dp 2-10) than that of 2.5%.

	Time (h)											
	0		1		2		3		4		5	
	TS (SD)	RS /TS	TS (SD)	RS / TS	TS (SD)	RS / TS	TS (SD)	RS / TS	TS (SD)	RS / TS	TS (SD)	RS/TS
Reaction 1	338 (19.1)	3.2	151.39 (13.7)	7.1	239.8 (8.5)	7.2	396 (50.7)	4.3	517.1 (18)	5.1	596.2 (50.3)	6.1
Reaction 2	277.7 (6.6)	4.4	105.3 (8.1)	12.8	197 (4.1)	11.3	248.3 (9.6)	15.1	267 (13.1)	20.5	305.2 (11.6)	19
Reaction 3	63.3 (2.8)	6.8	61.0 (4.8)	16	63.3 (3.4)	30	93.3 (11.4)	31	119.1 (2)	33.4	140 (7.5)	27
Reaction 4	185.4 (3.0)	6.3	96.6 (3.6)	16.5	127.3 (8.5)	22	179 (15.9)	20	216.1 (28.3)	18.7	211.6 (8.3)	20.4

Table 2: Total (TS) and reducing sugar (RS) of arabinan hydrolysis in batch reactor (mg/ml). Four mixing ratios of arabinan with viscozyme as mentioned in Table 1 were used in the reactor then permeates were measured after 1, 2, 3, 4 and 5 h of incubation. Total sugar and reducing sugar, equivalent to arabinose, were averaged for triplicate samples. Time 0 represents values in the feed. RS / TS were calculated by dividing average reducing sugar values by total sugar values for each time.

Production of AOS using 5% arabinan

The percentage content of reducing sugars under both conditions of substrate or buffer addition did not change significantly over time and remained less than 18.5% of the total sugar. Meaning that permeate contents of non-reducing sugars are the major component of the permeate (Table 4).

Structure of AOS under feed-batch condition

AOS (dp 2-10) in the permeate started after 2 h of reaction time. AOS of dp 2 and 3 but not dp 4-10 remained in the permeate up to 5 h of reaction time. AOS (dp 2-10) reappeared in the permeateat 6 h with smaller quantities than that of 2 h (Figure 2).

Time of reaction (hours)	Area under curve of each fraction(s)			
	V0	DP > 10	DP 2-10	Arabinose
3	0.24	0	0	0.14
5	0.28	0.06	0.12	0.18

Table 3A: Relative area of peaks of arabinan (5% or 2. 5%) hydrolysed by viscozyme (54 mg protein ml^{-1}), samples of the permeate were taken in different times and run through Bio-Gel P2 column at a flow rate of 2.2 ml min^{-1} then detected by a refractive index. Membrane reaction: Arabinan 5% was mixed with viscozyme and incubated at 35°C for 5 h.

Time of reaction (hours)	Area under curve of each fraction(s)			
	V0	DP > 10	DP 2-10	Arabinose
2	0.3	0	0.05	0.16
5	0.2	0	0.01	0.21

Table 3B: Membrane reaction: Arabinan 2.5% was mixed with viscozyme and incubated at 35°C for 5 h.

Time of reaction (hours)	Area under curve of each fraction(s)			
	V0	DP > 10	DP 2-10	Arabinose
3	0.3	0	0.06	0.3
5	0.6	0	0.01	0.17

Table 3C: Buffer addition: Arabinan 5% was mixed with viscozyme and incubated at 35°C for 6 h and buffer (pH 4.5) was added each hour as required.

Time	Feed batch (Substrate)		Diafiltration (Buffer)	
	TS (SD)	RS / TS (%)	TS (SD)	RS / TS (%)
0	480 (4.5)	5.5	509 (34)	3.3
1	142.8 (9.3)	8.6	248.1 (17.2)	3.9
2	199.2 (1.5)	18.5	223.5 (25.7)	7.5
3	270.0 (17.5)	13.5	258.3 (5.5)	7.7
4	226.5 (11.0)	16.4	287.1 (13.5)	9.4
5	331.4 (8.3)	17.2	335.2 (12.5)	8.7
6	318.5 (11.5)	16	450 (8.6)	4.9

Table 4: Total sugar (TS) and values of RS / TS ratios of the AOS production by addition of either substrate (arabinan 5% WV^{-1}) or of buffer (pH 4.5), arabinan mixing ratio was 50%. Substrate or buffer was added for 6 h. TS and RS values were means of 3 replicates. Time 0 represents values in the feed.

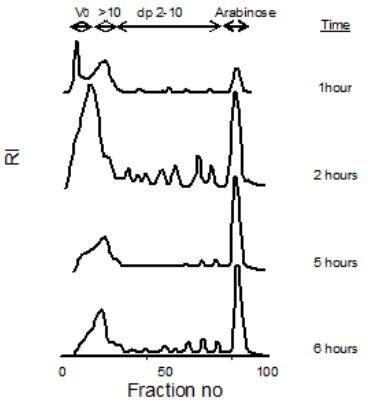

Figure 2: Chromatogram of the permeate of the substrate addition reaction. Arabinan (5%) hydrolysis by viscozyme (54 mg protein ml^{-1}) in a membrane reactor fitted with 5 Kda MWCO. Arabinan was mixed with viscozyme and incubated at 35°C for 6 h and substrate was added each hour as required. Samples of permeate were taken in each hour and run through Bio-Gel P2 column at a flow rate of 2.2 ml min^{-1} then detected by a refractive index.

Structure of AOS under diafiltration condition

AOS (dp 2-10) have been noted in the permeate only after 3 h of reaction time then discontinued. AOS of dps >10 were noted to remain in all reaction times (Table 3C).

Enzymatic hydrolysis of arabinose-containing compounds was reviewed by other researchers [25] using various enzymes which have been classified into groups depending on their mode of action. Endo-arabinases have a random mechanism of attack and generally produce an equilibrium mixture of oligosaccharides. Exo-arabinases cleave monomer units from the non-reducing ends and generally produce arabinose and arabinobiose as equilibrium products [26,27]. Arabinases that may exhibit both endo and exo hydrolysing activity were also reported [26]. In this study viscozyme could not hydrolyse arabinan totally and seemed to act as an endo-arabinase. However, this is not the only criterion to judge an enzyme's behaviour as the level of arabinan branching significantly affects the end products of hydrolysis [27] and some arabinases preferentially act on the side chains leaving the arabinan backbone unhydrolysed.

In the batch membrane reactor, substrate concentration affected the total sugar concentration in the permeate and the type of AOS produced. Initially, viscozyme debranched arabinan producing large fractions (dp > 10) and arabinose.If the substrate concentration was low (2.5%) then viscozyme catalysed further degradation of these large fractions and produced AOS of dp 2-10 within a shortperiod of time (2 h). When the substrate concentration was higher (5%) viscozyme took more time to debranch arabinan. As a consequence, production of AOS of dp 2-10 was achieved at 5 h.

In the substrate fed-reaction, substrate concentration remained constant with some variations in molecular weight, due to removal of hydrolysed substrate. Maximum production of AOS dp 2-10 was achieved after 2 h with a subsequent decline. AOS production then was restricted to dps 2, 3 and > 10. AOS of dp 2-10, in lower amounts,

reappeared after 6 h. This result is consistent with our speculation that viscozyme randomly debranched arabinan into fractions of different dps, favouring dps > 10.

This was supported by the second reaction where buffer was added to the reactor maintaining viscozyme concentration constant while arabinan was depleted. AOS of dp 2-10 were produced only at 3 h. AOS of dp > 10 appeared in the permeate at all reaction times. However, a complete hydrolysis of arabinan was not achieved by viscozyme.

In this experiment the overall results suggested that in a controlled reactor, hydrolysis of arabinan to produce short chain AOS was mainly affected by; substrate concentration and the time of reaction. We found that feed-batch conditions are the best condition to produce arabino-oligosachharides with dp less than 10.

Conclusion

Production of AOS in short period of time (2 h) has a remarkable application in the food industry. It has considerable potential to be developed for new applications other than dairy products, including bakery products, cereals and pasta. Consequently novel prebiotics with good thermal stability are of increasing interest [28]. Arabinan and its oligosaccharides can show such a property and be utilised commercially [4,29-32].

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