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Research paper

In vitro biodegradation study of acetyl and methyl inulins by *Bifidobacteria* and inulinase

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Abstract

The purpose of this study was to investigate if acetylated and methylated inulins can be degraded by inulinase from *Aspergillus niger* and by *Bifidobacteria*, in order to determine their potential in colonic drug delivery. Methyl and acetyl inulins were synthesized by the reaction of inulin (Raftiline HP) with methyl sulfate and acetic anhydride, respectively. The degree of substitution (DS) and the structure of the reaction products were confirmed by ^{13}C-NMR. Degradation by inulinase was investigated in a citrate buffer (0.05 M, pH 4) or with a mixture of citrate buffer and DMSO at 37°C. Biodegradation by *Bifidobacteria* was investigated under anaerobic conditions using an in-house prepared broth at 37°C for 2 days. The resulting products were analyzed chromatographically; the formation of short chain fatty acids was followed by measuring the pH of the incubation media. The results obtained suggest that acetylated and methylated inulins are not biodegradable, since no degradation product could be detected after incubation; a decrease in pH was clearly observed in control samples (pure fructose and inulin), but not in the derivatized inulin samples. The results can probably be explained by a change in conformation of inulin due to derivatization.

Keywords: Inulin; Methyl inulin; Acetyl inulin; Inulinase; *Bifidobacteria*; Colon targeting; Biodegradation

1. Introduction

Site specific delivery of drugs to the colon has recently attracted great attention, since this provides a possible approach for the oral delivery of drugs which are degraded in the upper part of the gastrointestinal tract, or drugs which have the desired local therapeutic effect on the colon. Indeed, many diseases of the colon, such as ulcerative colitis, Crohn's disease, irritable bowel syndrome, diarrhoea or constipation could benefit from colon specific delivery. Several approaches have been proposed to deliver drugs specifically to the colon, one of them using the bacterial population residing in the colon. Bacterial degradation of materials in the colon is well established [1], and a large number of polysaccharides are degraded by colonic bacteria [2], and may form the basis for a suitable colonic biodegradable carrier.

Inulin is a linear β (2–1) linked fructose polymer terminated by a sucrose unit residue with an average degree of polymerization ranging from 2 to 60, depending on the origin of the plant. It is non-toxic, almost totally degraded by colonic bacteria, but almost undigested by gastric or intestinal enzymes [3]. However, its fairly good water solubility makes it difficult to be used as a carrier for drug delivery to the colon. Currently, inulin is mainly used in the food industry as a dietary fibre or as a source of fructose, but the interest to broaden the applicability of inulin for pharmaceutical purpose is growing.

The aim of this study was to synthesize different derivatives of inulin with a reduced water solubility compared

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with inulin, and to investigate their biodegradable properties and their potential as excipients for colon targeting.

2. Materials and methods

2.1. Materials

Acetic anhydride and pyridine were obtained from UCB (Leuven, Belgium), methylsulfate from Acros Chimica (Geel, Belgium), sodium hydroxide from BDH laboratories (Poole, UK), Orcinol® from Sigma-Chemical (St. Louis, MO), *Bifidobacteria adolescentis* (ATCC 15705) and *Bifidobacteria longum* (ATCC 15137) from BCCM™/LMG (Gent, Belgium), glucose, fructose and methylglucose from Merck (Darmstadt, Germany), Labo-Lemco powder and Bacteriological peptone from Oxoid (Gent, Belgium), and Tryptic Soy Broth from Merck (Darmstadt, Germany). Inulin (Raftiline HP) was kindly donated by Orafti (Tienen, Belgium) and Fructozyme® by Novo Nordisk (Bagsvaerd, Denmark). All other materials were of analytical or reagent grade.

2.2. Methodology

2.2.1. Synthesis of acetyl inulin

Acetyl inulin was synthesized according to Haworth et al. [4] (Fig. 1). In brief, 10 g (61 mmol) of dried (100°C) and finely powdered inulin was vigorously stirred with 100 ml of pyridine (dried with potassium hydroxide pellets) at 80°C for 45 min, and the colloidal solution was cooled under continuous stirring. A tube containing anhydrous calcium chloride was fixed on top of the round bottom flask so as to prevent any access of water into the reaction mixture. To the colloidal solution, 10 ml (105.79 mmol) of acetic anhydride was gradually added from a dropping funnel. The ingredients were thoroughly mixed at 25°C for 3 h. Afterwards, a second quantity of 10 ml acetic anhydride (105.79 mmol) was added dropwise, while stirring. The reaction mixture was left overnight. A small amount of unreacted inulin precipitated and was removed by filtration. The reaction mixture was then poured into 2 l of ice water. A white precipitate was formed which was filtered and repeatedly washed with distilled water to remove pyridine and acetic acid. Further purification of the product was obtained by dissolving it in chloroform, followed by drying with magnesium sulfate. The chloroform was finally evaporated under reduced pressure and acetylated inulin remained as a white solid. Using this procedure, acetyl inulin with a degree of substitution of 2.8 was obtained (see Section 2.2.3.).

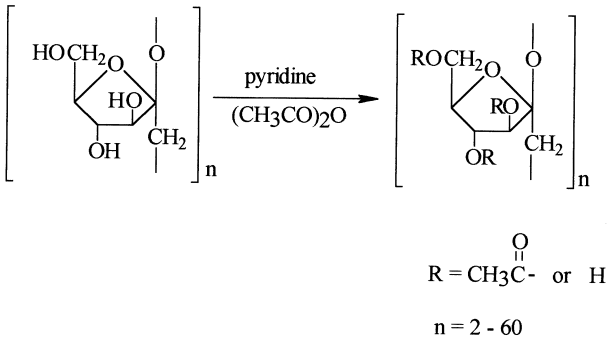

Fig. 1. Schematic representation of the reaction of inulin with acetic anhydride.

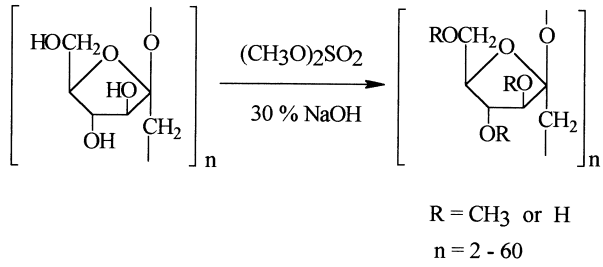

Fig. 2. Schematic representation of the reaction of inulin with methyl sulphate.

2.2.2. Synthesis of methyl inulin

Methyl inulin was synthesized according to Haworth et al [5] (Fig. 2). In brief, 10 g (61 mmol) of inulin which had been finely powdered, was dissolved in an aqueous solution of sodium hydroxide (15%; w/v). To that solution, 5.7 ml (60.1 mmol) of methylsulfate and 14.3 ml of aqueous sodium hydroxide (30%; w/v) were added. During this operation, the solution was vigorously stirred and the temperature was gradually raised to 70°C and finally lowered to 30°C. This procedure was repeated seven times under identical conditions. The methylated inulin remained suspended in the reaction mixture, which at the end of the reaction was heated at 100°C for 30 min to remove any excess of methylsulfate. The excess of alkali was neutralized in the presence of ice by addition of 1 M of sulphuric acid. The product was separated by decantation and then dissolved in chloroform. The soluble part was extracted three times with 500 ml of chloroform. All chloroform extracts were mixed together and dried with magnesium sulfate. The product was obtained by evaporation of the chloroform under reduced pressure.

2.2.3. Determination of the degree of substitution

The degree of substitution of acetyl and methyl inulin was determined by ^{13}C-NMR spectroscopy. The spectra were recorded on a Varian 500 MHz spectrometer using $CDCl_3$ as a solvent. The acetyl group content was calculated from the peak integrals of the acetyl group (δ = 20 ppm) relative to C-2 of inulin (δ = 104 ppm). The methyl group present was quantified by integration of methyl peak integrals (δ = 57–60 ppm) relative to C-2 of inulin (δ = 104 ppm).

2.2.4. Degradation study of modified inulin

2.2.4.1. Degradation by inulinase. Inulin, acetyl or methyl

inulin (0.1 g) was mixed with 10 ml of citrate buffer (0.05 M) of pH 4.7 or with a mixture of citrate buffer and DMSO. A predetermined amount of inulinase (from 2 INU (inulinase units) to 10 INU) was added. The samples were incubated at 37°C for 12 or 16 h, after which they were centrifuged and the supernatant was used for further analysis.

The presence of sugars in the solution medium was analyzed by thin layer chromatography (TLC) (Merck, Silica Gel 60) with a solvent system consisting of 1-butanol:acetic acid:water (3:3:2; v/v). The development proceeded for 7 h. After drying the plates, sugar spots were detected by spraying a reagent containing Orcinol® (10 mg/ml) in 50% (v/v) H_2SO_4 followed by heating for 15 min at 100°C.

In addition, high performance anion-exchange chromatography (HPAEC) was used to analyze the incubated methyl inulin. A 1 ml sample of the incubated methyl inulin solution was passed through a 1 ml bed volume of Dowex® - 50 H^+ and a 1 ml bed volume of Dowex® - 1 acetate. The resins were rinsed once with 1 ml of distilled water. From this neutral fraction, 50 μl were analyzed by HPAEC (Dionex, Sunnyvale, CA) on a CarboPac® PA1 anion-exchange column and quantified by a pulsed amperometric detector, equipped with a gold electrode (potentials: E_1: +0.05 V; E_2: +0.6 V; E_3: –0.8 V). The flow rate was 1 ml min^{-1}. The elution conditions were 90 mM NaOH with 50 mM Na-acetate for 1 min, followed by a linear gradient from 50 to 500 mM Na-acetate in 90 mM NaOH over a 60 min period. The column was regenerated with 1 M NaOH for 10 min and equilibrated for 20 min after every run. Fructose, glucose and methylglucose were used as references.

2.2.4.2. Biodegradation using Bifidobacteria. One loopful of freeze dried bacteria was suspended in 10 ml of either Tryptic Soy Broth (TSB), peptone water, VIP (Table 1), phosphate buffer or bactopeptone. The cultivation was performed at 37°C in the Compact Anaerobic Workstation (DW Scientific, West Yorkshire, UK), until the broth became cloudy. One millilitre of the cultured media was transferred into several vials containing 9 ml of either TSB, peptone water, VIP, phosphate buffer or bactopeptone and 0.1 g of methyl inulin (DS = 2.4) or inulin and then incubated for 2 days.

After incubation, the samples were centrifuged and then analyzed by TLC and HPAEC as described previously. In addition, the pH of the resulting solutions was also measured at the end of the experiment.

3. Results and discussion

3.1. Acetylation of inulin

Inulin was acetylated with acetic anhydride in the presence of dry pyridine. The intention of this chemical modification was to make derivatives with a reduced water solubility compared with inulin, but in such a way that enzymatic degradation of the modified inulin remained possible. Therefore, an optimization of the degree of derivatization was necessary. In a first approach, the molar ratio of inulin to acetic anhydride was varied between 1:3.4 and 1:1.56. Depending on the amount of acetic anhydride used, the degree of substitution of the isolated products (obtained by integration of the acetyl peaks from the ^{13}C-NMR spectrum) ranged from 1.6 to 2.8. Fig. 3 shows the ^{13}C-NMR spectrum of acetylated inulin with DS = 2.02. The signals for the carbons at $\delta = 20$ and $\delta = 170.581$ ppm are attributed by $-CH_2^*-CO-$ and $-CH_2-CO^*-$ groups, respectively.

All acetyl inulins with a degree of substitution from 1.6 to 2.8 were completely insoluble in water but soluble in chloroform, acetone, tetrahydrofuran, diethylether, dichloromethane and DMSO.

In a second step, we reduced the amount of acetic anhydride. This, however, resulted in products which could not be isolated by simple precipitation procedures. When the amount of acetic anhydride was reduced to 60 mmol so as to be in the same ratio as inulin, no precipitate was formed, even when stored in a refrigerator, suggesting that the formed product was still water soluble. Also, when other solvents such as chloroform, dichloromethane, acetone, ethanol and THF were added to the reaction mixture no precipitate was formed.

3.2. Methylation of inulin

Inulin was methylated using methylsulfate in alkaline medium. Methylation of many polysaccharides under these conditions normally requires several treatments with methylsulfate [6], before complete methylation is achieved. This is due to the fact that methylation of free hydroxy groups involves a nucleophilic attack of alkoxides, generated by action of a base on the hydroxy groups, on an alkylating reagent to form the ether. When the reaction is conducted in water, both the equilibrium in the formation of an alkoxide and the unfavourable solvent effects for the alkylation reaction preclude the stoichiometric formation of the methylated product in one single reaction.

In this reaction inulin was methylated seven consecutive times and a methylated product, which was soluble in water, was formed. It was not possible to determine the degree of substitution by ^{1}H-NMR spectroscopy, because the protons from the methoxy groups had similar chemical shifts as unmodified inulin. The only way to determine its degree of substitution was by ^{13}C-NMR spectroscopy. Fig. 4 shows the ^{13}C-NMR assignments of methylated inulin. The ^{13}C-NMR signals for methyl groups are found at $\delta = 57–60$ ppm. The total methyl groups present were quantified by using the chemical shift at C-2. The integration of the methyl peak integrals ($\delta = 57–60$ ppm) relative to those of C-2 of inulin ($\delta = 104$ ppm) gave a degree of substitution of 2.4.

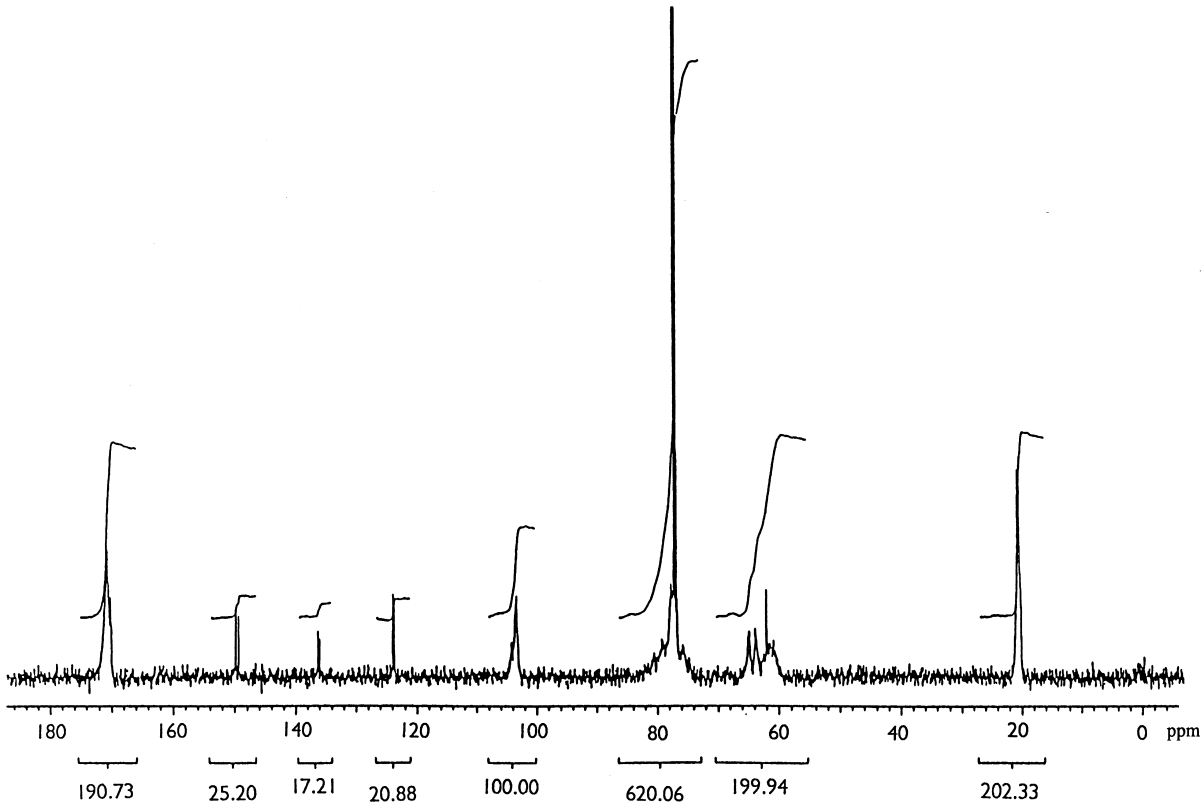

Fig. 3. ^{13}C-NMR spectrum of acetylated inulin (DS = 2.0). Peak assignments: δ = 20:CH_2CO–; 60 65 ppm:C-1 and C-6; δ = 74–82 ppm:C-3, C-4 and C-5; δ = 104 ppm:C-2; δ = 170–173 ppm:CH_2CO–.

Methyl inulin with a degree of substitution of 2.4 was soluble in water, chloroform and DMSO but insoluble in diethylether, and was used in further experiments.

3.3. Degradation by inulinase

Acetyl inulin (DS = 1.6) and methyl inulin (DS = 2.4) were incubated with Fructozyme® which is a mixture of exo- and endo-inulinase enzyme obtained from a selected strain of Aspergillus niger. The enzyme mixture hydrolyzes linear β-2,1-linked fructose polymers of inulin initiated by a glucose unit. Most inulinases are β-fructosidases and split-off fructose moieties from the non-reducing end of the inulin molecule or from certain sugars displaying a fructose unit at the terminal β-2,1-position: these enzymes can be designated as 2,1-β-D fructan-fructano hydrolases. Inulinases with β-fructosidase activity are encountered in plants and in micro-organisms, including, fungi, yeasts, and bacteria.

Several methods have been proposed for oligosaccharide analysis [7–9]: TLC, high pressure liquid chromatography (HPLC) or ion-exchange chromatography with pulsed amperometric detection (HPAEC). Some of the techniques reported require a prior chemical or enzymatic degradation to yield the monosaccharides. In order to study the products formed after enzymatic degradation of the chemically modified inulins, we performed TLC and HPAEC analysis.

The results of the TLC analysis are shown in Fig. 5. No spots corresponding to fructose or other degradation products were detected after incubating acetyl inulin with the enzyme, indicating that it was not degradable. For methyl inulin, a long band was obtained without appearance of distinct spots and no discrimination between test and control samples was possible. The same results were obtained with 2 and 10 INU. The source of inulinase used was *Aspergillus niger*, and eventhough it is mentioned in the literature [10] that this type of inulinase predominantly forms inulotrioses, inulotetroses, and inulopentoses when incubated with inulin, the inulin used as a control was always degraded into one spot corresponding to fructose. Other spots that could have reflected the presence of inulotriose or inulotetroses were never observed.

Acetyl inulin was not soluble in the degradation medium and this could explain why it was not degraded. Therefore,

Table 1

Composition of VIP broth

$CaCl_2$ (anhydrous)	200 mg
$MgSO_4.7H_2O$	480 mg
$K_2HPO_4.3H_2O$	1.3 g
KH_2PO_4	1.0g
NaCl	2.0g
$NaHCO_3$	10.0g
H_2O to	1000 ml

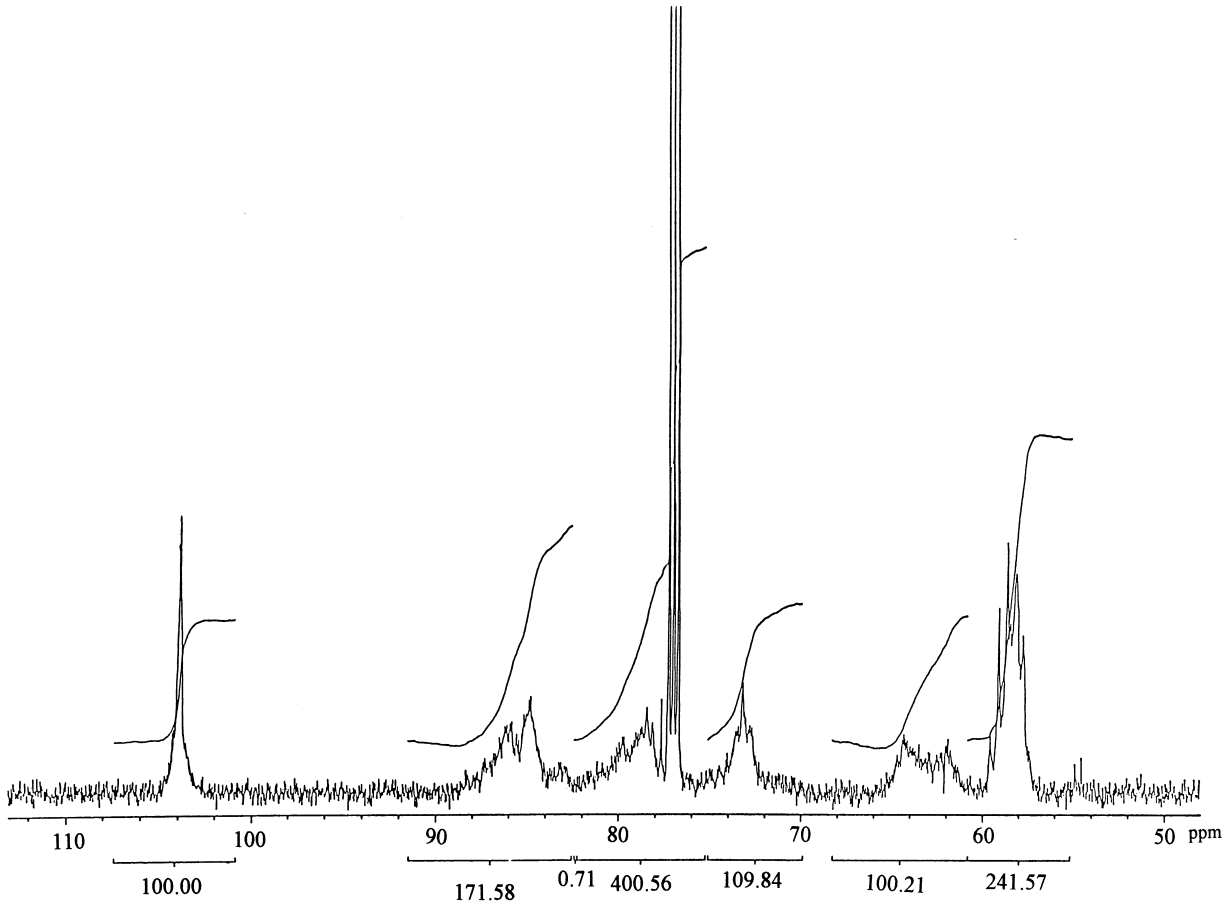

Fig. 4. ^{13}C-NMR spectrum of methylated inulin (DS = 2.4). Peak assignments: δ = 57–60 ppm –OCH_3; δ = 104 ppm:C-2.

supplementary experiments with inulinase were performed using mixtures of DMSO and citrate buffer in order to solubilize the acetylated compound. Using TLC, spots of fructose could be detected after incubating inulin with the enzyme in a mixture of DMSO and citrate buffer, indicating that the enzyme was still capable of degrading inulin in mixtures containing up to 50% (v/v) of DMSO. In the case of acetyl inulin, however, no degradation products could be found eventhough a substantial amount was dissolved in this mixture. Some modification studies by acetylation were also done on dextran [11], and it was found that acetyl dextran, although water soluble, was not degraded by colonic bacteria, the reason being structural changes of the molecule caused by derivatization. Most probably the same conclusion holds for acetylated inulin.

Fig. 6 shows a typical HPAEC pattern of methyl inulin after incubation with inulinase (10 INU). Clearly, methyl inulin has a short retention time due to the presence of methyl groups, making the polymer less accessible to the resin matrix. It is known that methylated (poly)saccharides have a shorter retention time compared to unmethylated products [12]; in the HPAEC chromatogram shown in Fig. 6, the retention time of glucose is 4.1 min while that of methyl glucose is 3.2 min. The obtained results did not allow us to conclude incontestably that enzymatic degradation took place, although the principal peak of incubated methyl inulin was slightly shifted to the left compared with unincubated methyl inulin. The chromatogram of incubated methyl inulin (B) also shows the presence of an extra peak with a retention time of 4.1 min which probably corresponds to that of glucose.

3.4. Degradation by Bifidobacteria

Degradation of carbohydrates by colonic bacteria mainly produces short chain fatty acids (SCFA) and gases [13]. SCFA are partly absorbed by the colonic epithelium that results in caloric contribution.

Figs. 7 and 8 show some HPAEC patterns of incubated inulin and methylated inulins, respectively. Inulin was used as a control to investigate if it can be metabolized by *Bifidobacteria*, and from Fig. 7 it is clear that enzymatic degradation took place. There was depolymerization of the polysaccharide and the spectrum shifted to the left after incubation (B), compared with unincubated inulin which contains more oligosaccharides (A). In the case of methyl inulin, the results were complicated by the sugars and other components from the broth medium. The broth alone gave

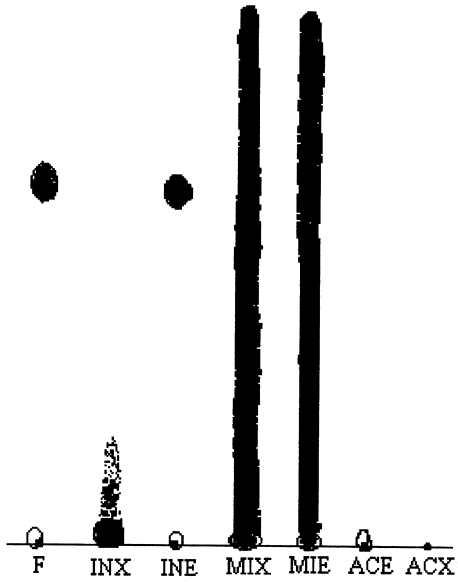

Fig. 5. TLC analysis of methyl and acetyl inulins: F, fructose; INX, inulin without an enzyme; INE, inulin with an enzyme; MIX, methyl inulin without enzyme; MIX, methyl inulin with an enzyme; ACE, acetyl inulin with enzyme; ACX, acetyl inulin without enzyme.

some peaks that were overlapping with those from methyl inulin. There were also other peaks that could not correspond to either fructose, glucose or methylglucose and it was difficult to interpret the results. Overlapping did not occur with inulin.

It is well established that the reduction of pH of the culture medium suggests the presence of SCFA [14] due to fermentation of sugars. The pH of TSB containing inulin or methyl inulin dropped from 7.3 to 5.3, but this was also not conclusive as the pH of the control medium (pure broth with bacteria) also decreased, and the results obtained cannot guarantee if there is any degradation of derivatized inulin.

In order to eliminate the interference of saccharides from TSB, other bacteriological media such as peptone water and VIP were also investigated to check if they are suitable for the growth of bacteria. Since *Bifidobacteria* were not able to grow in those media, they were suspended in phosphate buffer at pH 7 followed by sonication with the intention of releasing the enzymes from the bacteria. However, no change of pH could be detected and TLC analysis did not show any degradation compound after incubation of inulin or methyl inulin.

Finally, an 'in house' broth without sugars was formulated, with the following composition: bactopeptone 10 g; meat extract 5 g; Sodium chloride 5 g; Cysteine hydrochloride 0.5 g; purified water to 1000 ml.

One percent of methyl inulin, inulin or fructose was added directly to the broth under anaerobic conditions and then incubated for several days.

Samples containing fructose and inulin were turbid after 12 h showing the growth of bacteria, but in the case of methyl inulin, there was no growth observed and the sample mixture was not turbid even after being incubated for 5 days. A significantly large drop in pH was observed in the case of fructose and inulin; in the case of methyl inulin there was only a minimal change in pH. Using TLC, methyl inulin was still showing long bands in the test sample as well as in the control sample, so that discrimination between test and control was impossible. From these results one must conclude that methylation of inulin leads to products with reduced biodegradable properties.

4. Conclusion

The aim of the chemical derivatization of inulin was to obtain products having a modified solubility profile, but which are still degradable by enzymes. Under these conditions they could function as excipients for colon drug delivery.

The synthetic procedure to obtain acetyl inulin with a low degree of substitution resulted in a modified inulin which was water insoluble.

The results observed by TLC and HPAEC show that acetyl and methyl inulins are probably not degradable into fructose, methyl fructose, acetyl fructose or other intermediate polysaccharides. This could arise from difficulties of the enzyme to get access or to recognize the modified inulin chain. The insolubility of acetyl inulin may be the cause of its resistance to enzymatic breakdown, while the results with methyl inulin and those obtained with acetyl inulin in DMSO/water mixtures, suggest that the conformational

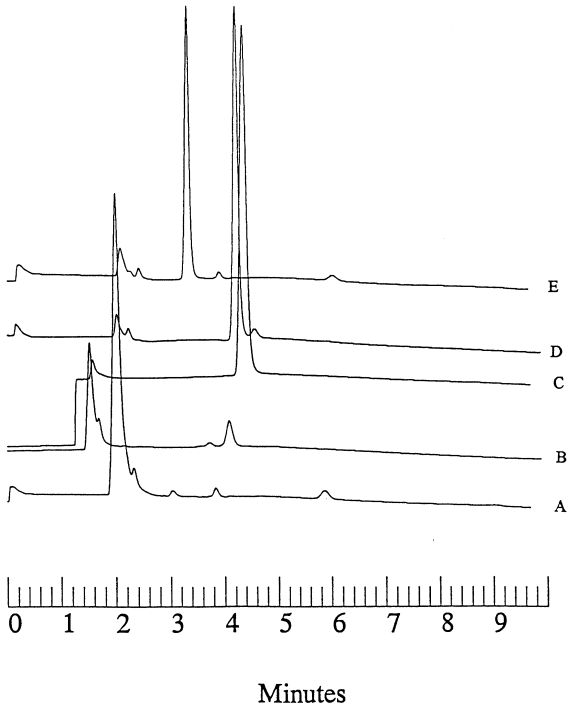

Fig. 6. Anion-exchange chromatography of methylated inulin (B) after being incubated with the enzyme, (A) methyl inulin not incubated with the enzyme, (C) fructose, (D) glucose and (E) methyl glucose.

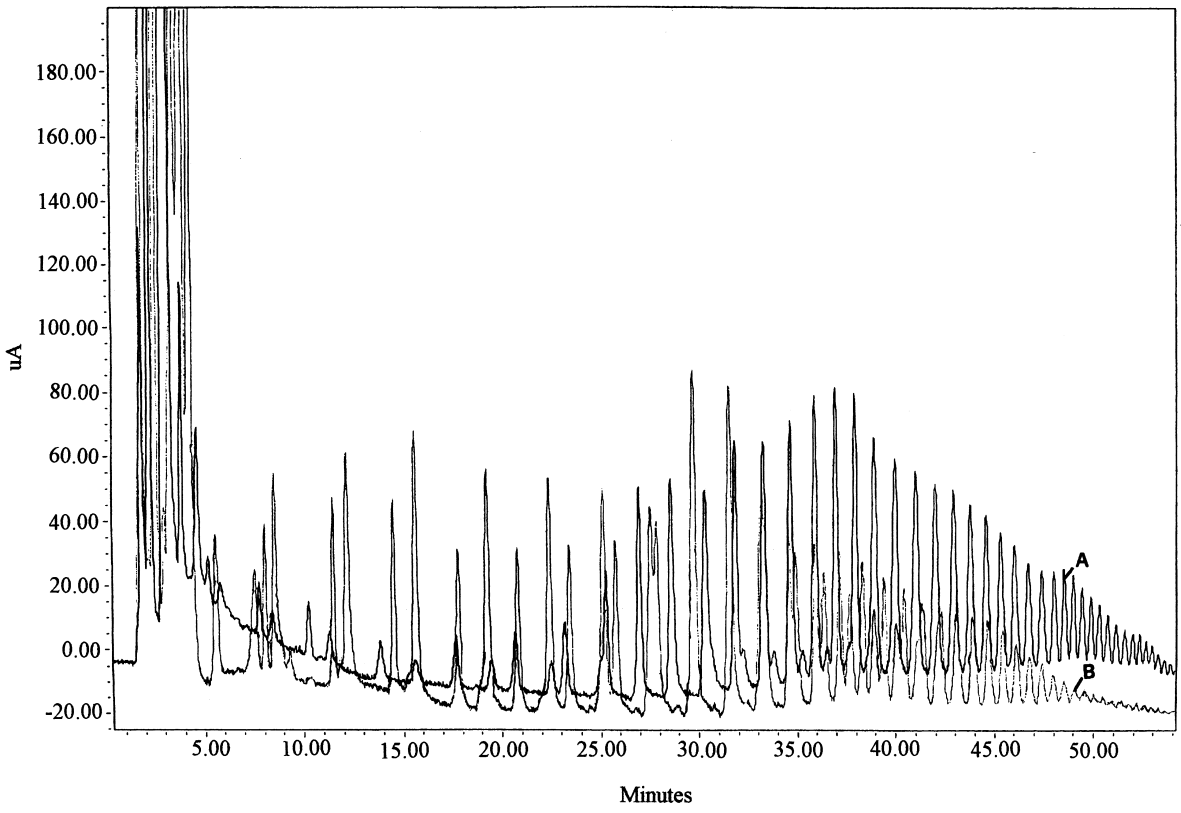

Fig. 7. High Performance Anion Exchange Chromatography of inulin incubated with *Bifidobacteria* (A) and without *Bifidobacteria* (B) at 37°C under anaerobic conditions.

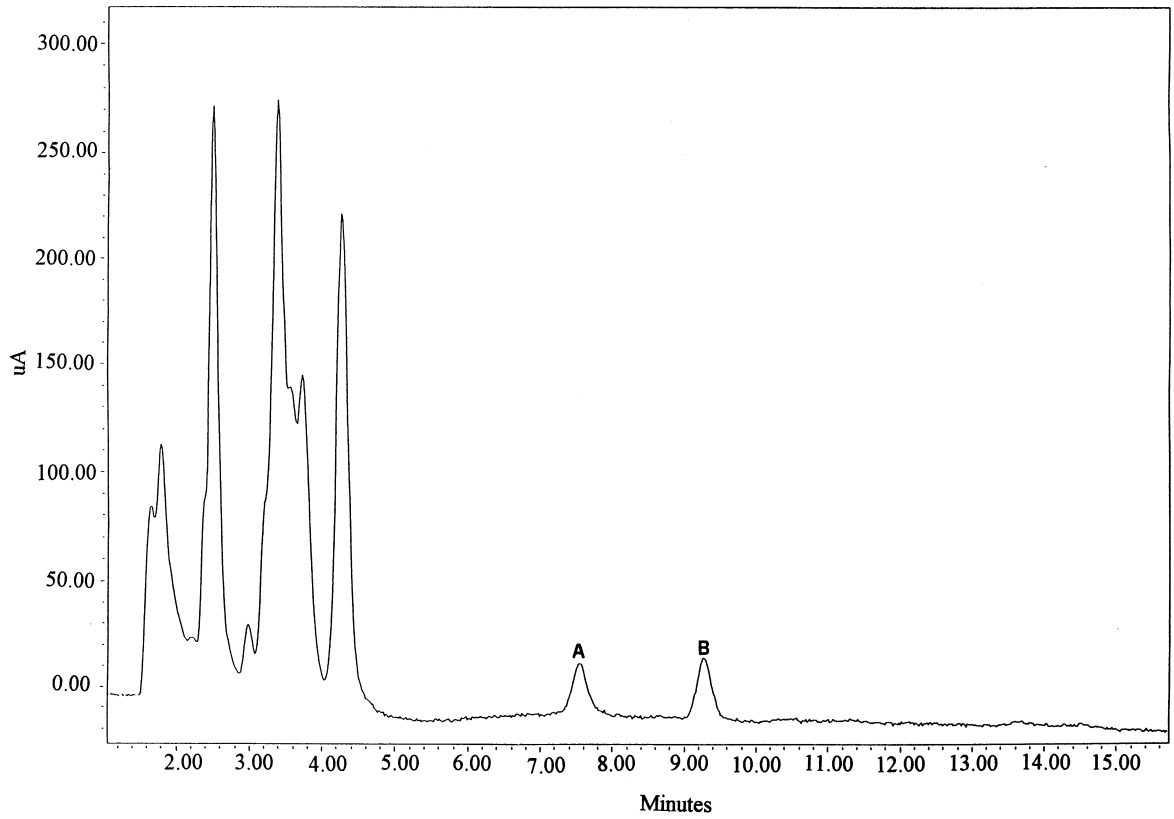

Fig. 8. High Performance Anion Exchange Chromatography of methyl inulin incubated with *Bifidobacteria* at 37°C under anaerobic conditions, (A) and (B) are the peaks which formed but did not correspond to any of the controls used.

changes of the inulin molecule due to derivatization might also be playing a role in its non-degradability.

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