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Carbohydrate — protein interactions: substrate specificity of enzymes used in the degradation of oligosaccharides related to starch and cellulose

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Abstract - Treatment of amylopectin with an α-amylase (Terma-myl) produces a branched pentasaccharide in good yield. The structure of this product has been determined by high field NMR spectroscopy, where particularly homonuclear 2-dimensional methods have been indispensable. A new sensitive and fast 1-dimensional experiment for the correlation of the carbon- and proton chemical shifts of the above mentioned pentasaccharide will be presented. Chemical modifications of maltose and cellobiose have shown that specific hydroxyl groups are required for the binding and subsequently for enzymatic reaction with the enzymes amyloglucosidase (AMG) and cellobiase, whereas removal of other hydroxyl groups enhances the affinity of the modified substrates towards the enzymes in agreement with the "polar gate" theory proposed by Lemieux. Furthermore, the preferred conformations of the oligosaccharide in solution can be correlated with experimental results in terms of hydrophobic and hydrophilic surfaces of the carbohydrate substrates. Thus X-ray data of an amylase inhibitor, acarbose, in the active site of an α-amylase from porchine pancreas, show that acarbose is bound to the enzyme very near its ground state minimum energy conformation proposed on the basis of experimental NMR data and HSEA calculations. Finally, it will be demonstrated how high field NMR spectroscopy can be used as a sensitive and powerful tool in the study of dynamic enzymatic reactions with oligosaccharide substrates.

INTRODUCTION

Carbohydrate chemistry and technology are classical fields due to the abundance and availability of sugars as energy storage (starch) or as structural components (cellulose or chitin) of plants and animals. It is, however, only during the last 15 years that studies have been performed on carbohydrates which have proved that these structures play an important role as biologically active compounds (ref. 1). These examples have often been carbohydrates which are specifically recognized by proteins, in which the interaction has led to a specific biological response. A well-known example is the recognition of blood group determinants by antibodies and more recently carbohydrates located at cell surfaces have been shown to be of importance as tumor associated antigens recognized by monoclonal antibodies (ref. 2 & 3). Plant lectins may induce selective responses in animal cells by specific binding to cell surface carbohydrates or oligosaccharides may act as hormones in plant regulatory mechanism (ref. 4).

The study of the interactions between carbohydrates and proteins has been intensified during the last several years due to the fact that the technical facilities available to study the functions of carbohydrates and their receptors, such as methods of detection, isolation, identification, chemical synthesis and conformational analysis, have improved rapidly during the last decade. Thus, modern high resolution isolation procedures in combination with analytical techniques such as high field NMR spectroscopy and FAB mass spectroscopy have made it possible to elucidate structures of complex carbohydrates at milligram levels. Furthermore, biological reagents such as monoclonal antibodies which recognize specific carbohydrate sequences have facilitated structural determination on submicrogram quantities (ref. 5). The appearance of high resolution X-Ray structures of protein carbohydrate complexes (ref. 6 & 7) has made it possible to draw detailed conclusions about the way

in which L-arabinose is bound to the arabinose-binding protein. Furthermore, extensive chemical modifications of oligosaccharides related to the Lewis-b blood group determinants have been carried out by Lemieux and co-workers (refs. 8-12), and the binding of these compounds to lectins and antibodies has been studied in great detail. The results from these studies have led Lemieux to propose the hydrated polar gate effect (ref. 13) on the strength and specificity of binding of oligosaccharides by proteins. The latter results have been based on an appreciation of the preferred conformation of the oligosaccharides in solution, as determined by high resolution NMR spectroscopy and simple computer calculations in which the importance of the exo-anomeric effect is well appreciated (HSEA calculations) (ref. 14 & 15). In the present paper the affinity of oligosaccharides towards glycolytic enzymes, particularly glucoamylase, has been investigated using the same approach as described by Lemieux and co-workers. The first part will, however, be devoted to a discussion of how modern NMR spectroscopic methods can be used to determine the structures of complex oligosaccharides produced during enzymatic degradation of starch with amylases.

DEGRADATION OF AMYLOPECTIN WITH AN α-AMYLASE

The degradation of amylopectin with an α-amylase (Termamyl, Novo A/S, Denmark) has been carried out under standard conditions in order to determine the structures of the oligosaccharides produced during this reaction using high field ^{1}H- and ^{13}C-NMR spectroscopic methods. The results will furthermore allow one to make conclusions about the substrate requirements of the enzyme.

A chromatogram of the resulting oligosaccharide mixture indicates that one product predominates. ^{1}H- and ^{13}C-NMR spectra of this fraction show that this substance must be a pentasaccharide. The compound is converted into its β-methyl glucoside (1) by conventional methods in order to facilitate spectral analysis. The ^{1}H-NMR spectrum of the resulting product is shown in Fig. 1a and the ^{13}C-NMR spectrum in Fig. 1b. These spectra clearly indicate

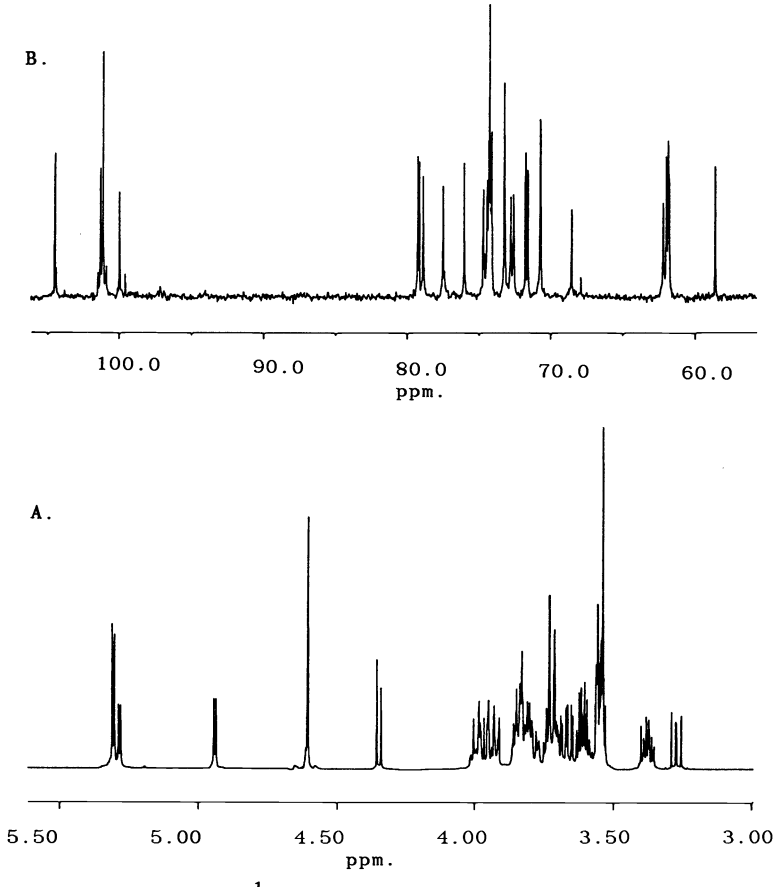

Fig. 1. A) 500 MHz ^{1}H-NMR spectrum of the pentasaccharide (1) in D_2O at 310 K. B) 125.7 MHZ ^{13}C-NMR spectrum of (1) in D_2O.

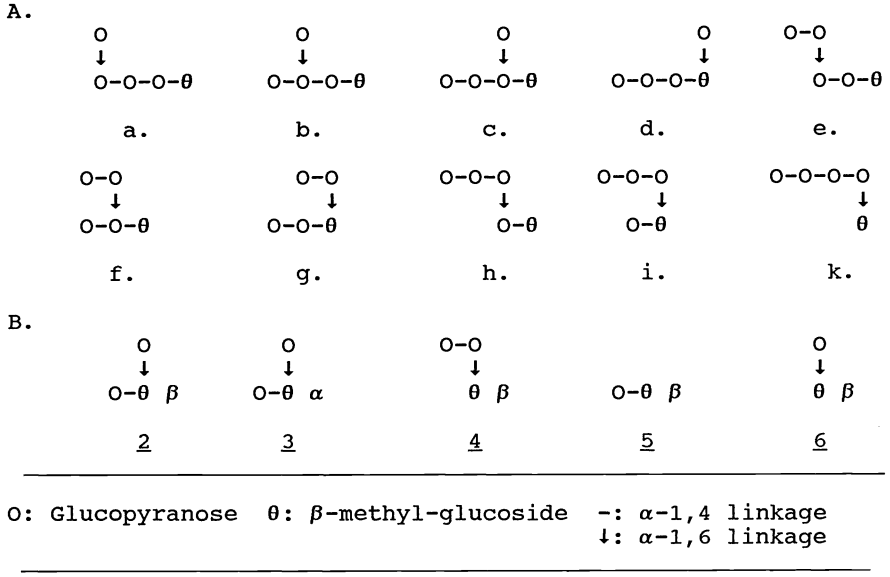

Fig. 2. A) Possible structures for the pentasaccharide (1) based on the spectra in fig. 1. B) Model compounds 2-6 used for the structural determination of (1).

that the product is a pentasaccharide with the reducing end as a β-methyl-glucoside and that it furthermore has three α-1,4-linked and one α-1,6-linked glucopyranosyl units. These can, however, be incorporated into ten different pentasaccharide structures as shown in Fig. 2a.

A complete ^{1}H- and ^{13}C-NMR spectroscopic analysis of the model compounds (2 - 6) indicated in Fig. 2b, has been carried out at 500 MHz and the results are presented in Tables 1 and 2. From these data, it is possible to extract the information shown in Table 3, which shows that the proton chemical shifts of both H-4 and H-3 in α- or β-linked glucosides are very sensitive to the presence of a substituent at the 4-position causing a downfield shift of H-3 and H-4 of about 0.3 ppm. Similarly a substitution in the 6-position with an α-D-glucopyranosyl-unit of an α- or β- glucopyranoside causes the H-5 proton to be downfield shifted about the same amount (0.3 ppm). Inspection of the ^{13}C-NMR spectral data shows that simultaneous substitution of O-4 and O-6 with α-D-glucopyranosyl units in a β-methyl glucoside (compound 2) causes C3/C5 to resonate at 76.7 and 72.6 ppm respectively, whereas the signals are found at 77.1/75.4 ppm and 76.7/74.9 ppm in the compounds with one substitute in either the 4-position (compound 5) or the 6-position (compound 6).

The observation of ^{13}C signals in Fig. 1b at 77.0 and 75.6 ppm therefore shows that the reducing end of the molecule only has one α-D-glucopyranosyl unit linked in either the 4- or the 6-position and this rules out possible structures d,g, and i shown in Fig. 2a.

The 2-dimensional ^{1}H-^{1}H correlated experiment (COSY) (ref. 16) is shown in Fig. 3 for the pentasaccharide and it is clearly seen that the chemical shift for the H-3 proton of the reducing unit is 3.75 ppm, which indicates that this unit has a glucopyranosyl unit at O-4 thereby ruling out the possibility of structure k (Fig. 2a).

The chemical shifts for the H-4 protons of the non-reducing units are found at 3.38 and 3.40 ppm respectively, and the COSY-spectrum (Fig. 3) shows that the neighboring protons H-3/H-5 resonate between 3.65 and 3.75 ppm. This indicates that none of the structures with a "terminal" glucose-unit can have substituent in the 6-position of this glucose, i.e., the possible structures a, e, h and k are eliminated, which leaves only b, c and f as possible structures (Fig. 2a).

Table 1. ^{1}H-NMR[a] Data for Model Compounds 2-6.

		H-1	H-2	H-3	H-4	H-5	H-6[b]	H-6[c]
2	α-Glc(1-4)	5.34 3.8	3.58 9.8	3.74[d] 9.8	3.44[e] 9.8	3.77[f]	3.87	3.78
	[α-Glc(1-6)]	5.01 3.8	3.56 9.8	3.70[d] 9.8	3.42[e] 9.8	3.74[f]	3.87	3.78
	β-GlcOMe	4.42 8.0	3.32 9.8	3.78 9.8	3.67 9.8	3.75	3.92 2.0	3.97 6.0,11.5
3	α-Glc(1-4)	5.36 3.6	3.58 9.8	3.73	3.42	3.74	3.87	3.79
	[α-Glc(1-6)]	5.01 4.0	3.53 9.8	3.71	3.46	3.74	3.87	3.79
	α-GlcOMe	4.82 4.0	3.63 10.0	3.96 10.0	3.68	3.95	3.88	3.99 5.0,11.0
4	α-Glc(1-4)	5.35 4.0	3.57 10.0	3.66 10.0	3.42 10.0	3.71	3.87 2.0	3.76 5.5,12.0
	α-Glc(1-6)	4.96 4.0	3.60 10.0	3.99 10.0	3.65 10.0	3.83	3.81	3.82
	β-GlcOMe	4.40 8.0	3.26 9.8	3.49	3.49	3.63	3.83 2.0	3.94 5.0,11.5
5	α-Glc(1-4)	5.40 3.8	3.58 9.8	3.68 9.8	3.42 9.8	3.72	3.85 2.0	3.74 5.0,12.0
	α-GlcOMe	4.39 8.5	3.29 9.8	3.76 9.8	3.60 9.8	3.58	3.95 2.0	3.74 5.0,12.0
6	α-Glc(1-4)	4.96 3.5	3.55 9.8	3.73 9.8	3.44 9.8	3.72	3.86 2.0	3.78 5.0,12.0
	β-GlcOMe	4.42 8.0	3.27 9.5	3.49 9.8	3.53 9.8	3.65	3.77 2.0	3.99 4.5,11.0

a. Measured at 500 MHz in D_2O at 310 K with acetone(2.22ppm) as internal reference.
b. Pro-S-proton. c. Pro-R-proton. d,e,f. Assignments may be reversed.

Table 2. ^{1}H-NMR[a] Data for Model Compounds 2-6.

^{13}C-NMR-Data		C-1	C-2	C-3	C-4	C-5	C-6
2	α-Glc(1-4)	100.5	72.6	73.8[b]	70.3[c]	74.0[d]	61.4[e]
	[α-Glc(1-6)]	99.2	72.2	73.7[b]	70.2[c]	73.5[d]	61.3[e]
	β-GlcOMe	103.9	73.6	76.7	78.7	72.6	67.5
3	α-Glc(1-4)	100.5	72.6	73.7	70.3[b]	73.7[c]	61.2
	[α-Glc(1-6)]	99.2	72.1	73.5	70.1[b]	73.9[c]	61.3
	α-GlcOMe	99.7	72.5	74.0	78.8	71.7	69.7
4	α-Glc(1-4)	100.4	72.3	73.2	70.0	73.6	61.0
	α-Glc(1-6)	98.1	71.8	74.0	77.9	70.8	61.1
	β-GlcOMe	103.9	73.5	76.5	69.9	74.9	66.1
5	α-Glc(1-4)	100.4	72.5	73.7	70.2	73.5	61.6
	β-GlcOMe	103.9	73.8	77.1	77.7	75.4	61.4
6	α-Glc(1-6)	98.4	72.1	73.8	70.1	72.4	61.1
	β-GlcOMe	103.9	73.8	76.7	70.0	74.9	66.0

a) Measured at 125.7 MHz in D_2O at 310 K with dioxane as internal referemce (67.4 ppm).

b,c,d,e) Assignments may be reversed.

Table 3. Conclusions from Analysis of NMR data for Reference Compounds 2-6.

Chemical Shifts for		Without α-glucopyranose	With α-glucopyranose
	Protons	in 4-position	in 4-position
A	H-4	3.44 - 3.53 ppm	3.60 - 3.68 ppm
	H-3 α	3.66 ppm	3.76 ppm
	H-3 β	3.49 -	3.76 -
		in 6-position	in 6-position
B	H-5 α	3.72 ppm	3.95 - 4.00 ppm
	H-5 β	3.58 -	3.75 ppm
	Carbons	in 4-and 6-positions	in 4-and 6-positions
C	C-3/5 β	77/75 ppm	77/73 ppm

Inspection of the data in tables 1, 2 and 3 shows that the only difference expected between these structures is the chemical shift of H-3 in the α-1,6-linked glucopyranosyl-unit, because the 4-substitution in structure f should lead to a downfield shift of about 0.3 ppm, whereas this should not be observed in the data from structures b and c. An analysis of the COSY-experiment (Fig. 3) cannot solve this problem because the chemical shifts for the H-2 protons for α-1,6 and α-1,4 linked glucosides are very similar. However, a new 2-dimensional experiment, relayed COSY (ref. 17) shown in Fig. 4, allows an unambigous assignment of the signals H-1 - H-2 to H-3 for the α-1,6 and α-1,4 linked oligosaccharides and clearly indicates that H-3 of the 1,6-linked unit resonates at 3.98 ppm and thus excludes structures a,b,c and d, which proves that the pentasaccharide has the structure shown in Fig. 5. It is possible to extend the relayed experiment to a double relayed experiment which allows an unambigous assignment of protons 1 to 4 in the pentasaccharide as shown in Fig. 6. This technique for the assignment of ^{1}H-NMR spectra for complex oligosaccharides is very useful because the "handle" is the anomeric proton which often resonates in an area devoid of severe signal overlap.

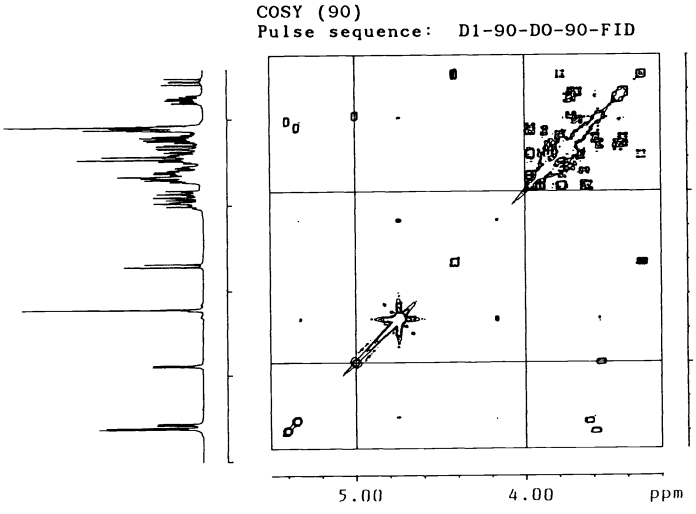

Fig. 3. 500 MHz ^{1}H-NMR COSY experiment of the pentasaccharide (1) in D_2O at 300 K.

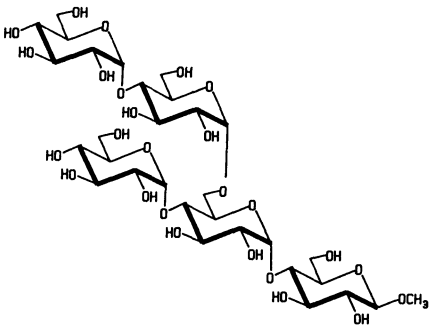

Fig. 5.
Structure of the pentasaccharide (1) resulting from the degradation of amylopectin with α-amylase.

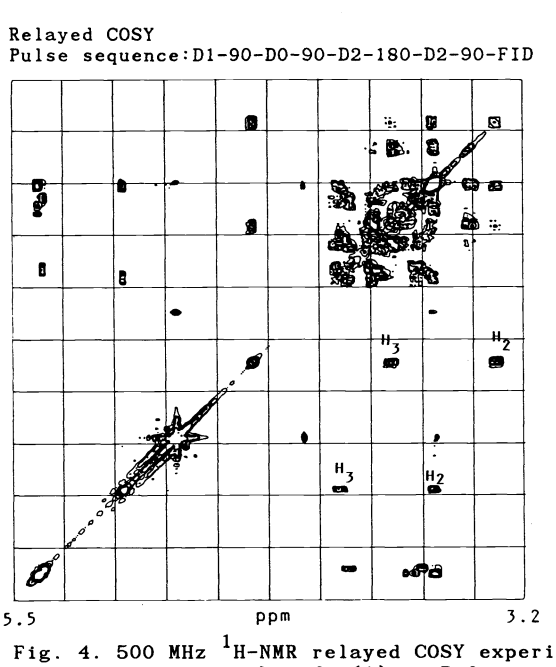

Fig. 4. 500 MHz ^{1}H-NMR relayed COSY experiment on pentasaccharide (1) in D_2O.

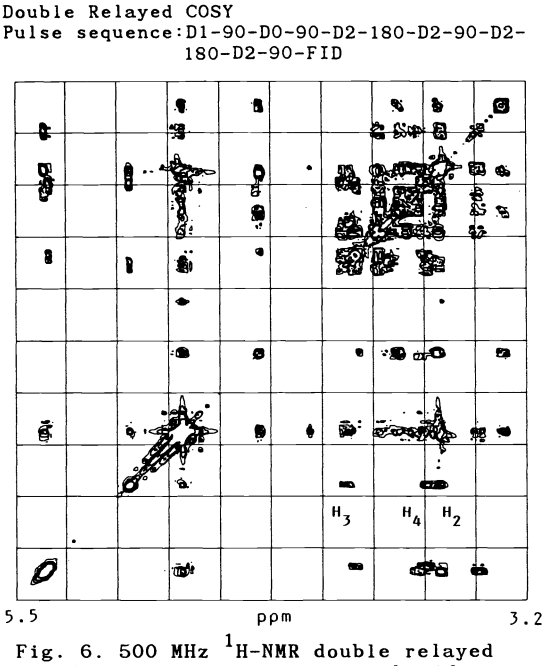

Fig. 6. 500 MHz ^{1}H-NMR double relayed COSY experiment on pentasaccharide

A complete structural proof furthermore requires that the ^{13}C-NMR chemical shifts are also in agreement with the proposed structure. The safest way to assign these is through the carbon-proton correlated 2-dimensional NMR experiment which can be carried out if a sufficient amount of compound is available (ref. 18). This experiment establishes the relation between the ^{1}H- and the ^{13}C-NMR chemical shifts and, based on the results discussed above, gives a complete assignment of the ^{13}C-NMR signals as seen from Table 4. This method, however, requires that an appreciable amount of compound (~ 20-30 mg) is available if the experiment is to be carried out in the phase sensitive mode (ref. 19) which gives most information. The experiment, however, can be carried out using a pulse sequence proposed by Bax (ref. 20) which causes decoupling in the proton dimension. This gives better signal to noise because the intensity from the multiplets are concentrated into singlets except for diastereomeric CH_2 groups which appear as AB-systems. When only a very small amount of material (2-5 mg) of an oligosaccharide is available, it is not possible to carry out these powerful correlated experiments, but a solution to this problem has been suggested by Pearson (ref. 21). This experiment, carbon hydrogen correlation from one-dimensional polarization-transfer spectra by least squares analysis, CHORTLE, yields results equivalent to those of the two-dimensional proton-carbon correlated experiment discussed above, but has the advantages (phasing) and speed of the one-dimensional experiment. A few (four to eight) ^{13}C spectra are acquired using a modified INEPT sequence, and from the intensity variations of the ^{13}C-signals, the chemical shift of directly bonded protons can be calculated very accurately by using a least-squares analysis. The accuracy of the proton chemical shifts is dependent on the signal to noise ratio but, in most cases, compares favorably to a two-dimensional experiment in accuracy but is almost a factor of 10 faster, or allows one to work on much more dilute samples. This experiment has also been carried out on the pentasaccharide and gave the same results as the two-dimensional experiment. One drawback of the CHORTLE-experiment is, however, that data analysis from overlapping ^{13}C-NMR signals normally does not give satisfactory results because the evolution of intensities is modulated by two different frequencies. We have developed a computer program which makes it possible automatically to transfer the data from our NMR spectrometer to a Personal Computer, on which the least squares analysis is carried out (ref. 22).

Table 4. ^{1}H-NMR[a] and ^{13}C-NMR[b] Data for Pentasaccharide (1).

^{1}H-NMR Data	H-1	H-2	H-3	H-4	H-5	H-6	H-6	OMe
α-Glc(1-4)	5.40[c] 3.6	3.56[d] 9.8	3.68 9.8	3.38[e] 0.9	3.71	3.77	3.84	
α-Glc(1-6)	4.96 3.6	3.58 9.8	3.98	3.58 9.8	3.84	3.77	3.84	
(α-Glc(1-4))	5.39[c] 3.6	3.57[d] 9.8	3.68 9.8	3.40[e] 9.8	3.71	3.77	3.84	
α-Glc(1-4)	5.36 3.8	3.62 9.8	3.92 9.8	3.64[f] 9.8	4.00	3.84	3.94	
β-Glc OMe	4.38 8.4	3.28 9.8	3.75 9.8	3.62[f] 9.8	3.58	3.80	3.96 1.2,12.2	3.56
^{13}C-NMR-Data	C-1	C-2	C-3	C-4	C-5	C-6	OMe	
α-Glc(1-4)	100.7	72.8	73.7	70.3	73.8[c]	61.6[d]		
α-Glc(1-6)	99.6	72.2	74.3	78.9	71.4	61.5[d]		
(α-Glc(1-4))	100.7	72.8	73.7	70.3	73.9[c]	61.6		
α-Glc(1-4)	100.9	72.4	74.0	78.8[e]	71.2	68.2		
β-Glc OMe	104.1	73.8	77.1	78.5[e]	75.6	61.8	58.2	

a) Measured at 500 MHz in D_2O at 310 K with acetone as internal reference (2.22 ppm).

b) Measured at 125.7 MHz in D_2O at 310 K with dioxane as internal referemce (67.4 ppm).

c,d,e,f) Assignments may be reversed.

The discussion above shows that it is possible to make a complete structural analysis of the pentasaccharide (1) with modern NMR spectroscopic technique even though all the component monosaccharides are glucopyranose units. The compound has previously been isolated by French et al. (ref. 23) as the free sugar. The structure has in this case been proven by enzymatic methods after methylation - analysis, periodate oxidation and partial acid hydrolysis failed to give an unambigous result. It is interesting to note that this compound, which accumulates during the enzymatic degradation of starch (amylopectin), is the only pentasaccharide that includes the branch point and does not have an unmodified maltose unit present. This suggests that the active site of the enzyme requires a maltose unit in order to function optimally.

SUBSTRATE SPECIFICITY OF THE ENZYME AMYLOGLUCOSIDASE (AMG)

Degradation of starch with α-amylases as discussed above results in mixtures of oligosaccharides which can be further degraded to glucose with amyloglucosidases. One of these enzymes produced by the microorganism Aspergillus niger, is commercially available in large amounts due to its use in the processing of starch. This enzyme is a glycoprotein and the amino acid sequence has been determined by Svensson and co-workers (ref. 24), but it has not been possible to crystallize the enzyme in order to obtain information about its three dimensional structure of particular interest is the structure of the active site. We have therefore made a study of the reaction mechanism of this enzyme and the forces involved in the recognition of substrates through chemical synthesis of modified substrates related to maltose.

As seen from the results shown in fig. 7 the accommodation of the substrate in the active site requires that both D-glucopyranose units of maltose are present and that the non-reducing unit exists in the $^4C_1$ conformation. The conformational requirements for the reducing glucopyranose-unit are less stringent since unsaturation between C-1 and C-2 or C-5 and C-6 does not prevent enzymatic cleavage, but complete inversion of the pyranose ring to a $^1C_4$ conformation, as in maltosan, gives a structure which is not a substrate for the enzyme.

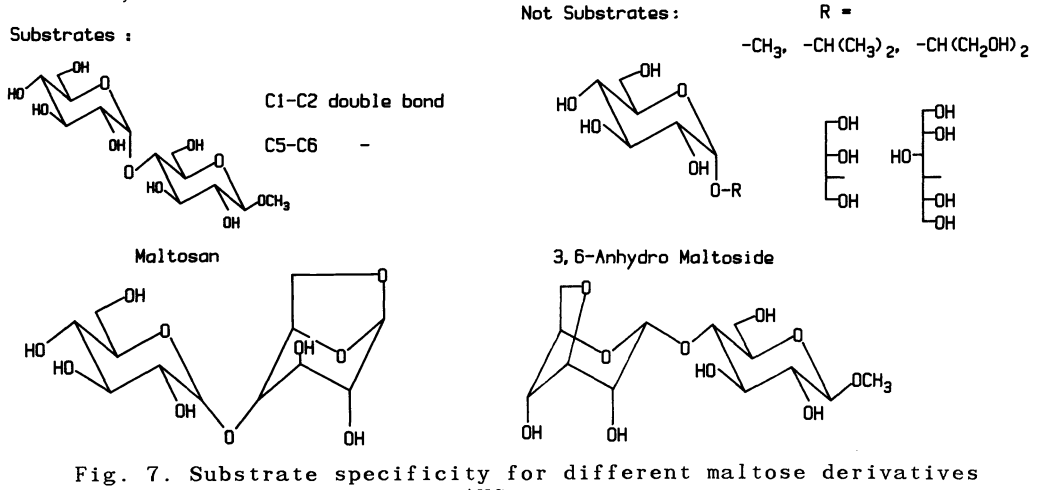

Fig. 7. Substrate specificity for different maltose derivatives towards the enzyme AMG.

The importance of the different hydroxyl groups for the enzymatic reaction to take place with maltose as the substrate, was next studied by chemical synthesis of all the possible mono- and several dideoxy derivatives of methyl β-maltoside. The results are shown in Fig. 8 and show that three hydroxyl groups (OH-3 in the reducing unit, OH-4' and OH-6' in the non-reducing unit) are essential for enzymatic activity. On the other hand, removal of the other hydroxyl groups does not prevent enzymatic reaction and in several cases even leads to better substrates than methyl β-maltoside. Synthesis of the three compounds with O-methyl ethers in the essential positions also gave compounds which do not appear to be active, suggesting that these hydroxyl groups are involved as donors of hydrogen bonds in the enzyme-substrate complex.

Several epimeric compounds were also synthesized, and as shown in fig. 9a, the 3-position in the reducing end still proved to be very important for the activity of the compounds as substrates.

These results are in excellent agreement with the model proposed by Lemieux (ref. 13) for the specificity of the substrates toward the enzyme AMG. An

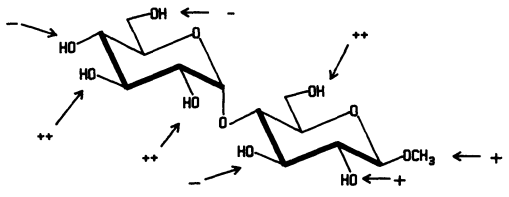

Fig.8. Substrate specificity of AMG on deoxy derivatives of maltose.
+ denotes positive reaction.
- " negative "
++ indicates substrates which are better than methyl β-maltoside

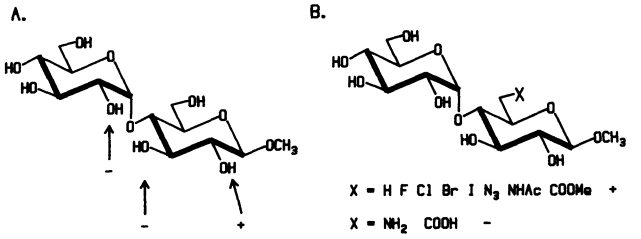

Fig .9. Substrate specificity for the enzyme AMG.
A) epimeric structures of methyl β-maltoside
B) 6-substituted methyl β-maltoside derivatives

investigation of the minimum energy conformation of methyl maltoside as determined by HSEA-calculations (ref. 14 & 25) and supported by ^{1}H-and ^{13}C-NMR data shows (ref. 26) that maltose has a hydrophobic area defined by H-2, H-4, H-1 and the ring oxygen of the non-reducing end of the molecule to H-4 and the CH_2OH group at C-5 in the reducing end. It is in agreement with this that the 6-deoxy compound should be a better substrate and that the 2'-epi compound is not a substrate. Substitution at the 6-position furthermore allows a wide range of neutral substituents like F, Cl, Br, I, N_3, NHAc and COOMe (fig. 9b) whereas introduction of charged groups like NH_2 or COOH cancels catalytical activity. All results support the theory proposed by Lemieux (ref. 13).

The reaction kinetics have been investigated for the above mentioned compounds using high field NMR spectroscopy, which allows one to follow both the disappearance of substrates as well as the formation of products as a function of time and thus to determine the anomeric specificity of the enzyme. But it is also possible to do this in competitive reactions in which both compounds can be substrates or one compound is a substrate and the other a (potential) inhibitor. It is possible to determine the $t_{\frac{1}{2}}$ and V_0 from progress curves as published recently by Duggelby (ref. 27 & 28), and the results for a selection of substrates and mixed substrates are shown in Table 5. It can be concluded from these results that 6-chloro-6-deoxy-maltoside is a poor substrate but a very potent competitive inhibitor, whereas the 3-deoxy compound, which is not a substrate, does not inhibit the reaction at all.

The same technique can also be used to study the affinity of larger substrates like maltotriose or branched compounds like the pentasaccharide (1). These results are presented in Table 5 and show that the enzyme has a higher affinity towards larger substrates, because maltotriose is cleaved almost completely to glucose and methyl β-maltoside before the latter is cleaved to glucose and methyl β-glucopyranoside. Similarly, cleavage of the branched compounds shows that the enzyme can cleave α 1-6 linkages, but much slower than α 1-4 linkages and normally other enzymes will have to be used to improve the efficiency in the cleavage of α 1,6-linkages in the processing of starch in technical applications.

SUBSTRATE SPECIFICITY OF THE ENZYME CELLOBIASE

The discussion above has focused on the enzymatic cleavage of an α 1,4-glucosidic linkage, but we have also investigated, using the same approach, the substrate specificity of cellobiase, an enzyme which cleaves β 1,4-glucosidic linkages. The enzyme is commercially available, but not so well characterized as AMG.

The results from these investigations show that the specificity for this enzyme is very different from AMG because as seen from Table 6 the enzyme requires only that the non-reducing glucopyranose-unit is present in the $^{4}C_1$ conformation, since cellobiosan and methyl β-D-glucopyranoside both are substrates. However, removal of the hydroxyl-groups in the non-reducing unit shows that the OH-3' and OH-4' both are essential for the enzymatic reaction and that the 6'-deoxy-cellobioside is a poor substrate. The 4'-epi compound (methyl β-lactoside) is not a substrate either. Removal of the 1,3 or 6-OH groups in all cases leads to compounds which are better substrates than methyl cellobioside (Table 6). Competitive experiments between methyl cellobioside and the corresponding 6' and 4'-deoxy derivatives shows that the former is good competitive inhibitor whereas the latter shows no inhibition at all (Table 6).

Table. 5 Kinetic Data for the Degradation of Maltose and Derivatives with Amyloglucosidase (AMG)[a]

Substrate	Concentration (mM)	v_o	$t_{1/2}$ min.
Methyl 6-chloro-6-deoxy-β-maltoside(7)	7.2	0.19	254
Methyl 6-deoxy-β-maltoside (8)	8.3	4.07	12
Methyl β-maltoside (5)	7.3	1.85	27
Methyl 3'-deoxy-β-maltoside (9)	8.3	4.16	13
Methyl 6-chloro-6-deoxy-β-maltoside (7)	3.6	0.20	215
Methyl β-maltoside (5)	3.8	0.32	127
Methyl 3'-deoxy-β-maltoside (9)	4.2	2.79	19
Methyl β-maltoside (5)	3.8	1.11	42
Methyl 6-fluoro-6-deoxy-β-maltoside (10)	4.0	0.45	106
Methyl β-maltoside (5)	3.8	0.47	102
Methyl 3-deoxy-β-maltoside (11)	4.2	0.00	~
Methyl β-maltoside (5)	3.8	4.90	9
Methyl 4,6-di-O-(α-D-glucopyranosyl)-β-D-glucopyranoside (2)	7.5		
α-1,4 linkage		1.79	24
α-1,6 linkage		0.39	244
Methyl β-maltotrioside (12)	7.3		
α-1,4 linkage (terminal)		17.06	5
α-1,4 linkage		2.11	28
Methyl 6"-O-(α-maltosyl)-β-maltotrioside (1)	14.3		
Formation of Methyl β-panoside		2.34	21

[a] At 27°, pH 4.3, and 5.5 10^{-10} M AMG.

Table. 6 Kinetic Data for the Degradation of Cellobiose and Derivatives with Cellobiase[a]

Substrate	v_o	$t_{1/2}$
Methy β-cellobioside	1.00	1.00
Methyl β-glucopyranoside	0.37	9.7
1,6 Anhydro-cellobiose	0.40	2.50
1-Deoxy-cellobiose	1.5	0.60
Methyl 6-deoxy-β-cellobioside	2.1	0.53
Methyl 3-deoxy-α-cellobioside	4.4	0.49
Methyl 3'-deoxy-β-cellobioside	not substrate	
Methyl 4'-deoxy-β-cellobioside	not substrate	
Methyl 6'-deoxy-β-cellobioside	0.18	5.6
Methyl β-lactoside	not substrate	
Methyl β-cellobioside Glucose	0.90	1.1
Methyl β-cellobioside Methyl β-glucoside	0.74	1.4
Methyl β-cellobioside Methyl 6'-deoxy-β-cellobioside	0.58	1.7
Methyl β-cellobioside Methyl 4'-deoxy-β-cellobioside	no inhibition	

a) At 27°C and pH 7.0

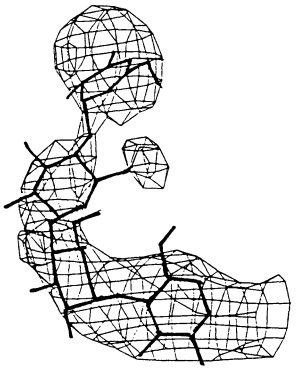

Fig. 10. Fitting of Acarbose in its minimum energy conformation (ref. 26.) bound to α-amylase into the electron density difference map at 5Å resolution (ref. 30.)

When the reaction course is followed by ^{1}H-NMR spectroscopy, it is observed that a product with a chemical shift about 4.1 ppm is formed during the enzymatic reaction, but later degraded again. This product is presumably gentiobiose i.e. the β 1-6 linked oligosaccharide which must be formed in a transglucosidation reaction. It is interesting to note that this enzyme which is capable of performing this transglucosidation reaction also shows product inhibitory effects (Table 6) since both glucose and methyl β-D-glucopyranoside inhibits the reaction of methyl cellobioside significantly.

PROTEIN - CARBOHYDRATE INTERACTIONS

The discussion above provides indirectly evidence about the interactions between carbohydrates and enzymes, but it would be desirable to have a more detailed picture of a carbohydrate substrate in the active site of an enzyme. This is normally not easy to obtain because the enzymes cleave the substrates before it is possible to crystallize the complex and it is therefore easier to work with inhibitors. This has been done with acarbose, a pseudo-tetrasaccharide, which is a potent inhibitor of amylases (ref. 29). The solution conformation has been determined by HSEA calculations and NMR spectroscopy (ref. 26) and it has been shown that the minimum energy conformation is very similar to that found in maltose. The latter compound includes all the requirements discussed above for the substrate specificity of AMG with the three polar gates and the hydrophobic surface including the 6-deoxy-function. Haser and coworkers (ref. 30) have crystallized an α-amylase, from porchine pancreas, with and without acarbose in the active site and determined the structure by X-ray crystallography to a resolution of 5 Å. The difference fourier map of the electron densities from those two structures are shown in Fig. 10 together with the calculated (HSEA) minimum energy conformation of acarbose fitted into the map. This shows a good agreement at the resolution obtained and work is in progress (ref. 30) to improve the resolution of the data.

CONCLUSIONS

It has been shown that modern NMR spectroscopic techniques combined with chemical synthesis of modified substrates are very useful in the study of the interactions between oligosaccharides and enzymes. A few hydroxyl-groups (the polar-gates) play a very important role for the binding of the carbohydrate by the protein whereas othersgenerally enhance the affinity of the binding.
The polar gates can be located in different units of oligosaccharides or only in one of them. It appears resonable based on the experimental results to assume that the oligosaccharides are bound to the enzyme in conformations quite similar to the ones experimentally determined in solution by NMR spectroscopy.

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