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Structural determination of some new oligosaccharides and analysis of the branching pattern of isomaltooligosaccharides from beer

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Abstract

Separation of the low molecular weight fraction of oligosaccharides from beer and subsequent NMR analysis led to the identification of several new derivatives of trehalose, sucrose, maltooligosaccharides glucosylated at O-2 of reducing end Glc, and linear Glc oligomers with α-(1→3) and α-(1→4)-linkages. Reducing oligosaccharides were labeled with 7-amino-4-methylcoumarin by reductive amination, separated according to their molecular weight and the branching pattern was studied using enzymatic (pullulanase) degradation in combination with MALDI-TOF mass spectrometry and HPLC analysis. It was found that up to DP 10 isomaltooligosaccharides mostly consisted of a linear reducing α-(1→4)-linked Glc chain substituted by single maltose or maltotriose residue at O-6 of any residue except the reducing one.

Keywords: Beer; Isomaltooligosaccharides; Pullulanase; HPAEC; MALDI-TOF mass spectrometry

1. Introduction

Carbohydrates are the major non-volatile components of beer. Therefore, monitoring of the carbohydrate composition in wort and beer is important for modern brewing technology and particularly in the development of new sorts of beer and in the selection of raw materials and yeast strains. Three polysaccharides, starch, pentosan (arabinoxylan) and β-glucan together with sucrose are the primary sources of carbohydrates in beer. During the malting and mashing operations in the production of wort the polysaccharides under the action of enzymes (particularly amylases) are converted into malto- and isomaltooligosaccharides together with Glc and cell-wall fragments from pentosans and glucans. During the fermentation process the lower maltooligosaccharides, sucrose and Glc are further hydrolysed and converted into alcohol and carbon dioxide, whereas the higher oligomers remain in solution. Pentosan and β-glucan depolymerized to some degree remain partially in beer, where they can cause undesired precipitation and filtration problems.

The composition of beer carbohydrates has been studied for many years [1–4] and a number of oligosaccharides has been identified, among them

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all the positional isomers of α- and β-glucobioses, xylobiose, trehalose, melibiose (α-Gal*p*-(1→6)-Glc), lactose, sucrose, turanose (α-Glc*p*-(1→3)-Fru), maltulose (α-Glc*p*-(1→5)-Fru), xylotriose, maltotriulose, cellotriose, raffinose (α-Gal*p*-(1→6)-α-Glc*p*-(1↔2)-β-Fru*f*), gentianose (β-Glc*p*-(1→6)-α-Glc*p*-(1↔2)-β-Fru*f*), isokestose (α-Glc*p*-(1↔2)-[β-Fru*f*-(2→1)]-β-Fru*f*), kestose (α-Glc*p*-(1↔2)-[β-Fru*f*-(2→6)]-β-Fru*f*), melezitose (α-Glc*p*-(1↔2)-[α-Glc*p*-(1→3)]-β-Fru*f*), stachyose (α-Gal*p*-(1→6)-β-Gal*p*-(1→6)-α-Glc*p*-(1↔2)-β-Fru*f*), together with different fragments of starch. However, finished beer contains many unknown oligosaccharides, and the structure of the starch derived products has not been thoroughly studied. We have therefore undertaken a detailed investigation of the oligosaccharide composition of beer using modern separation techniques and tools for structural determination, particularly NMR spectroscopy and MALDI-TOF mass spectrometry.

HPLC is the main method for the analysis of beer oligosaccharides. Separation of non-derivatized sugars on amino-columns with RI detection [5,6] is efficient but not accomplished with very high sensitivity. On the contrary reverse phase separation of fluorescent derivatives can be done with very high sensitivity [7]. Chromatography on a silver or calcium forms of cation exchangers [8] have also been used with some success. Recently attempts have been made to use capillary electrophoresis of fluorescent labeled oligosaccharides in the analysis of beer [9]. The electrophoretic conditions give very high resolution and sensitivity, but, as other methods using reducing-end labeling, it does not detect non-reducing oligosaccharides, which are present in beer in substantial amounts [2]. The best results both in sensitivity and resolution are today obtained by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC).

Below is described the separation and structural determination of some new oligosaccharides relatively abundant in beer and a novel method for the partial characterization of the isomaltooligosaccharides from starch.

2. Experimental

Materials.—For the analysis, samples of Carlsberg Pilsner (4.8% alcohol), Carlsberg Master Brew (10.5% alcohol), Carlsberg Dark Lager (4.4% alcohol), Tuborg Super Light (<0.09% alcohol) beers and a wort sample were used.

NMR.—NMR spectra were recorded on Bruker AM 600 spectrometer with standard Bruker software in D_2O at 300 K, and assignments were performed with the use of PRONTO program [10]. Chemical shifts are given relative to acetone (δ_H 2.225 ppm, δ_C 31.45 ppm).

Chromatography.—HPLC on reverse phase column (Waters Delta Pak, 2×20 cm) was performed in water at 6 mL min^{-1} with RI detection. Fractions were analysed by NMR and MALDI-TOF mass spectrometry with DHB matrix. Gel chromatography was performed on TSK HW40(S) gel on 1.6×80 cm column in water at 1 mL min^{-1} with RI detection. Analytical HPAEC was performed on a CarboPac PA100 column (0.4×25) in 0.05 M NaOH with a gradient of 0.25 M AcONa from 10 to 80% with PAD detection on a Dionex DX500 chromatography system. Preparative HPAEC was performed on a CarboPac PA 100 column (0.9×25 cm) in 0.1 NaOH with a linear gradient of 1 M AcONa (2–30% over 1 h). Samples (10–40 mg) were injected, 20 s fractions were collected, analyzed by HPAEC and desalted using Dowex 50×4 (H^+). HPLC on amino phase was performed on a Asahipak NH2P-50 column (25×0.9 cm) in a gradient from 80 to 50% of MeCN in water with UV detection at 360 nm.

Mass spectrometry.—MALDI-TOF mass spectra were obtained on Finnigan Lasermat 2000 or Bruker Reflex III mass spectrometers using 2,4-dihydroxybenzoic acid (DHB) matrix, samples were prepared by mixing 1 μL of sample solution with 1 μL of DHB in acetonitrile and drying at 40 °C. Beer samples were diluted with water (1:4), wort sample was diluted 1:16 before application, or passed through SepPak C_{18} cartridge and then diluted.

Preparation of beer solids.—Beer (330 mL) was passed through Dowex 50 W×4 (50 mL, H^+) and DEAE TSK gel (50 mL, AcO^-) and freeze dried to give 8–16 g of solid material.

Preparation of aminocoumarin derivatives.—The dried beer sample (500 mg) was dissolved in a water–methanol–acetic acid mixture (4:4:2 mL), 7-amino-4-methylcoumarin (1 g) and $NaBH_3CN$ (1 g) were added, the mixture stirred at 60 °C overnight, concentrated, and separated on a C_{18} column in a gradient from 5 to 25% MeCN in 0.1% TFA to give a mixture of aminocumarin derivatives of sugars (600 mg). A portion of it

(60 mg) was separated preparatively by HPLC on an amino column.

Pullulanase digestion.—Aminocoumarin derivatives (50 μL of the solution containing 1 mg mL^{-1} of oligosaccharide) were treated with 2 μL of pullulanase suspension (Sigma, catalog No. P 5420) overnight and analysed by MALDI-TOF and HPLC on a amino column.

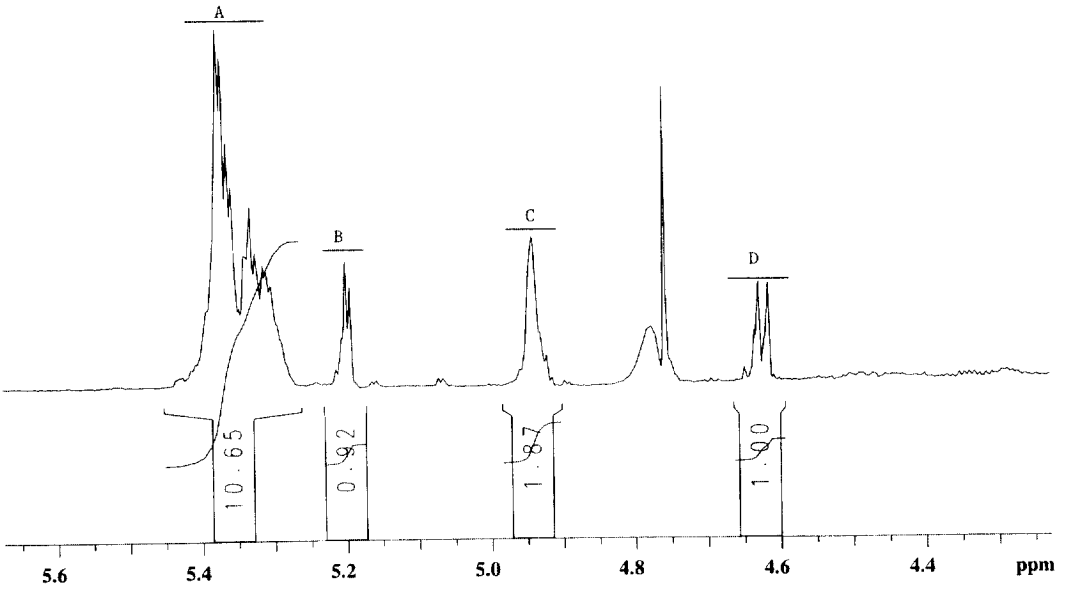


Fig. 1. Anomeric region of ^{1}H NMR spectrum of non-volatile components of Carlsberg Pilsner beer. Letters designate H-1 signals of α-Glc*p*-(1→4)-(A), reducing α-Glc*p* (B), α-Glc*p*-(1→6)-(C) and reducing β-Glc*p* (D) from isomaltooligosaccharides.

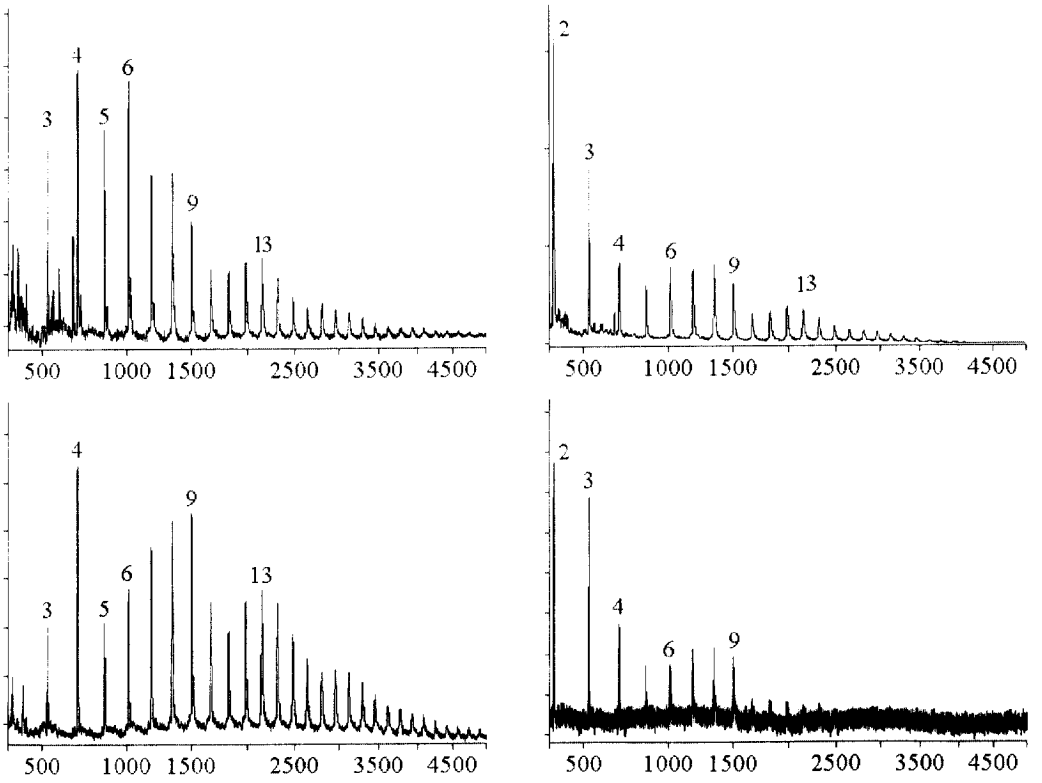


Fig. 2. MALDI-TOF mass spectra of different sorts of beer and wort. (a) Carlsberg Master Brew (10.5% alcohol); (b) Carlsberg Pilsner (4.8% alcohol); (c) Tuborg Super Light (< 0.09% alcohol); (d) wort.Peaks correspond to $[M+Na]^+$ ions, numbers indicate number of Glc units in oligosaccharides.

3. Results and discussion

Analysis of intact beer samples.—The ^{1}H NMR spectra of the non-volatile components of beer showed the expected signals for anomeric protons of starch oligosaccharides; signals from other oligosaccharides are also present at the noise level (Fig. 1).

Analysis of three different sorts of beers by MALDI-TOF mass spectometry gives a characteristic picture of molecular weight distribution of the Glc oligomers (Fig. 2). The dominant product in medium strong beers (alcohol content 4–6% by vol.) was a tetrasaccharide, and the degree of polymerisation (DP) showed maxima at 8–9, 13 and 18–19 and minima at 10–11 and 17 Glc residues, above these values the content of higher oligomers decayed smoothly (Fig. 2b). The highest visible molecular weight peaks using the current technology corresponded to DP 30–40. Strong beers (alcohol content >9%) showed an increased concentration of penta- and hexasaccharides, in

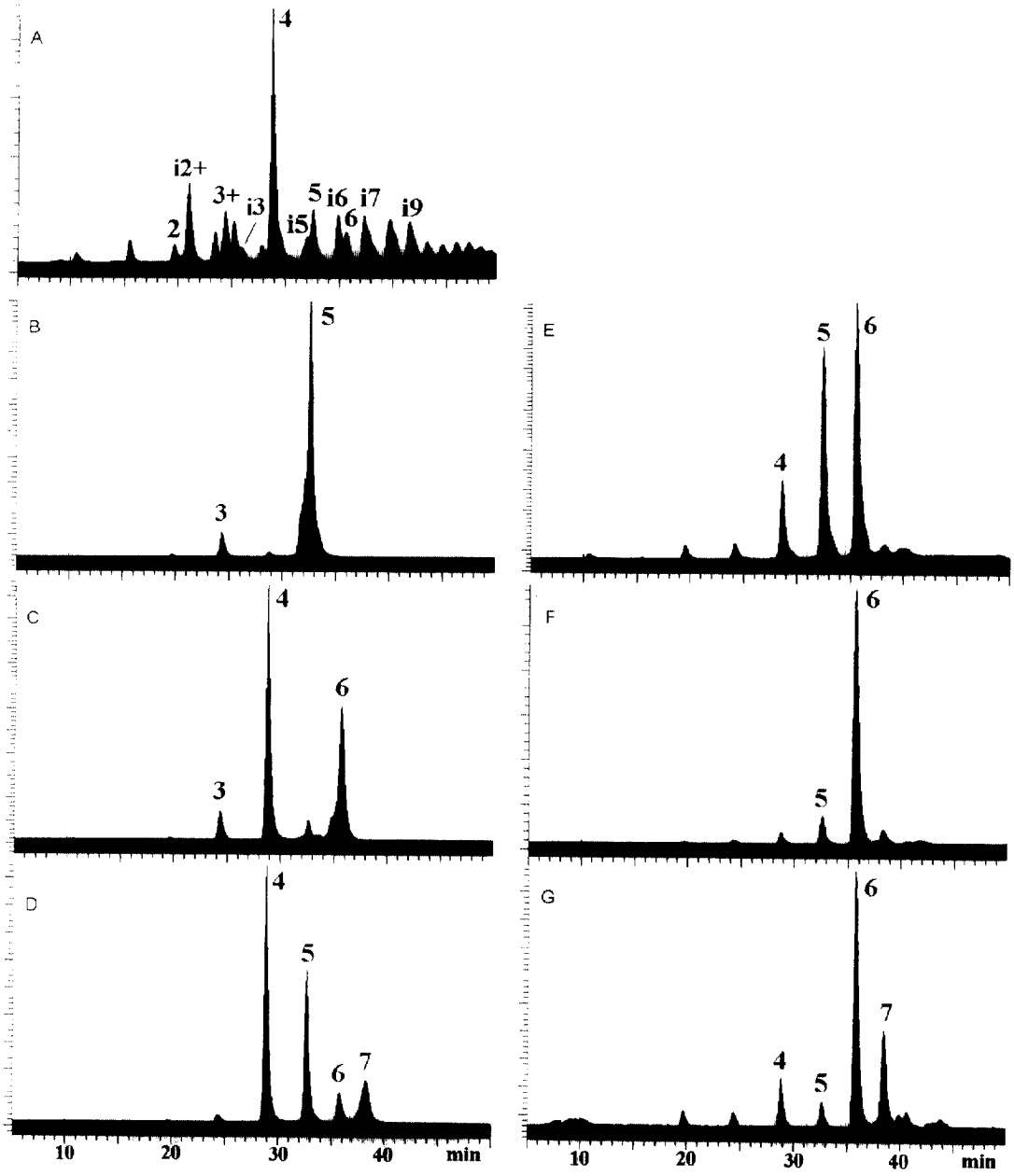


Fig. 3. HPLC on an amino column (UV detection at 360 nm) of AMK derivatives of beer oligosaccharides (a) and of pullulanase treated AMK labeled oligosaccharides of DP 5–10 (traces b–g); the numbers indicate the DP of oligosaccharides, "i" indicates isomaltooligosaccharides. Peak i2+ contain AMK derivatives of isomaltose and α-Glc*p*-(1→2)-Glc, peak 3+-derivatives of maltotriose and panose.

some beers the hexasaccharides were the most abundant oligomers (Fig. 2a). Analysis of low alcoholic beer showed dominance of di- and trisaccharides, with a similar picture of the higher mass distribution (Fig. 2c). This is most likely a result of the low activity of alcohol producing enzymes, which normally consume maltose and maltotriose during the fermentation of normal beers. A spectrum of wort showed an increased amount of the lower oligomers, whereas higher oligomers with DP about 10 were hardly visible in the experimental conditions used (Fig. 2d). In all MALDI-TOF spectra the intensity of the disaccharide peaks was decreased, but oligosaccharides of DP 3–10 gave a very similar and linear response.

Separation of beer samples.—HPAEC efficiently separated the mono- to trisaccharides, but was unable to separate the mixtures of malto- and isomaltooligomers of DP > 4; only a separation according to molecular weight with discrimination between linear and "branched" (containing α-(1→6)-linkages) oligomers was observed. The difference in branching degree was observed between strong beers, containing a higher proportion of linear oligomers, especially Glc_6, and the lighter beers. Light beer contained mostly Glc, maltose and maltotriose.

HPLC analysis of the 7-amino-4-methylcoumarin (AMK) labeled oligosaccharides on an amino column (Fig. 3a) gave partial separation of the trisaccharides, whereas higher oligosaccharides

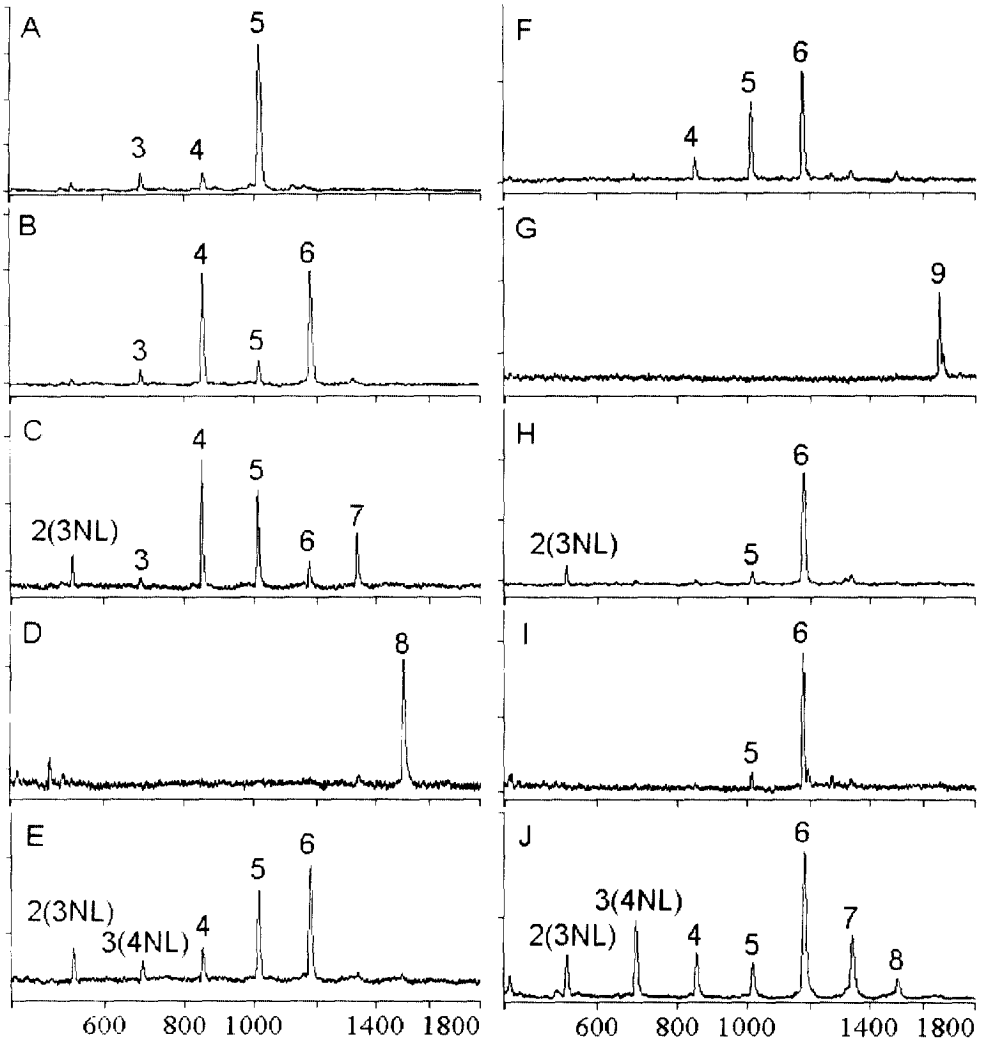


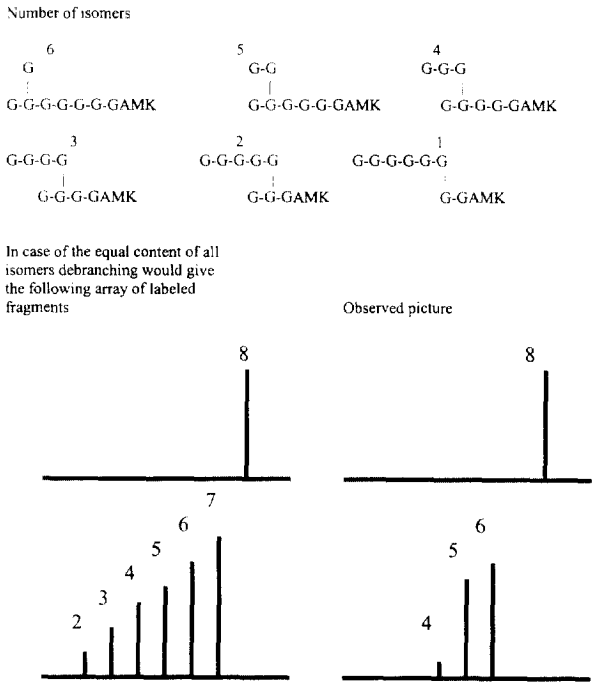
Fig. 4. MALDI-TOF mass spectra of pullulanase treated AMK labeled oligosaccharides of DP 5–7 (traces a–c), DP 8 (E-reaction mixture, F-isolated labeled products), DP 9 (H-reaction mixture, I-isolated labeled products), DP 10 (J). Traces D and G show mass spectra of isolated AMK derivatives of oligomers of DP 8 and 9, respectively, before pullulanase treatment. Peaks are labeled according to DP of AMK derivatives or (NL)-free oligosaccharides. *x*-Coordinate-*m*/*z*.

were separated according to molecular weight with the discrimination between linear and branched fractions, as was observed earlier for similar compounds [6]. The molecular weight distribution observed by this method was similar to that obtained by MALDI-TOF mass spectrometry. Preparative HPLC gave the mixtures of oligomers with the same degree of polymerisation of DP 2–16, the purity was controlled by MALDI-TOF mass spectrometry (Fig. 4d,g). The trisaccharide fraction contained mostly a mixture of the aminocoumarine derivatives of α-Glc-(1→6)-α-Glc-(1→4)-Glc (panose), α-Glc-(1→2)-[α-Glc-(1→4)]-Glc (centose) and α-Glc-(1→3)-α-Glc-(1→4)-Glc in nearly equimolar amounts, with a lower amount of isomaltotriose derivative. Fractions of DP >4 contained mostly malto- and isomaltooligosaccharides. From the integral intensity of the anomeric signals in NMR spectra of the isolated AMK derivatives it was concluded that the major part of the branched oligosaccharides up to DP 10 contained on average only one (1→6) bond. In agreement with this observation the overall structure of amylopectin also suggested that the probability of the presence of two branching points inside the fragments of less than 10 Glc units is low.

Branching pattern of isomaltooligosaccharides.— The fractions of aminocoumarine derivatives with defined DP from 4 to 16 Glc units, isolated by HPLC, were treated with the enzyme pullulanase to cleave specifically (1→6)-linkages. The products were analysed by MALDI-TOF and HPLC on an amino column with UV detection (Figs 3 and 4). Alternatively, AMK derivatives and reducing sugars were separated by reverse phase HPLC and analysed using HPAEC for the free sugars and MALDI-TOF for the labeled derivatives. The DP of the labeled products from the pullulanase degradation of oligosaccharides with DP < 10 identifies the length of a linear chain of α-(1→4)-linked Glc residues, which was substituted predominantly by a single side chain.

The tetra- and pentasaccharide fractions consisted mainly of linear structures. A minor branched pentasaccharide contained reducing maltotriose substituted with maltose, probably linked to O-6 of the middle residue. This compound has previously been shown to be the final product of starch degradation by α- and β-amylases [11]. Products with DP 6–9 on debranching with pullulanase gave mainly AMK derivatives two or three units shorter than the starting oligosaccharide. The observed results for the octasaccharide are summarized on Scheme 1. Debranching of the octasaccharide fraction (Figs 3e and 4f) produces almost equal amounts of linear penta- and hexasaccharide fractions, i.e. octasaccharide have structures b and c as indicated in Scheme 1, with only minor amounts of structures of the type d, and the remaining types hardly existing. The hexasaccharide fraction contained mostly a linear structure and a branched structure with a linear tetrasaccharide reducing chain substituted by maltose (Figs 3c and 4b). The nonasaccharide fraction consisted mostly of maltohexaose subsituted with maltotriose (Figs 3f, 4h and 4i). Thus, starch derived products of DP 6–9 are built mainly from maltooligosaccharide with a single maltose or maltotriose residue attached to O-6 of any Glc residue except the reducing one (this was shown by methylation analysis). Larger oligomers (DP 11–16) contained several branches and on pullulanase treatment gave complex mixtures of AMK labeled derivatives with a maximum content of G5–G7AMK, but not longer than G9AMK.

The branching pattern and DP of beer isomaltooligosaccharides is thus a result of the natural branching in starch [12] and the activity of starch degrading enzymes.



Scheme 1. Possible combinations of reducing and side chains in single branched labeled isomaltooligosaccharides with 8 Glc residues (only one isomer of the position of branching point is shown for each structure).

Structures of isolated compounds.—In order to isolate pure oligosaccharides from beer a combination of reverse phase, size exclusion and anion-exchange (HPAEC) chromatography was used. To facilitate NMR analysis oligosaccharides were reduced with $NaBH_4$. A sample of dried deionized beer (Carlsberg Pilsner) was passed through a preparative C_{18} column in water to remove higher oligosaccharides and non-polar compounds. The front fraction, which contained oligomers up to DP 7, was collected and reduced with $NaBH_4$. Separation on a TSK HW40 column gave four fractions, subsequently separated on a C_{18} column in water, to give 5–7 sharp peaks near the solvent front. This gave a pure form glucosylated trehalose **2**. All other fractions were further separated on a CarboPac PA-100 column. The structure of the products was subsequently determined by NMR (for each product COSY, TOCSY, ROESY, HMQC spectra were recorded) (Tables 1 and 2) and confirmed in some cases by methylation analysis [13].

Four groups of compounds were identified (Scheme 2): trehalose derivatives **1** and **2** (as well as known α,α- and α,β-trehalose); sucrose derivatives **3–5**; maltooligosaccharides, glucosylated at O-2 of reducing Glc (**6–8**); linear Glc oligomers with (1→3) and (1→4) linkages (**9,10**).

Several di- and trisaccharides containing β-linked glucopyranose residues were also detected in the mixtures in minor amounts, but none of them was obtained in pure form. The starch derived oligosaccharides could be separated according to molecular weight by the methods used, but no individual product of DP > 4 except linear maltooligosaccharides was obtained.

α,α-Trehalose is known to be produced by yeast in large amounts, and has been identified in beer before [4]. The synthesis and NMR spectra of compound **2** have been described previously [14]. Several sucrose derivatives, which were identified in beer previously [2], were not isolated in the present work. However, several others were isolated, all being glucosylated sucrose derivatives. Among the oligosaccharides **6-8** the trisaccharide **6** (with the trivial name centose) has previously been identified in honey and also synthesized [15,16]. The

Table 1
^{1}H NMR data for oligosaccharides (ppm relative to acetone δ 2.225)

Substance, unit	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
1A	5.34	3.50	3.61	3.34	3.65	3.68	3.76
1B	5.16	3.55	3.93	3.61	3.97	3.72	3.76
1C	4.57	3.32	3.45	3.33	3.40	3.63	3.78
2A	5.29	3.46	3.56	3.29	3.59	3.63	3.77
2B	4.54	3.33	3.69	3.53	3.49	3.65	3.71
2C	5.11	3.47	3.62	3.33	3.82	3.64	3.73
3A	5.34	3.44	3.63	3.36	3.72		
3B	4.92	3.46	3.63	3.32	3.56		
3C	3.57		4.18	3.95	3.82	3.70	3.70
	3.78						
4A	4.53	3.24	3.38	3.23	3.31	3.64	3.83
4B	5.45	3.59	3.81	3.41	3.75	3.72	
4C	3.72		4.11	3.92	3.79	3.70	
	3.72						
6A	3.76	3.88	3.98	3.86	3.90	3.59	3.71
	3.76						
6B	5.12	3.49	3.67	3.34	3.76	3.70	3.80
6C	5.04	3.50	3.65	3.37	3.83	3.70	
7A	3.76	3.88	3.98	3.86	3.90	3.60	3.72
	3.76						
7B	5.12	3.49	3.67	3.34	3.76	3.66	−3.82
7C	5.05	3.53	3.92	3.60	3.95	3.75	
7D	5.33	3.50	3.61	3.34	3.65	3.66	−3.82
8A	3.76	3.88	3.99	3.86	3.89	3.60	3.72
	3.76						
8B	5.12	3.49	3.67	3.34	3.76		
8C	5.05	3.53	3.93	3.60	3.95	3.75	
8D	5.32	3.55	3.89	3.58	3.78		
8E	5.33	3.50	3.61	3.34	3.64		
9A	5.27	3.49	3.66	3.36	3.93	3.69	3.75
9B	5.05	3.59	3.80	3.59	3.82	3.71	3.75
9C	3.58	3.78	3.84	3.82	3.90	3.60	3.72
	3.61						
10A	5.26	3.47	3.65	3.34	3.93		
10B	5.30	3.59	3.75	3.60	3.86		
10C	5.03	3.51	3.91	3.58	3.75		

Table 2
^{13}C NMR data for oligosaccharides (ppm relative to acetone δ 31.45)

Substance, unit	C-1	C-2	C-3	C-4	C-5	C-6
1A	100.1	72.5	73.6	70.1	73.5	61.0
1B	100.8	72.0	74.2	77.4	72.1	61.0
1C	104.0	74.1	76.3	70.0	76.8	61.2
2A	100.0	71.9	73.2	69.8	73.1	60.9
2B	103.2	73.4	76.3	76.9	75.2	60.7
2C	100.7	71.9	73.2	69.8	73.1	60.8
3A	93.1	72.0	73.4	69.9	73.0	61.0
3B	99.3	72.0	73.4	69.9	73.0	61.1
3C	66.6		77.1	74.9	81.9	62.9
4A	104.3	74.3	76.0	70.3	76.9	61.0
4B	92.9	79.7	72.4	69.9	73.0	61.0
4C	63.3		76.6	74.4	81.8	63.7
6A	61.0	77.8	71.6	82.0	72.2	62.5
6B	98.1	72.1	73.6	70.2	73.1	61.0
6C	101.2	72.1	73.6	70.2	73.1	61.0
7A	61.0	77.8	71.2	81.7	71.9	62.3
7B	97.7	72.0	73.1	69.7	73.3	61.0
7C	100.9	71.7	74.0	77.2	71.2	61.0
7D	100.3	71.9	73.0	69.7	73.2	61.0
9A	99.9	72.4	74.2	69.9	72.3	60.9
9B	101.4	70.8	80.3	70.8	73.1	61.0
9C	63.3	72.2	70.8	82.8	73.3	63.3

Trehalose type

α-Glc*p*-(1→4)-α-Glc*p*-(1↔1)-β-Glc*p* 1
A B C

α-Glc*p*-(1→4)-β-Glc*p*-(1↔1)-α-Glc*p* 2
A B C

Sucrose type

α-Glc*p*-(1→1)-[α-Glc*p*-(1↔2)-]-β-Fru*f* 3
A B C

β-Glc*p*-(1→2)-α-Glc*p*-(1↔2)-β-Fru*f* 4
A B C

α-Glc*p*-(1→6)-α-Glc*p*-(1→1)-[α-Glc*p*-(1↔2)-]-β-Fru*f*
+ (mixture) 5
α-Glc*p*-(1→1)-[α-Glc*p*-(1→6)-α-Glc*p*-(1↔2)-]-β-Fru*f*

Others

α-Glc*p*-(1→4)-[α-Glc*p*-(1→2)-]-Gol 6
C B A

α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-[α-Glc*p*-(1→2)-]-Gol 7
D C B A

α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-[α-Glc*p*-(1→2)-]-Gol 8
E D C B A

α-Glc*p*-(1→3)-α-Glc*p*-(1→4)-Gol 9
A B C

α-Glc*p*-(1→3)-α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-Gol 10
A B C D

Scheme 2. The structures of the oligosaccharides (Gol = glucitol).

origin of the compounds **1–8** could be enzymatic glucosylation of α,β-trehalose, sucrose and maltooligosaccharides during the fermentation process.

Starch also contains a small amount of 3-linked Glc, which was found as a part of oligosaccharides **9** and **10**. It is expected that more compounds of this type could be identified among the higher molecular weight products, but they are difficult to separate from isomaltooligosaccharides.

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