Preparation and Separation of Food Grade Cellooligosaccharides

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Abstract: The chromatographic separation of cellooligosaccharides using cellulose as a stationary phase was studied. The driving force of the work is the current interest in using cellooligosaccharides as functional non-digestible oligosaccharides in foods. The studies presented here illustrate the potential of using ethanol-water mobile phases in conjunction with cellulose stationary phases for cellooligosaccharide fractionation. The interaction between cellulose and cellooligosaccharide in ethanol-water mixtures and their elution order from cellulose-based columns using ethanol-water mobile phases were shown to be in line with their degree of polymerization (DP); the higher DP cellooligosaccharides being less soluble and having longer retention times. Microcrystalline cellulose was shown to work as chromatographic stationary phases. The results demonstrated that cellotetraose and cellopentaose could be obtained in relatively pure form using a cellulose stationary phase with a 20% ethanol mobile phase at room temperature.
Key words: cellulose, cellooligosaccharid, non digestible oligosaccharides, functional food, chromatography

Gıda Amaçlı Sellooligosakkaritlerin Hazırlanması ve Saflaştırılması [1]

Özet: Bu araştırmada sellooligosakkaritlerin selüloz sabit fazı ile ayrıştırılması çalışılmıştır. Bu çalışmanın motivasyonu ise gıdalarda sellooligosakkaritlerin fonksiyonel sindirilmeyen oligosakkaritler olarak kullanımıdır. Burda selüloz sabit fazı kullanarak sellooligosakkaritlerin etanol-su hareketli fazı ile ayrıştırılmasına bakılmıştır. Selüloz ve sellooligosakkaritlerin etanol-su karışımında interaksiyonu ve selülozdan elusyon sırası polimerizasyon derecesi ile orantılıdır yani uzun sellooligosakkaritler daha az çözünebilirliğe ve uzun alınkonma süresine sahiptir. Mikrokristal selüloz sabit faz olarak kullanılabileceği bu çalışmada gösterilmiştir. Sonuçlar sellotetroz ve sellopentozun %20 etanolu hareketli faz olarak kullanılmasıyla oludukça saf bir formda elde edilebileceği gösterilmiştir.
Anahtar kelimeler: selüloz, sellooligosakkarit, sindirilmeyen oligosakkaritler, fonksiyonel gıdalar, kromatografi.

1. Introduction

Functional food is defined by the Food and Nutrition Board of National Academy of Sciences as "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains" (Thomas and Earl, 1994). Nowadays, within the general class of functional foods, prebiotics receive enormous attention and they have become a popular food ingredient for the world food industry (Milner, 2000; Sloan, 2000; Shah, 2001). Prebiotics are defined as non-digestible food ingredients that beneficially effect the host by selectively stimulating the growth or activity of one or a limited bacteria in the colon and thus improve the health (Roberfroid, 1998; Macfarlane and Cummings, 1999). Non-digestible oligosaccharides (NDOs) are oligosaccharides containing between 3 and 10 sugar moieties that are resistant to digestion by human gastric and pancreatic enzymes. They are considered candidate prebiotics (Cummings et al., 2001). NDO's naturally occur in various plants, processed foods and human breast milk. They have been commercially produced by extraction, chemical condensation, transglycosylation reactions, or controlled hydrolysis of parent polysaccharides (van Laere et al., 2000). A number of such preparations have been investigated (van Loo et al., 1999).

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[1] Bu çalışma doktora tezimden hazırlanmıştır.

Cellooligosaccharides (COS) are sugar oligomers, which are made up of β-D-glucose units linked together by 1,4-glycosidic linkages. They are potentially the most widely available of the NDO's, in that the parent polysaccharide is the largest carbon sink in the biosphere. Studies on sugar tolerance tests of cellooligosaccharides showed that cellobiose served as an indigestible sugar in healthy humans, so it did not increase blood glucose levels (Satouchi et al., 1996; Watanabe, 1998). The digestibility, absorptive, and physiological effects of cellooligosaccharides in human beings showed that cellooligosaccharides affect carbohydrate metabolism and have potential application in the prevention of diabetes and obesity (Watanabe, 1998).The widespread technological application of cellooligosaccharides in foods is dependent on practical routes for the preparation of products with defined chemical/physical properties.

Cellooligosaccharides are produced from cellulosic materials by fragmentation of cellulose using acid or enzyme. The resulting mixed cellooligosaccharide are then separated by based on their degree of polymerization (DP) by using suitable chromatographic methods (Miller et al., 1960; John et al., 1969; Streamer et al., 1975; Sabbagh and Fagerson, 1976; Ladisch et al., 1978, Hamacher et al., 1985). However, food-grade oligosaccharides are not pure products, rather they are mixtures containing oligosaccharides with different degrees of polymerization. The aim of this study was the potential of using cellulose as the chromatographic stationary phase for cellooligosaccharides fractionation.

2. Materials and Methods
2.1. Materials

The Aminex HPX-42A column (dimension: 300x7.8 mm; average particle size: 25 μm) was purchased from Bio-Rad Laboratories, Hercules, California, USA; fibrous cellulose powder (CF 11) and LK5D (150 Å) TLC plates from Whatman Chemical Division, Clifto, New Jersey; microcrystalline cellulose (Avicel PH 105) from FMC Corp., Rockland, ME, USA; 4,4' dicarboxy-2,2'-biquinoline (Disodium salt of Bicinchonic

acid), from Sigma Chemical Co., St Louis, MO, USA; p-anisaldehyde from Aldrich Chemical Co, Milwaukee, WI, USA.

Cellooligosaccharide standards, cellotriose (G3) through cellohexaose (G6) were was obtained using a charcoal-celite column chromatography method based on that of Miller et al. (1960) after controlled acid hydrolysis of cellulose.

2.2. Preparation of Water Soluble Cellooligosaccharides

Soluble cellooligosaccharides were prepared according to Miller et al. (1960) with slight modification. Ten grams of fibrous cellulose powder was dissolved in 100 mL concentrated HCl, pre-cooled to –30 ^{0}C, and stirred for 15-20 min. The temperature was then raised to 25 ^{0}C and the stirring continued for 2-3 hr. The solution was then slowly added to 725 mL 4 ^{0}C 1-propanol and stirred for 15 min. The resulting precipitate was collected after centrifugation at 5,000xg for 5 min. The pellet was washed with technical grade ethanol until the pH reached 5-6. Finally, the pellet was extracted with 400 mL cold double-distilled water. The insoluble material was removed by centrifugation and decantation. The cellooligosaccharides in the aqueous phase were concentrated by rotary evaporation and freeze dried. The DP profile of the resulting cellooligosaccharide mixture was determined by HPLC as described below.

2.3. Effect of Ethanol on Cellooligosaccharide Solubility

One mg of COS preparation was added to 1 ml of 0, 20, 40, 60, 80 or 90 % (v/v) ethanol-in-water solutions. These test solutions were mixed thoroughly and allowed to stand at ambient (~22 °C) temperature for 1 hour. The resulting solutions/suspensions were centrifuged at 5000 xg for 5 min and the DP profile of the soluble phase determined by HPLC as described below.

2.4. Cellooligosaccharide Chromatography with Cellulose Stationary Phases

Microcrystalline celluloses were tested as chromatographic media in conjunction with

water/ethanol mobile phases. Cellulose preparations were washed repeatedly to remove impurities and fines prior to their being packed, using gravity feed, into 2 x 25 cm columns. The columns were then equilibrated with the mobile phase. A representative cellooligosaccharide preparation, ~100 mg, was dissolved in 10 ml of the same mobile phase and then introduced onto the column. Column eluate was monitored for the presence of cellooligosaccharides, as reducing sugars, using a 2,2'-bicinchoninate-based assay (Garcia et al., 1993). Fractions (1 mL) showing the presence of reducing sugars were further analyzed by thin layer chromatograpy (silica plates, ethyl acetate:methanol:water 40:20:15 mobile phase, p-anisaldehyde-sulfuric acid visualizing reagent) to determine the DP of the cellooligosaccharides. On the basis of the TLC results, fractions containing homologous cellooligosaccharides were pooled and cellooligosaccharides quantified via HPLC analyses, as described below. Following each chromatographic run, columns could be regenerated by washing with water at room temperature until no further reducing sugars could be detected.

2.5. HPLC Analyses of Cellooligosaccharides

Samples were chromatographed on a Water's HPLC system equipped with a differential refractometer (Model 410), autosampler (Model 717) and column oven (Waters, Milford, MA, USA). Before injection samples were filtered through 0.45 μ filter. Aliquots of filtered sample (50 μL) was injected to the HPLC system. COS were eluted using distilled-deionized water as the mobile phase from an ion-mediated stationary phase in the silver form (Aminex HPX-42A, Bio-Rad Inc.) (Schmid and Wandrey, 1986). The column (300 x 7.8 mm), which was preceded by its complimentary de-ashing cartridge (Bio-Rad), was used at 85 ^{0}C and a flow rate of 0.4 mL per minute. A completed analysis of cellooligosaccharide was carried out less than 30 min. After 30 min, since some of the samples were injected in ethanol solution, ethanol peak was eluted. Sample running time was 60 min, 30 min for data collection and 30

min for post-run. A computing integrator determined the start, retention time and end of the peak, and integrated the area under each peak as a function of height and width of the peak. Concentration of oligosaccharide was quantified using average peak areas compared with mixture of standard oligosaccharide (G1 through G6) and expressed as mM oligosaccharide.

3. Results and Discussion
3.1. Preparation of Water Soluble Cellooligosaccharides

The general method to produce cellooligosaccharides is fragmentation of cellulose by direct acid hydrolysis (Miller et al., 1960; Pereira et al., 1990). After cellulose hydrolysis, excess acid was neutralized with consecutive washing with ethanol. The main hydrolysis products, glucose and cellobiose, were soluble in the acid-alcohol-water phase while the higher DP cellooligosaccharides (DP3-8) tended to precipitate with the insoluble cellulose. A mixture of salt-free cellooligosaccharides mixture was obtained by water extraction of this solvent-washed precipitate (Table 1).

Table 1. The concentration of individual components of cellooligosaccharides mixtures in water

COS	G3 (mM)	G4 (mM)	G5 (mM)	G6 (mM)
1 mg/mL	0.23	0.40	0.33	0.23

3.2. Ethanol Effect on Cellooligosaccharide Solubility

Initial experiments focused on the extent to which ethanol, a relatively nontoxic and inexpensive solvent, affects COS solubility. Figure 1 illustrates the large decline in cellotetraose, cellopentaose and cellohexaose solubility as ethanol concentrations were increased above 60%; cellopentaose and cellohexaose being essentially insoluble at ethanol concentrations $\geq$ 80%. The results demonstrate that moderate-to-high ethanol concentrations profoundly influence COS solubility, the extent of the effect being dependent on the degree of polymerization of the COS. This lends credence to ethanol-based clean-up protocols applied to heterogeneous COS preparations (Hamacher et al., 1985) and

indicates that ethanol-water mixtures have potential as mobile phases for the chromatographic fractionation of COS differing only with respect to degree of polymerization.
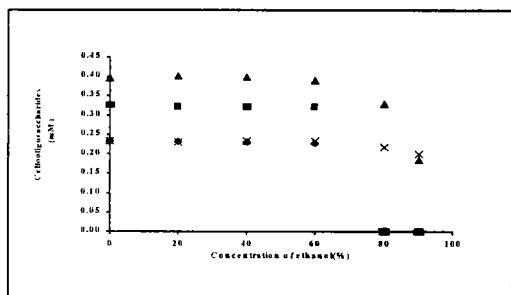

Figure 1. Effect of ethanol on cellooligosaccharide solubility. Test solutions were prepared by adding 1 mg mixed cellooligosaccharide preparation per ml solvent. Replicate measurements were within 7%. (cellotriose, ; cellotetraose, ▲; cellopentaose, ■ ; cellohexaose, ♦).

3.3. Cellooligosaccharide Chromatography with Cellulose Stationary Phases

The chromatogram presented in Figure 2 illustrates the potential of using water/ethanol mobile phases in conjunction with cellulose stationary phases for the fractionation of cellooligosaccharides. The stationary phase in this case was a commercially available microcrystalline cellulose. The results demonstrate that a 20% ethanol mobile phase can be used to effectively separate the lower DP cellooligosaccharides (DP ≤3) from the higher DP cellooligosaccharides. Under these conditions, cellopentaose was obtained with baseline resolution. The elution volume of the lower DP cellooligosaccharides (predominantly cellotriose) was found to be greater than the void volume for the column; the implication being that cellooligosaccharides as short as cellotriose have demonstrable affinities for cellulose stationary phases. This is in agreement with the results of Chitumbo and Brown (1971), who reportedly observed chemisorption between lower DP cellooligosaccharides and cross-linked cellulose gels.
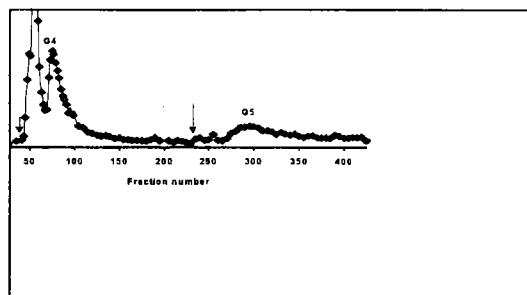

Figure 2. Elution profile of cellooligosaccharides from microcrystalline cellulose (Avicel PH105) with 20% ethanol-water. Arrow indicates that mobile phase was replaced with water (G3: cellotriose; G4: cellotetraose; G5: cellopentaose).

The effect of temperature on the partitioning of the cellooligosaccharides was studied using the microcrystalline stationary phase with water as the mobile phase (Figure 3a). Increased temperatures corresponded with increased retention volumes for all cellooligosaccharides. The temperature effect was slightly more pronounced for the longer cellooligosaccharides (cellotetraose and cellopentaose), thus improving the resolving power of the column (Figure 3b). The retention volume of the lower DP cellooligosaccharides, which appear to have relatively low affinities for microcrystalline cellulose, also increased with temperature. Again suggesting that at least minimal chemisorption occurs between the lower DP cellooligosaccharides and microcrystalline cellulose. In nature cellulose forms microfibrils where the molecules are packed tightly together by intra and inter molecular hydrogen bonds and van der Waals interactions. The intermolecular hydrogen bonding and van der Waals forces cause cellulose chains to pack very tightly together and form highly ordered structures. Similarly, the structural identity and the formation of hydrogen bonds between cellulose and cellooligosaccharides causes strong chemisorption. Adsorption between a solute and a adsorbent is related to the solubility of the solute. Typically, among a homologous series, adsorption increases strongly and regularly with increasing molecular weight of the solute (Freundlich, 1926). Figure 2 illustrates that the adsorption of cellooligosaccharides to cellulose becomes more important with increasing chain length of

the cellooligosaccharides since the solubility of the cellooligosaccharides decreases with increasing chain length. This is thought to be the result of an increased number of intermolecular interactions (Chitumbo and Brown, 1971; Huebner et al., 1978).
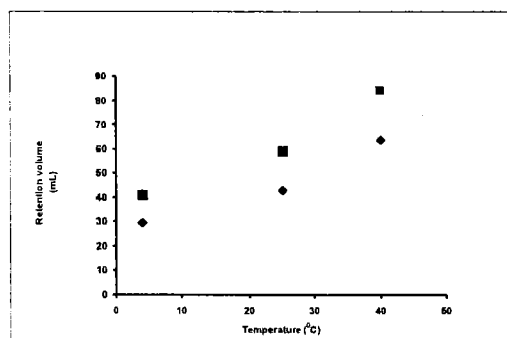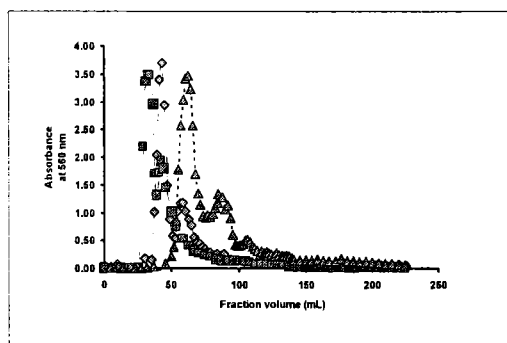

Figure 3.(a) Effect of temperature on elution profile of cellooligosaccharides chromatographed on microcrystalline cellulose with water as the mobile phase (4°C, ■; 25°C, ♦; 40°C, ▲). (b) Effect of temperature on retention volume of cellotetraose (▲) and cellopentaose (■) chromatographed as in part "a".

The choice of solvent is very important for interaction of cellooligosaccharides and cellulose. Solvents that favor the solubility of cellooligosaccharides such as DMSO (dimethylsulfoxide), interfere with the adsorption of cellooligosaccharide to the cellulose support. In such a case, interactions between the solute and the solvent are stronger than the interactions between the solute and the adsorbent. The separation observed in these latter cases are based on liquid-liquid partition

chromatography (Chitumbo and Brown, 1971; 1973). The opposite effect occurs when water or ethanol-water is used as the mobile phase. Incorporating ethanol into eluting solvent makes cellooligosaccharides less soluble, thus causing cellooligosaccharides to bind strongly on cellulose chains (Figure 2). This effect becomes more noticeable with increasing molecular weight.

The partition of solute with adsorbent is determined by the net interaction between adsorbent-solute and solute-solvent. Therefore, when the temperature is altered, it may favor or inhibit adsorption to the stationary phase (Brown, 1972). The interaction between cellooligosaccharides and cellulose decreased as the temperature decreased (Figure 3a and 3b). It is difficult to interpret this relationship because there may be unobserved changes in the character of the cellulose stationary phase as a function of temperature (Chitumbo and Brown, 1973).

4. Conclusion

This study showed that cellooligosaccharides at chain length greater than three units showed adsorption on cellulose support and it could be used as a stationary phase for the preparation of cellotetraose and cellopentaose. Cellulose has been used in food industry to stabilize foam, to replace fat, to control ice crystal growth and to form a gels (BeMiller and Whistler, 1996). The use of it as a support for the separation of cellooligosaccharides with different chain length provide as an advantage for preparation of food grade cellooligosaccharide. They could be obtained with low cost and these mixture can be useful to be used in the food industry as non-digestible oligosaccharides.

Acknowledment

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