Carbohydrate Polymers 115 (2015) 88–92

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Direct study of fluorescently-labelled barley β-glucan fate in an *in vitro* human colon digestion model

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ARTICLE INFO

Article history:
Received 26 April 2014
Received in revised form 5 August 2014
Accepted 10 August 2014
Available online 1 September 2014

Keywords:
β-Glucan
Prebiotics
Capillary electrophoresis
Glucanase

ABSTRACT

β-Glucans from cereals are β(1–3)(1–4)-mixed linkage linear homopolysaccharides of D-glucopyranosyl residues, recently recognised as functional components of foods with benefits in maintaining the health of the digestive tract not least through a prebiotic effect. Here we describe the development of methodology to facilitate the study of β-glucans as prebiotics. Relatively short β-glucan fragments (DP 6–50) were produced by partial hydrolysis of β-glucan fibres with Lichenase then functionalised at their reducing end with a tetramethylrhodamine dye. Their enzymatic break down by human colon microbiota in an *in vitro* fermentation model was examined. Digestion products were isolated by virtue of their fluorescence labels, identified and characterised using capillary electrophoresis and mass spectrometry. Complete digestion of the labelled substrates was indicated, as fluorescently labelled glucose was obtained as the final product. Furthermore, a pathway of enzymatic breakdown was proposed on the basis of a time course experiment; initial fast hydrolysis with an *endo*-1,3(4)-β-glucanase was followed by slow degradation with an *exo*-1,4-β-glucanase and finally slow action of an *exo*-1,3-β-glucanase.

1. Introduction

β-Glucans derived from cereals are increasingly being recognised as functional, bioactive components of foods and beverages (Lazaridou & Biliaderis, 2007; Charalampopoulos, Wang, Pandiell, & Webb, 2002). The consumption of β-glucans has been implicated in several health properties: the lowering of plasma cholesterol levels, reducing glycaemic index, reduced risk of coronary heart disease and of colon cancer (Brennan & Cleary, 2005). Prebiotics are food components that are not hydrolysed by human digestive enzymes in the upper gastrointestinal tract but instead are fermented by and thus stimulate the growth of beneficial bacterial in the colon with a health benefit for the human host (Patel & Goyal, 2012, Gibson & Roberfroid, 1995). In particular a prebiotic effect is characterised by an increase in the population and/or activity of *Bifidobacterium* and *Lactobacillus* species. A prebiotic effect of β-glucans from cereals has been suggested from *in vitro* studies (Vasiljevic, Kealy & Mishra, 2007; Jaskari et al., 1998), *in vivo* animal studies (Snart et al., 2006; Drzikova, Dongowski, & Gebhardt, 2005; Dongowski, Huth, Gebhardt, & Flamme, 2002) and recently in human studies (Mitsou, Panopoulou, Turunen, Spiliotis, & Kyriacou, 2010).

β-Glucans from barley are found in the starchy endosperm cell walls of the grains (Tosh, Wood, Wang, & Weisz, 2004a). They are a linear homopolysaccharides of D-glucopyranosyl residues joined *via* a mixture of β(1–4) and β(1–3) glycosidic linkages. The glucose monomers are arranged in blocks of several consecutive β(1–4) linked residues (mostly trimers and tetramers, and some longer) connected *via* single β(1–3) linkages (Fig. 1). They are typically high molecular weight polymers with reported sizes for β-glucans from barley of 3×10^4 to 3×10^6 g/mol (Lazaridou & Biliaderis, 2007) Their physicochemical properties such as solubility, viscosity and gelation, as well as their physiological function in the gastrointestinal tract are related not only to the size of the polymers but also to their molecular features, in particular the arrangement of β(1–4) and β(1–3) linkages (Tosh et al., 2004a; Tosh, Brummer, Wood, Wang, & Weisz, 2004b; Izydorczyk & Dexter, 2008). Fermentation of β-glucans in the human colon by gut microbiota requires first the hydrolytic breakdown of these polysaccharides to glucose monomers by enzymes endogenous to gut bacteria followed by metabolism to short chain fatty acids and carbon dioxide. The specific pathways of enzymatic degradation have not yet been well-studied.

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http://dx.doi.org/10.1016/j.carbpol.2014.08.056
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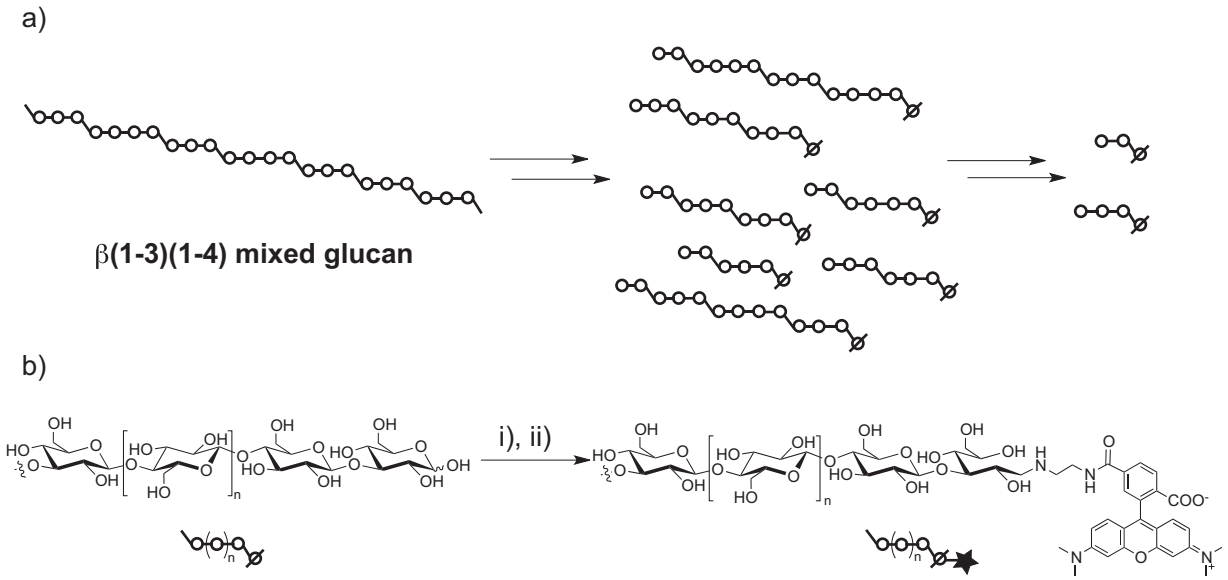

Fig. 1. (a) Action of Lichenase on cereal β(1–3)(1–4)-glucans. (b) Reducing-end labelling with TMR: (i) ethylene diamine, Et_3N, CH_3COOH; (ii) 6-carboxy-tetramethylrhodamine-*N*-hydroxy-succinimidyl ester, Et_3N, DMSO. In the cartoon representations horizontal lines represent β(1–4) linkages, diagonal lines represent β(1–3) linkages, circles represent glucopyranose residues, circles with a line through them represent the reducing end glucose unit and the star indicates the TMR label.

Studies of β-glucan fermentation in digestion models primarily detect β-glucan degradation *via* indirect methods, for example the increased production of short chain fatty acids and the stimulated growth of bacterial populations (Hughes, Shewry, Gibson, McCleary, & Rastall, 2008). In this article we describe a method to follow directly the degradation of β-glucans in such models by labelling their reducing ends with a stable fluorescent tag prior to introduction into an *in vitro* fermentation model simulating human colon digestion. Following fermentation, detectable fluorescent products were recovered and then characterised by fluorescence-monitored capillary electrophoresis. We further show how it is possible to monitor the degradation of the β-glucans over time, and thereby examine the specificity of β-glucanases secreted by human gut microbiota.

2. Material and methods

2.1. Materials and instrumentation

β-Glucans used in this study were Barliv barley beta fibres (200–300 kDa) obtained from Cargill Health and Nutrition. Tetramethylrhodamine-*N*-hydroxysuccinimidyl ester (TMR–NHS) was purchased from Life Technologies, Carlsbad, Ca., USA. Lichenase (EC 3.2.1.73) was obtained from Megazyme, Bray, Ireland. Other reagents were obtained from Sigma-Aldrich. D_2O was obtained from Cambridge Isotope Laboratory (Andover, MA, USA).

NMR spectra were recorded on an 800 MHz Bruker DRX NMR spectrometer equipped with a TCI cryoprobe and processed in Topspin 2 UV/vis spectra were acquired using a Saveen Werner nanodrop spectrophotometer. Electrospray ionisation mass spectra were recorded on a Bruker Daltonics Esquire Ion Trap instrument. MALDI–ToF–MS and LIF–CE analyses were performed as previously described (Johannesen et al., 2012) on a Bruker Daltonics Microflex instrument and PrinCE 500 CE system, respectively.

2.2. Preparation of TMR-labelled β-glucans

Short β-glucans were prepared by Lichenase-catalysed partial hydrolysis of 200–300 kDa β-glucan fibres. Lyophilised β-glucan fibres (1 g) were wetted in EtOH (1 ml/g) and dissolved in 20 mM Na_2HPO_4/NaH_2PO_4 buffer at pH 6.0 by boiling for 1 h. The resolubilised fibres were equilibrated at 50 °C before 1.5 U of Lichenase was added. After 30 min, the reaction was stopped by addition of NaOH to a final concentration of 10 mM, freezing and lyophilisation. The β-glucans were then functionalised at their reducing end with an ethylenediamine linker using a previously described method (Johannesen et al., 2012).

To a solution of amine-functionalised β-glucans (13 mg) in DMSO (1.3 ml) was then added triethylamine (130 μl) and then a solution of TMR–NHS (3.3 mg, 6 μmol) in DMF (1.3 ml). After stirring at room temperature for 2.5 h, the reaction mixture was concentrated *in vacuo* and the residue was washed with MeOH (4 × 7 ml). The pink solid obtained was dissolved in H_2O (4 ml) then purified by careful chromatography (double connected Seppak C18 plus cartridge (Waters): gradient elution from 2% MeCN in 0.2% CH_3COOH (aq.) to 14% MeCN in 0.2% CH_3COOH (aq.), with 10 ml fractions and 2% increments, then isocratic elution at 14% MeCN in 0.2% CH_3COOH with 50 ml of eluent). The pink fractions eluted with 14% MeCN were combined and concentrated *in vacuo* to give 5.6 mg of TMR-labelled β-glucans.

2.3. β-glucan fermentation in an in vitro human colon model

β-Glucan fermentation in an *in vitro* human colon model was performed at Alimetrics Ltd, Espoo, Finland. Intestinal digests from pigs fed with a humanised diet for 7 days were recovered and pooled to prepare the authentic growth medium for the simulation. The growth medium (10 ml per fermentation vessel) was kept anaerobic, treated with the partially digested β-glucans (1.3 mg, including 10% of the TMR-labelled samples) and then inoculated with pooled fresh faecal samples from three volunteer human donors. β-glucans were included at a concentration equivalent to a daily human dose of 9 g. Five replicate simulations were carried out in separate fermentation vessels. Fermentation was continued for 18 h. The fermentation mixtures from five simulation vessels were combined and centrifuged. Half the supernatant was lyophilised and then redissolved in 50 ml H_2O to give an approximately 0.4 g/ml solution.

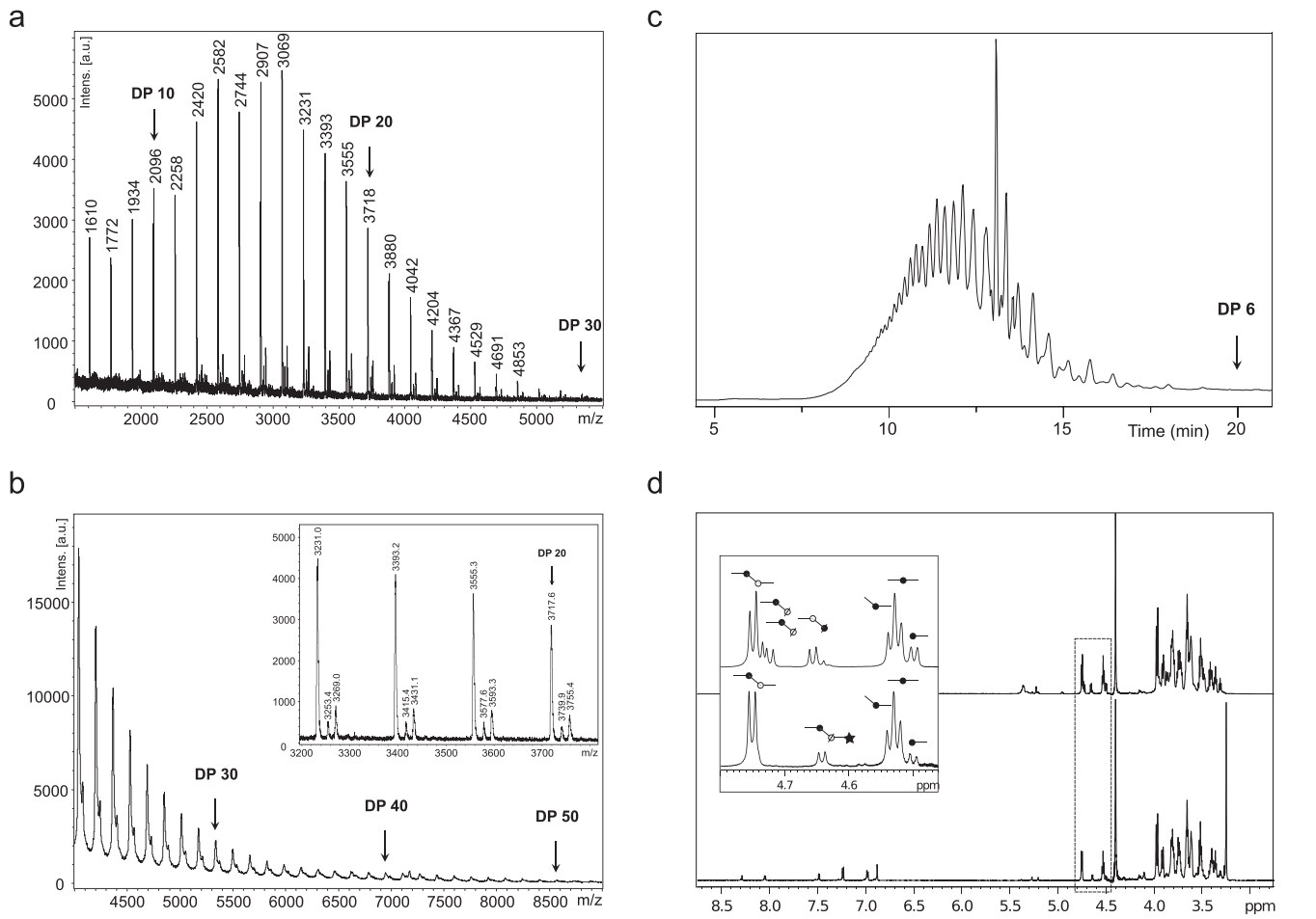

Fig. 2. Characterisation of partially Lichenase-digested TMR-labelled β-glucans. (a) and (b) MALDI spectra of the sample obtained in reflective, and linear modes, respectively; $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions are observed (inset). (c) Capillary electropherogram showing the distribution of different length β-glucan fragments. (d) ^{1}H NMR spectra in D_2O at 323 K of the partially digested β-glucan (top) and TMR-labelled β-glucan (bottom) and a magnification of the β-anomeric region of the spectra (inset). In the schematic, filled circles indicate the glucopyranose to which the anomeric ^{1}H signal corresponds.

2.4. Recovery of fermented β-glucans

A 2 ml aliquot of supernatant solution (0.4 g/ml) was diluted to 10 ml with H_2O then loaded onto a preactivated Seppak C18 plus cartridge (Waters) for reverse-phase chromatographic purification. The cartridge was eluted with a gradient: from 0.2% CH_3COOH (aq.) to 10% MeCN in 0.2% CH_3COOH (aq.) (5 ml fractions, 2% increments) and then to 20% MeCN in 0.2% CH_3COOH (aq.) (10 ml fractions, 2% increments). Pink fluorescence was observed in the fractions containing 14–20% MeCN. These were combined and analysed by CE.

2.5. Time course analysis of β-glucan degradation

A solution of undigested TMR-β-glucan (0.5 mg/ml) was prepared in H_2O. In micro-reaction tubes, 10 μl of this solution were combined with 190 μl of diluted supernatant (0.4 g/ml) from the human colon digestion model study. The samples were allowed to react for varying lengths of time after which they were quenched by addition of 1 ml 0.1 M NaOH and then frozen in dry ice/acetone. The reaction times were: 2 min, 15 min, 1 h and 6 h. After thawing the reaction mixtures were purified on Seppak C18 plus cartridges (Waters) as described above, but with smaller fraction volumes. The fractions containing 12–20% MeCN were combined, evaporated, redissolved in 200 μl H_2O and analysed by CE.

3. Results and discussion

3.1. Preparation of TMR-labelled partially-digested β-glucans

Lichenase obtained from *Bacillus subtilis* is an *endo*-1,3(4)-β-glucanase, which digests cereal β-glucans by hydrolysing β(1–4) glycosidic linkages of the 3-*O*-substituted glucose residues occurring in cereal β(1–3)(1–4)-glucans (Fig. 1a). Lichenase has frequently been used for the partial degradation of cereal β-glucans to produce samples with reduced average molecular weights (Hughes et al., 2008). Furthermore if β-glucans are digested exhaustively with Lichenase the polysaccharides are broken down to reveal their constituent building blocks - primarily cellotriose and cellotetraose (specifically the digested products are 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose) as well as some longer cello-oligosaccharides (Wood, Weisz, & Blackwell, 1994). The ratio of cellotriose and cellotetraose units is characteristic of cereal β-glucans from different sources and this, as well as the relative fraction of longer cello-oligosaccharide building blocks has been considered for its effect on β-glucan solubility and gelling properties (Jiang & Vasanthan, 2000; Lazaridou & Biliaderis, 2007). The molecular weight of the β-glucans has been suggested to influence colonic fermentation (Hughes et al., 2008). Here we briefly treated β-glucan from barley with Lichenase to obtain a partially digested sample with a suitable molecular weight range that would allow characterisation of the sample and monitoring of its further

degradation by gut microbiota using capillary electrophoresis and mass spectrometry following fluorescent labelling at the reducing end.

A fraction of this sample was labelled at the reducing end with a highly fluorescent tetramethylrhodamine dye (TMR) ($\lambda_{ex.}$ 550 nm, $\lambda_{em.}$ 575 nm) in a two step procedure (Fig. 1b). The reducing end hemiacetal was functionalised with an ethylene diamine linker *via* a reductive amination in a manner described previously (Johannesen et al., 2012). The resulting primary amine was then coupled under basic, anhydrous conditions to the *N*-hydroxy-succinimidyl (NHS) ester of 6-tetramethylrhodamine.

The stability of the TMR-labelled compounds upon incubation under acidic conditions was examined to confirm that the fluorescent label would likely withstand the conditions of the digestive tract. It was observed that the UV–vis spectra of solutions of TMR-β-glucans (0.02 mg/ml) in 100 mM sodium citrate buffer at pH 2,3, and 4 were unchanged following incubated for 24 h at 37 °C.

3.2. Characterisation of the TMR-labelled β-glucan mixture

Characterisation of the labelled sample by matrix assisted laser desorption ionisation (MALDI) mass spectrometry operating in positive mode, revealed oligosaccharides ranging from at least DP7 to DP50 (Fig. 2a and b) The dominant signals in the MALDI spectra are the $[M+H]^+$ ions, while Na^+ and K^+ adducts are also visible. Sample distribution of oligosaccharide lengths can also be assessed from the capillary electropherogram (Fig. 2c) The average degree of polymerisation of the partially digested and labelled sample was at least 14.5, as determined by integration of the anomeric proton peaks in the NMR spectrum. (Fig. 2d). Assignment of the various anomeric signals was achieved by reference to published chemical shift values (Petersen, Olsen, Beeren, Hindsgaul, & Meier, 2013). The ratio of cellotriose to cellotetraose building blocks in the oligosaccharides was determined by HILIC chromatography of the products of exhaustive digestion with Lichenase, which were fluorescently labelled with 2-aminobenzamide, as described previously (Beeren & Hindsgaul, 2013). The ratio DP3:DP4 was 2.9, which is similar to previously reported values of β-glucans from barley (Jiang & Vasanthan, 2000).

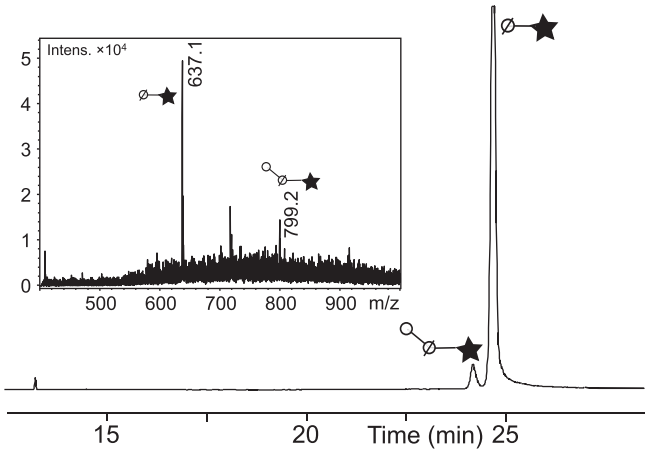

Fig. 3. Capillary electropherogram showing the TMR-labelled products isolated following the fermentation of TMR-labelled β-glucans in an *in vitro* human colon digestion model. Inset: ESI–MS spectrum of the same material. Two products are identified: the major product is TMR-glucose and the minor product is TMR-β-nigerose (β-*O*-3-glucosyl-D-glucose).

3.3. Fate of fluorescently-labelled β-glucans in the human colon in vitro fermentation model

The action of human gut microbiota on the fluorescently-labelled short β-glucans was examined using an *in vitro* fermentation model (Payne, Zihler, Chassard, & Lacroix, 2012). The human colon environment was simulated by inoculating intestinal digesta from pigs fed with a humanised diet with samples of human faeces. Fermentation under anaerobic conditions then followed for 18 h during which time bacterial metabolism of β-glucans was evidenced by the production of gas, the lowering of pH and the production of short chain fatty acids in line with previous studies (acetate > propionate ≥ butyrate (Topping & Clifton, 2001). Following fermentations, solids were removed by centrifugation and the supernatant, expected to contain the TMR-labelled digestion products, was collected and lyophilised.

The TMR-functionalised products were isolated using reverse-phase chromatography and could be identified by eye, from their pink fluorescence. The material was then analysed using CE (Fig. 3). One major species was identified; a single peak with a long retention time was observed and with it, a small peak with a shorter retention time. Analysis by ESI–MS (Fig. 3, inset) revealed that the major species was TMR functionalised glucose and the minor product identified as the TMR-functionalised β-nigerose (β-3-*O*-glucosyl-D-glucose). As only the products of fermentation that were attached to the TMR-label are detectable using this method it is not directly possible to see what has become of the rest of the glucan material once detached from the labelled reducing end. However, since short chain fatty acid fermentation products were detected it is highly probably that the rest of the β-glucan must have been hydrolysed to digestible fragments and then fermented. Based on the presence of only TMR-glucose and a small amount of

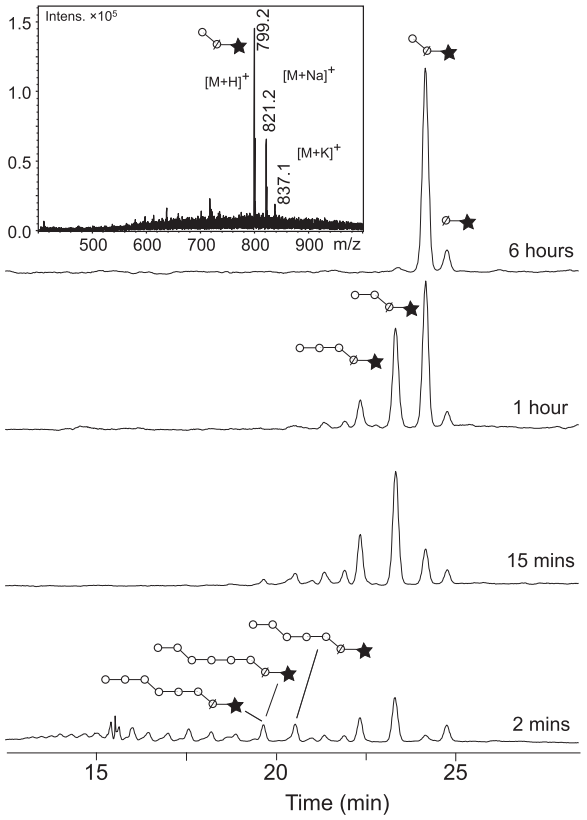

Fig. 4. Time course analysis by capillary electrophoresis of the degradation of TMR-labelled β-glucans by enzymes from colonic microbiota. Inset: ESI–mass spectrum of the product mixture obtained after 6 h.

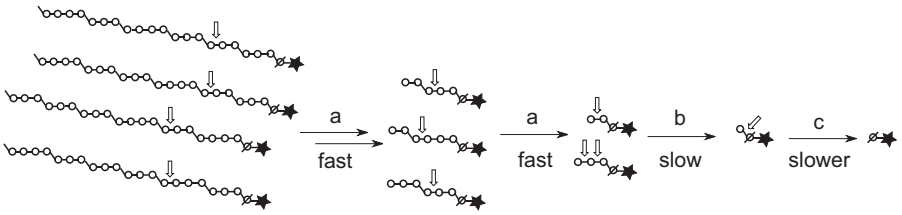

Fig. 5. Proposed route of enzymatic degradation of fluorescently-labelled β-glucans by human colon microbiota: (a) Lichenase-like *endo*-1,3(4)-β-glucanase activity; cleavage of (1–4) linkages on 3-*O*-substituted glucose residues (b) *exo*-1,4-β-glucanase activity, (c) *exo*-1,3-β-glucanase activity.

TMR-β-nigerose we can conclude that both 1,3-β- and 1,4-β-glucanases are active in this human colon model.

In order to explore further the specificity and selectivity of enzymes secreted by microbiota in the human colon, it was of interest to investigate the pathway of enzymatic degradation of the TMR-labelled β-glucans in a time-course experiment. Enzyme activity was still apparent in the lyophilised supernatant collected from the fermentation vessels. We therefore chose to examine the action of these still active enzymes on a fresh sample of TMR-labelled β-glucan, analysing the product mixture over time.

Fig. 4 shows the analysis by CE of TMR-labelled β-glucans exposed to colon microbial enzymes for 2 min, 15 min, 1 h and 6 h. After 6 h, the major product is TMR-β-nigerose, which was confirmed using ESI–MS. The minor species, therefore, is TMR-labelled glucose. The electropherogram collected after 15 min, shows the accumulation of intermediates with DP3 and DP4 *via* which TMR-labelled β-nigerose likely forms. On the basis of the knowledge that barley β-glucans are primarily composed of cellotriose and cellotetraose units, these two intermediates are almost certainly TMR-3-*O*-β-cellobiosyl-D-glucose and TMR-3-*O*-β-cellotriosyl-D-glucose. The predominance of these two species as intermediates suggests that there is an active 1,4-β-glucanase with Lichenase-like selectivity, that is, an *endo*-1,3(4)-β-glucanase that hydrolyses the β(1–4) glycosidic linkages of the 3-*O*-substituted glucose. Working backwards, therefore, at 2 min, the two peaks eluting just before and just after 20 min have DP 6 and 7. These are most probably 3-*O*-β-cellobiosyl-3-*O*-β-cellotriosyl-D-glucose (DP6) and a mixture of 3-*O*-β-cellotriosyl-3-*O*-β-cellotriosyl-D-glucose and 3-*O*-β-cellobiosyl-3-*O*-β-cellotetraosyl-D-glucose (DP7), which would be expected as earlier intermediates resulting from *endo*-1,3(4)-β-glucanase activity.

The enzymatic breakdown of β-glucans in the human colon proceeds most likely, therefore, according to the reaction scheme shown in Fig. 5. An *endo*-1,3(4)-β-glucanase reacts quickly to breakdown the polymer into trimer and tetramer building blocks. Slower hydrolysis by an *exo*-1,4-β-glucanase follows to generate 3-*O*-β-glucosyl-D-glucose. For the TMR-labelled substrates the final degradation step to give TMR-glucose by an *exo*-1,3-β-glucanase is particularly slow, which may be a consequence of modification of the natural substrate with the TMR label.

4. Conclusions

In conclusion, we have demonstrated that the inclusion of fluorescently-labelled β-glucans in an *in vitro* human colon digestion model experiment provides a means to obtain direct evidence of β-glucan digestion and can be used to monitor the pathway of enzymatic breakdown of β-glucans in time course experiments. Tetramethylrhodamine proved to be a suitable fluorescent label: functionalisation of the reducing ends of β-glucans was facile; fluorescent digestion products were easily recoverable; characterisation of these products was possible using capillary electrophoresis and ESI–mass spectrometry; and TMR was stable under model fermentation conditions as well as non-toxic to gut microbiota.

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