J. of Supercritical Fluids 53 (2010) 25–33

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

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

Supercritical fluid purification of complex carbohydrate mixtures produced by enzimatic transglycosilation and isomerized with complexating reagents

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ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form 26 January 2010
Accepted 6 February 2010

Keywords:
Supercritical
Prebiotic
Carbohydrate
Borate
Aluminate

ABSTRACT

Food-grade complex oligosaccharide mixtures manufactured commercially via transglycosylase reactions using disaccharides, such as sucrose or lactose as the raw material, are, in general, not pure products. Reducing oligosaccharides may be isomerized using borates and aluminates as complexating agents to obtain prebiotic oligosaccharides. Complexating agents are used to avoid side-reactions but are difficult to remove from mixture. In the present work, a new process based on the extraction with supercritical CO_2 plus a co-solvent has been developed to purify reaction mixtures leading to pure prebiotic oligosaccharides. A three-step supercritical extraction process has been designed to purify carbohydrates according to its degree of polymerization; the appropriate selection of the co-solvent employed, together with the most suitable extraction conditions allowed the almost complete removal of monosaccharides, and disaccharides from the mixture, leading to pure oligosaccharides as a residue of the extraction process. In this process, not only complete fractionation of mono-, di- and trisaccharides was achieved but also elimination of borates and aluminates from the prebiotic carbohydrates mixture.

1. Introduction

The gastrointestinal flora plays an important role in digestion and in maintaining gastrointestinal health by stimulating the immune system, thereby preventing harmful bacteria from establishing a home on the gastrointestinal wall and promoting acidification of gastrointestinal tract. Foods or ingredients such as prebiotics that encourage the growth and activity of beneficial bacteria in the gastrointestinal tract help maintaining a balanced gastrointestinal flora [1].

A prebiotic carbohydrate can be defined as a "non-digestible carbohydrate that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [2]. Currently, for a food ingredient to be classified as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial bacteria in the colon such as the bifidobacteria, and (3) fermentation of the substrate should induce beneficial effects within the host [3].

Although commercially available, the main drawback of prebiotic carbohydrates is that they contain a mixture of products; for example, when obtained from lactose or sucrose, they contain glucose, galactose and unreacted lactose, that do not have prebiotic properties since they are absorbed in the small intestine and their presence may not be desirable when dietary restrictions are prescribed [4]. Removal of simple sugars provides oligosaccharide products several advantages as they can be used to confer prebiotic properties in food where they are added and to decrease caloric value.

The separation of carbohydrates plays an important role in food production and in cosmetic and pharmaceutical industries, accounting for around 90% of the cost in food production [5]. Among the different techniques available, membrane separation has been used for OS (oligosaccharides) separation, although some problems have been associated with their use, for example, membrane fouling caused by highly concentrated solutions [6] and/or chemical or mechanical problems attributed to pH, temperature and mechanical resistance [7]. As an example, Iwasaki and Matsubara [7] separated most of monosaccharides, 92%, from a complex mixture of carbohydrates; however, 80% of lactose remained in the solution.

Among the prebiotic carbohydrates special attention has been put on galacto-oligosaccharides (GOS) which are commonly synthesized from cheese permeate and other lactose-rich streams by a transgalactosylation reaction catalyzed by the enzyme β-galactosidase (EC 3.2.1.23 from International Union of Biochemistry and Molecular Biology nomenclature recommendation) from different sources [8]. Transgalactosylation involves intermolecular as well as intramolecular reaction that may give rise to disaccharide

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doi:10.1016/j.supflu.2010.02.011

isomers of lactose as well as di-, tri- or higher oligosaccharides [9]. The ratio of transferase and hydrolase activities of the enzyme along with the enzyme source, the concentration and nature of the substrate and the reaction conditions (pH, temperature and time) are the main factors affecting the amount and nature of the formed OS [10]. The yeast *Kluyveromyces lactis* is an important commercial source of β-galactosidases [11], with Lactozym 3000L HP G being one of the most used enzymatic preparations. GOS are usually simple mixtures of tri- and tetra-saccharides made up of galactose and glucose molecules along with residual lactose. Some disaccharides are also present in GOS (e.g. allolactose and galactobiose) [12]. But synthesis reactions not only produce GOS but also unreacted lactose as well as small amounts of by-products such as galactose and glucose [9]. The presence of glucose and lactose is not recommended for people with diabetes or lactose intolerance. Therefore, GOS isomerization is performed, under basic conditions, to modify the properties of fermentable prebiotic OS for conversion into the corresponding ketoses having different fermentation characteristics.

Isomerization of reducing sugars is a well known reaction in carbohydrate chemistry. First discovered by Lobry de Bruyn and van Ekenstein [13], the reaction has been used to prepare unavailable carbohydrates by isomerization or epimerization of naturally occurring carbohydrates. Methods for producing carbohydrates by isomerization involve use of complexation reagents such as aluminate [14–17] and borate [18–22]. These compounds shift the equilibrium established during base catalyzed isomerization in favor of ketoses and prevent degradative side-reactions. These reagents allow formation of ketoses in acceptable high yields, but both are impractical at industrial level since they are difficult to remove [21] and a huge excess of complexation reagents is necessary to obtain an optimal reaction.

GOS are especially suitable in food products for special target groups, such as infant nutrition, clinical nutrition and foods for elderly people. GOS are available as powders or syrups, typically with about 75% solids, of which GOS comprise about 55–60%, lactose 20%, glucose 20% and galactose between 1 and 5% [23]. Also, the fact that the current market price for GOS is more than $17 per kg, makes it a high-value product [24].

GOS provide several health benefits, which make their use as food ingredients particularly attractive: (1) reduction of detrimental bacteria; (2) production of nutrients [25]; (3) increase in absorption of different minerals in the intestine [26]; (4) prevention of pathogenic and autogenic diarrhea [27]; (5) prevention of constipation [28]; (6) reduction in serum cholesterol [29]; (7) reduction of blood pressure [30]; and (8) anticancer effect, mainly the gut cancer [27].

Supercritical fluid technology has gained much attention for fast and effective extraction of a wide variety of compounds. Previous studies carried out in our laboratory have demonstrated that when using polar co-solvents the solubility of carbohydrates in supercritical carbon dioxide can be considerably enhanced [31] and that it is also possible to selectively fractionate carbohydrates according to its degree of polymerization [32]. Therefore, the goal of the present work has been to develop a new process based on the extraction with supercritical CO_2 plus a co-solvent to purify reaction mixtures leading to pure prebiotic oligosaccharides. A three-step supercritical extraction process has been designed to purify carbohydrates according to their degree of polymerization in order to remove monosaccharides and disaccharides from the mixture, leading to pure oligosaccharides as a residue of the extraction process. In this process, not only was saccharide fractionation attempted but also the elimination of borates and aluminates from the prebiotic carbohydrates mixture.

2. Materials and methods

2.1. Reagents

Lactose was acquired from Scharlau (Barcelona, Spain). Sodium aluminate, boric acid and sulphuric acid were purchased from Merck (Divisione Chimica Industriale, Milano). Sea sand and glass wool washed chemically pure were acquired from Panreac Química S.A. (Barcelona, Spain). Ethanol absolute was from Prolabo (Fontenay sous Bois, France). The commercial enzyme Lactozym 3000L HP G, a soluble preparation of β-galactosidase from *K. lactis* was donated by Novozymes (Bagsvaerd, Denmark). Sea sand washed was acquired from Panreac (Barcelona, Spain). 18.2 MΩ cm Ultrapure water quality with 1–5 ppb TOC and <0.001 EU/mL pyrogen levels (Milli-Q) was produced in-house using a Laboratory water purification Milli-Q Synthesis A10 system (Millipore, Bellerica, MA, USA) and was used throughout. CO_2 liquefied at high pressure and used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

2.2. Synthesis of GOS from lactose using Lactozym 3000L HP G enzyme

The synthesis of GOS was carried out in the optimal conditions previously reported by Martinez-Villaluenga et al. [10]. Thus, a concentration of 3 U/mL of enzyme was added to a concentration of 250 g/L of lactose. Two different conditions were assayed: (1) incubation lasting 2 h at 40 °C in a buffer phosphate 50 mM and 1 mM $MgCl_2$ at pH 7.5 for maximizing disaccharides production; (2) incubation lasting 5 h at 50 °C in a buffer phosphate 50 mM and 1 mM $MgCl_2$ at pH 6.5 for maximizing monosaccharides production. The objective of working on two different conditions was to study if different GOS composition affected in supercritical extraction. After this period the mixture was immediately immersed in boiling water during 10 min to inactivate the enzyme.

2.3. Isomerization reactions

The assays of isomerization of carbohydrate mixture with aluminates and borates were based on the method of Zokaee et al. [22]. In all the experiments, total carbohydrate concentration was 10% (w/v) in deionized water. Thus, 8 g of total carbohydrates present in hydrolyzed mixtures was dissolved in deionized water and mixed with sodium aluminate in different sodium aluminate/carbohydrate (lactose) molar ratios, 1:1 and 2:1. The same molar ratios were used for experiments using boric acid. The mixture was immersed into a water bath adjusted to the required temperature (40 °C and 70 °C, respectively, for reactions with aluminates and borates) and heated for a specific time period. Samples of the isomerization mixture were taken at 2, 4, 6, 8 and 10 h for reactions with aluminates and at 60, 90, 120, 150 and 180 min for experiments with borates. Reactions were stopped by placing the tube in an ice-bath and then adding a few drops of sulphuric acid 25% (v/v) to neutralize the pH. Afterwards, samples were centrifuged at 10,000 rpm during 10 min. The supernatant was collected and freeze dried. These sample were used in supercritical extraction experiments with CO_2 using both ethanol and water co-solvent. Some samples from isomerization reaction were not neutralized and were freeze dried directly.

2.4. Supercritical extraction with CO_2 + (ethanol:water) co-solvent

The equipment employed to carry out solubility measurements is based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications [33]. It has a thermostatic oven