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1D, 2D NMR and various Spectroscopic Techniques for the Structural Characterisation of A2 Cow Colostrum Oligosaccharides

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Abstract: Cow milk is one of the most widely accessible types of milk worldwide. The milk that mammals produce in the first few days after childbirth is called colostrum. A modified method of Kobata and Ginsburg including deproteination, microfiltration and lyophilization was used to process the milk of the A2 cow. This led to the isolation of crude milk oligosaccharide mixture. The general methods of purification (gel filtration and column chromatography) and structure elucidation (^{1}H, ^{13}C, HMBC, HSQC, TOCSY and COSY) of the oligosaccharide found in the A2 cow colostrum have been incorporated in this paper.

Keywords: Milk oligosaccharides, Kobata and Ginsburg, A2 cow colostrum, Structure elucidation, 1D-2D NMR.

1. Introduction

Cow milk contains oligosaccharide that strengthen bones [1], develop brain, improve vision and stimulate the immune system. Biologically active oligosaccharides with immunostimulant [2], anti-tumor [3], anti-cancer [4], anti-inflammatory, hypoglycemic [5], anti-oxidant [6] and anti-lipidemic [6] properties are abundant in colostrum. An essential step in determining the structure of an oligosaccharide or oligoglycoside is locating the position of the glycosidic bond, which helps in writing the stereoscopic structure and indicates the biological activity of the compound also. In the Modified method of Kobata and Ginsburg [6], after collection of colostrum/milk, an equivalent quantity of ethyl alcohol is added in order to preserve it. The procedure used to get the crude colostrum/milk oligosaccharide after preservation involved; deprotination, microfiltration and lyophilization. As a result, these acetylated oligosaccharides had high resolution in their NMR spectra and were readily soluble in $CDCl_3$. The position of glycosidic linkage was determined simultaneously by combining the findings of HSQC, COSY, TOCSY [7] along with HMBC experiments.

2. Methods and Material

10 L of A2 cow colostrum/milk was collected and processed by the modified method of Kobata and Ginsburg involving deprotination, microfiltration, gel chromatography and lyophilization.

2.1.Structure Determination of Milk Oligosaccharides

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Traditionally, the structure was determined by chemical transformation and degradation, including acid hydrolysis to identify the monosaccharide units in the oligosaccharide.

2.1.1. Acetylation of Milk Oligosaccharides

252 g of oligosaccharide mixture were extracted from the crude compound. 10 g of the oligosaccharide mixture was acetylated for 24 hours at 60°C with constant stirring using 10 ml each of acetic anhydride and pyridine [8]. 8.68 g acetylated oligosaccharides mixture was isolated after it was dried under low pressure at room temperature, extracted with 250 ml of chloroform and dried over anhydrous sodium sulphate.

2.1.2. Column Chromatography of Acetylated Oligosaccharide Mixture

The most popular technique for isolating oligosaccharides is column chromatography, which involves packing a column with particulate matter such as silica and then passing a solvent through it at atmospheric pressure. TLC was used to examine each of these fractions and those displaying similar spots were combined for additional analysis. The table below provides the chromatographic details.

Fraction no.	Solvent	Eluted Residue Amorphous (mg)	Spots on TLC	Further Investigation
1-10	$CHCl_3$	_	_	_
11-20	MeOH:$CHCl_3$(0.1:99.9)	789 mg	Streaking	_
21-30	MeOH:$CHCl_3$ (0.2:99.8)	925 mg	a, b	CC-2
31-40	MeOH:$CHCl_3$ (0.5:99.5)	945 mg	b, c	CC-3
41-50	MeOH:$CHCl_3$ (1:99)	892 mg	c, d	_
51-60	MeOH:$CHCl_3$ (2:98)	349 mg	d	Physio-chemical Investigation
61-70	MeOH:$CHCl_3$ (5:95)	897 mg	Streaking	_
71-80	MeOH:$CHCl_3$ (8:92)	989 mg	Streaking	_
81-90	MeOH:$CHCl_3$ (10:90)	1013 mg	Washing	_

2.1.3. Deacetylation of Milk Oligosaccharides

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36 mg of the pure oligosaccharide were dissolved in 3 ml of acetone and 3 ml of NH_4OH in a Stoppard hydrolysis flask. The flask containing reaction mixture was kept in dark for the whole night and concentrated under low pressure after 24 hr [8].

2.1.4. Methylglycosidation / Acid Hydrolysis of Oligosaccharide

The process of methylglycosidation involves refluxing the molecule with MeOH at 70°C for 12–18 hr in presence of cation exchange IR-120 (H^+) resin, followed by acid hydrolysis [8].

2.1.5. Kiliani Hydrolysis of Oligosaccharide

Kiliani acid hydrolysis (AcOH: H_2O:HCl::7:11:2) is frequently employed to hydrolyze oligosaccharides constituted of regular monosaccharides [9].

2.1.5. Result and discussion

NMR spectroscopy is a sophisticated and powerful analytical technique that has a number of applications in scientific research. Proton NMR spectroscopy of oligosaccharides suffers from substantial spectrum overlap because most monomeric residues differ only in their stereochemistry and their magnetic properties are only little affected by their location in the chain. ^{13}C-NMR spectroscopy has been extensively used to assign the conformation and type of anomeric linkages in oligosaccharides. Determining the number of O-linked monosaccharides is made much easier by the presence of anomeric resonances in a well-separated chemical shift range of 90–110 ppm. 2D-NMR spectroscopy offers precise, excellent, and easily comprehensible data on the sugar molecule.

^{1}H-^{1}H TOCSYis connected to COSY by the detection of coupled proton cross-peaks. The TOCSY spectrum also reveals cross-peaks between nearly every spin in the spin system. A sequence of cross-peaks can be used to map out the connection between protons inside a sugar residue. The TOCSY spectrum also reveals cross-peaks between nearly every spin in the spin system. This characteristic is useful in determining if the configuration is manno, galacto, or gluco. TOCSY may be used to identify a particular residue in oligosaccharides and provide the total correlation of all protons in a chain with one another. The chemical shifts of the associated protons and carbons are shown by the cross-peaks. The cross peak of anomeric and ring protons to their directly connected carbon atom with links between monosaccharides in the oligosaccharide moiety is shown by the HSQC spectra. The HMBC experiment establishes the glycosidic bond that connects monosaccharide units. The correlation between a proton and its neighboring carbon may be determined using HMBC experiments and this information is particularly helpful in the structural elucidation of

oligosaccharides. Mass spectrometry is a crucial technique that provides accurate findings, analytical flexibility and extremely high sensitivity.

3. Conclusion

The process for isolating and clarifying the structure of the oligosaccharide that was extracted from A2 Cow Milk is thoroughly explained in this paper. We have described the process for isolating milk oligosaccharides. Spectroscopic methods such as 1D and 2D NMR techniques have also been defined for colostrum/milk oligosaccharide structure elucidation.

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