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New Methods for Enzyme Hydrolysis, Analysis, and Characterization of Modified Cellulose

Claes Melander



LUND
UNIVERSITY

Department of Analytical Chemistry
2006

Akademisk avhandling som för avläggande av filosofie doktorsexamen kommer att offentligas försvaras på Kemicentrum, Sölvegatan 39, Lund, Hörsal A, fredagen den 19 Maj 2006, kl 10.15, med vederbörligt tillstånd av matematisk-naturvetenskapliga fakulteten vid Lunds Universitet. Fakultetsopponent är docent Bodo Saake, Zentrum Holzwirtschaft, Holzchemie und chemische Technologie des Holzes, Universität Hamburg, Hamburg, Germany.

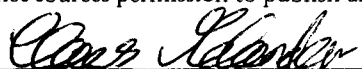
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Abstract <p>Cellulose can be chemically modified to produce derivatives with specific properties. Knowledge of correlations between the modification reaction, substituent distribution, origin of cellulose and properties are important in order to design processes so as to produce derivatives with the desired properties. This thesis describes research on newly developed analytical methods for the analysis of modified cellulose. Focus has been on developing new methods for the determination of the substituent distribution along the cellulose chain. This has been achieved by using specific enzyme hydrolysis of the modified cellulose. When the enzyme hydrolyses the modified cellulose, the substituents are considered as steric obstacles, making it more difficult for the enzyme to attack the cellulose chain. Thus, low substituted regions are generally hydrolysed to a higher extent than those that are highly substituted. By interpreting the formed oligomers after hydrolysis an attempt has been made to establish a picture of the substituent distribution of the intact polymer. A comparison to non-specific random acid hydrolysis has also been performed, making it possible to establish important information of the enzyme specificity to the substituents. The enzyme hydrolysis products have been analysed with several analytical techniques such as ESI-ITMS, MALDI-TOFMS, ESI-QqQMS, SEC-MALS/RI, HPAEC-PAD, and reducing ends. New ways of analysing the formed oligomers by selective marking of the formed oligomers, allowed for the analysis of how the substituents hinder the enzyme from gaining access to the modified cellulose chain. Also, a novel method using MS was developed using internal standard addition that allowed for quantitative analysis of the hydrolysis products. Several new considerations when working with enzyme hydrolysed cellulose are suggested.</p> <p>Micro immobilised enzyme reactors have also been used for the hydrolysis of the modified cellulose. These reactors made it possible to perform on-line hydrolysis of modified and unmodified cellulose connected to the techniques mentioned. The use of the reactors significantly reduced the amount of needed enzyme to perform the hydrolysis, reduced the amount of manual labour, and also significantly reduced the amount of time needed to perform the hydrolysis.</p>		
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ABBREVIATIONS

CBH	Cellobiohydrolase
CMC	Carboxymethyl Cellulose
DP	Degree of Polymerization
DS	Degree of Substitution
ECR	Effective Carbon Response
EHEC	Ethyl(hydroxyethyl) Cellulose
ESI	Electrospray Ionization
FAB	Fast Atom Bombardment
FID	Flame Ionization Detection
GC	Gas Chromatography
HEC	Hydroxyethyl Cellulose
HPAEC	High-Performance Anion Exchange Chromatography
HPC	Hydroxypropyl Cellulose
HPMC	Hydroxypropyl(methyl) Cellulose
IT	Ion Trap
LC	Liquid Chromatography
M_n	Number-average molar mass
M_w	Weight-average molar mass
μ IMER	Micro Immobilized Enzyme Reactor
MALDI	Matrix-assisted Laser Desorption/Ionization
MALS	Multi Angle Light Scattering
MC	Methyl Cellulose
MDS	Molar Degree of Substitution
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PAD	Pulsed Amperometric Detection
QqQ	Tripple Quadrupole
RI	Refractive Index
SEC	Size-Exclusion Chromatography
SeC	Sulfoethyl Cellulose
TOF	Time of Flight

LIST OF PAPERS

- I. **Investigation of micro-immobilised enzyme reactors containing endoglucanases for efficient hydrolysis of cellodextrins and cellulose derivatives**
Claes Melander, Martin Bengtsson, Herje Schagerlöf, Folke Tjerneld, Thomas Laurell, Lo Gorton
Analytica Chimica Acta, **2005**, 550, 182-190
- II. **Microchip immobilized enzyme reactors for hydrolysis of methyl cellulose**
Claes Melander, Dane Momcilovic, Carina Nilsson, Martin Bengtsson, Herje Schagerlöf, Folke Tjerneld, Thomas Laurell, Curt T. Reimann, Lo Gorton
Analytical Chemistry, **2005**, 77, 3284-3291
- III. **New approaches to the analysis of enzymatically hydrolyzed methyl cellulose.**
Part 1. Investigation of the influence of structural parameters on the extent of degradation
Roland Adden, Claes Melander, Gunnar Brinkmalm, Lo Gorton, and Petra Mischnick
Biomacromolecules, 7, **2006**, in press
- IV. **New approaches to the analysis of enzymatically hydrolyzed methyl cellulose.**
Part 2. Comparison of various enzyme preparations
Claes Melander, Roland Adden, Gunnar Brinkmalm, Lo Gorton, and Petra Mischnick
Biomacromolecules, 7, **2006**, in press
- V. **Determination of reducing ends with flow injection analysis with amperometric detection - Application on enzyme hydrolysed methyl cellulose**
Claes Melander, Emma Andersson, Sara Axelsson, Lo Gorton
Submitted to *Analytical and Bioanalytical Chemistry*, **2006**

Appendix (not included in defence)

- A. **Liquid chromatography mass spectrometry analysis of enzyme-hydrolysed carboxymethylcellulose for investigation of enzyme selectivity and substituent pattern**
Arieh Cohen, Herje Schagerlöf, Carina Nilsson, Claes Melander, Folke Tjerneld, Lo Gorton
Journal of Chromatography A, **2004**, 1029, 87-95
- B. **Hydrolysis of maltoheptaose in flow through silica wafer microreactors containing immobilised α -amylase and glycoamylase**
Claes Melander, Wiebke Tüting, Martin Bengtsson, Thomas Laurell, Petra Mischnick, Lo Gorton
Starch/Stärke, **2006**, in press

The following papers are not included due to the nature of material or the extent of my contributions

Automated liquid membrane extraction for high-performance liquid chromatography of Ropivacaine metabolites in urine

Jan Åke Jönsson, Malin Andersson, Claes Melander, Jan Norberg, Eddie Thordarson, Lennart Mathiasson

Journal of Chromatography A, **2000**, 870, 1-2, 151-157

Separation and characterisation studies of mixtures of starch and cellulose derivatives

Carina Nilsson, Anna Asplund, Herje Schagerlöf, Claes Melander, Anne Andersen, Folke Tjerneld, Arie Cohen, Lo Gorton, in preparation

Solid phase enzymatic alkyl glucoside synthesis, using immobilised β -glucosidase on μ -IMER with MS detection

Carina Nilsson, David Svensson, Claes Melander, Lo Gorton et al., in preparation

CONTRIBUTION BY THE AUTHOR TO THE DIFFERENT PAPERS

Paper I. The author performed all experimental work and calculations. The author wrote a substantial part of the paper

Paper II. The author performed most experimental work and wrote the major part of the paper.

Paper III. The paper was a collaboration where first and second author performed all experimental work and wrote major parts of the paper with assistance by co-authors.

Paper IV. The paper was a collaboration where first and second author performed all experimental work and wrote major parts of the paper with assistance by co-authors.

Paper V. The author performed most experimental work and wrote the major part of the paper.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Cellulosa är den mest vanliga polymeren i världen. Den tillverkas dagligen i naturen av alla växter och träd medan de växer. Vi är främst i kontakt med polymeren genom våra kläder (i form av bomull), när vi läser morgontidningen (papper) eller kanske då stoppar in en vedbit i brasan. Vad många inte känner till är att cellulosamolekylen har betydligt fler användningsområden. Sedan modern kemi utvecklades under 1800 och 1900 talen har vår kunskap om att använda oss av den mycket billiga och vanligt förekommande polymeren utvecklats enormt. Cellulosapolymeren används i stort sett överallt; i byggnadsmaterial, som betong och färg, förtjockare i mat och som en mycket viktig del i läkemedelsformuleringar. Den existerar då dock oftast inte i dess naturliga form utan den har kemiskt modifierats så att den får nya och bättre egenskaper såsom ökad vattenlöslighet och högre stabilitet mot nedbrytning.

En mycket stor fördel med den modifierade cellulosan är att den i många fall kan ersätta syntetiska polymerer som är framställda ur t.ex. råolja. Det är väl känt att dessa syntetiska polymerer påverkar naturen mycket negativt genom t.ex. att inte brytas ned. Med andra ord så påverkar cellulosa polymeren inte naturen i samma utsträckning och till skillnad från syntetiska polymerer så bryts cellulosa polymeren ned av bakterier och svampar.

Då den modifierade cellulosa tillverkas kan den få olika egenskaper. Egenskaperna beror bland annat på cellulosans ursprung (t.ex. bomullscellulosa är väldigt olik cellulosa från träd), hur den modifieras (t.ex. vilken typ av kemisk grupp som sätts på cellulosan) och under vilka reaktionsbetingelser som den tillverkas. Alla dessa faktorer påverkar de slutliga egenskaperna av den modifierade cellulosan. Idag vet man inte exakt hur de olika tillverkningsfaktorerna påverkar de slutliga egenskaperna hos den modifierade cellulosan. Detta är bakgrunden till varför arbetet i denna doktorsavhandling har gjorts. Avhandlingsarbetet har dock inte fokuserats på vilka fysikaliska egenskaper de olika cellulosaorna har utan har mer varit inriktat emot att ta fram nya, mer effektiva, metoder för att analysera den modifierade cellulosa och att utveckla metoder där små, men mycket betydelsefulla, skillnader mellan olika modifierade cellulosaer kan illustreras.

För att kunna finna dessa skillnader måste ett flertal analytiska metoder användas. Molekylviktsfördelningen av cellulosa polymeren måste bestämmas, typ av kemiska grupper och dess fördelning, hur mycket cellulosa är modifierad och även hur modifikationerna är fördelade längs polymeren. Det är mycket svårt att bestämma alla dessa faktorer som påverkar egenskaperna hos polymeren genom att analysera den intakt. Därför är det mycket vanligt att man bryter ned polymeren (hydrolyserar) till mindre små delar som kan analyseras med ett flertal olika metoder. T.ex. så kan man använda syror som hydrolyserar cellulosa slumpmässigt eller så kan man hydrolysera cellulosa genom att använda olika svamp- eller bakterieenzym. Genom att analysera och sedan pussla ihop dessa mindre delar som bildats kan man försöka få fram en bild om hur den intakta cellulosa polymeren ser ut. I detta doktorandarbete har främst enzym använts för nedbrytningen eftersom dessa hydrolyserar cellulosa enligt ett specifikt mönster. Mönstret är beroende på vilket enzym som använts. Dessvärre är kunskapen om hur enzymen ”angriper” cellulosa inte helt känt och därför så har även dessa undersökts.

Analyser av den modifierade cellulosa är idag väldigt tidskrävande och kräver mycket manuellt arbete. På grund av detta har nya, mer automatiserade metoder utvecklats under arbetets gång.

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1.0 INTRODUCTION

Cellulose is the most abundant biopolymer on earth. It is daily synthesized by plants and some organisms during their growth cycle. For us humans, cellulose is mainly used for clothes, paper and perhaps as an energy source when originating from wood. However, since the beginning of scientific chemistry and during its evolution, our knowledge about and ability to utilize cellulose have increased tremendously. Today we are in contact with cellulose, not only through our clothes and this thesis, but also through an increasing variety of applications. It may not be widely appreciated that cellulose plays an important role in pharmaceuticals, food products, paints, and construction materials, to name just a few examples. However, within these areas cellulose is not in its original form. Instead, it has been modified to suit our needs.

The easy access to cellulose as raw material and the possibility to modify cellulose to mimic synthetic polymers have resulted in greatly expanded usage. This is not only a great economical benefit but is also very beneficial for the environment. The bio-degradable cellulose, once it is consumed, is recycled through bacteria and fungi in nature, leading to less strain on the environment. Moreover, less petroleum-based polymers need to be used, easing consumption of a resource increasingly acknowledged being on the decline.

However, the correlation between the manufacturing methods for producing modified celluloses and their final properties is not entirely understood and therefore it is of great interest and utility to study such correlations. The properties of the modified cellulose change with the manufacturing process and type of modification. By improving the knowledge of cause-and-effect with studies of the products of cellulose modification, hopefully more efficient, cheaper and more reliable cellulose derivatives may be obtained, in turn leading to enhanced technological usage.

In this work modified cellulose has not been studied from an application point of view, but more from an angle, where different pieces of a puzzle could be laid together, establishing a more complete picture of the intact polymer. In essence the properties of the modified cellulose are determined by factors such as molar mass distribution of the cellulose polymer along with, type of modification (substituent type), extent of modification (degree of substitution and molar substitution), and if the modification is uniform over the cellulose polymer (heterogeneity). Most of these properties can be determined through different analytical techniques except for the uniformity of the modification.

Since the heterogeneity is one of the more important characteristics of modified cellulose new ways of determining this have to be established. Unfortunately, it is not possible to determine this factor through direct analysis of the intact polymer. Instead, the cellulose must be divided into more easily analyzable pieces, from which the information obtained should be reassembled to form an improved overall picture of the cellulose, its modifications and their distribution. The division process may be carried out either selectively using enzymes, or randomly using acids. The enzymes are natural catalysts that ensure the bio-degradability of cellulose in nature. Unfortunately, these enzymes are far from being rigorously characterized. For example, different enzyme multifunctions may be sensitive to cellulose modifications to different and as yet unknown or poorly understood extents. Thus the puzzle has a minor aspect: by understanding how the cellulose modifications impact the enzyme hydrolysis patterns information about the enzymes can also be obtained. To find out information both about modified cellulose and about the enzymes that hydrolyze the modified cellulose is an enormous challenge. Fortunately with combination of powerful analytical tools and chemical derivatization followed by mass spectral analysis, the puzzle is closer to being solved. Thus, if the enzyme attack on the cellulose is well characterized, information about the modification is revealed. In this work, several new considerations when working with enzyme-hydrolyzed modified cellulose are suggested and new knowledge about the enzymes has been established.

Today the demand for automated, sensitive and versatile chemical analysis has increased. Therefore, new ways of designing analytical systems have also been investigated with a particular emphasis on miniaturization. Immobilization of enzymes, capable of hydrolyzing the modified cellulose, has been performed on small micro-chips constructed from porous silicon. These have shown to improve the analytical methods available today by saving time and labor while also minimizing the consumption of enzymes.

2.0 CELLULOSE AND ITS DERIVATIVES

In this thesis the focus has been on the analysis of modified (derivatized) cellulose. However, since the structure of the unmodified intact cellulose molecule (raw material) also plays a significant role in the modification reaction, a short description of basic cellulose properties is given.

2.1 Cellulose

The word “cellulose” was first introduced in the literature in 1839 through a report of the French Academy [1]. This report was a summary of a discovery in 1838 by Anselme Payen [2]. The French Academy evaluated Payen’s work and described it as follows; “He [Payen] has performed an exact and successful separation of two organic elements of woods...this basic tissue, which is isomeric with starch, which we will call cellulose and also a substance which fills the cells and which constitutes the true ligneous matter.” The academy seemed to create the word by combining Robert Hooke’s early description of cells, *celluae*, with the common suffix for sugars, *ose*. The speculation that cellulose was built up from glucose molecules was first made by Braconnot in 1819, followed up by Ost and Qilkening in 1910, and later verified by Wilstatter and Zechmeister in 1913. They observed that the complete hydrolysis of cellulose by acid gives rise to glucose [3].

Cellulose is the most abundant macro molecule in the world. This is due to its high content in wood which is 35-50% cellulose [4]. Unlike the (1→4)- α -D-glucosidic linkages in starch, the (1→4)- β -D-linkages in cellulose cannot be broken by the human digestive system. In food products, cellulose thereby serves as a bulk material that increases the fiber content and also improves the properties by stabilization of emulsions, increased viscosity, and so on. The importance of cellulose in the world economy is well known. Hess estimated that 10¹¹ tons of cellulose-based materials are consumed annually [5]. Therefore the world disposal mechanism, mainly fungal but also bacterial, ensures that no long term build-up of waste products occurs. Commercially purified cellulose from higher plants life supplies an annual world consumption of about 150 million tons of fibrous raw material. Of this amount, 100 million tons, derived mostly from pulped wood, are used for paper; about 12 million tons, mostly from cotton, go to textiles; and 7 million tons are chemical-grade cellulose [6]. The ability to use a cheap, renewable and environmental friendly material that can compete with synthetically produced polymers has increased its use. Modern cellulose research is focused on the development of novel processes that yield little or no ecologically harmful by-products.

Cellulose is an isotactic (1→4)-β-D-polyacetal form of cellobiose, which in turn consists of two glucose units (Figure 1). The concept that cellulose is a linear macromolecule consisting of anhydroglucose units was debated for a long time before it was verified in the 1920s. The basic chemical formula is $C_{6DP}H_{10DP+2}O_{5DP+1}$ (DP is degree of polymerization), which gives an elemental composition of 44.4% C, 6.2% H, and 49.4% O. Indeed this composition was found by Payen in 1842 [7]. Haworth and Staudinger [8,9] later elucidated the covalent bonds inside and between the cellulose units and appreciated the macromolecular nature of the cellulose. Due to these (1→4)-β-D-glucosidic linkages, the most stable conformation for cellulose is that in which each glucose unit is rotated 180° relative to the preceding unit, so that cellobiose is the repeating unit. This discovery later won Staudinger the Nobel Prize in Chemistry in 1953.

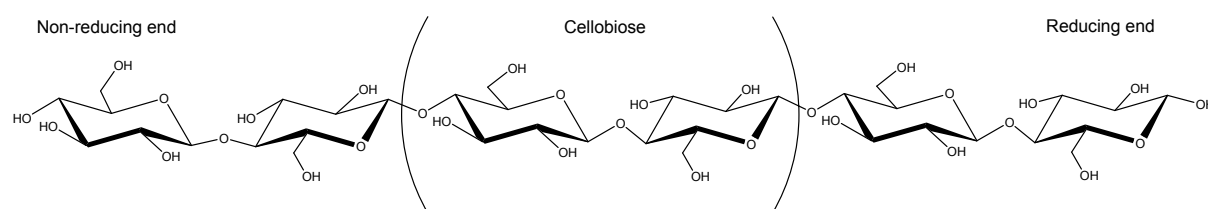


Figure 1. The structure of cellulose polymer

Each anhydroglucose unit has three free hydroxyl groups located respectively on carbons C-2, C-3 and C-6 (Figure 2). The right and left terminal glucose units also contain a free hydroxyl group on carbons C-1 and C-4, respectively. The right terminal glucose unit is called the reducing end and contains a potential aldehyde functional group and the left terminal glucose is referred to as the non-reducing end.

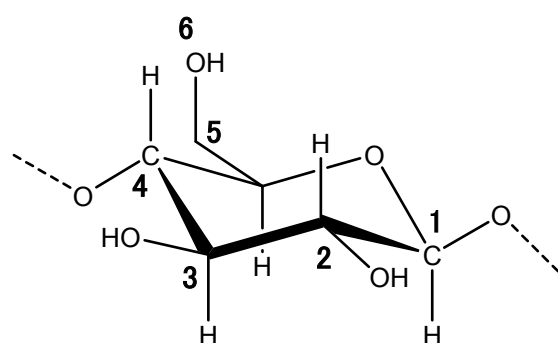


Figure 2. The anhydroglucose unit of cellulose

The length and the molar size of cellulose depend on its origin, but normally range from 1000 to 15000 glucose units (M_w of 160-2400 kDa). The number of glucose units in the chain

is normally called the degree of polymerization (DP) and is not a fixed value but rather a distribution over a range of DP (polydispersity). Thus, all celluloses are mixtures of cellulose chains with different lengths and may even have two or more distributions of different sizes. Knowledge of the molar mass and its distribution is very important for many applications. For instance, the increase of viscosity with DP affecting e.g. the consistency and the consumer appreciation of food products. The measurements of DP can be done by a wide range of instrumentation exploiting different physical chemical properties. Light angle scattering, osmotic measurements, rheological, and sedimentation measurements are some of the common techniques used [10]. More details are given below in section 3.0 *Analytical Techniques*.

In solution, the cellulose molecules exist as coils with a slight helical twist along the cellulose backbone. This twist is caused by intramolecular hydrogen bonds primarily between the hydroxyl group on C-3 and the pyranose ring oxygen in the adjacent glucose monomer, but also between the hydroxyl groups on C-6 and C-2 of the adjacent glucose unit. The intramolecular bonds are responsible for the rigidity of the cellulose molecule [10].

Solid cellulose exists mainly in high-order microcrystalline structures (“crystalline” or “supramolecular” regions) and lower order regions (“amorphous” regions). The crystalline structure can adopt several different crystal lattice structures and up to five different have been reported [11]. Depending on source the crystallinity varies between 50 and 90% [3]. Native cellulose is generally insoluble in water even though it can swell to some extent. Consequently native cellulose is not very reactive. The reactivity of cellulose can be greatly enhanced by various forms of treatment, such as swelling under basic conditions, acid degradation or mechanical grinding, which break down the fibrillar aggregations [10].

To extend the area of application, cellulose has to be modified. Modifications are most often performed under heterogeneous conditions in such a way that the water solubility of the cellulose is increased. This is performed through modification of the free hydroxyl groups along the cellulose chain under strongly alkaline conditions. The modification reaction takes place in a two-phase system, where the first phase involves reaction on the amorphous regions and the second phase involves reactions on the crystalline regions. Generally the latter reactions are slower due to the less favorable reaction conditions in the crystalline regions [10].

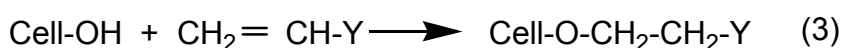
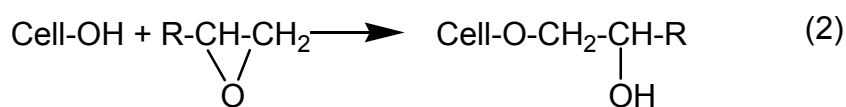
Cellulose nitrate and cellulose sulfate were the first two cellulose derivatives that were produced on an industrial scale [12]. To date many additional kinds of derivatives have been produced (ethers, esters, ionic, non-ionic, cationic etc.) but only a few of them have gained

any commercial importance. Since this work has been focused on the analysis of cellulose ethers, these are the only modifications that will be discussed here.

2.2 Cellulose Ethers

In 1905, Suida et al. reported the first cellulose ether, which was produced by methylation with dimethyl sulfate [10]. During the following ten to twenty years, many of today's commercial cellulose ethers were developed, but it was not until 1945, when ethyl(hydroxyethyl) cellulose (EHEC) was developed in Sweden, that production of cellulose ethers began to expand internationally. Cellulose ethers are generally non-toxic and are therefore often used as food and cosmetic additives [see Table 1]. They are degraded in nature to carbon dioxide through slow bioreactions with no known toxic metabolites.

Cellulose ethers are derivatives of cellulose where the free alcoholic hydroxyl groups on position C-2, C-3 and C-6 are partially or fully substituted. The substitution is usually achieved by alkaline swelling of the cellulose, a process in which the strong hydrogen bonds are overcome and supramolecular structures are opened up [12,13]. Under the strong alkaline conditions, the alcoholate ions are formed as nucleophiles that can attack e.g. alkyl halides, or oxiranes. For the preparation of mixed ethers, the reagents may be added simultaneously or in subsequent reaction steps. There are three main industrial reaction types:



Reaction (1) describes the Williamson etherification where R-X is an inorganic acid ester. X is normally a halide or sulfate. Unreacted NaOH is washed away after the reaction. The second reaction (2) shows an alkali-catalyzed addition of an oxirane. This reaction may proceed further since new hydroxyl groups are generated in the reaction. The final third (3) reaction shows the Michael-addition of Cell-OH to an activated double bond. All reactions are base catalyzed.

Industrial modification processes normally take place in heterogeneous systems, i.e. both the cellulose starting material and the corresponding final cellulose ether are present as solids, either as dry matter or in suspension. Most commonly, the industrial manufacture of cellulose derivatives is carried out by spraying NaOH solution onto the dry cellulose powder or plates, after which the modification reagent is added as a gas or liquid [12,13].

The average number of substituents per anhydroglucose unit is called degree of substitution (DS), and can be a value between 0 and 3. Derivatives with a DS below 0.1 are generally insoluble in water and differ from unmodified cellulose only in some physical and technical parameters. If hydrophobic ether groups predominate, the water solubility may again disappear at DS above 2. The reactivity of the alcoholic hydroxyl groups varies with the secondary C-2 group often being the most reactive under heterogeneous conditions followed by the primary C-6 and finally the secondary C-3 group. The substituent can be of one type; or alternatively, a combination of different ether groups can appear in the same macromolecule. Substitution that generates new free hydroxyl functions that can again be etherified are characterized by the molar substitution (MDS). This parameter gives the average number of moles of reactant added to one mole of anhydroglucose unit. An example is hydroxypropyl or hydroxyethyl cellulose. The MS value has no upper limit. If the DS value is also known, the average chain length of the ether side chain can be calculated from the ratio MDS/DS. If only one type of non-hydroxylgroup-containing substituent is introduced, 2³ different monomer combinations is possible with their molar ratios assigned the symbols s₀, s₂, s₃, s₆, s₂₃, s₂₆, s₃₆, s₂₃₆ by Spurlin [14]. Some of the cellulose ethers used in this work is described briefly below.

Table 1. Common industrial cellulose ethers and their main applications [12,13]

Substituent	Structure	Abbreviation	Application
Methyl	-CH ₃	MC	Building materials Surfactant
Hydroxypropyl(methyl)	-[CH ₂ CH(CH ₃)O]nH/CH ₃	HPMC	Paint Tablet Food
Hydroxypropyl	-[CH ₂ CH(CH ₃)O]nH	HPC	Paint Tablet
Hydroxyethyl	-[CH ₂ CH ₂ O]nH	HEC	Paint Emulsions Drilling
Carboxymethyl	-CH ₂ COOH	CMC	Detergents Food

Carboxymethyl cellulose (CMC) was manufactured in 1918 by Jansen et al. as the first water-soluble ionic cellulose derivative and was produced commercially starting in the 1920s [15]. It is most commonly prepared by the reaction of sodium chloroacetate with alkali cellulose leading to formation of the water soluble sodium salt. Production of CMC is easier than for most other cellulose ethers because all reagents are solid or liquid and the reaction

can be done at atmospheric pressure. Today it is the most widely produced cellulose ether and it is used in detergents, as thickeners, food additives, and water retaining agents. Many of these properties make the substance very useful in oral and topical pharmaceutical formulations due to its viscosity increasing properties and its ability to stabilize emulsions. Another very important property of CMC (and other modified celluloses) is the non-toxicity. Many of the modified celluloses are used in formulations to mask unpleasant taste or odour, improve product stability, facilitate handling, and modify drug-release characteristics [16]. After the discovery of methyl cellulose and CMC, other water-soluble cellulose derivatives such as hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC), hydroxypropyl(methyl) cellulose (HPMC) and sulfoethyl cellulose (SeC), as well as some graft copolymers of CMC or HEC with hydrophilic vinyl monomers such as acrylamide, acrylonitrile and acrylic acid were reported [17,18]. The DS of CMC normally ranges from 0.3 to 1.2. Due to the carboxyl groups the viscosity of CMC depends on the pH with a maximum at pH 6-7.

Methyl cellulose (MC) was first produced in the United States in 1937-38 but was described already in 1905 [17,18]. Today this modification (methyl) is often used together with a second functional group such as hydroxypropyl and hydroxyethyl groups. The second functional group gives the methyl cellulose new properties such as increased gelatinization temperature. MC exists in mainly two types; one with DS between 1.4 and 2.0 which is soluble in cold water, and one with DS ranging from 0.25 to 1.0 which is soluble in alkaline solution. Due to its excellent water retention, it is often used in cement and gypsum formulations, but also in water based paints and wallpaper adhesives. The high DS also inhibits microbial growth.

Hydroxypropyl(methyl) cellulose (HPMC) is manufactured through parallel reactions with propylene oxide, methyl chloride and methyl oxirane. The DS of the MC groups are generally between 1.3 and 2.2 and the hydroxypropyl MDS values range from 0.1 to 0.8. The addition of the second hydroxypropyl function gives a more clear solution compared to MC and also increases the gelatinization temperature. HPMC is widely used in pharmaceutical formulations such as oral products, tablet binders, film-coatings and as controlled-release matrix [16].

Hydroxyethyl cellulose (HEC) is typically prepared by reaction of cellulose with ethylene oxide. Industrially it has a MDS value between 1.7 and 3 with a corresponding DS between 0.8 and 3.0 [19]. HEC is also more compatible with high-electrolyte solutions than other non-ionic alkyl cellulose ethers. HEC is often used in paint formulations since it can be

manufactured in such a way (high and uniform substitution) that little or no enzyme attack occurs. The bioresistance is often measured as the viscosity change after enzyme attack. HEC is also used as thickener, binder, stabilizer, film former etc. As with HPMC, HEC is also widely used in pharmaceutical formulations [16].

Hydroxypropyl cellulose (HPC) is normally very highly substituted with a high MDS value and it is generally soluble in cold water. As for MC and HEC, the viscosity does not depend on pH in the range between 2 and 12. The more hydrophobic character of the hydroxypropyl functional groups makes this derivative soluble in many organic solvents such as methanol, ethanol, chloroform, and cyclohexanone. It is considered to be non-toxic and physiologically inert and therefore approved as a food additive and as a film-forming agent in pharmaceutical formulations. These properties are often controlled by the DP of the modified cellulose [16].

3.0 ANALYTICAL TECHNIQUES

The properties of the modified cellulose depend on several different factors. The molar mass (DP) and its distribution (polydispersity) influence the properties of the modified cellulose in different aspects such as solubility and viscosity. The type of substituent and its distribution on the monomer level (DS and MDS) and on the polymer level (heterogeneity) also play a significant role in determining the cellulose properties. Today, there is no single analytical technique that can provide information on all these aspects of modified cellulose. Instead, a combination of analytical techniques has to be employed to obtain this information. Each technique has its own advantages and disadvantages and can only provide a piece of the puzzle that is necessary to solve in order to completely characterize the cellulose. Preferably the analytical techniques employed should be both qualitative and quantitative while also being fast and sensitive. Often hydrolysis, using acids or enzymes, of the modified cellulose is required to obtain analyzable smaller oligomers and monomers. Some of the most common techniques employed will be discussed here and the techniques used throughout this work will be discussed in detail.

3.1 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC) also referred to as gel-filtration or gel-permeation chromatography (GPC), is a very powerful separation technique. The IUPAC definition of SEC is “A separation technique in which separation mainly according to the hydrodynamic volume of the molecules or particles takes place in a porous non-adsorbing material with pores of approximately the same size as the effective dimensions in solution of the molecules to be separated” [20]. SEC can be employed to determine the average molar mass and the molar mass distribution of both natural and synthetic polymers over a broad range ($10^2 - 10^8$ Da). In an ideal case, the species are separated according to the hydrodynamic volume (volume of a polymer coil when it is in solution) and elute from the SEC column in order of decreasing size. The separation can be seen as providing a measure of the overall distribution of molar masses of each single molecule size. Molecule-solvent, molecule-packing-material, and molecule-molecule interactions will affect the elution as well as the solubility of the analyte in the solvent [21]. SEC may also be employed for preparative purposes, where normally the inner diameter of the column is enlarged relative to the analytical format and thus the sample loading capacity is increased.

The stationary phase of an SEC column consists of a porous non adsorbing material, which is built up by a three dimensional network, into which the analytes penetrate to an extent depending on the ratio of their dimension to the average diameter of the pores. Molecules that are smaller than the pore size can enter the SEC particles and therefore have a longer path and longer transit time than compared to larger molecules that cannot enter the particles. Molecules larger than the pore size cannot enter the pores and elute together as the first peak in the chromatogram. The stationary phases is generally silica-based (Synchropak from Merck and TSK GSW from TosoHaas), polymeric sorbents such as methacrylate (TSK GPW from TosoHaas) or crosslinked gels based on e.g. agarose (Superose from Pharmacia) or dextran (Sephadex from Pharmacia) [22,23]. Depending on the porous material in the SEC columns, species within a certain limited range of modifications may be separated and therefore a combination of several columns in series may also be used to obtain a desired separation [23]. The length of an SEC column may be anything from one centimeter up to around one meter. The eluting solvent in SEC is normally aqueous but other solvents such as DMSO can also be used [21,24-26]. Salts are often added to increase the ionic strength, reducing electrostatic interaction of the analytes with the stationary phase and improving separation [27].

Depending on the analytes and the eluting solvent SEC can be employed with most detection techniques that can be used with liquid chromatography. However, for SEC analysis of polymers, the refractive index (RI) detector is the most widely employed. The RI detection principle involves measuring the change of the refractive index of the column effluent passing through a flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become for a given analyte concentration. Thus, the sensitivity will be higher when there is a larger difference in RI between sample and mobile phase. Changes in the eluent composition require rebalancing of the detector. Thus, gradient elution is not possible when employing RI detection. Determination of the molar mass distribution by SEC-RI requires calibration of the system with standards. This is a weakness since standards are not always available with molecular properties (different hydrodynamic behavior) similar to those of the polymer being characterized. If the sample is very polydisperse, variations in the range of 10 - 25% in the determination of number-average molar weight can occur [28]. SEC-multi angle light scattering (MALS) in combination with RI allows the direct determination of the molar mass by an absolute method. Normally calibration standards are not required, as the MALS and RI detectors give signals proportional to molar mass and concentration, respectively [29]. MALS involves measuring the amount of light scattered by a solution at

many angles relative to that of the incident laser beam. The intensity of this scattered light will be proportional to the product concentration (in g/l) times its molar mass. Since the signal from the light-scattering detector is directly proportional to the molar mass of the sample times the concentration, by combining this signal with that from a concentration sensitive detector (refractive index or absorbance) it is possible to measure the molar mass of each peak eluting from the column. Unlike the situation for conventional SEC analysis using standards, these molar masses from light scattering are independent of the retention time in the column (elution volume). By measuring the scattering at multiple angles (MALS) and combining that with an quantitative detector such as RI, direct absolute measurements of masses from approximately 1000 Da up into the MDa range can be made, and the radius of gyration (a measure of geometric size) can also be determined [30,31]. Thus, determination of number-average molar mass, M_n , weight-average molar mass, M_w , and polydispersity (M_w/M_n) can be made. Other detection techniques such as UV, viscosity and MS have been employed in combination with RI detection [21,23]. Melander and Vourinen also investigated the possibility of using SEC connected to a total organic carbon detector and a reducing-sugars detector. By hydrolysing CMC with an enzyme and combining the two detectors it was possible to determine the absolute number of DPs in the hydrolysate without any external calibration [32].

3.2 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Carbohydrates do not naturally contain any light-absorbing functionality making ordinary UV detection impossible. As explained above, RI detection may be employed for detection but due to its low sensitivity it is not always suitable. Several sensitive methods involving quantitative derivatization of the reducing end with a UV-absorbant ligand have been suggested, whereby quantitative analysis becomes possible [33-38]. However, separation problems with suitable columns of the carbohydrates prior to detection are generally an issue and it is also very difficult to ensure that the derivatization is completely quantitative. In the mid 1980s, development of high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) revolutionized carbohydrate chromatography yielding extremely fast analysis, high resolution and sub-nanomolar sensitivity [39,40]. In HPAEC a very high pH is used (>pH 12), which allows carbohydrates to be separated in their oxyanion state. At this pH, the carbohydrates behave as anions and can dissociate partially or completely. The separation mechanism is based on the differences in pK_a values of the

ionizable hydroxyl groups in the carbohydrate oligomer. Thus, retention times are inversely proportional to pK_a values and increase with chain length (DP). Also, acidic compounds such as mannose-6-phosphate are retained more strongly than mannose itself. The hydroxyl position on a monomer level also influences the elution, making it possible to separate not only monomers but also oligomers with exceptionally high DP (~80). The selectivity during analysis is influenced both by the choice of column as well as by the composition of the mobile phase. Normally the mobile phase consists of a solution containing NaOH in a gradient mixture with either water or NaOAc. The OAc^- ion interacts more strongly with the anion-exchange stationary phase and serves as a competitor to the sugars and thereby reduces the retention time of the carbohydrates. Nitrate may also be used as competitor and has been shown to yield more sensitivity and higher resolution compared to acetate as competitor [39].

Pulsed amperometric detection (PAD) is the most common detection technique employed with HPAEC separation of carbohydrates. The PAD is characterized by high sensitivity (down to the pg level), high selectivity towards electroactive species in a wide linear range, fast response, and relative low cost [41-43]. PAD is based on measurement of the anodic current caused by oxidation of the carbohydrates at a gold, carbon or a platinum electrode at high pH. A problem associated with electro-oxidative detection of carbohydrates is that the electrode surface becomes fouled by oxidation products (from intermediate oxidation products and solution impurities) that cause a rapid decrease in the sensitivity. This problem can be overcome by employing a triple-step waveform pulse (PAD) with anodic and cathodic polarization of the working electrode (Figure 3). Normally the waveform starts with a detection potential at which the analytes are detected by direct oxidation of the hydroxyl groups at the oxide free electrode surface (E_1). To minimize the background signal originating from double-layer charging, a time delay (t_d) is introduced. During this delay the charging current is allowed to decline before the measurement of the faradic current (t_1) is performed. The detection sequence is followed by cleaning of the surface, in which a high positive potential (E_2) is applied to fully oxidize the electrode surface. Finally, the surface is regenerated by a negative potential (E_3) strongly cathodic enough to reduce the previously formed gold oxide film. Optimization of the PAD waveform is very difficult and is normally achieved by the 'trial-and-error' method, where the signal-to-noise ratio is maximized while maintaining a reasonably high value of waveform frequency (1-2 Hz).

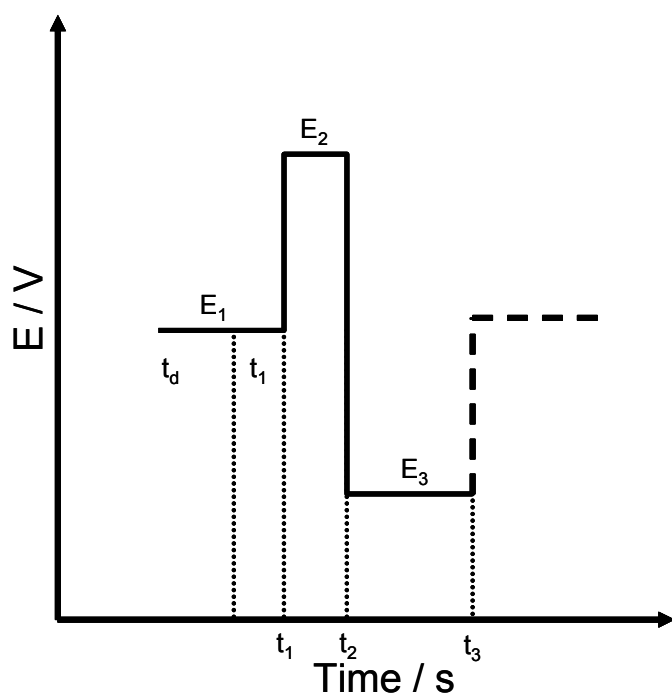


Figure 3. The waveform used in pulsed amperometric detection (PAD)

Analysis of cellulose, starch and corresponding derivatives using HPAEC-PAD has been investigated by several research groups. The chain lengths of amylopectin from various starches have been determined by selective debranching of the amylopectin, followed by analysis of the unit chain lengths [44-48]. It has been shown that HPAEC-PAD can provide analysis of starch up to DP 80 [44].

For determination of cellulose the PAD response varies for individual chains and free hydroxyl groups, and to obtain a quantitative analysis response factors have to be determined for each individual compound in order to compensate for variation in the PAD response [45,47,49-51]. Only semi-quantitative determination is possible without injection of standards. Since the number of free hydroxyl groups varies for modified cellulose, standards are also required in this case. This is in contrast to derivatization with UV-, radio- or fluorescently labeled sugars that show, if derivatization is performed quantitative, a quantitative response independent of chain-length or chemical structure. Thus, HPAEC-PAD analysis can sometimes be limiting due to lack of standards.

Heinrich and Mischnick developed a method for determination of the eight different monomers of methyl glucose using HPAEC-PAD and compared the results with those obtained by GC [50]. The substances eluted in order of decreasing number of methyl substituents with unsubstituted glucose eluting last. The quantitative determination required synthesis of the individual monomers, whereby the response factors could be determined

using nuclear magnetic resonance (NMR) as reference method. Heinrich and Mischnick suggested that the obtained response factors should be determined for each individual analytical HPAEC-PAD system, since the electrochemical response is very much dependent on factors such as NaOH added to column, individual detector sensitivity and concentration range in which analysis is employed. Kragten et al. also showed that HPAEC-PAD can be used to determine the eight monomers of carboxymethyl and sulfoethyl glucose [51]. These authors fractionated the eluting peaks and compared them with GC and mass spectrometric detection (GC-MS) thus, identifying the products. In contrast to MC, it was found that the peaks eluted in groups in order of increasing number of substituents, with unsubstituted glucose eluting first. To determine the responses in the PAD the eluting peaks were collected, and quantified using NMR. It was found that the PAD response decreased with increasing number of carboxymethyl substituents. With the developed method a new, less laborious analysis of the substituent pattern of CMC and SeC was established. The proposed method has also been successfully used in other works [52-55]. An important requirement is that the response factors for the analytes in HPAEC-PAD analysis must always be determined for the modified cellulose investigated, which can be done either by synthesis of the monomers [50] or through fractionation and further analysis of the hydrolysates [56].

3.3 Gas Chromatography (GC)

Capillary gas chromatography (GC) is a well known analytical technique that was developed in the early to mid 20th century [57]. GC analysis requires volatile and thermally stable analytes. The high separation efficiency of GC columns allows extremely sensitive and selective separation of molecules and can therefore be useful tool when analyzing modified cellulose. However, modified cellulose does not have the properties required to be directly analyzable with GC. Thus, analysis of modified cellulose must be preceded with suitable sample preparation. Depending on the cellulose polymer, different approaches have to be used.

For the determination of the substituent pattern of modified cellulose containing only hydroxyalkyl functions (e.g. HPC and HEC) the appropriate pre-treatment would comprise permethylation, hydrolysis, reduction and acetylation according to the well-known methodology of methylation analysis [58] (see Figure 4). In the initial (permethylation) step the free hydroxyalkyl functional groups are methylated (protected) (Figure 4). The permethylation procedure is most often performed according to Hakomori [59], where the base methyl lithium in DMSO is used to ionize the hydroxyl function, or according to

Ciucanu and Kerek [60] where solid NaOH is suspended in DMSO. After the addition of the base, permethylation is most often achieved using either methyl iodide or deuteromethyl iodide for reaction with the deprotonated hydroxyl functions. After this, complete degradation of the cellulose to monomers is performed using acid hydrolysis at high temperature. The hydrolysis step is followed by reduction of the monomers and finally a second derivatization step by acetylation to obtain glucitol acetates. Several different acids have been used for the hydrolysis, but trifluoroacetic acid (TFA) and HClO₄ are the most commonly used since they yield low amounts of side reactions [51,61-63]. By reduction of the α - and β -glucose derivatives with NaBD₄, open-chain alditols are obtained. The use of a deuterated reduction agent aids in identification of the hydrolysis products using GC-MS. Finally the monomeric units are acetylated with acetic anhydride and pyridine [58,61,64].

For the analysis of only alkylated modified cellulose like MC or ethyl cellulose, the initial permethylation step is generally not necessary. Thus, the alkyl pattern can be determined directly through hydrolysis, reduction and acetylation [65,66].

For the analysis of modified cellulose containing alkyl and hydroxyalkyl functionalities, the alkyl distribution can be determined without the initial permethylation step (Figure 5a). Information on hydroxyalkyl substituents is to some extent also revealed but very complex patterns of the combined alkyl and hydroxyalkyl functionalities are obtained and many hydroxyalkyl functionalities can therefore remain undetected. Hence, if two types of substituents are possible, e.g. methyl and hydroxypropyl, four different types of monomers are present in the hydrolyzed cellulose (-OH, -OMe, -OHP, and -OMP (methoxypropyl)). That means that there are $4^3=64$ different theoretical monomer patterns and even then, the tandem reaction of the hydroxypropyl functionality is not accounted for. Thus a virtually unlimited number of different monomers are possible. However, with very detailed GC analysis the complete monomer composition up to MDS 4-5 of the substituent distribution may be determined [67]. However, to be able to determine the hydroxyl functionalities of mixed alkyl and hydroxyalkyl cellulose a twofold approach has to be used. The alkyl pattern can be determined directly through hydrolysis, reduction and acetylation. However, for the determination of the hydroxyalkyl distribution, an initial permethylation of the free hydroxyl groups, similar to only the hydroxyalkyl modified celluloses, is required. These methods have been applied on ionic and non ionic derivatives, sulfoalkyl ethers and trialkylsilyl ethers [64,66,68,69]. The permethylation focuses all monomers with an alkyl/hydroxyalkyl group in a certain position in one peak in the GC chromatogram [70,71]. This increases the sensitivity multi-fold and allows the possibility to get detailed information of the hydroxyalkyl pattern of

the modified cellulose (Figure 5b). The permethylation step solves or at least decreases many problems such as discrimination during hydrolysis due to polarity effects of substituents and uncontrolled side reactions as hydroxymethylfurfural or reversion product formation, and has also been used to determine the substituent distribution of HPC [72], HEC [68], and EHEC [66].

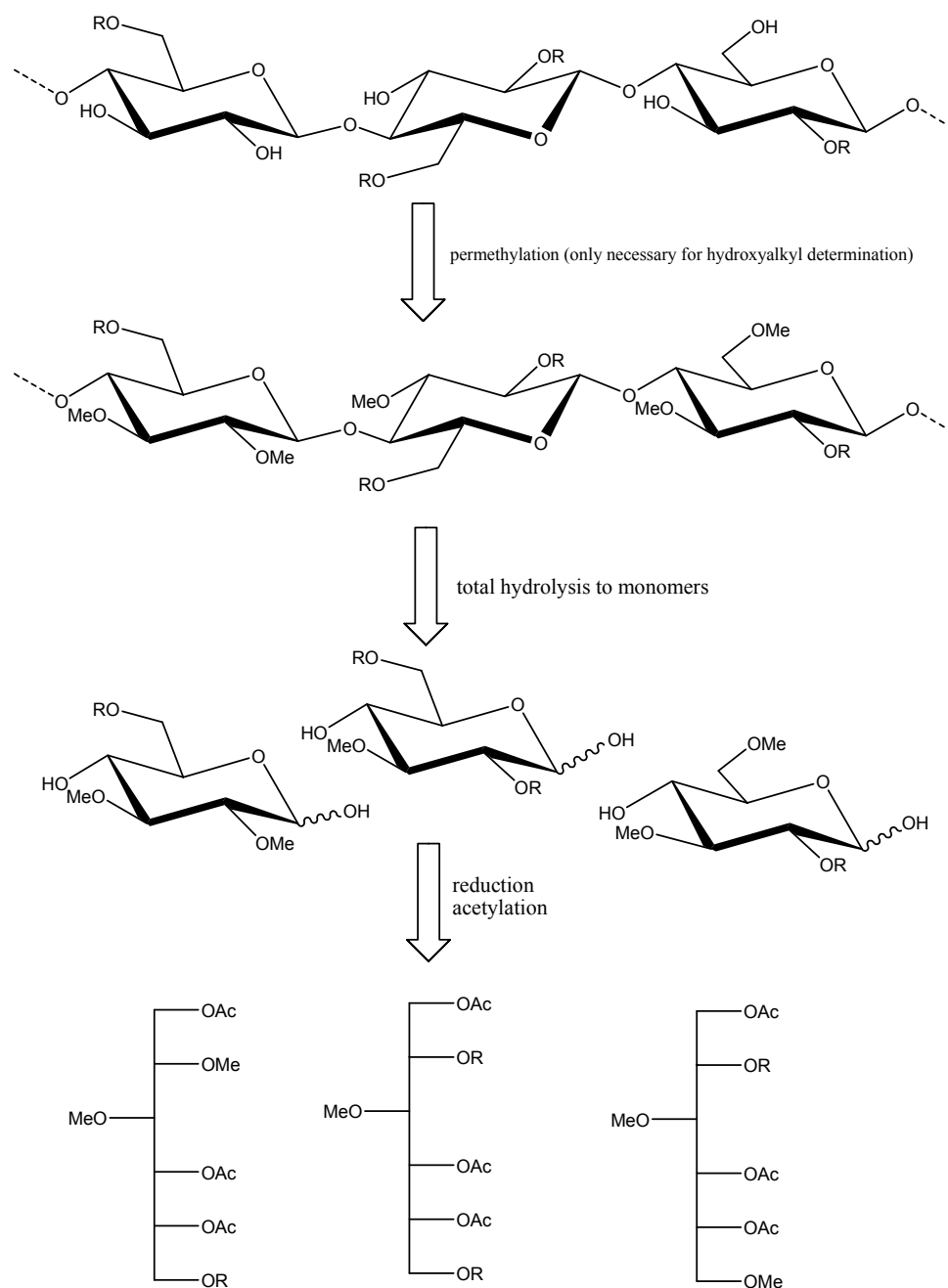


Figure 4. The permethylation, hydrolysis, reduction, and acetylation procedure as described by Lindberg et al. [68]

An alternative type of GC analysis is the reductive cleavage method [73], which has been successfully applied to the structural analysis of modified polysaccharides, especially samples containing components sensitive to acidic conditions [61,64,74-76]. This method is

based on the methylation analysis described above, but departs from it significantly with regard to the cleavage reaction and the types of fragments obtained after cleavage. Here, the glucosidic linkages of the fully permethylated samples are cleaved by ionic hydrogenation of the carbo-oxygen bonds, promoted by a Lewis acid, with triethylsilane as reducing agent [61,64,74,77]. The resulting partial anhydroalditols are acetylated and finally analyzed by GC-MS/FID as described above. The advantage over the methylation methodology is that the reductive cleavage method enables bond cleavage, reduction and acetylation to be performed in a “single-pot” reaction at room temperature.

Independently on sample pretreatment, the resulting partially alkylated alditol acetates are separated using GC and identified using MS. Ionization in MS is commonly achieved through chemical ionization (CI) or electron impact (EI) ionization [58,64,78-80]. Based on the characteristic fragmentation behavior, where structural differences and regioisomers can be determined and the elution order of the species, it is possible to deduce the substituent pattern among the C-2, C-3 and C-6 hydroxyl groups. Standards are then unnecessary, which is very beneficial since normally these are not available. Flame ionization detection (FID) is used for quantitative purposes (calculation of DS and MDS). The response of this detector in principle corresponds to the number and type (hydrocarbon, carbonyl or ether) of carbon atoms present in the compound that passes the detector. By summing all the contributions from the various carbons to the flame response, the “effective carbon response” (ECR) for certain structural features can be calculated [81-83].

Other GC methods are also available but they only provide the overall DS by determination of the total substituent content and comparison with an internal standard [84-86]. The later types of methods have not been used in this work since they do not provide information of the substituent distribution and will therefore not be explained here.

GC analysis has also been applied to elucidate information on enzyme specificity towards polysaccharide substrates. Mischnick [87] performed enzyme hydrolysis using α -amylase and glucoamylase of methyl amyloses prepared under different methylation conditions. To obtain GC-analyzable species along with the selective marking of reducing, non-reducing ends and the middle glucosidic units of the formed oligomers, extensive derivatization had to be performed. It was found that the reducing end of the formed oligomers was generally unsubstituted, while the non-reducing end could be un-, 2-*O*-, 6-*O*- or 2,6-di-*O*-methylated. All glucosyl units containing substituents in position 3-*O* were located in the middle glucose units of the formed oligomers [87]. Thus, neither α -amylase nor

amylglucosidase can hydrolyze a (1→4)- α -glucosidic linkage with a 3-*O*-methyl group on any of the adjacent glucosyl residues.

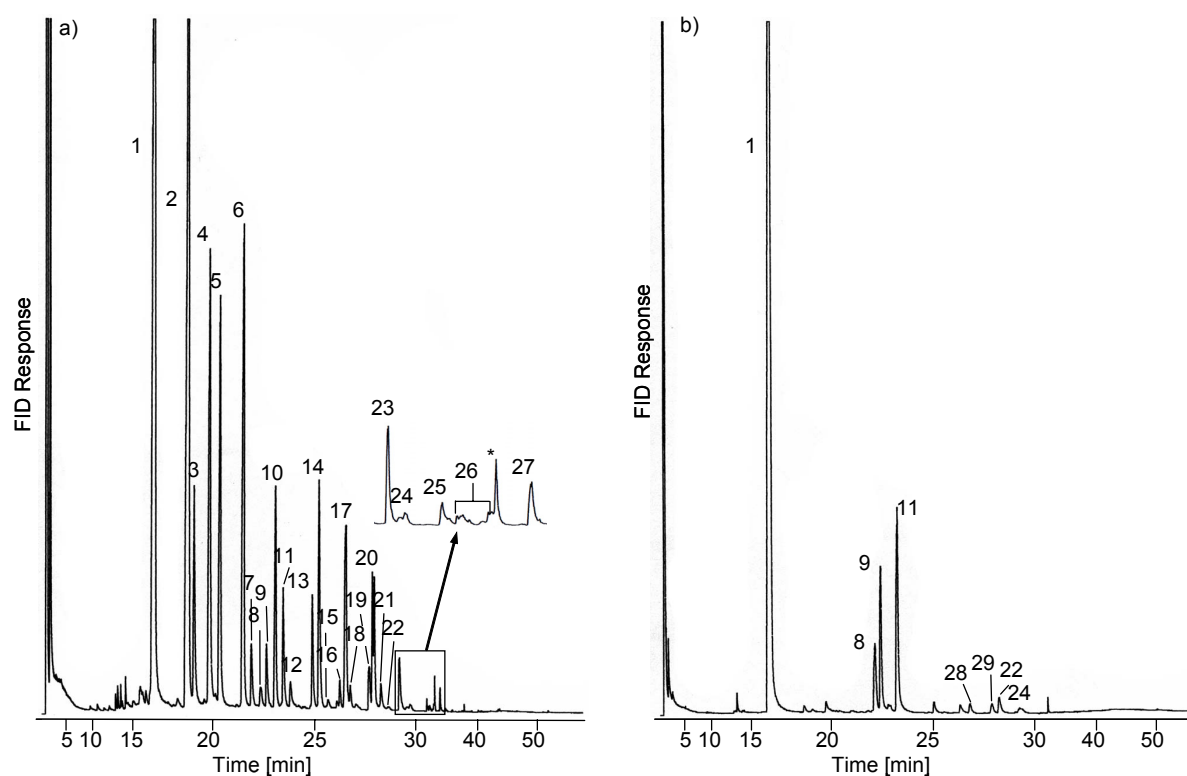


Figure 5. GC-FID chromatogram of *O*-methyl-*O*-(2-hydroxy)propyl-*D*-glucitol acetates obtained from HPMC after direct hydrolysis, reduction, and acetylation (a), and after additional methylation prior to hydrolysis (b). Peaks are assigned according to Table 2 [70].

Table 2. Peak assignment for Figure 5. ECR = Effective carbon response [70]

Number	Peak	ECR	Number	Peak	ECR
1	236-Me	745	18	6-Me-2HP/6-Me-3HP	1035
2	26-Me	790	19	2-Me-3-HP	1035
3	36-Me	790	20	2-Me-6HP	1035
4	23-Me	800	21	3-Me-6-HPb/2-Me-36-HPMe	1035
5	6-Me	835	22	3-Me-26-HPMeb/36-Me-22-HPMe	1145
6	2-Me	845	23	23-HP	1280
7	3-Me	845	24	23-Me-66-HPMeb/2-HPMe-3-HP-6-Me	1145
8	26-Me-3HPme	945	25	2-Me-3-HP-6-HPMe	1190
9	36-Me-2HPme	945	26	Di-HPc	1190
10	Unsubstituted	890	27	2-Me-66-HP	1235
11	23-Me-6HPme	945	1	236-Me	745
12	6-Me-2HPMe	990	8	26-Me-3-HPMe	945
13	2-Me-6HPMe	990	9	36-Me-2-HPMe	945
14	26-Me-3-HP	990	11	23-Me-6-HPMe	945
15	3-Me-2-HPMe	990	28	6-Me-23-HPMe	1145
15	36-Me-2HP	990	29	2-Me-36-HPMe	1145
16	6-HPMe	1035	22	3-Me-26-HPMeb/36-Me-22-HPMe	1145
17	23-Me-6HP	990	24	23-Me-66-HPMe	1145

Since GC columns have so high selectivity it may be possible to determine all the different original monomers, ranging from unsubstituted to all mono-, di- and tri-substituted glucose units. If two types of substituents are present, e.g. methyl and hydroxypropyl, the distribution pattern on the monomers can be determined using the methods described above (Figure 4). Thus, analysis of modified cellulose using GC can provide very useful information of the modified cellulose. However, there are some known problems involved in the GC analysis such as incomplete permethylation and hydrolysis and also side reactions of the monomers during the hydrolysis [88]. In this work GC analysis of the substituent distribution was performed on all samples, providing valuable reference data of the substituent distribution on the modified cellulose.

3.4 Mass Spectrometry (MS)

In this work, mass spectrometry (MS) has been an important tool in the characterization of modified cellulose. By taking advantage of the versatility of MS, it has also been particularly useful tool for the characterization of cellulose-hydrolyzing enzymes.

MS is a very exact technique to measure molar masses. The measurement is based on the separation of analytes in space or time according to their mass-to-charge ratios (m/z). The obtained intensity distribution as a function of m/z for a sample is called a mass spectrum. Normally, mass spectrometers consist of an ion source, in which the analytes are ionized in the gas phase, a mass analyzer, in which the ionized analytes are separated according to their m/z values, and finally a detector.

An ion source transfers the analyte molecules into the gas phase and ionizes them. The most common ionization techniques used today are based on desorption/ionization of the analyte molecules or formation (spraying) of small charged droplets in a strong electric field. Matrix-assisted laser desorption/ionization (MALDI) and fast-atom bombardment (FAB) are some common desorption/ionization methods, whereas electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are some examples of spraying techniques. Other ion sources are available but only the one used in this work will be described in detail.

3.4.1 Matrix-Assisted Laser Desorption/Ionization (MALDI)

MALDI-MS was developed in the middle to late 1980s (Nobel Prize winner 2002) [89-92] for the analysis of molecules with large molar weights and is considered to be 10-100 times more sensitive than FAB-MS [93]. FAB-MS concepts were important in the development of MALDI-MS but FAB is nowadays therefore very uncommon. In MALDI-MS

the sample is prepared by mixing matrix and analyte in solution at a ratio usually in the range from 100:1 to 50 000:1. The matrix is commonly a small aromatic organic solid or liquid with acidic and/or basic functional groups, and has often highly light-absorbing characteristics. The matrix is often dissolved in solvents such as water, ethanol, methanol, acetone, or mixtures of these [94,95]. It is believed that in the ion source, the analytes become ionized after the matrix molecules have been excited by a laser pulse of typically nanosecond length. The purpose of adding the matrix molecules is that when they are exposed to a laser, usually a nitrogen laser at 337 nm, they become excited and their absorbed energy causes translational motion and ionization of the analyte molecule [96,97]. The matrix strongly reduces decomposition of the analyte by absorbing most of the laser pulse energy. As a consequence of this, an “explosion” at the surface causes vaporization of the matrix and the analyte molecules are thereby ejected into the gas phase under vacuum or atmospheric pressure. The ejected material contains both neutral and charged species that are often analyzed with a time-of-flight analyzer but can also be analyzed with other mass analyzers. It should be noted that the nature of the ionization mechanism is still being discussed [97,98]. The mixture of matrix and sample (normally 0.1-2 μL) is placed onto a target plate, where the solvent is allowed to evaporate, whereby crystals are formed. The matrix and sample may also be deposited in separate individual steps. The drying of the solvent can either be performed at ambient pressure or at reduced pressure. The method used can influence the formation of crystals to a significant extent, which can impact performance and ease of use [94,95]. It is important that the matrix is relatively stable and not evaporate under low pressure conditions.

One great advantage with MALDI is that the most abundant species are usually singly-charged molecular ions such as $[\text{M} + \text{H}]^+$ (positive ions) or $[\text{M} - \text{H}]^-$ (negative ions), where M corresponds to the molar mass of the neutral molecule. The interpretation of the spectrum thereby becomes easier (no multiply charged species interfering). Doubly-charged species may occur but this is not as common as in other MS techniques such as ESI-MS.

As stated above, MALDI is often used together with a time-of-flight (TOF) mass analyzer (Figure 6). This analyzer technique was first described in 1946 [99]. In a TOF analyzer the ions are accelerated to about 5-30 keV in an acceleration region positioned between the MALDI target and a nearby grid at ground potential. The kinetic energy is directly proportional to the acceleration voltage and the number of charges of the ion. In an ideal case, all ions with the same charge will have the same kinetic energy, and ions will thus acquire a speed proportional to $(z/m)^{1/2}$. After exiting the acceleration region, the ions drift

through a field-free flight tube, where they separate (because of the different speeds) according to their mass-to-charge ratios.

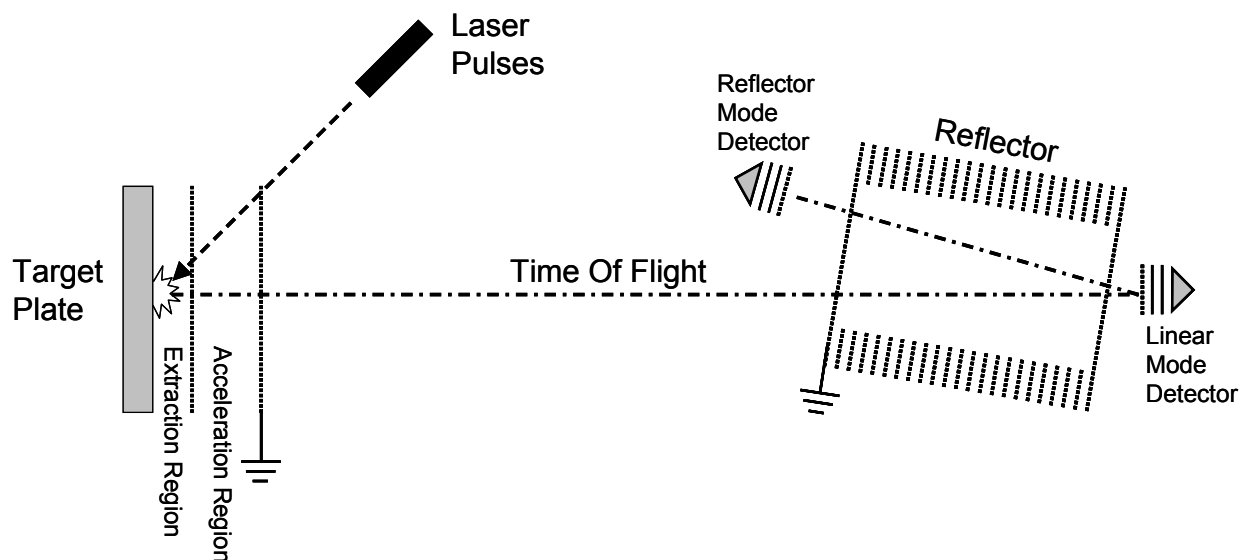


Figure 6. The principle for matrix-assisted laser desorption/ionization with time-of-flight separation.

To obtain high mass resolving power in a TOF analyzer there are several factors to consider. Operating at the lowest possible laser intensities will reduce differences in initial kinetic energy of the formed ions and thus will increase the resolution of the measurements. There are, however, two main modifications to the TOF analyzer that compensate for the natural variations in the initial velocity of the ions of the same m/z due to the ejection and ion formation process. The reflector is usually a part of the end at the flight tube where the direction of an electric field is set in such a way that the ions become decelerated, turn around, accelerate and exit the reflector in a near opposite direction [100,101]. If two ions with the same charge and same mass enter the reflector at different times because of slightly different speeds, this will be compensated for. The faster ion will penetrate deeper into the reflector, taking a somewhat longer flight path than the slower ion, which travels a shorter distance in the spectrometer. After exiting the reflector the direction, the ions will have obtained their original speeds again and the faster ions are now “behind” but catch up by the time they reach the detector. Analytes with a specific m/z will therefore reach the detector within a narrower time-window and the resolving power is thereby increased. The reflector can optionally be switched off, whereby detection is made directly after the first flight path (linear mode). The sensitivity can thereby be improved.

Another modification is so-called time-lag focusing, or delayed extraction [102]. It is based on using an additional grid in the ion acceleration region of the TOF tube. When the sample is excited by the laser pulse, this extraction region is initially kept field-free. But after a short time the sample plate and intermediate grid potential are adjusted for full extraction. However, the slower ions close to the sample plate will thereby be subjected to a higher potential different than the faster ions further away, and will therefore gain more kinetic energy and speed. Thus the originally slower ions will be the faster ones when finally exiting the extraction region, and they can “catch up” to the slower ions. With a proper setup, all ions with the same m/z will arrive at the detector in a narrower time interval than they would in the absence of delayed extraction. A combination of delayed extraction and a reflector further improves the quality of the TOF resolution in a synergetic manner.

MALDI-TOFMS can best analyze compounds in the m/z range of 500-10 000. Larger molecules can be detected but problems involving low sensitivity, broad isotopic distributions, matrix adduction and poor resolving power are issues that complicate the analysis. Detection of analytes below 500 Da is possible but matrix clusters may interfere and limit detection in this range. The technique may be connected semi-online to LC so that the analytes eluting from the column are sprayed onto a plate with already prepared matrix or perhaps are sprayed onto the target plate after being mixed with matrix solution (Paper II). MALDI-TOFMS has been employed for the analysis of modified cellulose as reported in the literature [65,94,95,103]. Momcilovic investigated different MALDI matrices and solvents on enzyme and acid hydrolyzed MC and CMC. The results showed that the solvent, in which the sample was dissolved, had a significant effect on the apparent degree of substitution (DS). For CMC only water was suitable as solvent since organic solvents gave poor solubility of the samples. For MC aprotic solvents produced higher apparent DS values, which was most likely due to poor solubility of species with low DS. The obtained signal intensity, however, did not correlate with the solubility but seemed to be more dependent on certain matrix/solvent combinations. The choice of matrix did not have any significant effect on the measured DS [94,95].

Adden et al. [19,65] suggested a novel method for the analysis of HEMC and HPMC using MALDI-TOFMS. By applying partial acid hydrolysis of perdeuteromethylated samples, followed by reductive amination and a second permethylation step, discrimination due to different distributions of substituents (thus a random hydrolysis was achieved) during the acid hydrolysis were minimized and discrimination problems during MS detection due to variations in polarity were minimized. Therefore quantitative measurements of the

hydroxyalkyl and alkyl functions could be obtained using MALDI-TOFMS. Comparison with results from ESI-ITMS showed that the ion trap is appropriate for quantitative analysis of methyl patterns but not for hydroxyethyl distributions of the formed oligosaccharides. A decrease in the apparent molar substitution was indicated (it should be constant) using ESI-ITMS but not for MALDI-TOFMS. This was explained by the limited optimal working range for quadrupole ion trap instruments, which causes discrimination over the broad hydroxyalkyl pattern.

Richardsson et al. [103] investigated the distribution of substituents of HPC using MALDI-TOFMS and compared the results with those from NMR. The DS results from MALDI-TOFMS were generally higher than the values obtained from NMR, which in turn were higher than the values provided by the manufacturer. The deviation in MALDI-TOFMS was suggested to originate from signal discrimination due to inhomogeneous sample preparation, poor ionization of low substituted monomers, and discrimination of the MALDI-TOFMS in the low molar mass range.

In the work reported in Paper II we used SEC connected semi-online to MALDI-TOFMS in order to increase the mass range analyzed by mass spectrometry. By spraying the eluting substance from the SEC column onto an MALDI-target plate it became possible to detect oligomers up to DP 30, significantly increasing the working range previously obtained using ESI-ITMS. In work presented in Paper III, we compared the discrimination effects that take place on modified cellulose when the samples are not properly treated and free hydroxyl functional groups are present. Three different MS techniques were investigated as described below (see section 3.4.4. *Electrospray Ionization Coupled to Ion Trap (IT) MS*)

3.4.2 Electrospray Ionization MS (ESI-MS)

Some of the pioneers of ESI-MS and MALDI-TOFMS was awarded the Nobel Prize in 2002 [89,104]. In ESI, the analytes are dissolved in a polar and volatile solvent, normally water in a mixture with some more organic solvent such as methanol, isopropanol or acetonitrile. Addition of weak acids, such as formic or acetic acid, can improve the ionization in positive mode (detecting positively charged ions). In negative mode, ammonia or a volatile amine can improve the deprotonation. Salts in too high concentrations are to be avoided, since this generates numerous adduct ions, diluting information over more peaks and increasing the danger of overlapping charge-state distributions and other disturbances in the mass spectra. The sample is transported through a needle to a needle tip placed at high positive or negative potential ($\pm 1\text{-}5$ kV) with respect to the sample inlet of the mass spectrometer (Figure 7) [104-

106]. A spray of charged droplets is ejected at the needle tip. Due to the strong electric field at the needle tip the emerging liquid will be electrically charged. With the aid of a turbulent flow of nitrogen gas (nebulizer gas) the droplets are sprayed [107]. A counter-flow of heated nitrogen promotes evaporation of the droplets and release of charged analytes. The charge density on the droplet surface thereby increases, which causes the Coulomb repulsion forces to increase. When the repulsive force approaches the same magnitude as the droplet surface tension, the droplets disintegrate in a so-called Coulomb explosion and a family of smaller ionized droplets is thereby formed [108-110]. This process proceeds until the droplets are so small that the ions are desorbed into the ambient gas and are swept in by the high vacuum of the mass analyzer. Electro spray ionization of oligosaccharides in positive mode usually produce ions of the type $[M+nH]^{n+}$, $[M+nNa]^{n+}$ or $[M+nLi]^{n+}$. The ESI technique is often used together with mass analyzers such as quadrupoles and/or ion traps and is the most common technique to be used in conjunction with liquid chromatography (Paper II) [107]. It has also been used together with TOF analyzers [111]. ESI can be used to analyze species in the m/z range of 500-2500 but can also be used to analyze larger proteins if multiple charged species is used. A technique called nano-spray can also be used instead of ESI and in this technique only a minute amount of sample is required [112].

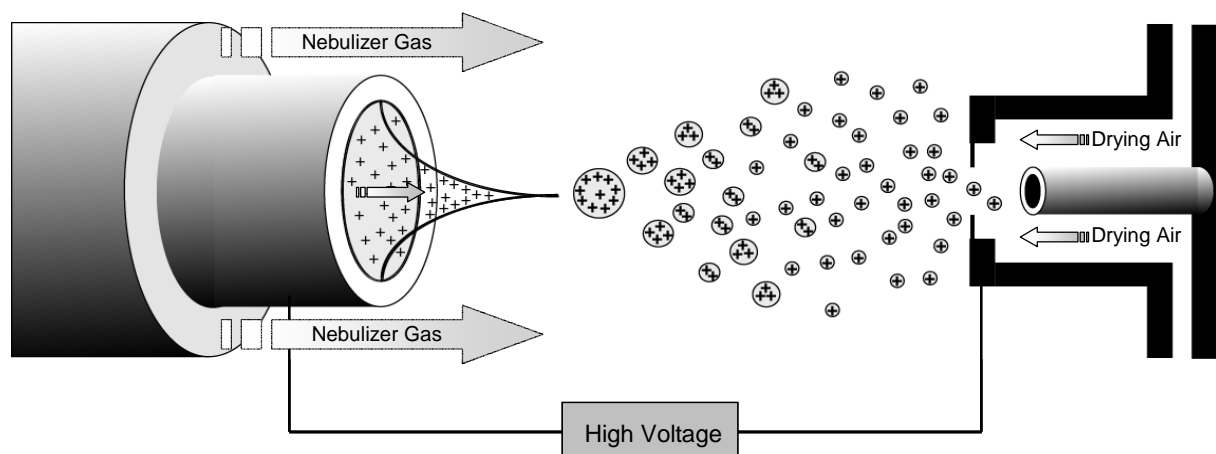


Figure 7. The principle of ESI MS spectrometry where the sample is sprayed towards the inlet of the mass spectrometer.

3.4.3 Electro spray Ionization Coupled to Triple Quadrupole (QqQ) MS

The basic principle of the quadrupole mass filter was published in 1952 [113]. In an ESI quadrupole system, the ions formed in the ESI source (atmospheric pressure) are guided into the first of three quadrupole or cylindrical electrode sets, which are held under vacuum. The ions pass through a volume between four metal cylinders and the injected ions respond to a

combination of radiofrequency and constant potentials applied to the cylinders. Depending on the potentials only ions with a very narrow m/z range take a stable path through the field while the others take an unstable path, hit the rods, and are thereby not detected. The fields are scanned rapidly in order to sequentially detect the ions, building up an m/z spectrum. Quadrupoles can be used as a mass selection tool together with other MS analyzers (e.g. QIT or Q-TOF) but can also be used either alone or in combination with several quadrupoles. In a triple quadrupole (QqQ) system the first quadrupole can be used as a mass selector where only a certain m/z is transmitted (let through). For tandem MS, the second quadrupole enclosed in a box provided with a background of e.g. Ar gas, fragmentation occurs through collisionally induced dissociation (CID). The formed fragments travel to the third quadrupole, where they are analyzed. In this way a one-stage tandem mass spectrum is acquired. Quadrupoles are generally cheaper and less demanding of floor space than other MS equipment, which has led to a consistent usage. Although it suffers from lower resolving power compared with other mass analyzers it is generally sufficient for routine analysis. Typical m/z values that can be analyzed in a quadrupole instrument are in the range of 100-4000.

3.4.4 Electrospray Ionization Coupled to Ion Trap (IT) MS

When combining electrospray with an ion trap analyzer an octopole is often used prior to the injection of the ions to the ion trap. The octopole then works as a guide for the flight path of the ions towards the ion trap. The ion trap consists of two end cap electrodes and a ring electrode. The ions normally enter the trap through an opening in one of the endcaps or in the ring electrodes. The trap captures ions with m/z greater than a certain adjustable cut-off (determined by the trap drive parameter). When the trap gets filled up, the ion beam from the octopole is deflected away and the captured ions oscillate inside the trap. Selected ions can be ejected from the trap and detected by applying an additional dipole resonance frequency that matches their oscillating frequency. By scanning this frequency one can read out the contents of the trap and acquire a mass spectrum. The IT is an excellent instrument for performing tandem MS. Since the trap allows residence of the ions for a long time, they can be manipulated several times, thus allowing consecutive stages of fragmentation (tandem MS, or MSⁿ). In tandem MS, the dipolar frequencies corresponding to all ions except for the m/z of interest are applied and thereby all other ions except for the ion of interest are ejected from the trap. After isolation, the amplitude of the frequency corresponding to the isolated ion can be increased, causing increased kinetic energy of the analyte. The excited ions collide with He atoms present in the trap, causing fragmentation of the mother ion and formation of daughter

ions. The fragmented ions can then be read out. Alternatively, selected daughter ions can be trapped and fragmented again. In a commercial instrument ions can be isolated and fragmented in up to ten cycles, but in practice the need for orders higher than five is rare. The normal working range of an ITMS is between 150 and 3000 m/z but some commercial instruments can allow detection of ions with m/z up to 20 000.

In the work presented in Paper III we compared ESI-IT, ESI-QqQ, and MALDI-TOF MS for analysis of acid and enzyme hydrolyzed MC, with the purpose of investigating the discrimination effects in ionization and detection in MS analysis that occur due to free hydroxyl functional groups of the modified cellulose. To measure the extent of discrimination, methyl derivatives were selected, since they can easily be transformed into isotopically labeled and chemically uniform oligomers, which give equal response in MS. The DS values for the non-deuteromethylated oligomers (free hydroxyl groups still present) varied with the strongest deviation for ESI-IT, followed by ESI-QqQ, and essentially no deviation for MALDI-TOF MS. A shift of relative intensities to the higher methylated oligosaccharides for the non-deuteromethylated samples was observed to a different extent for each technique thereby giving an overestimation of the measured DS. The polarity of the oligomers had a big influence in electrospray ionization. The results indicate that in MALDI-TOFMS, the polarity of constituents is of less influence compared to electrospray ionization and/or that mass analysis by time-of-flight does not discriminate ions with respect to their m/z value. Thus, the free hydroxyl function influences the ionization less in MALDI-TOF compared to ESI-IT and ESI-QqQ MS. However, to completely eliminate discrimination, perdeuteromethylation of the free hydroxyl functions of the MC was necessary which has also been reported for FAB-MS, ESI-ITMS, and MALDI-TOFMS after random acid cleavage [114].

3.4.5 Tandem Mass Spectrometry

Tandem mass spectrometry is an extremely valuable tool in characterization and structural chemistry [98,115-117]. The technique has and can be used for everything from detecting metabolic disorders of humans to human genome mapping and of course carbohydrate characterization. Tandem mass spectrometry can be thought of as two mass analyzers in series connected by a chamber that can “break” or fragment a molecule into more easily characterized pieces which must then be reassembled, just like a puzzle. In the first stage of a fragmentation experiment, a collection of ions is created in the ion source of the mass spectrometer. The ions are allowed to pass through the mass analyzer and their m/z values are measured. On the basis of the mass spectrum measured in the initial measurement,

the mass analyzer is set to isolate just one m/z value of interest (the mother ion). Just these ions enter a stage, where the fragmentation occurs. The kinetic energy of ions is converted to vibrational (internal) energy during collision and the ions fragment into smaller pieces (daughter ions). The m/z values of fragment ions are then determined in the mass analyzer. The fragmentation can be performed in one single mass analyzer (e.g. ESI-IT) or using a combination of several spread out in space (e.g. QqQ).

The hope is that knowledge of the fragments can give better elucidation and that the information obtained can be recombined to form a better picture of the original molecules. This is indeed often possible, and tandem MS of oligosaccharides and derivatized cellulose for characterization purposes has become more and more important and prevalent [62,114]. The fragments of oligosaccharides that are formed in MS/MS have been described by Domon and Costello [118] who suggested the nomenclature shown in Figure 8.

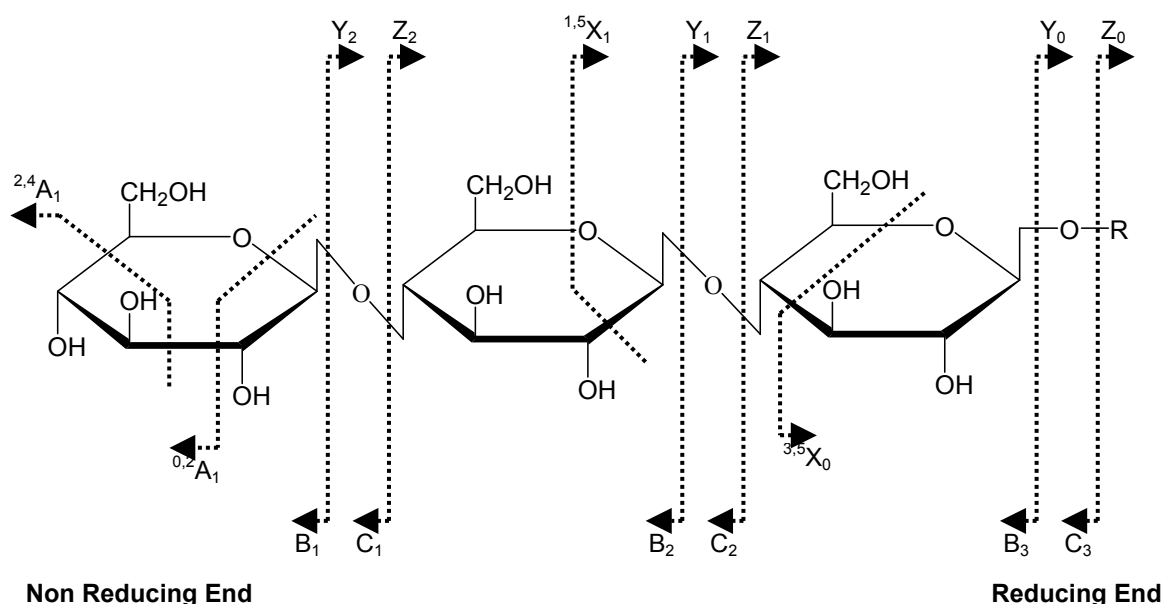


Figure 8. Synopsis of the nomenclature for oligosaccharide fragments observed in tandem MS; suggested by Domon and Costello. Fragment ions that contain a non-reducing terminus are labelled with uppercase letters from the beginning of the alphabet (A, B, C...) while fragments that contain the reducing end of the oligosaccharide are labelled with a letter from the end of the alphabet (X, Y, Z...). Superscripts indicate the site of the cleavage. A and X fragments are results of internal glucosidic ring cleavage and are labelled by assigning each ring bond a number and counting clockwise [118].

Tandem MS of carbohydrates was first studied with FAB desorption-ionization [119]. The analysis was not very sensitive since neutral and basic carbohydrates were very poorly ionized in FAB. The tandem MS analysis was also limited to molecules of mass < 1000 and the FAB desorption process sometimes caused unwanted additional fragmentation forming

other kinds of ions confusing the analysis [119]. Fragmentation is most frequently performed through post-source decay (PSD), or through collision induced fragmentation (CID). In PSD, most commonly used with MALDI, the fragmentation occurs spontaneously due to the high internal energy of a fraction of the ions. Fragmentation with CID is performed through collision of the ions with an inert gas (often He, N₂ or Ar). Low-energy CID primarily fragments the polysaccharides by breaking the glucosidic linkages. Fragmentation across the sugar rings is less pronounced since it requires two covalent bonds to be cleaved. However, by increasing the energy causing fragmentation this kind of fragmentation can be induced, providing important additional information in characterization studies.

Conditions during ESI-MS fragmentation of oligosaccharides in positive mode (detection of positive ions) can be manipulated to favor the production of protonated, alkaliated, or ammoniated ions [120]. Normally this is performed through addition of the corresponding salts. Alkaliated fragmentation is commonly performed through addition of singly charged alkali metal ions such as Li⁺, Na⁺, and K⁺. Fragmentation yields are inversely related to the cation size, following the order H⁺>Li⁺>Na⁺>K⁺>Rb⁺>Cs⁺. The fragmentation of protonated ions requires more energy than for the cations, and the energy required increases with cation size. Protonation is likely to be localized to the glycosidic oxygen, whereas metal ions can undergo coordination with several atoms simultaneously, resulting in less destabilization of the glucosidic bond [121]. Thus, the choice of adduct will influence the fragmentation behavior of the ions by favoring fragmentation of different bonds.

There has not been that much published work on tandem mass spectrometry of derivatized cellulose. On the other hand, extensive work has been published on tandem mass spectrometry of shorter substituted oligomers and other carbohydrates [98,116,117,122-127].

Tüting et al. investigated the fragmentation mechanism and the substituent distribution of regioselectively *O*-methylated maltooligosaccharides. Through the selective methylation and the fragmentation analysis, it was found that the substituent position has a significant influence on the relative abundances of the charged fragments. For instance it was shown that formation of ^{0,2}A fragments (Figure 8) was enhanced for malto-oligosaccharides that were methylated in the C-2 position, whereas methylation on the C-6 position did not influence the fragmentation [128]. Adden and Mischnick continued this study by developing a quantitative method, in which the fragments could be used for determining the complete substituent distribution along the cellulose chain of both α - and β -linked (1 \rightarrow 4)-linked glucose polymers. The method is based on perdeuteromethylation of the free hydroxylgroups of *O*-methyl celluloses and amyloses with a DS of 1.1 to 1.9 and 0.7 to 0.9 respectively, followed by a

partial hydrolysis, tandem MS analysis and finally calculation of the distributions. The method required ESI-MS² and MS³ data from both sodium and lithium adducts to obtain sufficient information for the analysis. The method correlated well with those results obtained from GC analysis [62].

Momcilovic et al. [129,130] performed tandem MS using MALDI-TOFMS and ESI-ITMS of acid and enzyme hydrolyzed samples (see section 4.5 *Characterization of Cellulases* for more details). By coupling the reducing end with dimethylamine, forming a Schiff base, MS fragments obtained from protonated adducts yielded predominantly Y-fragments, from which the substituent distribution of the oligomers could be estimated. On the other hand, fragments obtained from sodiated adducts provided information regarding the position of the substituents within the anhydroglucose units. Although the approach is not completely quantitative due to the existence of free hydroxyl functionalities, it was demonstrated that a combination of DMA derivatization and tandem MS could be used as a tool to elucidate information of the enzyme selectivity [130]. It was also shown that derivatization using DMA and other alkylamines improved the sensitivity in MS by a large factor [129,130].

In the work presented in Papers III and IV we took a somewhat different approach to quantitatively obtain information on the methyl position and its distribution after enzyme hydrolysis of MC. Instead of performing reductive amination of the reducing end, we reduced it, performed perdeuteromethylation and thus obtained chemically uniform oligomers where only the isotopic pattern differentiated between the original methyl substituents. The non-reducing and reducing ends were selectively marked, making it possible to distinguish between the C and Y fragments. By analysing the formed oligomers using tandem MS, the specificity of the cellulases was investigated (see section 4.5 *Characterization of Cellulases* and Papers III and IV for further details). By comparing with reference data the method showed good reproducibility. However, due to problems with fragment formation it was difficult to obtain information on the position of the substituents within each anhydroglucose unit.

3.5 Determination of the Amount of Reducing Ends

The measurement of the amount of reducing ends can be valuable in the characterization of carbohydrates, cellulose, food and biological samples. It gives estimation on the amount of free reducing ends in the sample thus providing information on the amount of carbohydrates in the samples. Most available methods for the determination of reducing ends have been applied on food or biological samples [131-139]. For the analysis of pure and modified

cellulose, these methods have most commonly been employed to estimate the efficiency of different enzymes to degrade the modified cellulose [140-142]. Each time the cellulose chain is cleaved, a new reducing end is formed. Thus, if the amount of reducing ends can be determined before and after hydrolysis, information on the efficiency of the enzyme and the resistance of the cellulose to enzyme attack can be determined. Several different methods have been suggested in the literature for the determination of the reducing ends. Typically the amount is determined by colorimetric procedures based on the reduction of a metal ion [138,143,144] but other methods are also available. The method proposed using bicinchonic acid (BCA) as reagent has been used extensively since it is very sensitive. It may also be applied for the measurement of endoglucanase activity where K_m values may be determined [142]. Unfortunately this type of reductometric assay is not specific for sugars. Proteins (such as the endoglucanase itself) in the sample will also react with BCA giving erroneous values of the amount of reducing ends. Other methods have been developed to circumvent this problem using aldehyde-specific reagents [138,142-144]. One issue with determination of reducing ends after enzyme hydrolysis has been problems regarding the reliability of the measurements. Many methods, such as that using dinitrosalicylic acid (DNS) as reagent, easily give an overestimation of the amount of reducing ends formed due to a non-stoichiometric response [141]. Another issue is that it has not been investigated how the presence of substituents on modified cellulose may influence the response of the methods.

The common procedure to automate the determination of reducing ends is flow-injection analysis [136,139]. The normal detection is based on UV absorbance but potentiometric detection has also been investigated successfully [145]. In Paper V we suggest a fast and sensitive novel method based on amperometric detection. Ferricyanide was reduced to ferrocyanide during reaction with the reducing ends under alkaline conditions. The ferrocyanide was then re-oxidized at the detecting electrode and the current was measured. The chemical parameters were optimized using factorial design. The method showed very good reproducibility, high sensitivity, and was very stable. One great advantage, found also in a few other reducing end measuring methods, was that the response seemed to be independent of the length of the oligosaccharide. Thus, the detection seemed to give a stoichiometric response. The method was successfully applied for the determination of reducing ends formed after hydrolysis of MC with different endoglucanases. With this method it was also possible to measure the amount of reducing ends in real-time using a lab robot. Injection of standards between each sample injection compensated for random errors during the analysis.

3.6 Other Methods used for Cellulose Analysis

Capillary electrophoresis (CE) has been applied for the analysis of cellulose derivatives. CE is often employed in conjunction with UV detection but since cellulose does not possess any natural UV absorbing functionalities, quantitative derivatization of the reducing end with a UV absorbing ligand is therefore necessary. Several methods have been proposed for both pure cello- and malto-dextrins as well as for derivatized cellulose and starch [146-151]. The detection limits are in the range of ng/l. CE can also be used together with MS and RI detection [152,153].

Nuclear magnetic resonance (NMR) is also very useful in cellulose analysis and can provide a partial distribution pattern of the substituents. By ^{13}C -NMR and ^1H -NMR studies it is possible to determine the average degree of the substitutions in position C-2, C-3 and C-6 which have been assigned x_2 , x_3 and x_6 by Spurlin [154,155]. Tezuka investigated mixed acetates/butyrate by ^{13}C -NMR [156], Kern et al. investigated regioselectively substituted 2,3-di-*O*-methyl cellulose, and several other applications are available employing NMR for structural elucidation of modified cellulose [53,56,157-162]. The advantage of using NMR for determination of the substituent distribution is the smaller amount of work that is needed for sample preparation compared with for instance GC-FID. However, NMR may so far only provide the partial substituent distribution by providing the x_2 , x_3 and x_6 and normally suffers from poor sensitivity. Also, in-house experiments on the DS determination of HPMC have shown that NMR can give overestimated values of DS compared with GC-FID.

Alvarez-Lorenzo et al. [86] compared GC, NMR and Raman spectroscopy for the determination of DS for non-ionic cellulose ethers (HPC and HPMC). Using a GC method, very different from the one described above and only providing the total DS of the sample, it was found that GC was the most accurate, closely followed by NMR. Raman Spectroscopy was found to be somewhat inaccurate but had the advantage of requiring very little sample pre-treatment.

Erler et al. [38] developed a method using reversed phase HPLC with a C18 or an amine-based column with the purpose of determination of the substituent pattern of MC. After total hydrolysis, separation of the eight monomers, and RI detection they showed that significant separation took place of the formed monomers and thus, it became possible to obtain reliable information for the determination of substituent distribution. Significant differences were found in the separation between the two columns.

4.0 ENZYMES FOR THE HYDROLYSIS OF MODIFIED CELLULOSE

Enzymes (in Greek *en* = at or in and *zyme* = leaven or yeast) are proteins that accelerate and promote repeatedly a chemical reaction, without themselves being damaged by the reactions [163]. The word itself was introduced by the German researcher Wilhelm Friedrich Kuhne in 1876. In the beginning it was debated whether the catalytic activity of enzymes could exist independently of living cells. Kuhne thought so, and the opposing view led by Louis Pasteur, was eventually proved wrong [164]. In general, an enzyme catalyzes only one reaction type (reaction selectivity) and operates on only one type of substrate (substrate selectivity). Enzymes are generally named by adding the ending "-ase" to the name of the substance on which the enzyme acts. Because enzymes, like all catalysts, do not affect the relative energy between the products and reagents, they thus do not affect the equilibrium of the reaction. The selectivity of an enzyme depends on its active site, which is the three-dimensional region that contains the binding and catalytic sites [165].

The potential of cellulose as a renewable resource was realized during the Second World War. The U.S. Army was alarmed at the rate of deterioration of cellulose materials including clothing, tents and sand bags, in the South Pacific. To solve their problems, laboratories were set up to find a solution. It was discovered that the deterioration was due to a fungi (*Trichoderma reesei*) that converted the cellulose to small water-soluble sugars such as glucose. Hence, the cellulose-degrading enzymes were discovered. The discoveries lead to a world wide expansion for the use of cellulases ranging from improving food products to “stone washing” of jeans [166-169]. It has later been found that cellulases have very interesting properties, including extreme thermostability, high acidic and basic stability, and also low risk of contamination by other pathogens [170]. All these properties have led to an increased usage. Therefore, cellulases are today used in a variety of applications such as detergents, ethanol production, brewing, and food [167-169,171].

The activity of cellulose-degrading enzymes is measured in units (U). The definition of a unit varies between different commercial manufacturers and should therefore be interpreted with care. Normally, 1 U of cellulase is defined as that amount of enzyme that liberates 1 micro-equivalent of reducing ends per minute. However, when this is determined, the starting material can vary between different sorts of CMC to Avicel and filter paper, producing very different results.

In this work, enzymes capable of hydrolyzing the (1→4)- β -linkage in cellulose have been investigated. Their specificity and selectivity have been studied with several new

techniques on both native and modified cellulose. The main purpose was to find a correlation between the substituent distribution of modified cellulose and the hydrolysis products formed by the enzymes. Extensive investigations of the enzyme selectivity on modified cellulose were therefore performed. It was found that it is very important to have detailed knowledge about the enzyme mechanism before conclusions or correlations can be drawn.

Cellulose, and thus modified cellulose, degrading enzymes are divided into three major groups of enzymes, which belong to the EC 3.2.1.X class; Endoglucanases, Exoglucanases (cellobiohydrolases) and β -glucosidase. According to modern concepts most cellulolytic enzymes comprise multidomain proteins containing at least three separate structural elements of different functions, i.e. catalytic domain, cellulose binding domain and interdomain linker. Cellulases can be obtained from several different origins both from aerobic and anaerobic organisms and both from bacteria and fungi. Many cellulases have activity for several substrates such as chitin, mannan, xylan and cellulose [172].

4.1 Endoglucanases

Endoglucanases (endo-cellulases), are enzymes of the EC 3.2.1.4 class that have a cleft-shaped active site [173] and hydrolyze the internal (1 \rightarrow 4)- β -D glucosidic bonds in cellulose, lichenin and cereal β -glucans [174]. They are produced as many multiforms and other variations in fungal and bacterial systems. The former generally produce different kinds of cellulases (endo, exo, β -glucosidase) whereas the latter mainly produce endoglucanases [175]. These enzymes act fundamentally by two different mechanisms, which are characterized by the stereochemical outcome of the degradation reaction. If the stereochemistry of the linkage at the anomeric centre is inverted in the product, forming terminal α -glucose, then the enzyme is an inverting enzyme (Figure 9). On the other hand, if the stereochemistry of the linkage at the anomeric centre is retained in the product, forming terminal β -glucose, then the enzyme is a retaining enzyme (Figure 9) [176]. The inverting enzymes use a single-displacement mechanism, in which water attacks directly at the anomeric center, displacing the leaving group in a general acid/base-catalyzed process via a transition state with considerable oxocarbenium character [177]. Retaining enzymes employ a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate. The first step involves attack of an enzyme nucleophile at the anomeric centre with general acid-catalyzed displacement of the leaving group to yield a covalent glycosyl-enzyme acylal intermediate. The second step involves a water attack on the anomeric centre of this intermediate in a general base-catalyzed

process [177-179]. The water in the second step can be substituted for a glucosyl group which then, instead of an attack, will result in the formation of another oligosaccharide. This capability of retaining enzymes is called transglucosylation and has been investigated and reported on several occasions in the literature [180].

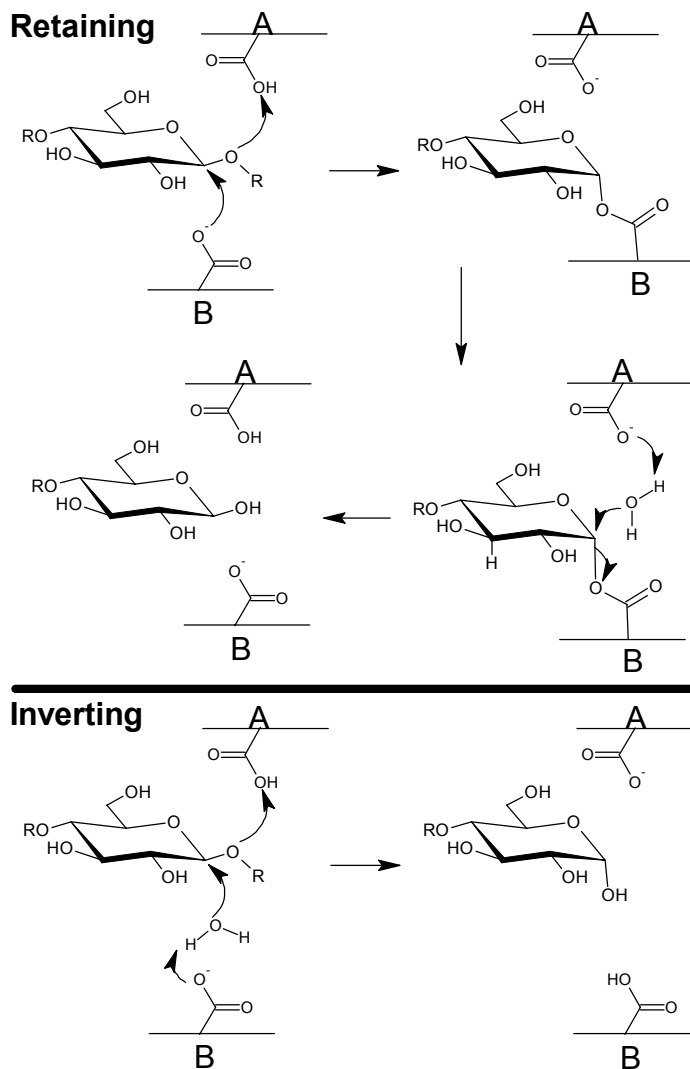


Figure 9. Reaction mechanisms for cellulases. In the retaining mechanism (a) an enzyme-substrate complex is formed where the substrate is covalently bound to the enzyme. In this mechanism the anomeric carbon will retain its β -configuration. The inverting mechanism (b) changes the anomeric configuration to α -configuration. The figure is redrawn from McCarter and Withers [181].

When unmodified cellulose is the substrate, the endoglucanases have lower activity towards the crystalline regions of the cellulose. This is caused by pervasive hydrogen bonding between the cellulose chains in these regions, which the endoglucanases have problems to overcome. Amorphous regions of cellulose allow great penetration of the enzyme and thus, the endoglucanases can effectively hydrolyze the internal bonds in these regions randomly,

creating short cello-oligomer chains with low DP. The oligomers formed might also tend to be longer in presence of crystalline domains [174,182].

Endoglucanases have been shown not to have any activity towards smaller oligomers, i.e. for oligomers with $DP \leq 3$. However, depending on the endoglucanase, the cellulose chain might have to be even longer for hydrolysis to occur. The knowledge of this “prerequisite” in chain length for effective hydrolysis reflects the degree of specificity of the enzyme.

4.2 Exoglucanases

Exoglucanases (exo-cellulases), also called cellobiohydrolases abbreviated CBH, belong generally to either EC 3.2.1.91 or 3.2.1.74 classes, and usually contain a tunnel-like active site [173] and attack the cellulose from the reducing or non-reducing end, most commonly producing cellobiose but also glucose and longer oligomers can be formed [175,183-185]. Since the active site is shaped as a tunnel, it is believed that the substituents around the linkage to be hydrolyzed in effect pose steric obstacles, which limit the extent to which these enzymes can hydrolyze a given polymer. Thus, since this type of enzyme attacks the ends of the cellulose chains hydrolysis may progress until a substituent appears. By contrast, endoglucanases with a cleft-shaped active site are believed to be more tolerant towards the presence of substituents since they attack the cellulose “from the side” of the chain instead of from the ends. Three-dimensional structures of several enzymes have confirmed this hypothesis [186-188]. Exoglucanases are distinguished from β -glucosidase (described below) by the inversion of the products [167,189].

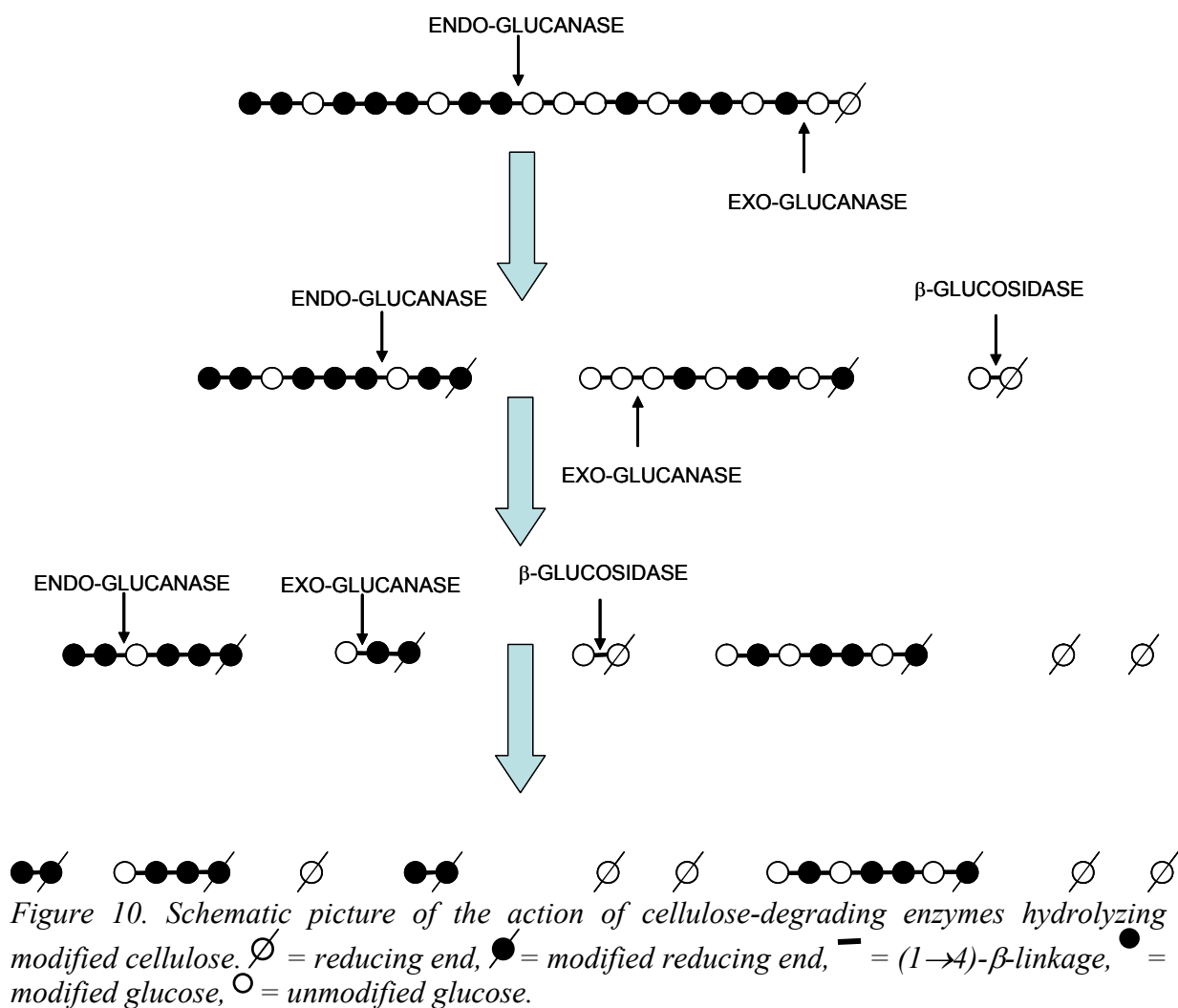
4.3 β -Glucosidase

The third type of enzyme that can hydrolyze a cellulose chain is called *β -glucosidase* and is an exo-enzyme that hydrolyzes unmodified cellobiose to glucose starting from the non-reducing end. The activity of cellobiose is crucial for the hydrolysis of cellulose to individual glucose units [190]. This is mainly done as β -glucosidase further hydrolyzes the final products of the endo- and exo-glucanases that otherwise may hinder their hydrolysis through product inhibition [191]. Since endoglucanases only hydrolyze cellodextrins with chain length of DP 3 and higher, the presence of β -glucosidase in a cellulase batch can be verified by hydrolysing cellobiose. If the cellobiose remains intact and no traces of glucose are found, the cellulose batch is considered to be free from β -glucosidase. Distinguishing between β -glucosidase and exoglucanases is more difficult since inversion and activity towards longer substrates is the only difference.

4.4 Synergism in a Multi-Enzyme Approach

The complete hydrolysis of unmodified cellulose to glucose requires a combination of enzymes (endo-, exo-glucanase and β -glucosidase). During the hydrolysis of native unmodified cellulose, the endoglucanases attack the cellulose in a random manner creating low amount of new reducing ends. This reaction is followed by the hydrolysis with exo-glucanases, which attack the cellulose from either end, depending on which type of enzymes, releasing a higher number of new reducing ends. Finally, the β -glucosidase completes the hydrolytic process through the formation of glucose from cellobiose. It is considered that all three enzymes work in a synergistic manner for hydrolysis of both native and modified cellulose [167,192]. The endoglucanases can hydrolyze the interior parts, liberating free modified and/or unmodified glucose chains which in turn will be attacked by the exo-glucanases and the β -glucosidase. Thus, the use of a combination of all three enzymes will hydrolyze modified cellulose to a higher extent than the use of only one of the enzyme types by itself (Figure 10). An important fact that has to be addressed is that the substituents of modified cellulose in most cases hinder the enzyme from gaining access to the cellulose chain and forming an adequate transition state for hydrolysis to occur. Hence, complete degradation of modified cellulose to monomers using a combination of these three enzyme types is not possible.

Evidence of synergism between endo-endo and exo-exo enzymes has also been found, suggesting that e.g. not all endo-enzymes are equivalent [175,193]. Synergism is not always evident when using combinations of endo- and exo-enzymes. Shepard et al. [194] found that the combination of enzymes did not improve the hydrolysis yield and that one of the investigated enzymes could independently carry out the entire hydrolysis. Sequential separate hydrolysis with an endoglucanase followed by an exoglucanase has been shown to give much less extensive cellulose hydrolysis than that achieved by incubation with both enzymes simultaneously [195]. This effect has been suggested to be due to a special synergistic effect, where each enzyme speeds up the action of the other, with a resulting increase of hydrolysis yield.



4.5 Characterization of Cellulases

There are many ways of characterizing the activity of cellulose-hydrolyzing enzymes. Various model cellulose substrates have been used for the purpose of studying the mechanism of action and interaction of individual cellulose enzymes. The influence of different characteristics such as varying degree of polymerization and crystallinity of the substrate has been investigated. Reference substrates include filter paper, cotton, Avicel, and soluble cellulose derivatives (mainly CMC) [140,192,196,197]. Several different assays have been suggested and developed for characterization of cellulases and their mechanisms [140,198-200].

Previously the influence of each amino acid residue in the active site of the enzyme has been investigated through analysis with X-ray crystallography of experiments, during which the enzyme has been soaked with possible hydrolysis products. The catalytic activity of cellulases is affected by substrate-binding sites distant from the bond actually undergoing hydrolysis (Figure 12). These additional substrate-binding sites may also be revealed by X-

ray crystallography, which can also reveal the 3D structure of the catalytic domains [186,187,201-207].

The viscometric method is based on the ability of endoglucanase to rapidly cause a decrease in chain length, and hence in viscosity. The viscosity change of modified cellulose in the solution is then measured prior to and after addition of the enzyme; the reaction can be followed until completion [200]. The method is very specific and but only provides a general estimation of cellulase activity. Martinez-Richa [208] investigated how HEC changed in viscosity while being hydrolyzed by glucanases. It was found that the hydrolysis and the viscosity change were highly dependent on the DS and DP of the sample, and a simple model relating DS and the resistance towards enzyme hydrolysis was suggested.

The measurement of reducing ends after hydrolysis of CMC or other soluble cellulose derivatives is another method that is frequently employed to monitor enzyme activity. Each time a glucosidic bond is cleaved a new reducing end is formed, and therefore the activity of cellulases can be estimated. However, this method has a number of pitfalls since the reducing end assays are not always stoichiometric, the assay is not specific for endoglucanases, and the substrate CMC is not always the same, having different DS and anhydroglucose composition and perhaps different substituent distributions, causing irreproducible results [200]. Also, the presence of extraneous enzyme activity when determining endoglucanase activity, i.e. presence of β -glucosidase, will lead to excess production of ends and overestimation of the endoglucanase activity.

The use of chromatography such as SEC combined with RI or/and MALS detection has gained an increased interest, since it provides a nearly complete picture of the molar mass distribution prior to and after enzyme hydrolysis [140,198,209-211]. Eremeeva [212] recently reviewed the area of SEC for characterization of unmodified polysaccharides. Vlasenko et al. [213] investigated the activity of different cellulase preparations on CMC. They compared reducing capillary viscometry, end-group analysis, and MALS detection after SEC and tried to employ the information obtained to establish a relationship between the enzyme hydrolysis and the obtained results. They found that the viscometry method gives big errors since it did not account for the hydrolysis of CMC near the chain ends. It was also shown that SEC-MALS easily gives an overestimation of the hydrolysis compared with reducing-end analysis.

The results presented in Paper IV show the variation in hydrolysis efficiency of MCs of different qualities hydrolyzed with five different cellulases. The molar mass distributions of the samples were investigated prior to and after hydrolysis, and it was found that the different enzymes hydrolyzed the MCs differently (Figure 11). The enzyme preparations could be

divided into three classes. One was considered as an outlier (denoted reesei) due to bad signals, two seemed to be less affected by the substituents producing a lower average molar mass (denoted viride and BaCel 5A), and two seemed to be more affected by the substituents producing a higher average molar mass (denoted longi 1 and 2). It was also found that if the DS of the MC was too low, then the results was an exhaustive hydrolysis of the MC, and thus SEC could not be used to distinguish between the efficiency of the enzymes batches due to the very low molar masses. Instead a methodology based on internal standard addition in combination with mass spectrometry had to be used (see below).

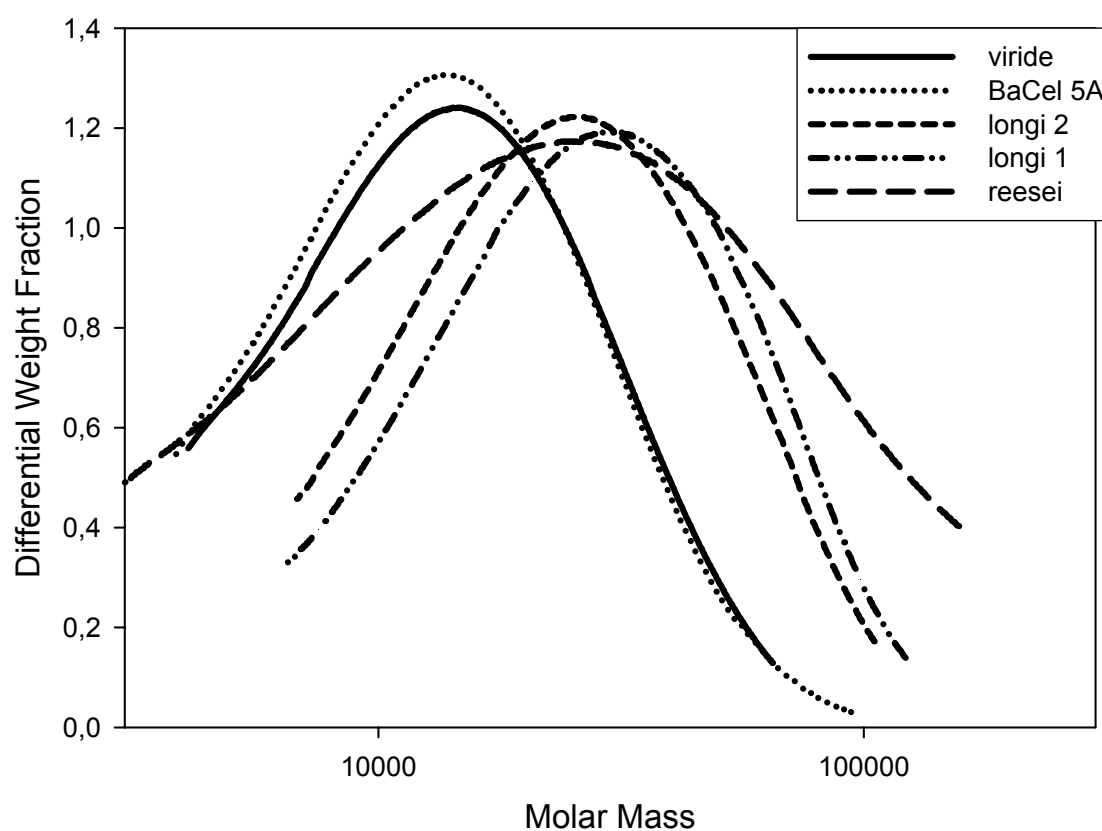


Figure 11. SEC analysis plot (differential weight fraction) of MC (DS 1.8) hydrolyzed with five different enzyme preparations. The curve for reesei has been renormalized to make the signals comparable with the other curves.

HPAEC-PAD has also been employed to determine the amount of unsubstituted cellulose released by endoglucanases after hydrolysis of modified cellulose [209,210]. The general problem with using HPAEC-PAD on modified cellulose is that a very low amount of unsubstituted cellulose oligomers are formed after hydrolysis and that the solubility in water, the normal solvent for enzyme hydrolysis, of the cellooligosaccharides is limited to a size not bigger than DP 6. In homogeneously modified cellulose, with a DS generally above 1, the statistical

presence of parts of unmodified cellulose corresponding to oligomers above DP 3 is below 0.5% (Papers III and IV) and as the DP increases the probability of the existence of longer unsubstituted chains decreases. Thus, determining the release of only unmodified cellodextrins after hydrolysis of modified cellulose provides very limited amount of information on the mechanism of the enzymes and the structure of modified cellulose. Techniques complementary to HPAEC-PAD need to be developed in this case.

If cellodextrins with a specific DP are hydrolyzed by pure endoglucanases, detailed information on the specificity and selectivity of the enzymes may be obtained [140,214] (Papers I and IV). By incubating the enzyme with cellodextrins with DP 2-6 (commercial water-soluble cellodextrins) individually, and by studying the product pattern obtained, information on how the enzyme attacks the cellulose can be acquired. Endoglucanases are generally unable to hydrolyze DP 2 (cellobiose) and thus if DP 2 happens to be hydrolyzed to glucose, the endo-glucanase preparation is expected not to be pure and contain β -glucosidase activity. Since endoglucanases have an active site, which functions most effectively with a certain minimum DP, pure endoglucanases may produce glucose, however, additionally oligomers of various lengths may also be produced (Figure 12). In the work presented in Paper I, we investigated the product formation patterns of two pure endoglucanases by immobilizing them on a μ IMER and passing substrate through the reactor. The use of a μ IMER made it possible to perform the hydrolysis of the cellodextrins and modified cellulose completely online connected to a chromatographic system, thus creating a completely automated system for combined hydrolysis and analysis of modified cellulose. In the study, pure cellohexaose and CMC were used as substrates and were hydrolyzed in the μ IMER online connected to either an HPAEC-PAD or a SEC-RI system, respectively. By studying the product formation pattern of the DP 6 it was found that the reaction mechanism of the two enzymes had different active sites involved in the hydrolysis. One enzyme functioned most effectively when there were at least two glucose units on either side of the cleavage point. The other enzyme hydrolyzed when there were at least one on one side and three on the other. It is believed that when the endoglucanases hydrolyze modified cellulose, the same demands on a minimum chain length have to be fulfilled for hydrolysis to effectively take place. In the study, the most efficient enzyme was used for hydrolysis of CMC. The influence of varying the flow rate through the μ IMER was investigated showing that low flow rates allowed a more extensive hydrolysis of the CMC.

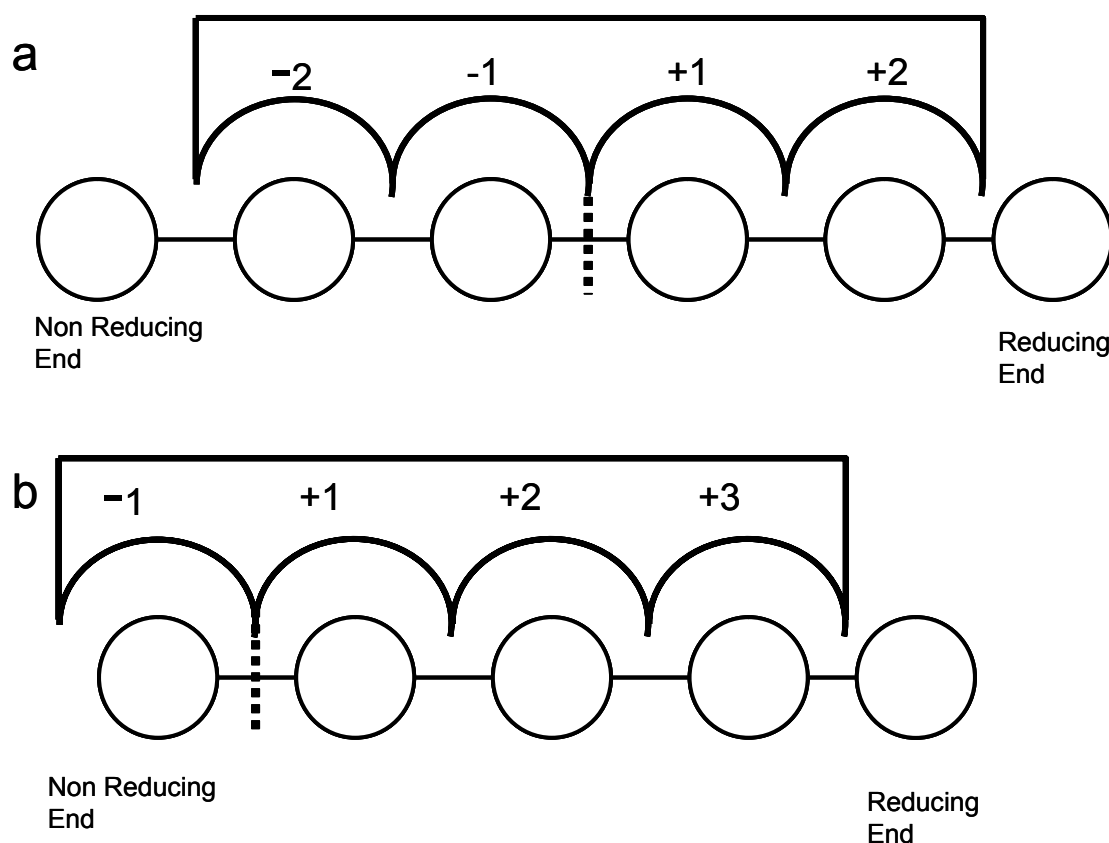


Figure 12. Schematic illustration of the binding of the sugar in the active site, illustrating the sub-sites as suggested by Davies et al. [215]. The glucosyl unit that interacts with the -1 sub-site will hold the newly formed reducing end and the one in the +1 sub-site will hold the new non-reducing end. The amount and configuration of sub-sites involved in the hydrolysis is different for the various cellulases. For example, BaCel 5A makes use of scheme (a) whereas TrCel 12A makes use of scheme (b) (Paper I)

In Paper IV we utilized a similar approach to investigate the purity of five different endoglucanase preparations. By incubating cellodextrins of DP 2 to DP 6 with the enzymes individually and studying the product formation, it was possible to determine which enzyme preparations contained pure endoglucanase and which ones were mixtures of endoglucanases and exoglucanases/ β -glucosidase.

Lately, mass spectrometry has also gained an increased interest in the field of enzyme characterization due to its high sensitivity, specificity and versatility (See section 3.4 *Mass Spectrometry*). MS is ideal since it can be applied on several enzyme substrates for determination of the hydrolysis products with high specificity without extensive sample preparation. The possibility of performing tandem MS adds even another dimension to the field of cellulase characterization.

The general problems with MS characterization are the limited working range (~100-10000 Da) and the non-quantitative detection caused by differences in polarity of the

modified celooligomers and molar mass. As found the work reported in Paper III the non-quantitative detection (discrimination) was dependent on which MS technique that was used with MALDI-TOFMS giving the least discrimination. Therefore chemical pre-treatment and derivatization are required to obtain a quantitative approach without discrimination in the mass-selective detection. Another question that arises is whether the hydrolysis products are actually representative of the enzyme activity. If the DS of the cellulose is very high, the enzyme may only hydrolyze the modified cellulose to a very low extent, producing only minor amount of oligomers with molar mass below 10 kDa. Hence, only a small fraction of the hydrolysis products would be analyzable using MS. A combination of several analytical techniques therefore generally gives the best overall picture of the cellulase enzyme activity.

When cellulose derivatives are hydrolyzed by the endoglucanases, the introduced substituents in the cellulose may prevent hydrogen-bond formation or constitute steric hindrance for the sugar-binding sub-sites of the enzyme and in both cases hinder enzyme-substrate binding and cleavage. The extent of hindrance by the substituents depends on the active site, which in turn is effected by the amount of substituents, the type of substituents (size, shape, length, charge and so on) and the substituent position on each glucosyl unit. Few studies concerning on how the substituents hinder the enzyme hydrolysis have been performed. When the influence of substituents was first studied, it was believed that hydrolysis is only possible between two adjacent unsubstituted glucosyl units [191]. It was later found by Nojiri et al. [216] that this was not true. Nojiri et al. studied an endoglucanase from *Trichoderma viride* that was used to hydrolyze regioselectively-substituted MC standards. In this study it was found that the enzyme can cleave glucosidic bonds between glucosyl units which were methylated at the C-6 position. It was also found that the rate of catalysis of the MC was extremely poor if there were substituents positioned at both C-2 and C-3.

To obtain quantitative results in MS without discrimination after enzyme hydrolysis of MC (Papers III and IV), we employed perdeuteromethylation. It has previously been shown by Adden et al. [62] and Arisz et al. [217] when investigating partially acid hydrolyzed MC that perdeuteromethylation of partially methylated cellulose minimizes discrimination in MS due to polarity differences. Some in-house experiments have shown that if perdeuteromethylation is not performed of the MC, the content of an oligomeric peak of MC can be overestimated over 100 times. This methodology gives us the possibility to determine the DS of each DP and thus obtain quantitative DS-DP plots for six MCs hydrolyzed with five enzyme preparations individually (Figure 13). Once again it was found that the enzymes

could be divided into different groups with one group producing oligomers with a higher DS, indicating that these enzymes are competent even when substrates have a larger degree of substitution.

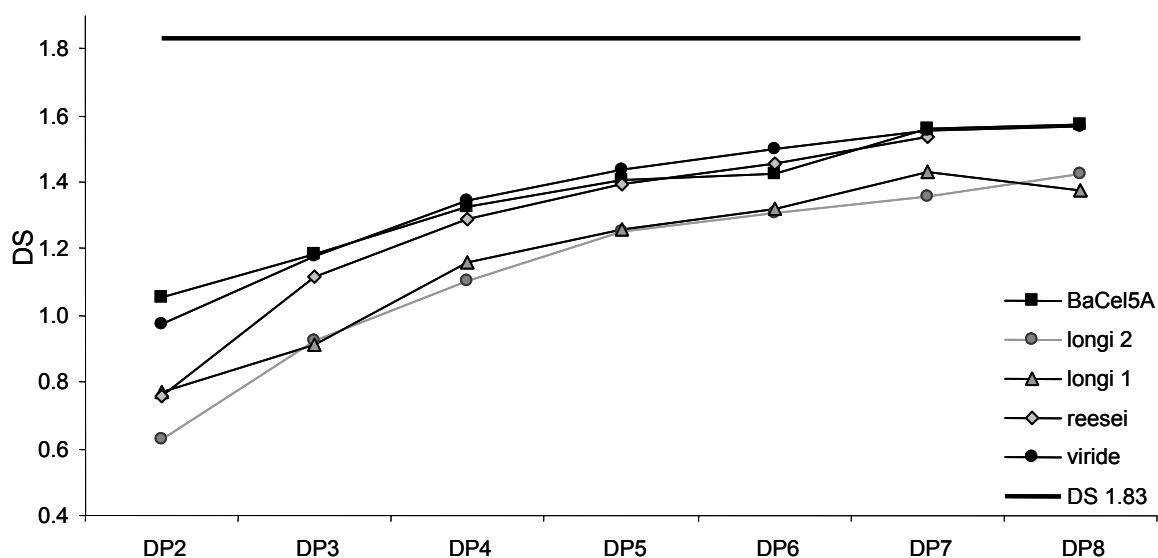


Figure 13. DS vs. DP plot of MC (DS 1.83) measured using MS of degradation products after enzyme hydrolysis combined with perdeuteromethylation.

Despite the fact that perdeuteromethylation eliminates discrimination in MS within one DP, it does not eliminate discrimination between oligomers of different DPs. To overcome this problem and to be able to quantify the amount of the hydrolysis products, it was necessary to develop a new method. The method was carried out by adding cellodextrins of a known amount to the enzyme hydrolyzed MCs and then subsequently performing perdeuteromethylation (Figure 14). By using this internal standard addition methodology, we determined the amount of formed DP 1, DP 2 and DP 3 of two MCs with DS 1.3 and 1.8, hydrolyzed with five different enzyme preparations. For the MC with DS 1.3, DP 2 and 3 constituted between 7 and 15% and for MC with 1.8, DP 2 and 3 constituted between 1 and 2% of the total hydrolysis products respectively. No formation of DP 1 could be detected. The developed method does not limit the analysis to unsubstituted glucose units, as in the case for HPAEC-PAD (with which standards can be used to determine substituted monomers). Moreover, it becomes possible to determine substituted oligomers up to DP 6, which is the largest commercially available water-soluble cellodextrin. The method might also be applicable for analysis of other types of hydrolyzed modified celluloses, such as HPMC, HEMC, and HEC, if a quantitative MS analysis is obtained.

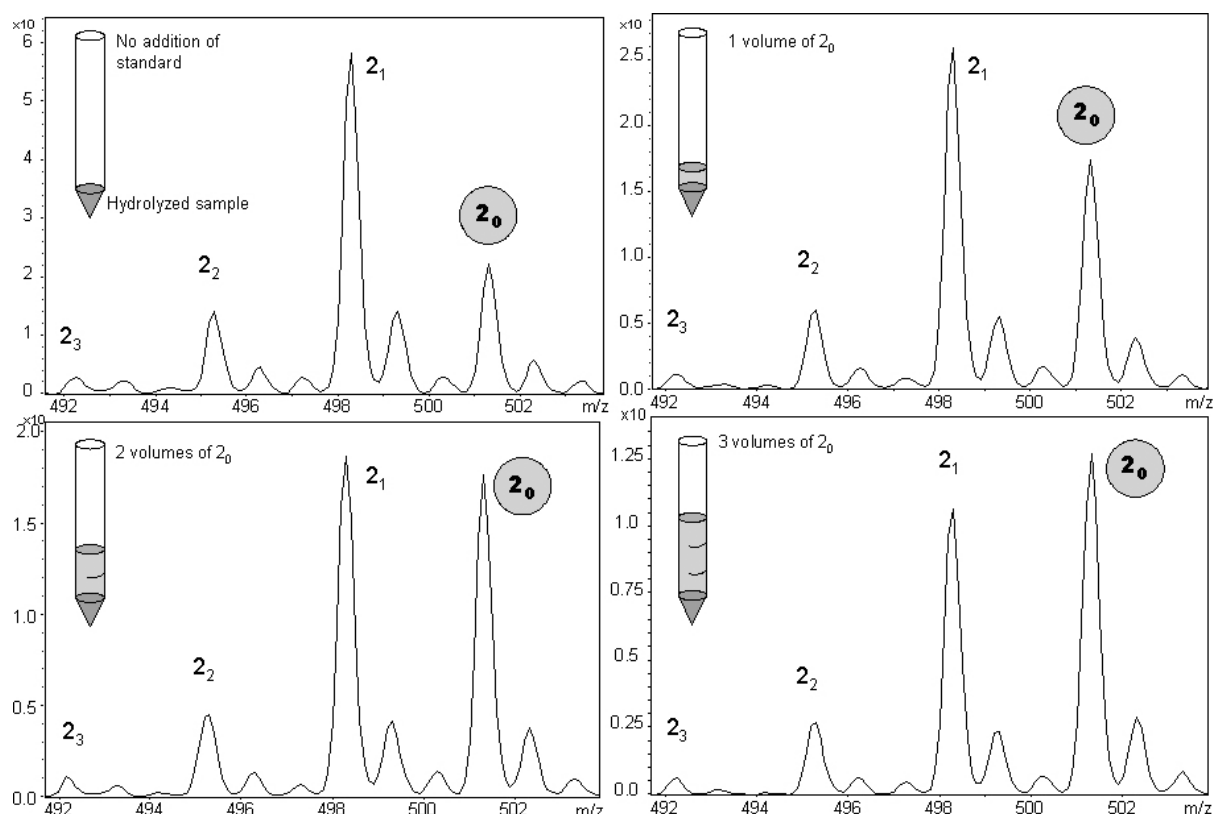


Figure 14. Determination of hydrolysis products DP 2 with methyl substituents after enzyme hydrolysis using internal standard addition. The overall signal intensity in each spectra does not affect the determination of the amount.

By using tandem MS, Momcilovic et al. [218] investigated the specificity of endoglucanase BaCel 5A on MC using regio-selective marking of the newly formed reducing ends and investigating these using tandem MS. In this study it was found that the endoglucanase BaCel 5A could cleave adjacent to a fully substituted glucose unit in +1 position in the active site. Although this study was not quantitative these findings were verified by us, as reported in Paper IV, using a quantitative approach for tandem MS investigation of the enzyme specificity (how the methyl substituents affect the hydrolysis) to methyl substituents. When investigating the specificity of five enzyme preparations we used specific marking of the hydrolyzed MC using reduction, followed by perdeuteromethylation of the reduced reducing end, non-reducing and middle pieces. Using tandem MS it was possible to distinguish between these different parts, revealing significant information about the enzyme specificities. In our study it was found that the reducing end (-1 site) of the investigated enzymes most likely has only one substituent, and that the glucosyl unit in the -2 position also influences the enzyme to a significant extent.

Cohen et al. [198] used a different approach to characterizing how different enzymes hydrolyze CMC. An LC system containing a graphitized carbon column connected with the

ESI-ITMS allowed detection of the hydrolysis products. It was shown that the enzymes produced different hydrolysis products and that the enzymes could hydrolyze adjacent to a substituted glucosidic unit. Due to lack of standards, the analysis was not quantitative. SEC-RI was used in the work to verify that hydrolysis occurred, while HPAEC-PAD and reducing end analysis were used to estimate the degree of hydrolysis.

5.0 MICRO ANALYSIS

Micromachining and miniaturization have gained more and more interest within the analytical field. Chip-based micro-analytical systems have been employed for everything from CE, GC and LC separations to total analysis systems (μ TAS), in which separation of species and detection is performed in the same micro-scale system [219]. The advantages of using micro-based analytical systems are several. Normally when utilizing a micro-based system, the economic benefits are improved due to low manufacturing costs, and reduced energy and chemical consumption. One also strives towards an increased analytical and technical performance. The analytical performance and the economical benefits are interrelated in that sense that if the miniaturized system offers a faster analysis, higher sample throughput and lower reagent consumption, there are direct economical improvements [220-223]. The most important aspects and implications, which normally are referred to when downscaling analytical systems (microsystem) are listed below;

- Ultra-sensitive analysis
- Multiplexing
- Automation
- On-line analysis
- Integration
- Portability
- Low manufacturing costs
- Disposability
- Low energy consumption
- Reduced chemical consumption
- Improved separation efficiency
- Short diffusion paths
- Increased surface-to-volume ratio
- Faster heat dissipation
- Fast analysis time
- Low manual labor
- Laminar flow

Several of the aspects above are intimately related to each other. A short analysis time may be the consequence of shorter diffusion paths i.e. increased mass transfer, improved separation efficiency or increased reaction rates may be increased due to improved thermal transport. Automation and on-line analysis also provide a low manual input from the user, which in turn minimizes the risk of external contamination and also decrease the costs.

5.1 Micro Immobilized Enzyme Reactors (μ IMERs)

The first immobilization of an enzyme was reported in 1916 by Nelson and Griffin, who immobilized invertase [224]. Although immobilized enzyme reactors have been used successfully in research-based systems, today there are some commercial systems available,

which are mostly used in the food industry. The immobilized enzyme systems permit selective changes of the substrate that allow increased usage of the raw material. In industrial applications the immobilized enzyme systems permit continuous and more automated production with better quality control e.g. production of corn syrup [224].

In a micro immobilized enzyme reactor (μ IMER) system many of the aspects of a micro scale system are utilized. Biospecific, ultra-sensitive detection and monitoring become possible when the μ IMER is used in conjunction with suitable detection techniques such as electrochemical, optical, conductometric or mass spectrometric detection. The μ IMER then serves as a vital component in the analytical process that normally ensures a high catalytic conversion of the substrate and thus generates a high concentration of products that subsequently can be monitored and measured.

In the μ IMER environment a high local concentration of the enzyme may be obtained through immobilization to the walls of the μ IMER. The immobilization of the enzyme may be performed in several different ways (see section 5.4 *Immobilization of Enzymes on Solid Supports*) that increase the efficiency of usage of the enzyme. It is assumed that the enzyme is uniformly distributed on the reactor walls, which can be everything from a porous silica support to magnetic beads covered with a porous material [225]. The enzyme coverage of the support does not need to be complete for the enzyme to work optimally. The high local concentration of the enzyme combined with the low diffusion paths for the substrate (due to miniaturization) ensures high catalytic conversion and thus decreased analysis time. Since the enzyme is attached to a solid surface (in this work silica), a change in the enzyme activity is often observed. Often the activity is decreased but at the same time the long-term stability of the enzyme may also be improved [226]. Thus, the structural stability of the enzyme can be enhanced due to immobilization [227,228]. The changes in the enzyme activity are often attributed to conformational changes in the enzyme structure or to steric hindrances in the immediate vicinity of the enzyme [229]. The increase in the enzyme stability is believed to be due to the prevention of autolysis or the lower susceptibility to denaturation or microbial attack [230]. Generally, the immobilization allows for the enzyme to be used repeatedly with only minor loss of activity. This should be put in contrast to the situation with a normal batch system, in which the enzyme is only used once and then normally considered as waste. Since enzymes generally are expensive, especially novel enzymes used for research purposes, the μ IMER provides a cheaper solution that not only ensures continuous conversion of perhaps several substrates and samples (by flow-through format) but also consumes orders-of-magnitude less of enzyme. Another interesting feature of the μ IMER format is that it is very

easy to connect online to many popular analytical techniques like MS, which is not volume dependent [225,230] (Papers III and IV). The characterization analysis of for instance proteins [231,232] or sugars can therefore be performed in completely automated miniaturized systems with low solvent, sample, and enzyme consumption [233,234].

Although the μ IMER format provides many advantages there are still many aspects that have to be considered when working with enzymes. The enzyme may be sensitive to several factors and denaturation should of course be avoided. Chemicals and the immobilization protocol should therefore be chosen in such a way that they are as harmless to the enzyme as possible. The linkage (covalent, entrapment, adsorption) between the solid surface, possible linker and the enzyme should also be as stable as possible, otherwise the outcome would be loss of enzyme and decreased long-term stability of the μ IMER. Since all flow in the μ IMER system is laminar the mass transport in the μ IMER format is accomplished by diffusion (low Reynolds numbers) of the substrate to the fixed enzyme. Therefore, the depth, width and porosity of the μ IMER wall and the shape of the interior of the reactor have to be optimized carefully. Some successful efforts have been made using ultrasonic standing waves to try to increase the mass transport of substrate to the immobilized enzyme [235]. Since diffusion decreases with increasing viscosity, diffusion becomes more and more important as the viscosity of the substrate solution increases. The diffusion affects the rate of the enzyme not only by influencing how rapidly the substrate reaches the bound enzyme, but also through influencing the diffusion of the formed products away from the enzyme. Both these processes determine the hydrolysis rate (turn-over). The immobilization protocol for the enzyme should also be considered since the active site of the enzyme should be affected as little as possible. Different immobilization protocols also offer different stability and coverage of the solid surface [236]. Finally, clogging of the porous solid material by contaminants and other side products should also be considered and avoided as much as possible.

5.2 Silicon and Silica for μ IMERS

Monocrystalline silicon wafer in principle displays the same surface chemistry (SiO_2) as controlled pore glass (CPG), and thus it is easy to modify the silicon surface with the same immobilization chemistry as described for glass beads [237]. The advantage of using a solid support as immobilization matrix is mainly the inherent chemical and rigid, mechanical stability.

The benefits of using porous silicon for micro reactors are multi-fold. Silicon possesses excellent chemical properties for immobilizing enzymes. Numerous available methods for

protein coupling to silica are all applicable [236,238-241]. The chemical and mechanical stability of silicon surfaces makes them suitable for on-line use over a long period of time, allowing for on-line connection to other instrumentation. Silicon can be made highly porous, which in turn means that a high amount of enzyme can be immobilized compared to conventional solid-phase matrices. The manufacture of microreactors is also cheap since a great number of reactors can be fabricated simultaneously on the same wafer, using well-established technology.

5.3 Manufacturing of μ IMERs

The manufacturing procedure for the μ IMERs used in this work is based on the same principles as the ones used in the integrated chip (IC) industry for production of electronic components. The process, which is used for the creation of structures in the silicon material, is called etching, and it is based on chemical or physical removal of unwanted material on the starting silicon, a sort of sophisticated sculpting process. The etching process used in this work is called wet etching. In this process the substrate is immersed in a liquid mixture of chemicals, which react with (etch) and remove material. To obtain selectivity and the desired pattern in the etching process some areas of the silicon can be coated with a layer of another chemical composition that does not react with the etching solution, thus protecting the underlying bulk material from being etched. This protective coating is commonly called a “mask” [242,243]. The etching procedure can either be isotropic, indicating that the material is removed at equal rate in all directions on the exposed area, or anisotropic, indicating that the etching rate depends on the direction in the material. The etching speed is also dependent on the silicon crystal structure, since the different crystalline planes in the silicon are etched at different rates. By exploiting this knowledge, complex structures can be created in the silicon. A second important feature of silicon is that the surface can be turned into a highly porous state down to a controlled depth by electrochemical etching [244,245]. This enhances the local surface area, the surface-to-volume ration, and ultimately the available area on which enzymes can be immobilized.

The fabrication process for the μ IMERs, using chemical wet etching, can be divided into four parts (Figure 15).

1. *Deposition*: A photoresist is deposited on the SiO₂ surface, which will serve as a mask to prevent the silicon dioxide layer from reacting with the etching solution in the third step.

2. *Photolithography*: A glass plate template (lithographic mask) with the desired pattern drawn on it is placed in front of the photoresist. By irradiating the mask with UV light the

photoresist polymer breaks down in the exposed areas. Next the exposed polymer is removed in a chemical developing step.

3.*Etching*: Some areas of the silicon now consist of exposed silicon dioxide, whereas other areas are still coated with photoresist. In a first etching sub-step, the silicon dioxide is removed in the exposed areas. In the second step the photoresist is removed. The final structuring is anisotropic deep etching of the silicon to form the final desired structure. The silicon dioxide will serve as a mask in this step. The newly etched silicon is oxidized to silicon dioxide (silica) when exposed to air.

4.*Making the silicon porous*: The silicon is placed in an electrochemical cell containing hydrofluoric acid and ethanol, which serves as etchant. When a suitable voltage is applied, an etching process starts creating pores in the silicon surface. The etching process can be terminated when the desired thickness is attained.

A more detailed manufacturing procedure has been presented by Bengtsson et al. [246].

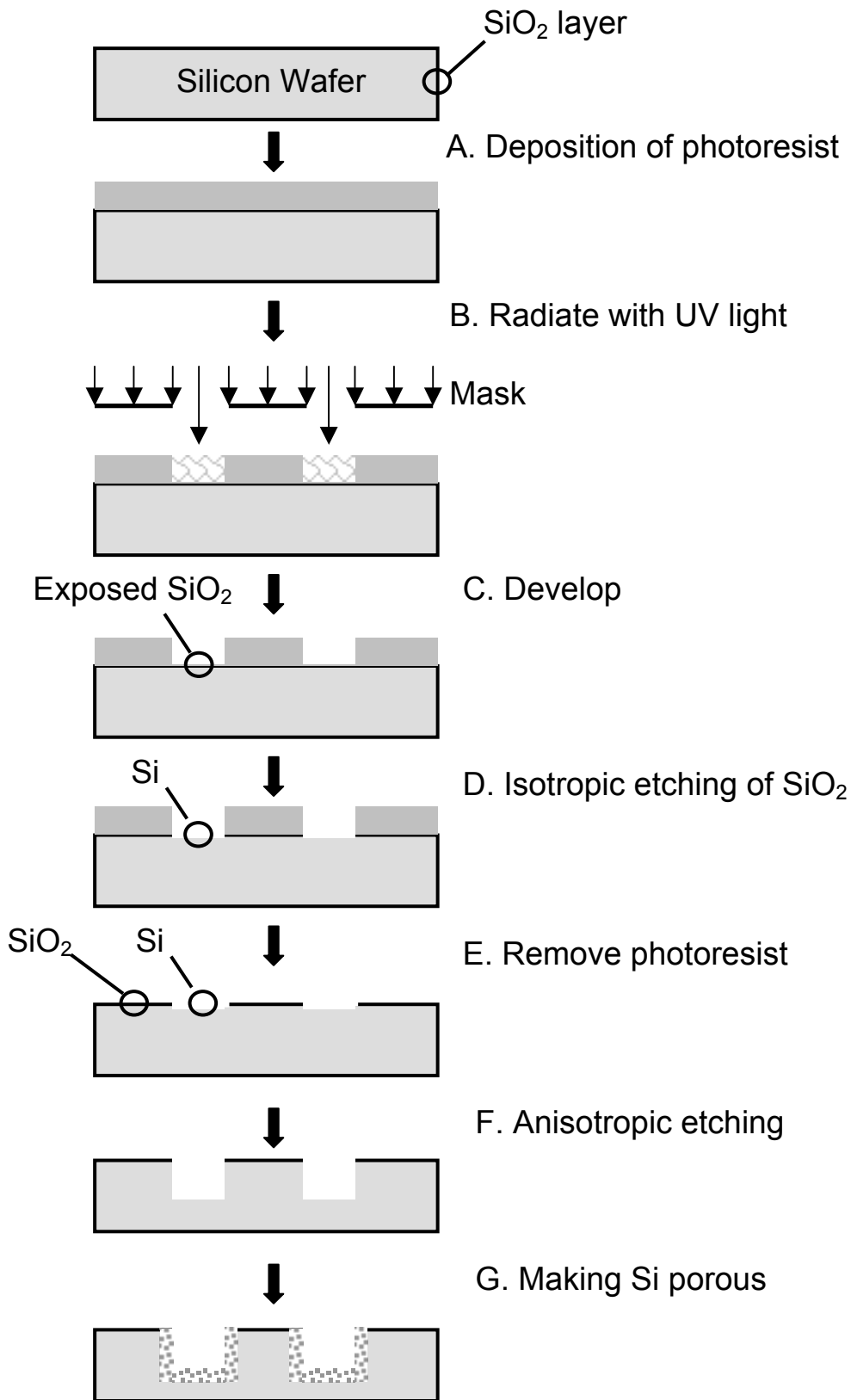


Figure 15. Basic manufacturing procedure for the μ IMER used in this work

5.4 Immobilization of Enzymes on Solid Supports

The enzyme reactors employed in this work were made of highly porous silicon and have been developed by Laurell et al. since 1994 (Figure 16) [247,248]. The latest developments have involved optimizing the manufacturing procedure with respect to its analytical performance. Silicon dopant type, thickness of porous layer, and anodization conditions for generating the final porosity have been thoroughly investigated [246].

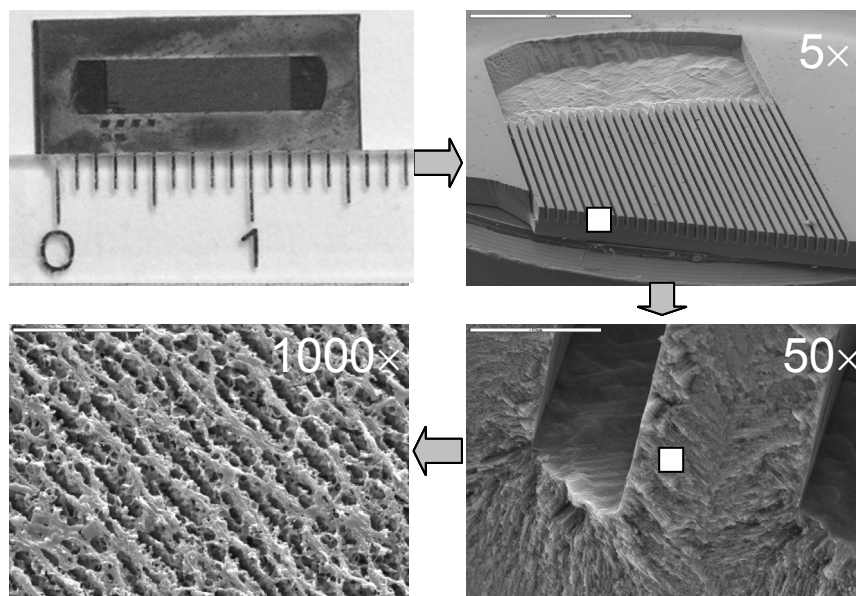


Figure 16. The micro immobilized enzyme reactors used throughout this work (size shown in centimeters). The squares symbolize the magnification window.

Immobilized enzymes have many advantages over their in-solution counterparts. As stated above, they are more resistant to denaturation and therefore maintain their activity for a longer period of time. Generally, the time for the enzyme to act on the substrate time can be adjusted simply by varying the flow-rate of substrate through the reactor. In addition due to the small diffusion paths and high local concentration of the enzyme, the reactors are very effective even with small amounts of enzyme. The enzymes do not contaminate the substrate solution and therefore do not pose a problem in inactivation or removal, and since the immobilization chemistry is focused on specific functional groups on the enzyme, other extraneous components in the enzyme solution do not become immobilized and an enzyme without disturbing components is thereby obtained.

There are three basic methods for immobilization of enzymes in a reactor: entrapment in a gel-like structure, adsorption to a solid, and covalent attachment to a solid. These methods are summarized in Table 3 and have been reviewed extensively [249]. Enzymes immobilized

by entrapment are more subject to diffusion inhibition, since by definition the matrix pore size must be sufficiently small to prevent enzyme loss by diffusion. The key distinction in the μ IMER is that the substrate and products must be able to diffuse freely in the matrix, whereas the enzyme must not. In adsorption, immobilization of the enzyme is the result of ionic, affinity, electrostatic, van der Waals and hydrophobic interactions. Generally immobilization of this kind is less stable than equivalent covalent immobilization, so there is a greater tendency for leaching of the enzyme. To have a stable adsorption, the enzyme needs to be closely connected to the matrix. This is normally achieved by using an ion exchange matrix, and since most enzymes have a net negative charge in the pH range of 7-9, anion exchangers are often used. Adsorption of special enzymes with long non-polar chains can also be performed through hydrophobic interactions [250-252]. In general, since covalent attachment is the most secure and stable method and is the only immobilization method that is considered as permanent, this was the method used throughout the work presented in this thesis.

Table 3. Different immobilizations of enzymes

Entrapment	Gels (organic and biological polymers) Vesicles Semi-permeable membranes
Adsorption	Ionic adsorption Hydrophobic adsorption
Covalent bonding (reactive functional groups)	Enzyme amino groups Enzyme carboxyl groups Enzyme tyrosyl groups Enzyme sulfhydryl groups

5.5 Covalent Immobilization of Enzymes

Covalent attachment of the enzyme to the solid matrix has to be performed through a nonessential functional group located on the surface of the protein molecule. The matrix is most often solid and is always insoluble in the system in which it is used. It should also be chemically inert, resistant towards pH, temperature, pressure and mechanical stress and if possible, easy to handle. The surface should have good capacity for the target molecule, have a large surface area, and resistant towards microbial and enzyme attack [238]. Finally cheapness of materials and process is desirable. Good matrix supports exist in mainly two different forms. Natural supports include agarose, cellulose, silica, and alumina. These normally require considerable human engineering and processing before they are suitable for use as affinity matrices. Synthetic matrix supports such as acrylamide, methacrylate and polystyrene derivatives are produced by polymerization of functional monomers to yield

matrices suitable for affinity-based separations. Generally, these types of materials have superior physical and chemical durability, can withstand the rigors of process-scale purification and manufacturing, and are also cheaper, all factors which have led to their increased industrial use.

When the enzyme is immobilized, the active site of the enzyme should not be affected and should not be hindered in any way by the matrix or the immobilization chemicals. A multitude of covalent immobilization protocols have been suggested [225,239,253-256]. Targeting of a special functional group on the enzymes can be used, i.e. the α -amino group of the peptide chain and the ϵ -amino groups of lysine and arginine, the carboxyl groups of the polypeptide chain end and the β - and γ -carboxyl groups of aspartic and glutamic acids, the phenol ring of tyrosine, the thiol group of cysteine, the hydroxyl groups of serine and threonine, and so on [225]. Unfortunately, reactive groups suitable for immobilization are often inconveniently situated in the active site of the enzyme, thus inactivating some or all of the enzyme during the immobilization. This problem can be sometimes eliminated by immobilization in the presence of the substrate or a competitive inhibitor of the enzyme [257,258]. This also helps to stabilize the tertiary structure of the enzyme during the immobilization.

Primary hydroxyl groups, primary amines or carboxylic acids are generally the functional groups of the matrix that is targeted. On most matrices these are no naturally available functional groups, and therefore the surface has to be chemically modified. Since the solid matrix may affect the activity of the enzyme due to sterical hindrance, spacers (“arms” that increase the distance between the enzyme and the matrix) are often used to minimize its influence [259]. The spacers have also greater flexibility, allowing them to more easily move to the correct position on the enzyme [238]. The spacers also permit the enzyme, although it is immobilized, to have an environment very similar to that of the solution. Spacers usually consist of linear hydrocarbon chains with functionalities on both ends for easy coupling, generally in two subsequent steps, to the matrix support and ligands.

When the enzyme has become immobilized (or during this process), unreacted active groups of the matrix must be blocked by reaction with inert moieties providing the same functional groups (amine, carboxylic and so on) that was used for the enzyme immobilization. This blocking reaction is necessary to prevent further non-specific reactions between the support and polypeptide that could decrease the stability and specificity.

5.6 Immobilization of Enzymes on Silicon

As mentioned above, silicon possesses excellent features for protein immobilization. Since microstructures of silicon are not stable in air, the surfaces will oxidize and form silicon oxide. Once this oxidized surface comes into contact with moisture and/or water it will become hydroxylated.

Silica can exhibit strong non-specific protein adsorption via ionic interaction, van der Waals forces, and polar-polar interactions and this is a well known fact [260]. Although enzyme adsorption may be stable, a low surface coverage of the reactor with the enzyme is generally a problem [261]. Since covalent immobilization to silica is considered more stable, the focus of this work has been to use this kind of immobilization.

The immobilization of proteins on silica has been performed in a one- or two-step procedure. In the one-step procedure a bi-reactional linker is used to coat the silica surface. As shown in Figure 17 below, the surface is reacted with glucidoxypypropyltrimethoxysilane (GOPS), which is a molecule that contains groups that react both with the silica surface and the enzyme. This one-step procedure using GOPS has shown to be very efficient, giving high yields of immobilized active enzyme [240,262].

The normal procedure used in his work for covalent immobilization to silica is the two-step procedure where in the first step involves covalent coating of the surface. Many different methods have been suggested. Weetal et al. [237] suggested a coating by incubating the silica with aminopropyltrimethoxysilane (APTES) and then further reaction and immobilization of the proteins through Schiff base formation between glutardialdehyde and the aminogroups of the enzymes (see Figure 17). This method has successfully been used for different kinds of enzymes, antibodies and cells [232,236,263,264]. The use of a silane for functionalizing the surface has shown to be advantageous due to its simplicity and stability. It has also the possibility to react with a variety of surfaces that are rich in hydroxyl groups including glass, silica, germanium, alumina, and quartz.

The coating of the silica surface has also been performed in several other ways. Gölander et al. [265] showed that polyethyleneimine (PEI) forms a thin layer of bound amine groups at silica oxide. Adsorption of the PEI to the silica is driven by electrostatic interactions between positive charges on PEI and negative charges on the silica surface and has been shown to be practically irreversible [266]. Both PEI and APTES can be used in combination with glutaraldehyde giving a long-lasting stable immobilization [236] (Papers I and II).

Glutaraldehyde (GA) is by far the most common cross-linking agent providing high yields of immobilized proteins (enzyme) on the support. GA has the advantage that Schiff base formation with the enzyme is executed under mild conditions (pH, T , and stirring), thus causing very little stress for the enzyme. As described above, the procedure for immobilization with GA includes activation of the support followed by treatment with GA and finally incubation with the protein. The procedure may be followed by a reduction of the Schiff base to a stable secondary amine. The reduction step may prove to be disadvantageous since it may harm the immobilized enzyme. Although the true mechanism of immobilization with GA has been debated in the literature, it generally provides a stable active immobilization of the enzyme [267,268]. The optimum conditions for immobilization with GA have shown to be around pH 6.8 at 4°C. Activation at higher pH has led to unfavorable side reactions, presumably due to increased GA polymerization. A Lower pH than pH 6.8 during the immobilization using GA has sometimes been shown to be favorable [268].

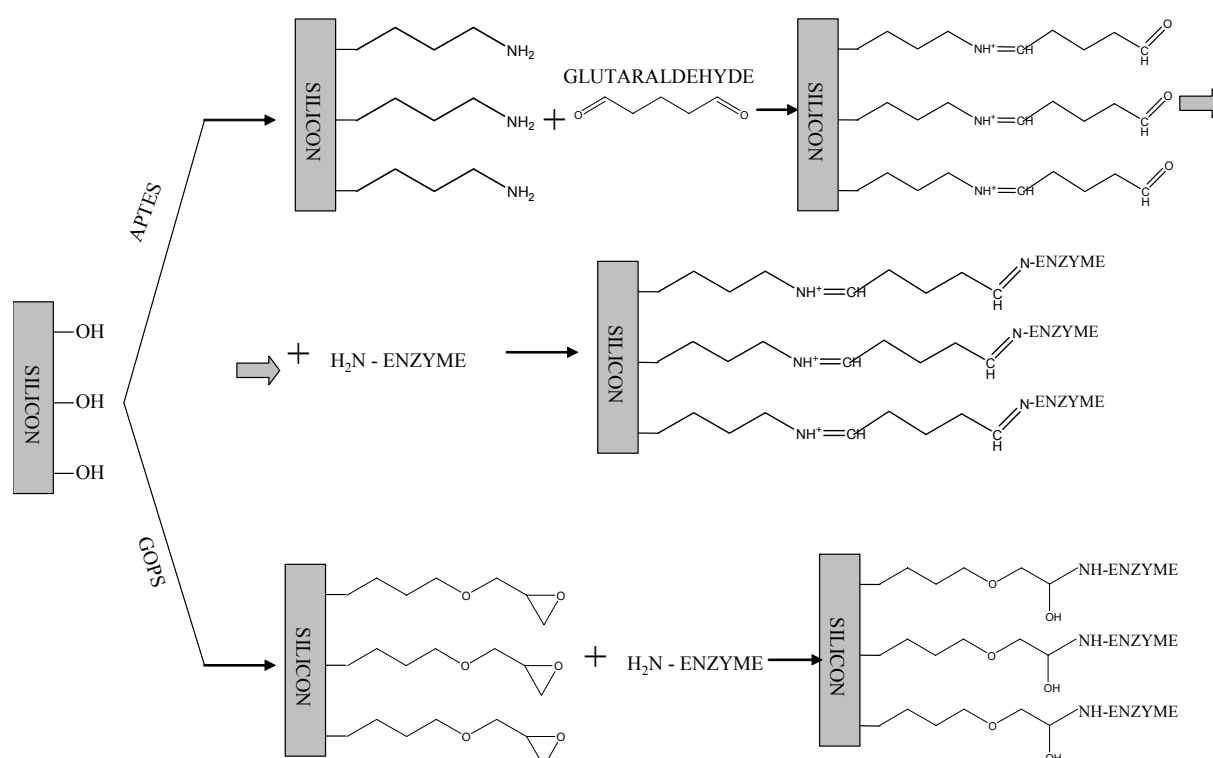


Figure 17. Two-way approach for immobilization of enzyme using APTES to silica surface (a). One-way approach using GOPS (b). The APTES method is followed by a reduction of the double bonds.

In the work presented in Papers I and II we investigated how μ IMERs could be used for hydrolysis of pure cellodextrins and modified cellulose. In a previous work [236,254], immobilization based on PEI as spacer and covalent immobilization using glutardialdehyde has been shown to be very efficient. Therefore we adopted this method in all our work. In the work presented in Paper I, two different endoglucanases were immobilized and the hydrolysis product pattern from passing cellodextrins through the reactor was investigated. It was seen that the reactors were stable for over ten days. Moreover, the reactors could be used for hydrolysis of cellodextrins with the output connected on-line to an HPAEC-PAD system. The influence of factors such as pH, concentration, temperature, flow-rate and so on was investigated and it was also investigated how the degree of hydrolysis of CMC can be controlled by varying the flow-rate of substrate through the reactor. Additionally, it was seen that each immobilized enzyme had a specific hydrolytic pattern of the substrate (*See section 4.5 Characterization of Cellulases*). An additional feature that was discovered was that sugars in the enzyme preparation solution were successfully removed from the enzyme after the immobilization. The presence of these sugars would give misleading values during the interpretation of the enzyme hydrolysis pattern.

In the work presented in Paper II we used one particular immobilized enzyme type, which was then used for hydrolysis of MC. Three different commercial MC preparations were investigated by connecting the μ IMER online to ESI-ITMS, offline to SEC-MALS/RI and semi-online to SEC MALDI-TOFMS. The μ IMER once again allowed for online hydrolysis of modified cellulose and thus decreased the hydrolysis time tremendously. The manual labor required in the conventional batch hydrolysis was also drastically reduced. It was also shown that the μ IMER format could be used together with immobilization of cellulose-hydrolyzing enzymes, for verification of differences between MCs through analysis of the hydrolytic products.

6.0 ANALYSIS AND CHARACTERIZATION OF SUBSTITUENT DISTRIBUTIONS

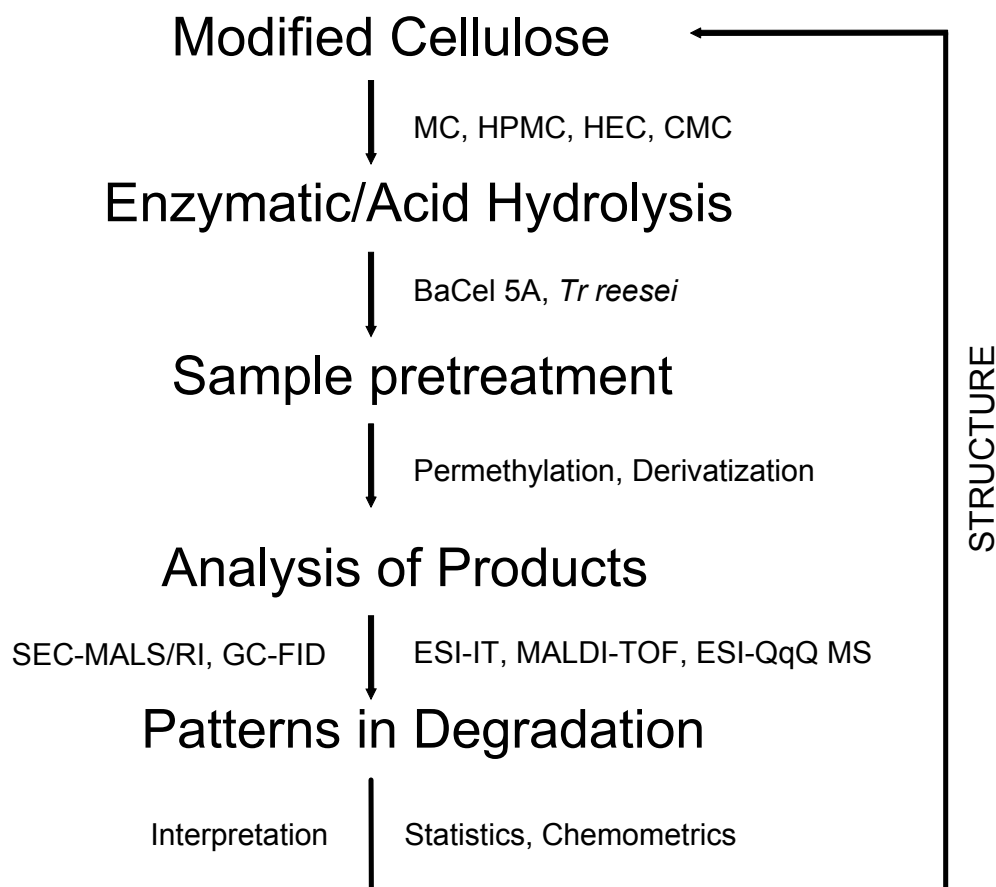


Figure 18. The general plan used throughout the work presented in this thesis.

The physical and chemical properties of the modified cellulose depend on several factors, such as the modification reaction, type of substituent, average molar mass, molar mass distribution, degree of substitution, and the distribution of the substituents on monomer level and along the polymer chain. Since the properties of the modified cellulose are correlated with their technological usefulness, techniques to accurately characterize them are of great importance. Today, many of these characteristics such as average molar mass and degree of substitution can be determined by a variety of techniques. However, determination of the distribution of the substituents along the cellulose chain is a more troublesome task. One might ask how the distribution of the substituents along the chain can affect the properties. Actually it has been found that the substituent distribution affects the properties to a great extent. Takahashi et al. [269] investigated the relationship between the modification reaction of cellulose modified with methyl substituents, the distribution along the chain and the final properties of the MC. It was found that the manufacturing procedure greatly

influenced the distribution of substituents which in turn affected the solubility and gelation properties. The degree of substitution seemed to have a lower effect on these properties than the manufacturing procedure. Hence, it is of great importance to estimate the distribution of the substituents along the cellulose chain if correlations to the manufacturing procedure of the MC and the final properties can be drawn. It has also been shown in other work that the substituent distribution also affect solubility, flocculation, gel formation and biodegradability [16,269-272]

As explained above there are many aspects of the modified cellulose that determine its properties and use. The type of functional groups (substituents) is most of the time known and therefore does not have to be determined, although the area of *de novo* analysis of unknown cellulose ethers has always been of some industrial interest. This is true both in the area of mixed cellulose ethers and for cellulose ethers mixed with starch ethers. In this area the use of enzymes can be very valuable. By using an enzyme that can only hydrolyze the starch or the cellulose part only one of the polymers is hydrolyzed and thereby becomes distinguishable from the other. The quantitative and qualitative analysis of the substituents can be performed by for instance modern methods in MS, GC or NMR, and the molar mass distribution can be determined using SEC.

The estimation of the distribution of the substituents on a polymer level is a complex task. The great importance of having knowledge within this area is motivated by the fact that there are correlations between manufacturing procedures, substituent distribution and final properties of the modified cellulose. How these aspects are correlated is not entirely known. To be able to determine this distribution several different analytical methods have been applied. The general idea of the methodology used throughout the work reported here is illustrated in Figure 18. Several different modified celluloses, all with an industrial relevance, have been analyzed. Hydrolysis of the modified cellulose using enzymes has been one of the main topics addressed, but comparison with statistical hydrolysis using acid has also been applied. After the enzyme hydrolysis, carried out either in μ IMER or in batch, analyzes have either been performed directly or after sample pre-treatment including derivatization. The hydrolysis products have thereafter been analyzed with a variety of analytical techniques and the data have been treated in numerous ways. By combining all the gathered data, an attempt was made to get an overall picture of the original modified cellulose. In the process knowledge about the enzyme specificity and action was also established.

6.1 Hydrolysis of Cellulose Ethers

As mentioned earlier, it is very difficult to obtain information on the derivatized cellulose without hydrolyzing the cellulose into smaller oligomers that can then be analyzed with some analytical technique. In this work mainly two kinds of hydrolysis procedures have been applied. Partial acid hydrolysis of the cellulose may, under correct circumstances, provide a random hydrolysis. After analysis of the formed oligomer fractions, this experiment gives a picture of the substituent distribution within each oligomer which in turn can be compared with the theoretical distribution. Acid hydrolysis may also be performed to completion yielding only monomers which can provide information on the reactivity of the hydroxyl functions in the polymer. In contrast to acid hydrolysis, the hydrolysis may also be performed with enzymes that selectively hydrolyze the modified cellulose producing monomers, oligomers and also polymers in a mixture. Enzymes hydrolyze the modified cellulose at specific sites, depending on the pattern of substituents, in the cellulose chain, and if the properties of these sites are known information about the substituent distribution can be obtained.

6.2. Random/Acid Hydrolysis

Total hydrolysis of modified cellulose down to only monomers may only be achieved by acid hydrolysis. The hydrolysis mechanism, a first-order reaction, is achieved through the protonation of the acetal oxygen of the glucosidic linkage, followed by formation of an intermediate carbonium ion, which causes chain splitting, and finally reaction of the carbonium ion with water. The condition to achieve total depolymerization varies from one material to another. At optimal conditions a maximum depolymerization is obtained with a minimum of side reaction that otherwise may destroy the carbohydrates. Hydrolysis may be performed with acids such as trifluoroacetic acid (TFA) [62,63,65,217] sulfuric acid (H_2SO_4), [273,274], perchloric acid (HClO_4) [275] or through methanolysis using methanolic hydrochloric acid. The methanolysis reaction yields the corresponding methyl glucosides and causes less destruction of the monomeric units [273,276]. Other methods for degradation are also available such as the “Smith degradation” which uses an oxidation reaction with periodate and sodium borohydride [277,278]. Mosier et al. investigated how a dicarboxylic acid such as malic acid hydrolyzes cellulose and found that it causes less side reactions compared to dilute sulphuric acid [279]. TFA was the acid that was mainly used in the work

reported here, since it is easier to handle, causes less non-specific degradation and is fairly easy to remove through co-distillation or evaporation after hydrolysis.

After hydrolysis to monomers the hydrolysis products may be analyzed directly with HPAEC-PAD (see section 3.2 *High Performance Anion Exchange Chromatography*) or after permethylation, reduction and acetylation, with GC (See Section 3.3 *Gas Chromatography*). When employing any of these two methods it becomes possible to obtain the complete monomer distribution of the substituents. In Figure 19 an example is given of results from nine different HPMC samples, which have been analyzed using GC-FID. When knowing the distributions of the substituents on the monomer level, the reactive constants k_2 , k_3 and k_6 can be determined. In the example below (HPMC) the reactivity of C-2 and C-6 is found to be higher than that for C-3. By comparing the statistical and experimental substituent distribution an initial indication of whether the substituent distribution is homogeneous or heterogeneous can be given.

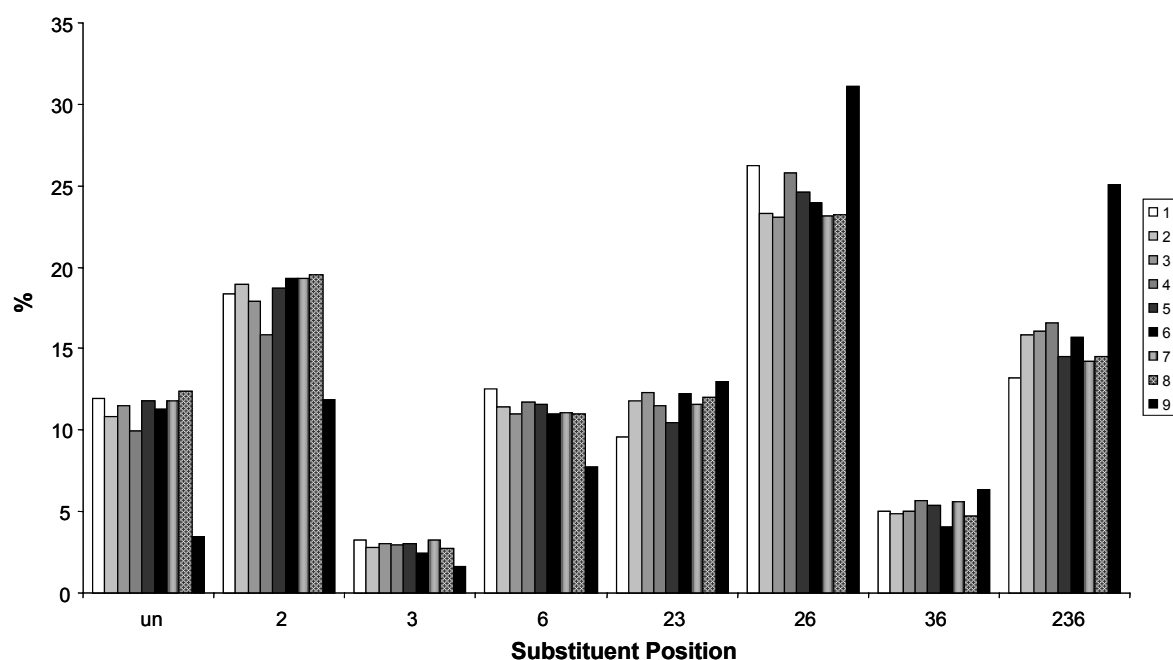


Figure 19. Substituent distribution of methyl groups in nine different HPMC samples. The percentage values give total amount in sample.

6.2.1 Statistical Evaluation after Acid Hydrolysis

If the cellulose is acidically degraded completely, analysis of the monomers should give detailed information on the derivatized cellulose. The type of substituent(s) can be determined, DS and MDS can be determined, and by statistical calculations, the reactivity of the hydroxyl groups can be determined and compared with statistical models.

The original statistical and kinetic model for substitution was proposed by Spurlin in 1939 [14]. This model assumes that the substituent distribution and thus the monomer composition is governed by the relative rate constants for reaction of the three hydroxyl groups (on C-2, C-3 and C-6), k_2 , k_3 and k_6 . By determining the monomer composition of the derivatized cellulose, these rate constants can be determined and thus the composition can be fitted to the model. If the distribution fits the model, a perfect random distribution (homogeneous) of the substituents is indicated. However, the Spurlin model does not take into account the possibility that the reactivity constants for some substituent types are not constant during the modification reaction but rather change in a specific manner. The model also assumes that all D-glucose units are equally accessible during the modification reaction and that end-groups have negligible reactivity. However, the modification procedure does not always follow a random distribution reaction. In the mid 1980's Reuben et al. [280] proposed a refined model, which includes an interrelation of 2-OH and 3-OH (differentiates between the reactivity of the hydroxyl groups). It takes into account that for methyl and some hydroxyalkyl celluloses, the reactivity of 3-OH is increased (up to three fold) when the 2-OH position of the same glucosyl moiety is substituted. In contrast, when comparing Reubens model with CMC it has been found that there was no correlation between the reactivity of the C-3 and C-2 groups and that CMC followed the Spurlin model within experimental errors [281]. However, in other works CMC did not follow the Spurlin model [52]. Hence, the Spurlin and Reuben models have to be used with consideration to the manufacturing procedure of the modified cellulose. Different manufacturing procedures will generate different fits to the models.

Mischnick and Hennig developed the Reuben approach even further and developed a mathematical model, in which the quantitative influence of substitution of a neighboring glucosyl residue is estimated [282]. This model estimates the amount of substituents next to a tri or unsubstituted unit depending on the original DS. The model uses two different approaches, one for a DS below 1.5 and one for DS 1.5 and above. The model has been applied successfully on different amylose and cellulose derivatives and has also been compared with the Spurlin and Reuben models [61,63,65](paper IV).

Adden et al. [62] developed a method involving perdeuteromethylation and statistical partial hydrolysis of the MC and investigating the possibilities for determining the complete monomer composition by performing MS and tandem MS experiments on the formed oligomers. Previously they had investigated how methyl-substituted starch and cellulose were fragmented [283]. By using sodium adducts and lithium adducts for MS^2 and MS^3

experiments, and combining their previous knowledge of fragmentation patterns, it was possible to determine not only the statistical distribution of the substituents but also the complete monomer composition of the methyl substituents. Comparison with GC data confirmed the results.

If only partial random hydrolysis is performed, producing a mixture of monomers and oligomers, information about the connectivities to neighbouring glucosyl units are retained and additional information is available. In 1995, Arisz et al. [217] introduced the heterogeneity parameter H_n where n denotes the number of monomers in an oligomer fraction. Arisz et al. analyzed the substituent distribution of trimers using MS formed after perdeuteromethylation of partially hydrolyzed MC. Arisz et al. compared these patterns with the calculated random distribution of the monomers (as determined by monomer analysis) in trimers and thus could see if the substituents were distributed in a random manner. The difference between the theoretical and the experimental models provided the heterogeneity parameter (H_n) of the MC. It was seen that heterogeneity of a sample was not only reflected in the data at the oligomer level, but also in the data at the monomer level [217]. Thus, not only do the substituents already positioned on the glucosyl unit affect the reactivity of substitution during modification (k) but also the composition of the neighboring glucosyl unit has a strong bearing.

For MS analysis of the statistical distribution with the aid of the heterogeneity parameter on mixed cellulose alkyl ethers such as HPMC, HEMC and hydroxyalkyl ethers such as HEC, a somewhat different approach has to be used. Adden et al. [19,63,65] showed that it was necessary to perform perdeuteromethylation and partial hydrolysis, followed by reductive amination and methylation to obtain quantitative results in MS. Using the method, the reducing ends became quarternized with permanently charged amine groups, allowing for elimination of discrimination in MS due to free negatively-charged hydroxyl functionalities. The results of this method were compared with the monomer composition obtained from GC data and it was shown that it was possible to determine the distribution of substituents for the various samples.

The statistical calculations can be used to estimate the distribution of substituents along the cellulose chain through monomer analysis and comparison with the oligomer substituent distributions. It has been suggested that the distributions can be divided into three major classes [64]. These are summarized in Figure 20. A random distribution of the substituents along the cellulose chain will be the outcome if all glucose monomers in the chain are equally accessible for substitution during manufacture. If the glucose units are not accessible during manufacturing in the same way, e.g. due to crystalline inert regions that have not been

activated, then a more heterogeneous pattern compared to random pattern will be obtained. The inert regions will then be less substituted, while the easy substituted regions will be characterized by a random distribution. The reactivity of monomers might also change during the reaction, e.g. because the local reactivity is enhanced when first changes in polarity have modified the “local accessibility”, or it may be decreased due to sterical effects or electrostatic repulsion. In the latter case a more regular or narrower distribution of substituents in the polymer chain would be expected, compared to the random pattern, which is always the reference model. The substitution may also be block-like. Block-like regions can either be fully substituted or be regioselectively substituted over a wide set of adjacent glucose units. By comparing the statistical distribution with the experimentally obtained ones, it is possible to determine if there are deviations from the random distribution that would indicate the presence of a heterogeneous distribution.

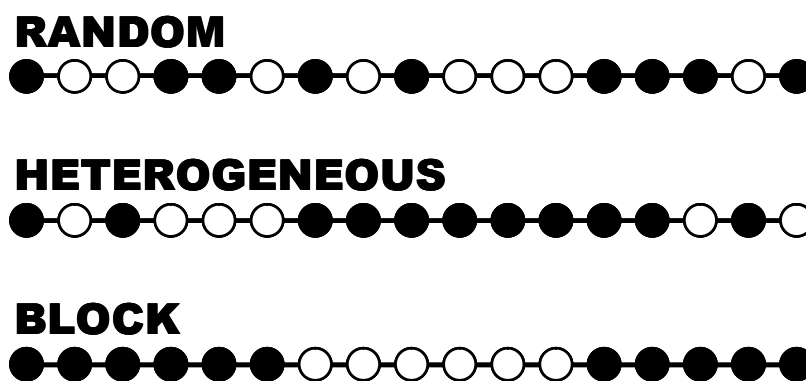


Figure 20. The distribution pattern of modified cellulose as described by Mischnick et al. [64]

6.3 Selective/Enzyme Hydrolysis

Enzyme hydrolysis of the modified cellulose is a lot different from random acid hydrolysis. The main difference is that since the hydrolysis cannot be carried out to completion, producing only monomers, and thus there are no statistical models available to fit to the obtained results. Instead the interpretation of the hydrolysis pattern relies on the specificity of the enzymes that only cleave the modified cellulose at sites with specific characteristics. These sites are specific for not only the different enzyme types such as endo- and exo-glucanases, but also for each individual enzyme. Some endoglucanases may require two adjacent unsubstituted glucose units to be able to gain access to the chain, while others may still be able to gain access even when one of the two glucose units is fully substituted. There may also be other factors such as type of substituent, substituent amount, molar mass, buffer, enzyme batch, and so on, that influence the enzyme activity on the modified cellulose. The analytical concept is thus to study the products formed from the partially hydrolyzed

modified cellulose and thereby gains information about the distribution of substituents along the chain. To the best of our knowledge, there is no standardized method for obtaining reliable information on the substituent distribution using enzyme hydrolysis. This has encouraged us and several other research groups to investigate how enzymes attack modified cellulose, and to ascertain how this information can be used to estimate the substituent distribution along the cellulose chain.

In this work comparison between statistical acid (random) and enzyme hydrolysis has been performed, using the former as a reference method when evaluating the results. The enzyme hydrolysis has been performed either in batch format or in micro immobilized enzyme reactors.

6.3.1 Characterization of the Substituent Pattern Using Enzymes

Several ways of analyzing the substituent distribution pattern of enzyme-hydrolyzed modified cellulose, have been proposed involving techniques such as HPAEC-PAD, SEC and MS. Once this information is obtained it can be used to form an accurate picture of the substituent distribution pattern of the intact cellulose.

A number of articles have appeared reporting use of SEC to determine the molar mass distribution prior to and after enzyme hydrolysis of modified cellulose (Papers II, III and IV) [52,53,210,284]. Several enzyme preparations have been used and many different arguments have been expressed about the interpretation of the results and the conclusions that can be drawn about substituent distributions. The prevailing view is that SEC as a single analytical technique cannot provide enough information to draw definite conclusions about the substituent distribution, but it may provide hints that can be useful during an interpretation involving wider data-sets. For a precise determination of the complex polymer composition, including chemical composition and molar mass distribution of the components, a separation step is necessary. A good analysis with regard to chemical composition, especially over the entire polymer, can only be achieved through fractionation of the SEC or LC eluates. This may be performed on the intact polymer to investigate whether the distribution of substituents is independent of the molar mass. More extensively employed is the technique of fractionation of enzyme hydrolyzed modified cellulose. This approach takes advantage of the steric hindrance of cellulose hydrolyzing enzymes by the substituents. When using enzymes for hydrolysis, the idea is that the enzyme can only gain access to the cellulose chain if the DS is below a certain specific value. The lower the DS, the higher the extent of hydrolysis. In 1992 Iijima et al. [285] were the first ones to perform enzyme-aided characterization of

cellulose acetate (CA) using SEC. Since then several procedures using SEC combined with other techniques has been suggested for numerous cellulose derivatives.

Experiments performed by Saake et al. [56] also indicate that the type of substituent plays a certain role even when the enzyme gains easy access to the chain. By comparing CA, cellulose sulfate and MC, it was found that methyl substituents influence the enzyme least. Our work also shows that the extent of hydrolysis is dependent not only on the modified cellulose, but also on the enzyme (Paper IV).

Saake et al. [56] performed enzyme hydrolysis of MC followed by fractionation using SEC and chemical characterization. Standard substances were isolated with preparative SEC, whereby their structures were confirmed by ^{13}C -NMR and their response factors in an HPAEC-PAD system were determined. Four different MC samples with DS ranging from 0.51 to 1.96 were then enzyme hydrolyzed and fractionated whereafter each fraction was hydrolyzed with acid to monomers and analyzed with HPAEC-PAD. For the samples with low DS, problems occurred in the analysis due to poor solubility of the hydrolysates. Nonetheless, it was possible to obtain excellent information by these means. It was shown that with their method, it was possible to determine the DS over a wide range of molar mass. Slight differences between the MCs of different qualities could also be detected in the substitution pattern.

To investigate how CMC was hydrolyzed by endoglucanases Horner et al. hydrolyzed two different qualities of CMC with DS of 0.6 and 1.2 and followed this treatment by SEC fractionation and analysis, total hydrolysis of the fractions, and finally HPAEC-PAD analysis [275]. Due to lack of standards, it was not possible to perform any absolute determination of the monomer composition in each fraction, but it was still demonstrated that 6-*O*-carboxymethylglucose and 2-*O*-carboxymethylglucose were the major constituents in all fractions. By contrast, 3-*O*-carboxymethylglucose was only found in small quantities. It was also shown that, similar to MC analysis, the DS decreases with molar mass for the fractions. Thus, as the degree of substitution increases, the efficiency of the enzyme in hydrolyzing the CMC becomes more limited. The distribution of substituents in CMC was also investigated by Saake et al. [52] using a similar approach. By investigating CMC according to solubility and fractionation after SEC it was revealed that a non-statistical (non-random) distribution of the substituents existed in some of the investigated samples.

Enzyme hydrolysates of cellulose derivatives (CMC, CA, cellulose sulfate etc.) have been fractionated using SEC, where after the DS have been determined with different techniques such as ^{13}C -NMR and GC-FID/MS [56,286]. We have also investigated HPMC

with a similar approach, in which the fractions were analyzed with GC-FID (unpublished results). The general problem with HPAEC-PAD analysis of cellulose derivatives seems to be the lack of standards. Such standards would need to be synthesized or fractionated in order to determine the detector response factor in PAD detection. GC-FID/MS detection could therefore be more beneficial since standards are not necessary. For both these methods it is also necessary to perform total hydrolysis of the fractions, which in turn limits the analysis to an average DS over a wide range of DP rather than analyzing each DP individually. However, the method makes it possible to analyze the DS of molecules with a very high molar mass. This is not possible with e.g. mass spectrometry, where the limit is up to approximately 10 000 Da.

Richardson et al. [287] also investigated the possibility of connecting HPAEC-PAD to ESI-ITMS for analysis of the distribution pattern of EHEC that was hydrolyzed with endoglucanases. The strong alkaline eluate was desalted online prior to injection into the MS. The connection of the MS to the HPAEC system gave the possibility to investigate whether there were co-eluting compounds in each peak of the chromatogram and identification of unknown peaks also became possible. Although no quantitative data were obtained, results obtained from the proposed method suggested a promising future for interconnecting two analytical techniques for characterization of modified cellulose.

During the progress of this work it has been investigated whether the substituent pattern could be estimated, or perhaps even determined, with an enzyme approach, using approaches other than the classic fractionation, hydrolysis and chemical characterization.

In the work presented in Paper II we used a μ IMER with immobilized endoglucanase BaCel 5A to hydrolyze MC of three different qualities. The use of the μ IMER allowed fast hydrolysis of the MC with up to ten days of continuous use. The μ IMER was connected on-line to MS detection and also produced hydrolyzed samples off-line for further analysis using SEC-MALS/RI and SEC-MALDI-TOFMS. The immobilization of the enzyme did not affect the hydrolysis specificity of the enzyme. It was found possible to establish differences in the methyl distributions amongst the formed oligomers. Further investigation showed that one of the MCs had such low molar mass that it significantly influenced the DS of the detected oligomers (end effects). An overestimation of the DS of the formed products was seen in MS and the SEC profiles provided underestimated values on the degree of hydrolysis. It was also found that SEC profiles could be used to verify that the substituent distributions for two of investigated MCs were very similar. This was achieved by exploiting the fact that if the substituents had been distributed in the same manner, the enzyme would have been able to

access the MCs with the same probability, and the final average molar mass would therefore be the same. This was indeed what was found. The use of the μ IMER substantially decreased the necessary time for hydrolysis and also allowed an automated hydrolysis which could be connected online to MS.

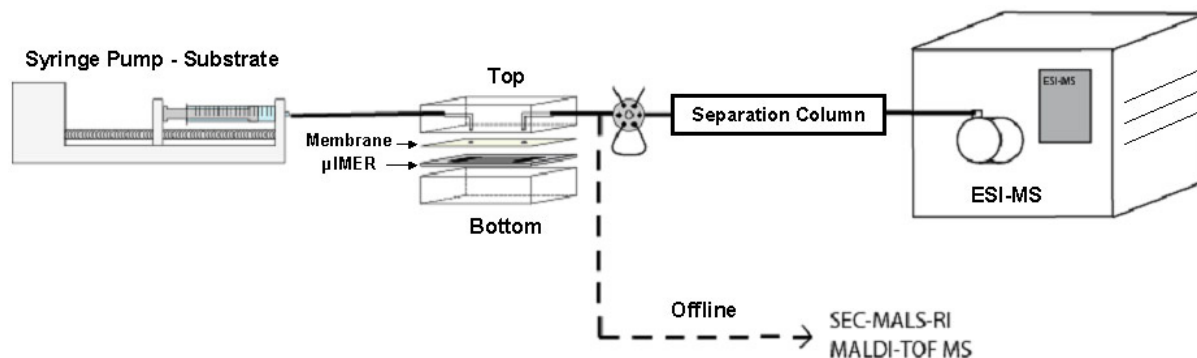


Figure 21. The setup for hydrolysis of MC using a μ IMER.

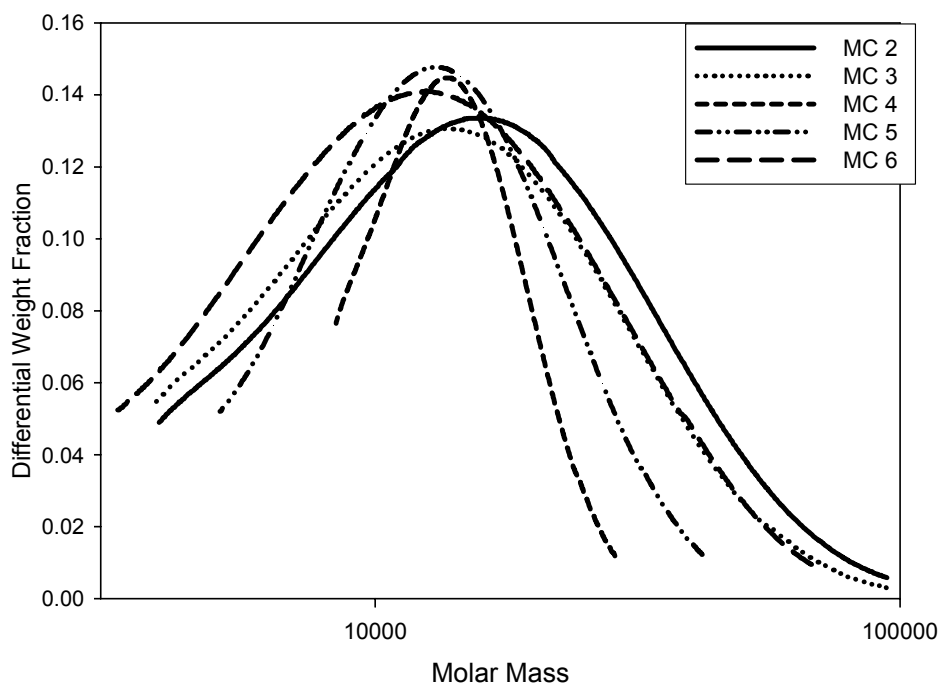
As presented in the literature, analysis of enzyme hydrolyzed modified cellulose using MS has been popular [94,130,140,210,284,288](Papers II, III and IV). Unfortunately, as explained above, due to the free hydroxyl functional groups of the modified cellulose, discrimination does occur, and no reliable quantitative data can be obtained. Thus, only a set of samples with the same or very similar DS can be compared, unless proper sample pre-treatment is performed. In the work presented in Paper III we therefore developed a new method with which enzyme hydrolyzed MC was pre-treated through perdeuteromethylation, thereby minimizing the discrimination. After this treatment, it became possible to compare MCs of different qualities and DS, and both qualitative and quantitative data were obtained.

In the study, it was found that when interpreting the mechanism of enzyme cleavage, the molar mass of the original material played a significant role. If a substrate with too low a molar mass was chosen, too few cleavages occurred and the outcome was hugely influenced by the original end groups and the DS of the original material. Hence, a misinterpretation of the analysis gave an overestimation of the amount of substituents on the oligomers formed after hydrolysis. However, by subtracting the distribution of the random distribution of substituents, it became possible to estimate the true amount of substituents on the formed oligomers.

By comparing the enzyme hydrolyzed MCs, a correlation between the SEC profiles and the monomer/oligomer substituent distribution statistics could be found (Figure 22). The SEC profiles were divided into two groups with one group having a higher average molar mass. Our argument for this was that the group characterized by a higher average molar mass had a

more regular or nearly random distribution (as shown by statistics) of the substituents thus having less sites in the polymer chain with low DS making it more difficult for the enzyme to gain access to the chain. The other group with a lower molar mass was characterized by a less regular distribution, thus having more sites that were sparsely substituted so that the enzyme

a) BaCel 5A



b) Tr longi

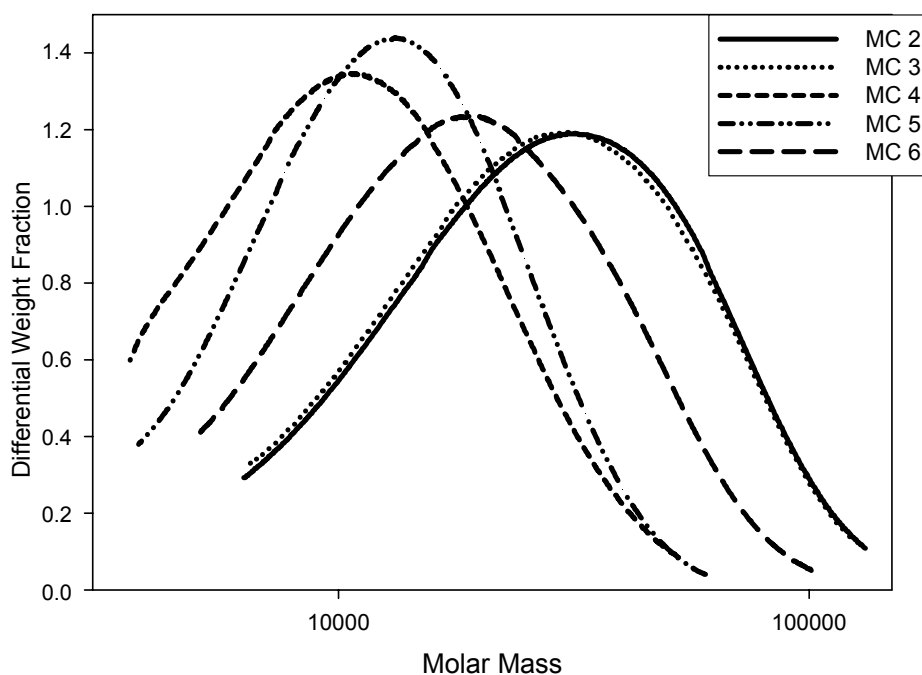


Figure 22. SEC analysis plot (differential weight fraction), comparing two different enzymes hydrolyzing five different MCs. a) BaCel 5A and b) Tr. longi

could gain access to the cellulose chain more effectively. When performing SEC analysis of MC hydrolyzed with several different enzyme preparations, it was also found that only some preparations gave results that could be used to distinguish between the more heterogeneous and the more random distributions (Figure 22b). Hence, it was believed that some enzymes were too efficient in hydrolyzing (low specificity) the MC that they gained access to the cellulose chain even at positions that had a more highly random distribution of substituents (Figure 22a).

Interestingly, we also discovered that by careful analysis the MS spectra of the oligomers from the enzyme hydrolyzed MCs, correlations with the statistical distribution of the substituents (heterogeneity) were found. In Figure 23, a comparison between the distributions of methyl substituents at DP 5 after hydrolysis with two enzymes is given. Three different MC qualities with different heterogeneity have been hydrolyzed by each enzyme. For the MCs with a more homogeneous distribution (more random), the methyl content was shifted towards the higher methylated constituents, while the heterogeneously substituted MCs were shifted towards lower methyl content. This was explained by the fact that there are less highly substituted regions in the polymer at the expense of regions with average substitution, i.e. there is a certain heterogeneity concerning the DS in and/or between the chains. Thus, the sparsely substituted areas are hydrolyzed to a higher extent and consequently form more oligosaccharides with a lower DS. On the other hand, the more substituted areas are less accessible to the enzymes, reducing the probability that short, more highly methylated oligosaccharides are delivered. Unfortunately, this observation was only seen for the most efficient enzymes. Hence, this effect can only be seen when the enzymes have high accessibility to the modified cellulose chain.

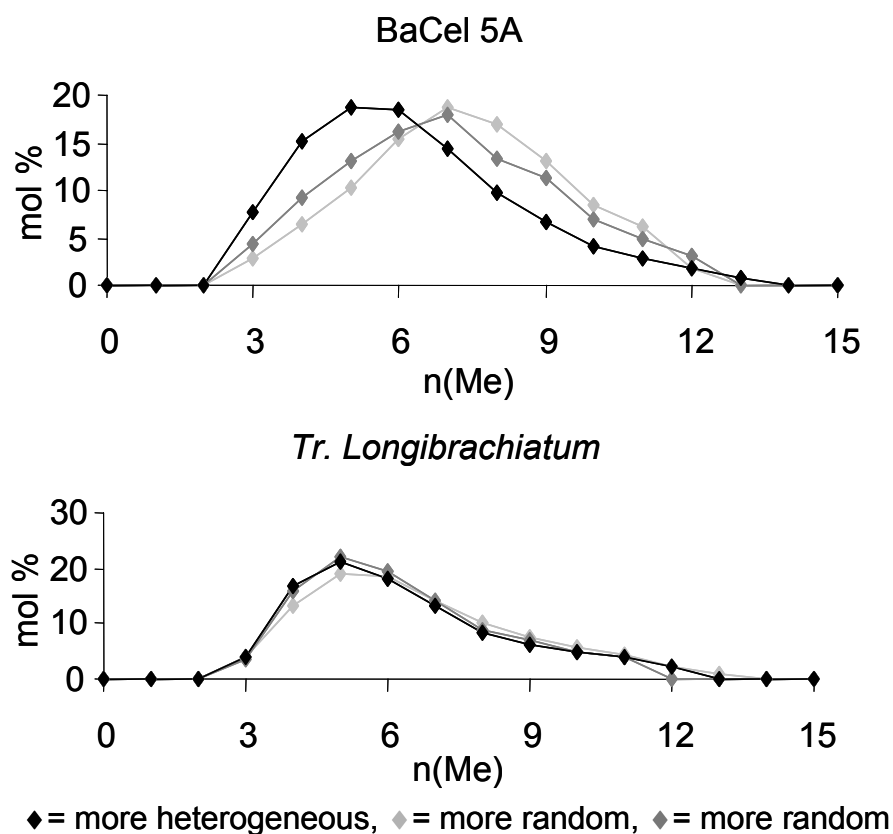


Figure 23. Hydrolysis pattern of DP 5 of three MCs with different heterogeneity hydrolyzed with two enzymes. Tr longibrachiatum gives overall the same distribution of substituents after hydrolysis while BaCel 5A gives differences between them.

7 DISCUSSION, THOUGHTS AND FUTURE IDEAS

There are several concerns that have to be addressed when using enzymatic hydrolysis for characterization of modified cellulose. As described, fractionation of the degradation products using SEC, followed by chemical characterization of the fractions, is a strategy that can be geared towards finding differences in the distribution pattern of substituents when comparing different celluloses. However, after any enzyme hydrolysis, the only way of truly knowing how to interpret the results is to compare them with the outcome of a statistically random hydrolysis, which is highly reproducible. An important issue that we have found in this work is that if too efficient enzymes are used for the hydrolysis, the modified cellulose can be hydrolyzed to an excessive extent, severely restricting the information content (e.g. through SEC analysis). Even after fractionation of the hydrolysates it will be hard to deduce the substituent distribution in this case.

One problem with enzymes is that they can vary from batch to batch both in purity and content. As shown in this work, commercial enzymes sold under the same name showed significant variations in activity on modified cellulose substrate. Hence, very reproducible results are impossible to obtain. One possible way of improving this situation is to always use enzymes of the same specificity and carry out hydrolysis to the same degree and the only way of achieving this is to either have very pure or else very well characterized enzymes.

Another approach could be to have a well characterized mixture containing enzymes of different type (endo- and exo-cellulases) and origin (fungal and bacterial). The mixture would provide a higher degree of hydrolysis, be less specific, and hopefully would be less sensitive to batch-to-batch variations. As shown in this work, less specific enzymes provide a higher degree of hydrolysis and may therefore produce oligomers that when measured, provide a reasonable ability to differentiate between different substituent distributions.

Modified cellulose is often very highly substituted ($DS > 1$). The accessibility of the polymer chain for the cellulases is therefore restricted to the relative few sites with low degree of substitution. We found that (Paper IV), the detected fraction of oligomers of DP 1 – 3 in MS is below 5% for a sample (MC) with DS 1.8 and below 20% for samples with DS 1.3. The question then arises whether the relatively small fraction of the sample that is observable is actually representative of the entire substituent distribution. In our work (Paper IV) we actually verified that this was the case. We found clear differences in the MS profiles related to the substituent distribution among the formed oligomers. Enzymes with low specificity hydrolyzed closer to the substituents and could thereby differentiate between the different

substituent distributions. However, once again, parallel experiments involving statistical hydrolysis were necessary in order to be able to draw any clear conclusions as to the substituent distribution.

Although SEC-MALS/RI may provide quantitative information on the substituent distribution, it has been shown in this work that, contrary to MS analysis, more specific enzymes may be used to differentiate between different substituent distributions. Thus, the more specific enzymes were hindered to a higher extent by the substituents. However, to obtain reproducible results, it is again very important to have very pure and well-characterized enzymes otherwise the results would vary to such an extent that it would not be possible to draw any conclusions. Hence, the method cannot provide enough information for precise determination of the substituent distribution, but may be used as a complementary qualitative method.

Since there are no direct mathematical or statistical methods for determination of the substituent pattern after enzyme hydrolysis such methods need to be developed and validated. Throughout the course of this work, several attempts using multivariate data analysis, polynomial fits, and statistical correlation analysis were made to compare samples and enzymes. Although it has been possible to find trends, no absolute correlations have been found. Perhaps a wider dataset using very well defined enzymes and substrates is necessary. Genetic modification of the organisms producing the enzymes could perhaps be performed, producing extremely well-defined enzymes with high cellulose specificity and selectivity. It is also of significance to determine how a wider variety of different substituent types (size, charge, length) influence the enzyme hydrolysis. In this work at least some of the questions have been answered, and we have also discovered facts that, when this work started, might have seemed to be less important than we now believe.

In this work new approaches using μ IMERs have been investigated. It was found that a given reactor could function continuously for several days with only minor loss of activity. Moreover, the stability of the enzyme immobilization was such that the reactors could be stored for several months with no significant loss in activity. The reactors hydrolyzed modified cellulose and cellodextrins effectively using only minute amounts of enzyme. Moreover, they could also be coupled online to both size exclusion chromatography with refractive index detection and mass spectrometry, allowing fully automated procedures. It was also shown that it was possible to determine sample differences with the reactors. Thus, information that otherwise would be much more laborious to find out was determined rapidly in a completely automated system. When theoretical methods have been developed and

validated, the reactor might be an extremely valuable tool for the characterization of modified cellulose and other (modified) polysaccharides, providing a cheap, automated analytical procedure.

To summarize, enzyme hydrolysis of modified cellulose can be a valuable tool. It may provide information about the modified cellulose that otherwise would not be possible to extract. For instance the enzymes can be used to investigate the degradability of “bioresistant” modified cellulose, to characterize modified starch and cellulose mixtures, to extract cellulose from crude sample preparations, and to provide information about the substituent distribution. Although random statistical degradation so far is still required as a reference method, use of very well characterized enzymes or enzyme mixtures may in the future provide faster and easier ways of determining the substituent distribution using only one or a few analytical techniques. Perhaps micro immobilized enzyme reactors with online derivatization connected to MS will become a method of choice.

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