Approaches for molecular characterization of modified biopolymers

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Approaches for molecular characterization of modified biopolymers

Ruud ter Haar

Thesis

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Abstract

In this thesis, research on the molecular characterization of products obtained after structure modification of oligosaccharides, starch, model peptides, and bovine α-lactalbumin is described. The research goals comprised the development of analytical tools as well as the elucidation of molecular structures by using these tools.

The structure of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-oxidized gelatinized potato starch was analyzed after degrading it to fragments, using acid hydrolysis and methanolysis. Mass spectrometric analysis of the resulting oligomers revealed that the TEMPO oxidation proceeds in a block-wise manner. Also, a high acid-resistance of especially the α(1→4) glucuronic acid-glucuronic acid glycosidic linkage was observed, making oxidized starch a possible dietary fiber. The structure of epoxidized granular 1-allyloxy-2-hydroxypropyl-waxy maize starch was characterized by a similar approach using enzymatic hydrolysis. This showed that the created epoxy groups took part in subsequent reactions to form cross links and/or diols.

Discrepancies in literature concerning the specificity of immobilized *Candida antarctica* lipase B in the acylation of oligosaccharides were explained. Molecular sieves, used to remove free water from the organic reaction medium, were found to be responsible for the catalysis of side-reactions. Next, hydroxy-aryl esters of various oligosaccharides were chemically produced. Via a peroxidase-mediated reaction, these esters could subsequently be coupled to the model peptide Gly-Tyr-Gly, as a *proof-of-principle* for enzymatic protein glycosylation.

In addition, α-lactalbumin was glycated with various saccharides via the Maillard reaction. Products were studied using UPLC-ESI-TOF MS and size exclusion chromatography. A detailed view on the extent of glycation and the dispersity of the products was obtained. The glycation rate, extent of protein cross-linking, and the foam stability of the glycated α-lactalbumin depended on the type of saccharide used. Similar saccharide structures led to similar behavior. The glycation rate and the extent of protein cross-linking decreased when the degree of polymerization of the saccharide increased. Dehydrated Amadori products, as identified by UPLC ESI-TOF MS, were found to be an indicator of the formation of cross-linked protein.

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Chapter 1

General Introduction

Project outline

Nature offers a wide variety in biopolymers to be used in food and non-food products. They are formed in the growth cycles of all organisms *(1)*. Examples of biopolymers used as food and non-food ingredients are starch, cellulose, pectins, hemicelluloses, and proteins. Several biopolymers are modified to tailor the structure of these molecules in order to improve their functionality, after isolation, in specific applications. When excluding backbone hydrolysis reactions, the goals of these modifications comprise changing or adding functionality by modification of the polymer backbone, by the addition or removal of functional groups, or by cross-linking. In order to understand the relation between modification procedure, structure, and functionality, proper analysis of the structures involved is essential. This also applies to modification of oligomeric fragments derived from biopolymers. In the research presented in this Ph.D. thesis, advanced chromatographic and mass spectrometric techniques are used for separation and structure characterization of a number of the above mentioned molecules. In particular, the research is focused on:

1) Protein-saccharide conjugates
2) Saccharides conjugated with functional groups by transesterification
3) TEMPO-oxidized starch and epoxy starch derivatives

In this chapter, the synthesis, characterization, and in some cases the application of these structures will be discussed.

I Protein-saccharide conjugates

Proteins are usually built up out of a combination of 20 different amino acid types. They have several biological functions such as reaction catalysis (enzymes), nutritional and energetic storage, process regulation (hormones), and structure formation. Among the major sources of proteins used as food ingredients are milk, meat, potatoes, legumes, cereals, and eggs, which contain mainly storage proteins *(2)*. Part of the proteins may be glycosylated *in vivo*, and the resulting glycoproteins have important functions, including a broad range of cell-matrix and cell-cell recognition events *(3)*. Native 'food' glycoproteins have been observed a.o. in potatoes *(4)*, meat *(5)*, and milk *(6)*. Industrial protein-saccharide conjugation may be applied to change food protein properties. Glycoproteins can be formed easily via the Maillard reaction during food processing or during directed ingredient modification. Enzymes may also be applied for directed protein-saccharide

conjugation. Enzymatic protein glycosylation as well as protein glycation via the Maillard reaction will be discussed.

Enzymatic protein glycosylation *in vitro*

The ability to create protein-polysaccharide complexes *in vitro* by using enzymes may lead to materials with interesting rheological and mechanical properties *(7)*. For this, several routes have been developed. The basis for one of these routes was established when ferulic acid (FA) could be coupled to a tyrosine containing tripeptide (GYG) using horseradish peroxidase *(8)*.

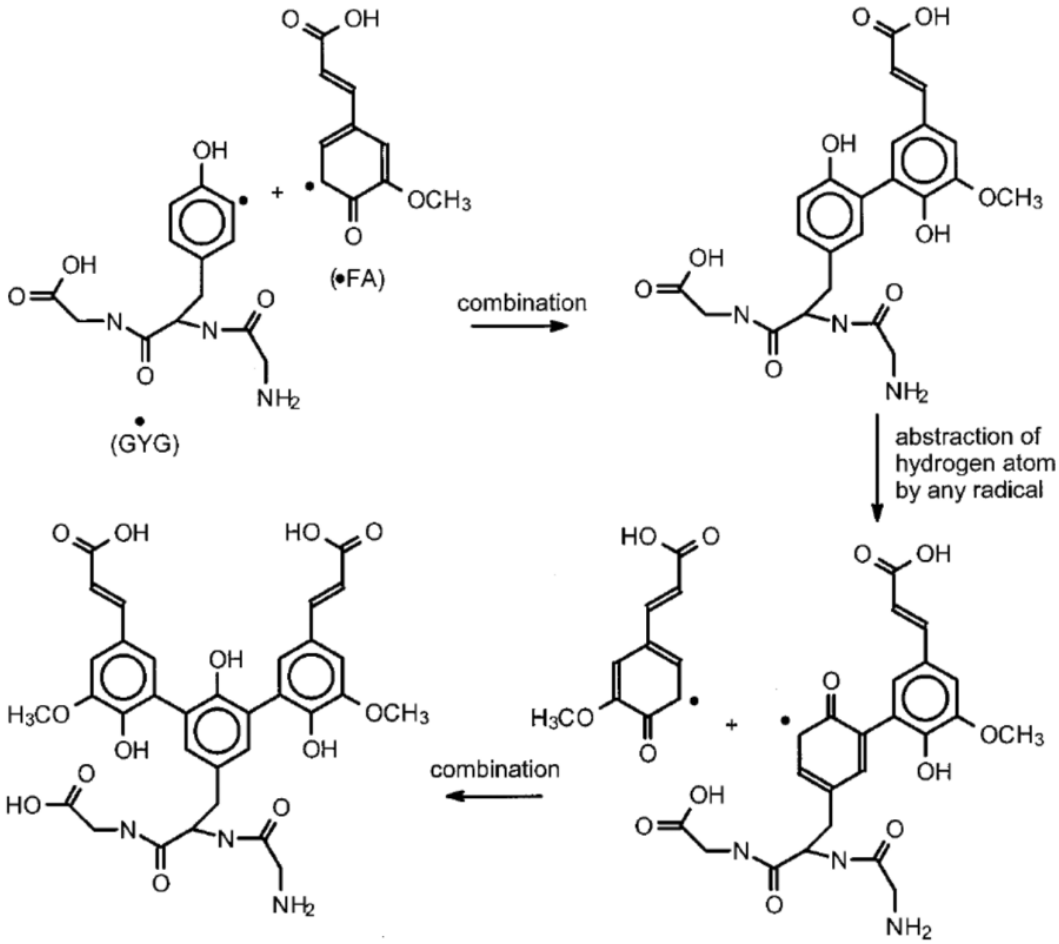


Fig. 1 Mechanism proposed for the formation of heteroadducts of ferulic acid (FA) and the tripeptide GYG, using horseradish peroxidase for generation of radicals. GYG-$(FA)_2$ was the most predominant hetero-adduct formed (taken from *(8)*).

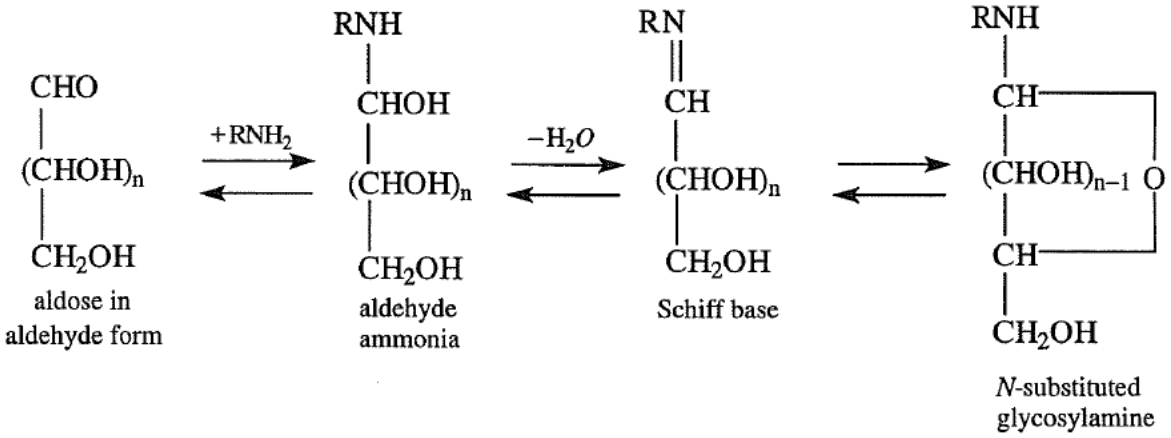
During this reaction horseradish peroxidase is applied in combination with hydrogen peroxide to generate radicals from phenolic substrates by oxidation (Fig. 1). The phenolic

substrates are in this case ferulic acid and the tyrosine moiety of the tripeptide glycine-tyrosine-glycine (GYG), the latter serving as a model for a protein. Two phenolic substrate molecules are typically oxidized by horseradish peroxidase while one hydrogen peroxide molecule is consumed *(8)*. When an FA radical and a GYG radical combine, a heteroconjugate is formed. Based on this horseradish peroxidase-mediated reaction, feruloylated arabinoxylans have also been linked to tyrosine moieties in β-casein *(9)*. This may create opportunities for the production of heteroconjugates with new properties. The reaction in Fig. 1 is, therefore, the basis of a possible route for protein glycosylation. To date, the application is, however, limited to saccharides that are naturally carrying a phenolic moiety, such as sugar beet pectins and cereal arabinoxylans *(10)*.

Other attempts to link proteins to polysaccharides using oxidative enzymes have been made using a fungal laccase. Using this enzyme, arabinoxylans were tried to be linked to gluten or BSA in order to form a gel system. Weakening effects on the gelation of wheat arabinoxylan/laccase systems were reported when reduced proteins were present, but no clear conclusions about the mechanism could be drawn *(11)*. Next to this, the oxidative enzyme tyrosinase has been used to create a link between gelatin and chitosan *(7)*. The activity of the enzyme had an influence on the thermal behavior of the resulting gels. A combination of covalent and physical interactions was suspected to be the cause of this, but could not be proven.

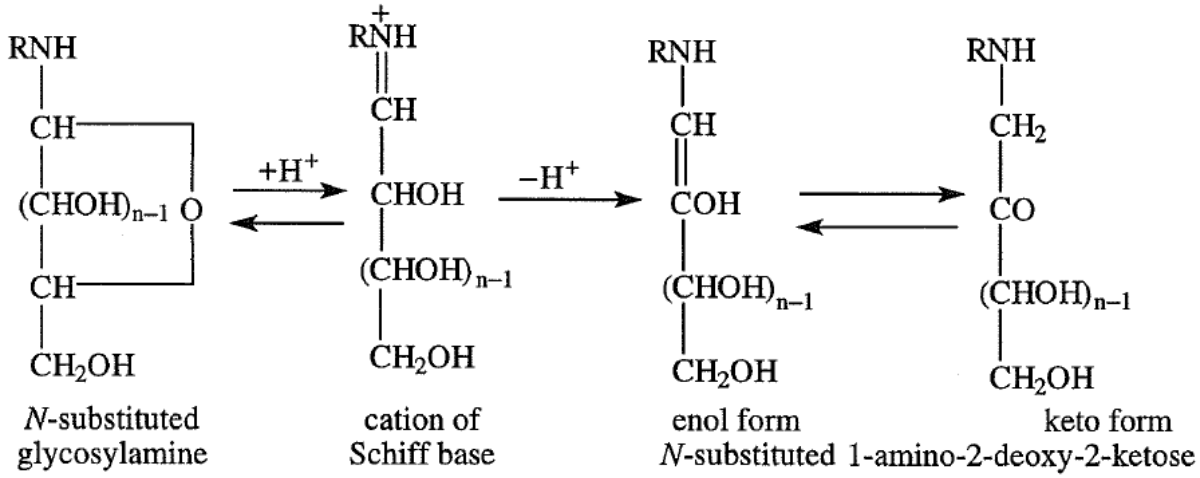
Protein glycation via the Maillard reaction

Another way to create conjugates of proteins and saccharides is application of the Maillard reaction. This reaction is induced by heating and occurs between a carbonyl compound, mostly a reducing saccharide, and an amine. The latter is usually part of an amino acid, peptide or protein structure *(12)*. The Maillard reaction can be performed in both dry (powder) and wet (solution) conditions. It may occur spontaneously during food processing, but can also be used for directed protein modification. The reaction has been divided into three stages, being the initial, intermediate, and final stage *(13)*.

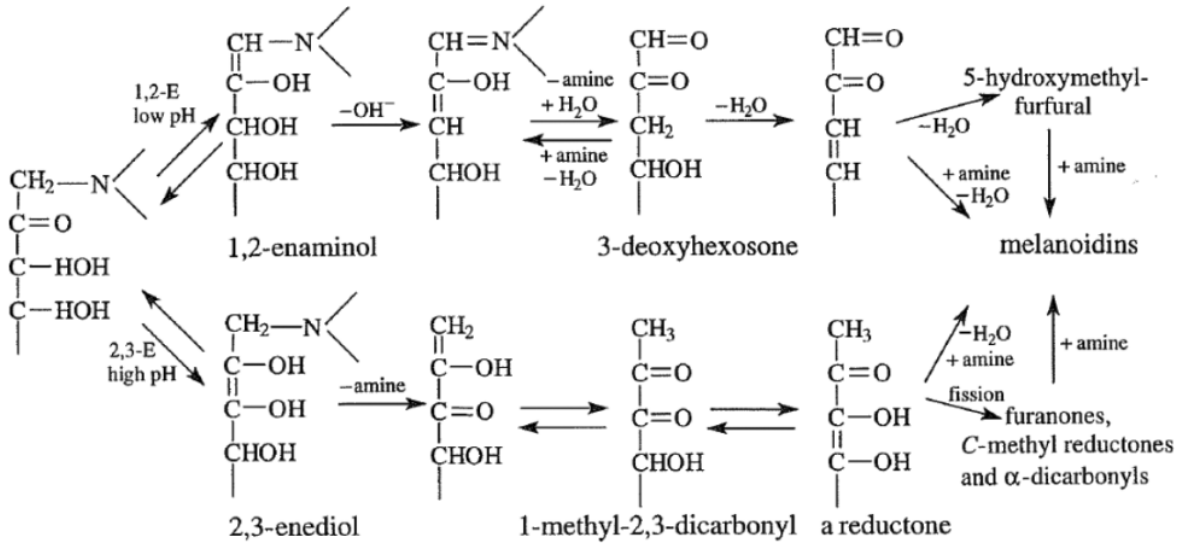


Scheme 1 Condensation of a reducing saccharide with an amino group, to form the N-substituted glycosylamine (*(12) Reproduced by permission of The Royal Society of Chemistry, UK)*

The initial stage is started by saccharide-amine condensation (Scheme 1): a nonprotonized amino group is added to the electrophilic carbonyl carbon of a reducing saccharide *(14)*. Via a water molecule split-off and the formation of a Schiff-base, an N-substituted glycosylamine is formed. This may be followed by the Amadori rearrangement (Scheme 2), which contains irreversible steps and was found to be acid-catalyzed *(12, 14)*.



Scheme 2 The Amadori rearrangement (*(12) Reproduced by permission of The Royal Society of Chemistry, UK)*



Scheme 3 The two major pathways from Amadori compounds to melanoidins, based on Hodge (*(12, 13) Reproduced by permission of The Royal Society of Chemistry, UK)*

The Amadori rearrangement products are quite stable at neutral conditions, but their stability is much lower compared to the original saccharide, especially when the Amadori product exists in the acyclic form *(14)*. As visualized in Scheme 3, degradation reactions of the Amadori compounds in the intermediate stage may comprise saccharide dehydration, deamination, saccharide fragmentation, and amino acid (Strecker) degradation. It yields products that can be yellowish *(13)*. In the final stage, aldol condensation and aldehyde-amine condensation reactions are observed *(13)*, leading to a very complex set of products, including melanoidins. The latter are defined as dark-colored, nitrogen containing polymers *(15)*. This polymer formation implies that in the final stage of the Maillard reaction, protein cross-linking may also occur *(16, 17)*. This can be a result of the generation of highly active compounds from saccharides, possibly after hydrolysis of their connection to the protein *(12, 13)*. From research on Maillard-mediated protein cross-linking *in vivo,* however, it appeared that cross-linking can also occur without amino group re-liberation, by the formation of e.g. glucosepan and pentosidine via intermediate dideoxyosones *(18)*. It is not clear to which extent these types of cross-links are formed in food systems, which makes the precise structure of the protein cross-links in food systems resulting from Maillard reactions hardly understood *(17)*.

Factors influencing the Maillard reaction

External conditions have a major influence on the course of the Maillard reaction, of which temperature is an important one. It affects both the reaction speed and the precise reaction

mechanism *(14)*. This is partly caused by temperature-dependence of the relative concentration of the open-chain form of the saccharide and the rate of mutarotation, since these are the main determining factors for the reaction speed *(19)*.

Water activity is also an important factor *(19)*. At very low water activities, diffusion is difficult, resulting in a low reaction speed *(14, 19)*. When the water activity increases, reaction speed will increase up to the point where dilution negatively influences the reaction speed *(14)*.

The pH is another important parameter, because several steps of the Maillard reaction are acid/alkali catalyzed. Protonation of the carbonyl group increases its reactivity, while the reactivity of the amino group is decreased by protonation. As a result, the Maillard reaction with amines has a maximum rate in a slightly acidic medium, while the reaction involving amino acids reaches a maximum rate in a slightly alkaline medium *(14)*. The open-chain concentration of the saccharide is also pH dependent *(19)*. The pH also has an important effect on the progress of the reaction in the form of enolization after formation of the Amadori compound (Scheme 3) *(12)*. The presence of certain (buffer-) salts, such as phosphate, can also catalyze the reaction *(20)*.

Influence of the saccharide characteristics

An important factor determining the progress of the Maillard reaction is the type of reducing saccharide involved. As mentioned, the relative concentration of the open-chain form is a factor determining the saccharide reactivity. This relative concentration saccharide-dependent *(19)*. The acyclic form of a saccharide is more reactive than the cyclic form *(14)*. Some more rules of thumb have been established, including the fact that aldoses are more reactive than ketoses *(14)*. For monomeric saccharides, the reactivity order *fructose ~ glucose < arabinose < xylose < ribose* has been established *(21)*. In general, pentoses are more reactive than hexoses *(21)*. Also, the saccharide reactivity tends to decrease when the saccharide chain length increases *(22)*. Monosaccharides are apparently the most reactive in the Maillard reaction. They are also the most vulnerable to degradation. As a result, product browning can be prevented by the absence of low molecular weight saccharides in the reaction mixture and by careful control of the treatment time *(23)*.

Functionality of Maillard-modified proteins

Protein glycation via the Maillard reaction has been associated with a number of changes in terms of protein functionality. It has been reported that the gel-forming properties of whey

protein isolate (WPI) are affected as a result of the Maillard reaction, depending on the type of saccharide used *(24)*. Conjugates of WPI with maltodextrins have been proposed as an alternative to gum arabic in stabilizing low-pH emulsions *(25)*. The low pH-solubility, and consequently the emulsification properties of casein, also improved after conjugation with a maltodextrin *(23)*. Emulsifying properties of ovalbumin improved after conjugation with glucuronic acid *(26)*. Also, the emulsifying and antimicrobial properties of lysozyme improved after conjugation with galactomannan *(27)*. The improvement of the functional properties of β-lactoglobulin by glycation via the Maillard reaction has been reported to depend on the type of saccharide used. Glycation with arabinose and ribose improved the emulsifying properties, while foaming properties improved after glycation with glucose or galactose *(28)*. Dextrans have also been used for protein modification via the Maillard reaction. This resulted in improved emulsion stabilizing properties of WPI, which was mainly attributed to the steric stabilization provided by the hydrophilic bulky polysaccharide moiety *(29)*. Furthermore, the use of casein-dextran conjugates instead of casein in double emulsions resulted in smaller oil droplets, a narrower droplet size distribution, and an increased stability under acidic conditions *(30)*.

Analysis of Maillard-modified proteins

For the analysis of proteins glycated via the Maillard reaction, a number of techniques has been used. Analysis of the amount of free amino groups via several assays has frequently been applied to estimate the average degree of glycation. This is sometimes done in combination with gel electrophoresis and/or MALDI-TOF MS *(21, 22, 26, 28, 31-36)*. The application of advanced MALDI- and ESI-based MS techniques in studies of milk protein glycation has recently been reviewed *(37)*. This review shows that these techniques have gained a more prominent role in glycated milk protein research in the past years. They have been applied for the analysis of intact proteins for determination of the extent of glycation, but also for the analysis of protein hydrolysates to determine the exact glycation sites *(37)*. By using LC-ESI MS, the solid state glycation of intact β-lactoglobulin with lactose, galactose, and glucose has been followed using deconvoluted spectra *(38, 39)*. In mass spectrometry, the latter means that a mass spectrum is obtained by conversion of an *m/z* spectrum by using specific software. Especially the determination of the exact number of saccharides attached per protein molecule and the product heterogeneity, which is made possible by this method, represents a major step forward compared to determinations of the average extent of glycation by e.g. free amino group analysis, as mentioned before. The

high resolution MS-techniques have, however, not been further optimized and applied so far for monitoring of the Maillard reaction with other food proteins and/or saccharides.

The research on hydrolysates of glycated proteins has for example been performed on β-casein glycated by using glucose. In the first hour of incubation the formation of N^{ε}-(fructosyl)lysine, which is the Amadori product of glucose, was fast at two specific lysine moieties in the β-casein chain *(40)*. The formation of the advanced glycation end product N^{ε}-(carboxymethyl)lysine was also reported at both lysine residues mentioned. The remaining 9 lysine residues were not modified at all. This is in contrast to results of another study, where it was found that at least five lysine residues in β-casein were lactosylated during milk sterilisation *(41)*. This difference was explained as being a result from different conformational states of the protein, depending on the conditions used, which may lead to differences in lysine accessibility *(40)*. This example shows the importance of detailed analysis after protein glycation. A change in conditions may not only change the rate of the reaction, but may also have an influence on the moieties that will be modified. This can be expected to have an influence on the functionality of the resulting products.

II Carbohydrate modifications

A wide range of polysaccharides in the form of carrageenan, pectins, alginates, cellulose, starches, several gums, and konjac mannan is used as food ingredients. Their functions comprise thickening, stabilizing, gelling, and/or emulsifying *(42)*. Apart from these, also a number of oligosaccharides is used as an ingredient. Maltodextrin, a product of starch hydrolysis, is for example widely used for bulking, promotion of dispersibility, crystallization prevention, freezing control, and binding of fats, pigments, and flavorings *(43)*. In addition, some carbohydrates are active as prebiotics. Examples are fructooligosaccharides and resistant starch, which have been reported to provoke a possible synergistic prebiotic effect, due to their difference in rate of fermentability *(44)*. Carbohydrates, in the form of mono-, oligo- or polysaccharides, can be subjected to enzymatic as well as chemical modification. These modifications improve their properties and, therefore, extend their use *(15)*. Nowadays, most of the processes in industry where carbohydrates are involved as an ingredient contain (bio-)technological transformations *(45)*. A number of carbohydrate modifications will be described.

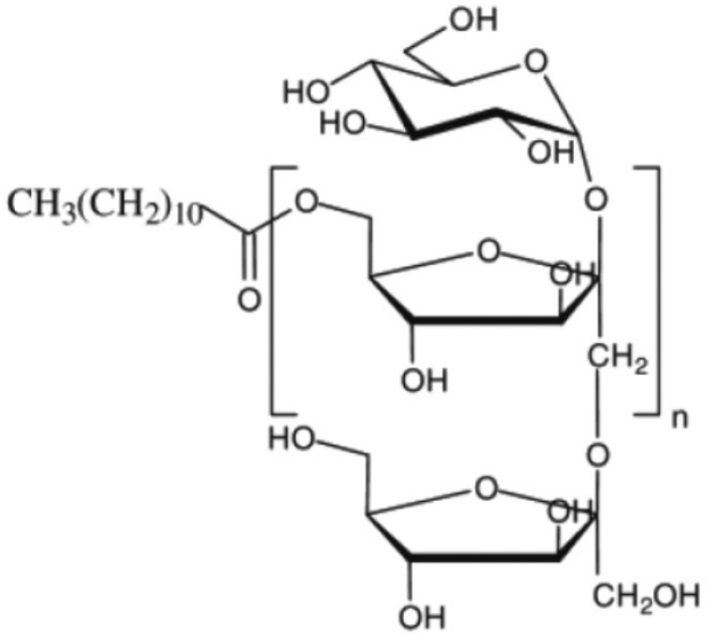


Fig. 2 Possible structure of a fructooligosaccharide-lauryl ester (Sagis et al. *(46))*

Enzymatic and chemical carbohydrate acylation

Regioselectivity and stereoselectivity together with the possibility of their low cost production have made enzymes attractive as potential catalysts in carbohydrate modifications. Due to their regioselectivity there is no need for group blocking and deblocking steps, which are common when chemical catalysts are applied *(47)*. An application of enzymes in the carbohydrate field is found in the lipase-catalyzed acylation of carbohydrates in organic media. Lauryl esters of fructo-oligosaccharides have been synthesized in a Bu^{t}OH/DMSO mixture, catalyzed by immobilized *Candida antarctica* lipase. The resulting conjugates (Fig. 2) showed enhanced surface-active properties and may represent a new generation of structuring agents to be used in food products *(46)*. The same enzyme has been applied in, e.g., the production of monolauryl maltose *(48)*, the acylation of saccharose, trehalose, maltose *(49)*, and konjac glucomannan *(50)*. A much wider range of lipases and substrates has been applied in the field of saccharide acylation, yielding products with a wide range of functionalities, as reviewed recently *(51)*. The enzymatic synthesis of acylated oligosaccharides is often limited due to enzyme inactivation provoked by the polar solvents needed to solubilize the saccharides *(52)*. This problem can be solved by using two miscible organic solvents *(52)*, enabling the adjustment of the hydrophobicity to a level where the oligosaccharide is partly soluble while the enzyme is still active. Molecular sieves are often added to the reaction mixture to remove

free water. Some water is needed to preserve the enzyme structure, but too much water would result in hydrolysis of the products formed.

Lipases have also been applied for functionality improvement of various polysaccharides. Cellulose has, for example, been acetylated using a lipase from *Aspergillus niger (53)*. Also, hydroxypropyl cellulose has been laurylated using immobilized *Candida antarctica* lipase *(54)*.

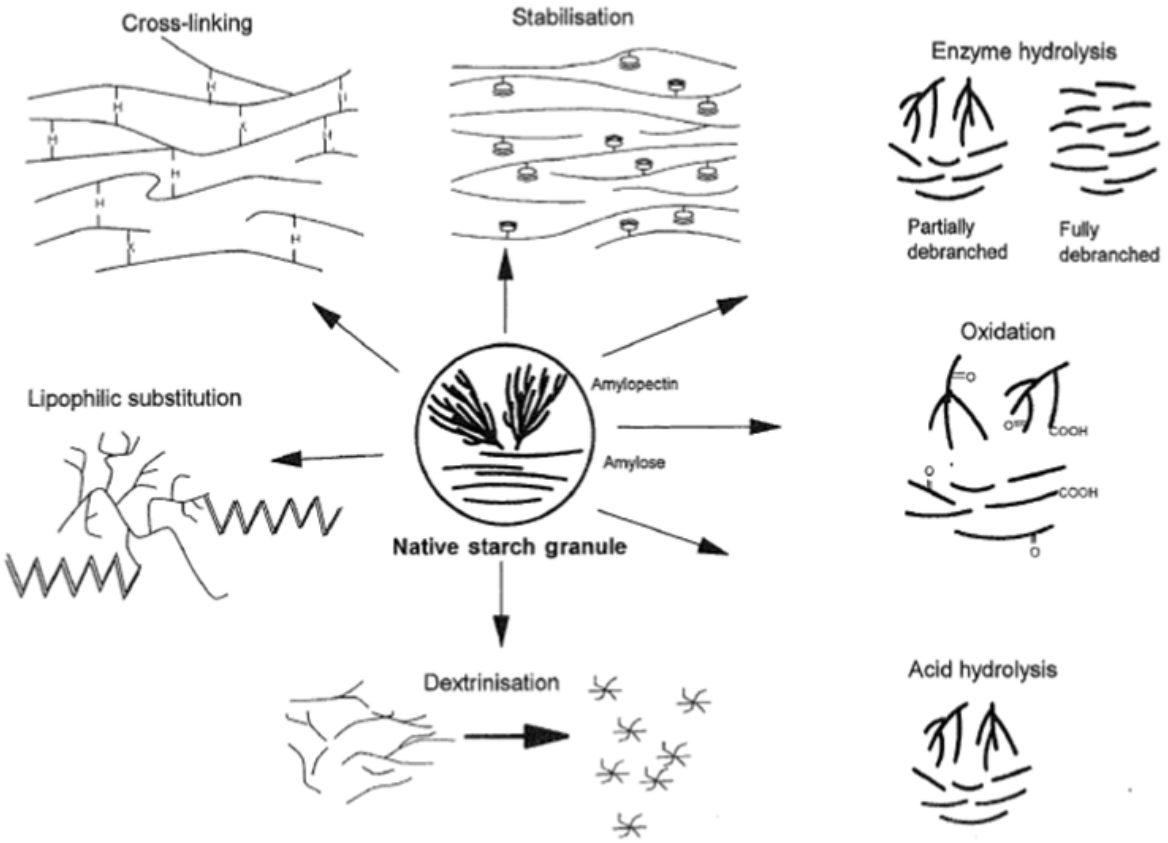
When less specific modification and/or multiple substitutions per saccharide unit are required, acylation of saccharides can be performed using chemical catalysts. This is for example the case in the production of Olestra™, which is a saccharose polyester carrying at least four fatty acid ester groups. This molecule can be synthesized in methanol, using potassium hydroxide and potassium carbonate as the catalysts, and was initially designed as a non-caloric cocoa butter substitute *(55)*.

Chemical starch modifications

Apart from chemical acylation, other chemical modifications are also widely applied in the carbohydrate field. A polysaccharide that can be subjected to a wide range of chemical modifications is starch. The modifications are performed to alter the functional properties and some of them may also increase the proportion of 'resistant starch' present *(56)*. A schematic overview of starch modifications is provided in Scheme 4, and a number of them will be discussed in this section.

Oxidation of starch and other starch conversions

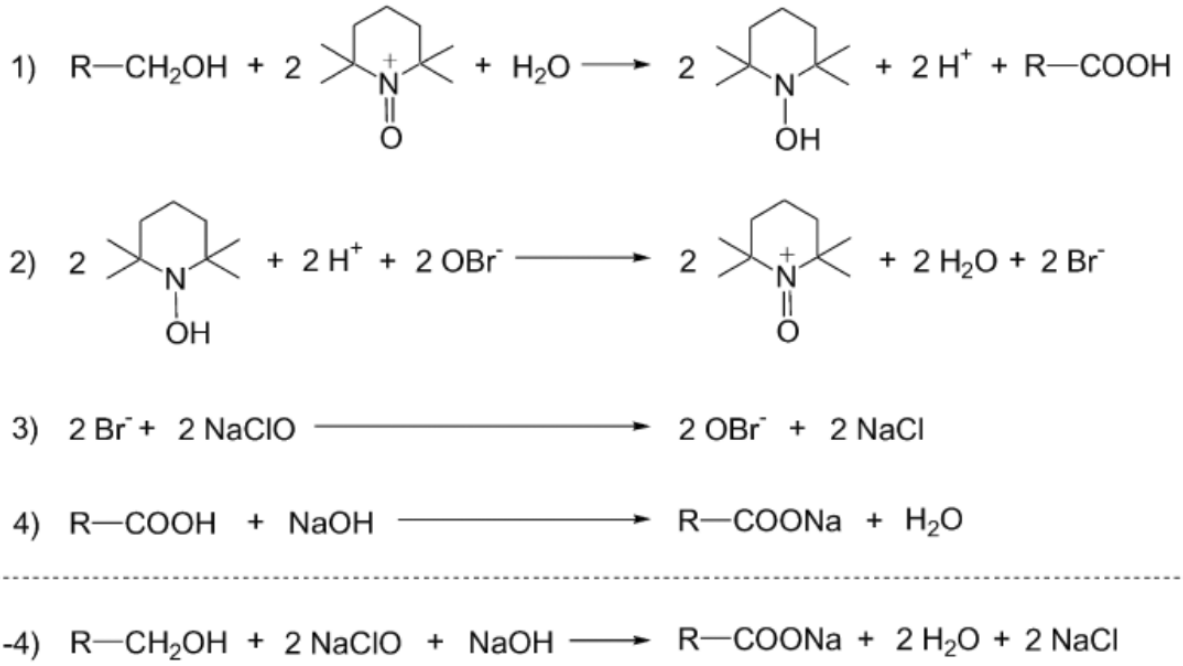
Several conversions of starch are known. One of them is (partial) *acid hydrolysis*, which lowers the hot viscosity of the starch and results in stronger gels upon cooling *(58, 59)*. Another starch conversion is *dextrinization or pyroconversion*. This consists of a heat treatment, and is resulting in hydrolysis, transglucosidation and/or repolymerization *(60)*. The influence of this process on physical properties of the starch vary from lower viscosity to altered gelling properties, which depend on the exact nature of the treatment *(59)*. Starch *oxidation* is another important conversion, as it decreases the interactions between starch molecules due to the generation of bulky and charged carboxyl groups. For this reason, it reduces retrogradation and decreases viscosity *(59)*. Oxidation can be performed by treatment with several oxidizing agents. When only a relatively low amount of oxidizing agent is applied to remove colored impurities, it is referred to as bleaching, which involves no significant chemical modification *(60)*.



Scheme 4 Chemical and biochemical modifications of starch (*(57)Reproduced by permission of Woodhead Publishing Ltd.)*

For more severe starch oxidation, including chemical modification to change the functional properties, starch can be treated with hypochlorite. This procedure is mostly performed under alkaline conditions. Hypochlorite will attack the molecules in the starch granules randomly and can lead to some backbone degradation. As a result of this, a number of oxidation types may occur *(60)*, including: 1) Oxidation of primary hydroxyl groups to carboxyl groups, yielding uronic acids; 2) Oxidation of secondary hydroxyl groups to ketone groups; and 3) Conversion of the glycol groups at C2 and C3 to aldehydo groups, leading to rupture of the linkage between C2 and C3. Aldehydo groups may then be oxidized to carboxyl groups. In the past years, carbohydrate oxidation via a TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl)–mediated reaction has gained interest *(61)*. This procedure is illustrated in Scheme 5. Step 2 in Scheme 5 shows the formation of the reactive nitrosonium ion from TEMPO by OBr^-. Two nitrosonium ions subsequently oxidize a primary hydroxyl group in starch to a carboxyl group, as shown in step 1 (Scheme 5), under formation of two TEMPO molecules. These TEMPO molecules are subsequently

regenerated using the oxidizing agents hypochlorite and bromide *(62)*. During step 1 in Scheme 5, C6 aldehydo groups are likely to be formed as an intermediate and are further oxidized to carboxyl groups if reagents and time suffice *(63)*. Benefits of the application of TEMPO for starch oxidation include *(61, 62)*: 1) A high reaction rate and yield; 2) A selectivity of more than 95% for the primary hydroxyl group; and 3) Only a modest depolymerization of the starch. Because the application of bromide is undesired from an industrial and environmental point of view, efforts have been made to perform the TEMPO-mediated reaction free from bromide *(64)*. Applications of TEMPO-oxidized starch have for example been found in microgels for controlled protein uptake and release applications *(65)*, and in particles for targeted drug delivery *(66)*.



Scheme 5 Formulae for the TEMPO-mediated oxidation of primary hydroxyl groups of e.g. starch to carboxyl groups, using NaClO and NaBr as the regenerating agents (*(63) Reproduced by permission of Elsevier*)

Substituted starches

Apart from conversion of the starch structure, starch can also be modified by the addition of various groups to the structure. Starches subjected to these modifications are often referred to as stabilized starches.

Starches can be *phosphorylated*, yielding an anionic polymer. *Carboxymethylation* of starch also introduces negatively charged groups, and is performed by reaction with

chloroacetic acid *(67)*. Other examples of stabilized starches carrying negatively charged groups are starch *succinates (15)*. Starches substituted with octenylsuccinate, having an 8-carbon chain, show an increased hydrophobicity, which is referred to as *lipophilic substitution (59)*. *Cationic starches* carry positively charged groups, that can be introduced by reaction with a quaternary ammonium reagent, and are widely used in the paper industry *(68)*.

Hydroxypropylation, which is the most often applied reaction for starch stabilization, can be performed by treatment with propylene oxide *(15, 69, 70)*. The modification inhibits retrogradation and increases textural and freeze-thaw stability *(70)*. *Acetylated starches* were the first commercialized modified food starches, and are produced by treatment of granular starch with either acetic anhydride or vinyl acetate *(60)*. By acetylation, the thickening capabilities of starch are improved *(71)*. Furthermore, improved stability against retrogradation, improvement of elasticity, and a lower gelatinization temperature are the result of this treatment *(72)*. Only acetylated and hydroxypropylated starches with a DS below 0.2 are of commercial interest for application in foods, they are mostly used in products with low amounts of unbound water *(58, 59)*.

Starches can be *allylated* to introduce reactive carbon-carbon double bonds *(73)*. They have mainly been applied in non-food applications *(74)*. For their production, a novel synthetic route using allyl glycidyl ether has recently been presented, to replace the less favorable routes involving allyl halogenides *(74)*.

Starch *cross-linking,* which is not in the starch stabilization category, can be performed via phosphate bridges, and alternatively via adipate bridges *(58-60)*. Starches can also be cross-linked via *epoxidation* of allylated starches, epoxidized starches are able to interact with a wide range of ligands *(75)*.

Structure analysis of oxidized and substituted starches

The properties of (modified) starches are determined by several factors such as the degree of branching, the molecular mass distribution, the degree of substitution, and the type of the substituents and their distribution within the starch molecules *(76)*. Several factors make the structure analysis of starch complicated. There may be a difference in the extent of modification between amylose and amylopectin, there may be variation in the extent of modification of differently sized starch granules, the outside of a granule may be differently modified than the inside, and the distribution of substituents along the backbone may be either random or more blockwise. Despite of this, quite some effort has been put in the

structure analysis of a number of starch derivatives that goes beyond analysis of the degree of substitution.

The structure of cationic starches has been studied by applying mass spectrometry after enzymatic degradation. It was for example found that slurry modification mainly results in cationization in the amorphous lamellae of the amylopectin growth rings of the potato starch granules *(76)*. A similar approach which also involved capillary electrophoresis was applied to study carboxymethyl starch. In this research, samples with a similar degree of substitution (DS) showed different amounts of unsubstituted glucosyl residues and high variations in accessibility for amylases. A DS gradient within the samples was supposed to be the cause of this *(77)*. Hydroxypropylated starch has also been studied to some extent using enzymatic and chromatographic techniques *(78)*. Granular acetylated starch has been analyzed in detail for the substitution pattern. The roles of the granule size and the location within the granule as well as differences between amylose and amylopectin populations have been taken into account in this research *(72, 79-82)*. Differences in extent of modification between amylose and amylopectin populations in acetylated granular starch were found to depend on the type of reagent used. Also, the distribution of acetyl groups along the backbones depended on the type of reagent *(81)*. Furthermore, it was found that the heterogeneous distribution of acetyl groups in both amylose and amylopectin populations is due to the crystalline and amorphous regions of the starch granule, which makes granule size also a determining factor for the properties of acetylated starch *(72)*.

Granular starches oxidized using hypochlorite have been analyzed using a range of chromatographic and enzymatic techniques *(83-85)*. These studies indicated that the composition of the starch and hence the starch granules influences the course of the oxidation. It was, furthermore, concluded that amylose present in the amorphous lamellae was preferentially depolymerized before amylopectin was oxidized. Amylose molecules might protect amylopectin molecules in this way *(84)*. In granular allylated starch, also a more dense substitution of allyl groups was found in the amylose molecules, which was concluded to be a result of the easier penetration of reagents in the amorphous regions of the granules *(74)*.

Thesis outline

The above has shown that detailed analysis of modified oligo- and polymers can provide valuable information on the relation between modification procedure, product fine structure, and product functionality. The aim of the present PhD thesis is the structure modification of oligosaccharides, starch, and α-lactalbumin by chemical or enzymatic methods, followed by a detailed characterization of the products.

In **Chapter 2**, the elucidation of the fine structure of TEMPO-oxidized, gelatinized potato starch is described. After acid hydrolysis of the modified polymer, the fragments are analyzed using MALDI-TOF MS and various chromatographic techniques. Making use of the resistance to acid hydrolysis of the various glycosidic linkages present, information about the distribution of the glucuronic acid moieties along the starch backbone is obtained. In **Chapter 3**, the synthesis and structure characterization of granular epoxy starch derivatives is discussed. Analyzable fragments are in this case created by applying a set of starch degrading enzymes. The substituent distribution as well as the presence of possible cross-links in the starch is evaluated.

Chapter 4 deals with the application of molecular sieves in the lipase-catalyzed synthesis of fructose oligosaccharide-lauryl esters in organic media. By varying the reaction conditions and by detailed analysis of the resulting products, the suspected catalyzing effect of these molecular sieves is investigated. In **chapter 5**, the synthesis of activated non-reducing saccharides and polyols for peroxidase-mediated protein glycosylation is described. After chemical hydroxy-arylation of the saccharides and polyols in an organic environment, products are characterized and purified. Subsequently, the enzyme-catalyzed coupling of a purified saccharose mono-ester to a tyrosine containing peptide is performed, followed by analysis of the resulting products.

Chapters 6 and 7 are related to the glycation of α-lactalbumin via the Maillard reaction, followed by detailed ESI-TOF MS analysis. The Maillard reaction rate, the dispersity of the products, the extent of protein cross-linking, and the foam stability of the products depending on the type of saccharide applied is provided and discussed. By application of a model peptide, additional information is obtained about the factors playing a role in the Maillard reaction.

In **Chapter 8**, the implications of the results presented in this thesis are discussed, and the importance of detailed product analysis after synthesis is articulated.

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Chapter 2

TEMPO oxidation of gelatinized potato starch results in acid-resistant blocks of glucuronic acid moieties

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Abstract

Chemical derivatization is often applied to improve polysaccharide functionality. Primary hydroxyl groups in starch can, for example, be oxidized to aldehydes by using a 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated reaction. The major part of the aldehydo groups is subsequently converted to carboxyl groups by NaOCl. The exact structure of TEMPO-oxidized starch was studied to promote a better understanding of the TEMPO oxidation mechanism and the functionality of oxidized starches. By using weak and strong acidic hydrolysis, and methanolysis, at elevated temperatures, oxidized starches with different degrees of oxidation (DO) were broken down into oligomers and monomers. Analysis of the oligomers by chromatographic and mass spectrometric techniques revealed that blocks of glucuronic acid moieties are present in the oxidized starch polymers. The α(1→4) glucuronic acid-glucuronic acid bond was found to be very resistant to breakdown by acids. The α(1→4) glucuronic acid-glucose bond also showed increased resistance to acids compared to α(1→4) glucose-glucose bonds. The size of the blocks of glucuronic acid moieties increased when DO increased. Furthermore, the presence of clusters of aldehydes close to carboxyl groups directly after oxidation was proven. This implies that TEMPO, which is positively charged in its active state, is apparently attracted by the negatively charged carboxyl groups. Because of this, TEMPO tends to be active in areas where carboxyl groups have already been formed, which leads to a block wise distribution of the glucuronic acid moieties.

Introduction

Chemical and enzymatic derivatizations are commonly used to improve polysaccharide functionality. Starches can be oxidized using hypochlorite under alkaline conditions *(1, 2)*. These oxidized starches find various applications in the food industry because of their high stability, low viscosity, clarity and film-forming properties *(3)*.

Polysaccharides can also be oxidized by using a 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated reaction. Hypochlorite and bromide are used as the regenerating agents in this case. One of the first published procedures for such an oxidation was applied on starch *(4)*. This reaction is more than 95% specific for the primary alcohol groups present in the starch polymer *(4)*. The TEMPO-mediated oxidation reaction was, furthermore, shown to have yields of almost 100% when reagents suffice *(5)*. The yield and selectivity of the oxidation reaction were found to depend on the temperature and the concentration of NaBr and TEMPO *(6)*. Kato et al. performed a study on the formation of intermediate structures during the TEMPO oxidation process. They found that significant amounts of intermediate aldehydo groups at the C-6 position are likely to be formed during the TEMPO oxidation of water-soluble starch. These groups can form hemiacetals with water or with hydroxyl groups of the starch itself, and will be further oxidized into C-6 carboxyl groups if reagents and reaction time suffice *(7)*.

TEMPO-oxidized polymers find their application in the non-food area. Carboxyl and carbonyl functionalities are known to be important in the pulping process and have an influence on paper properties *(8)*. Recently *(9)*, potato starches were TEMPO-oxidized to several degrees of oxidation (DO) and subsequently cross-linked into a micro gel using sodium trimetaphosphate. It appeared that the DO determined the cross-linking efficiency and could, therefore, be used to adjust the density of the gel. This was applied in order to make the most suitable gel for a specific controlled-release application.

In this study, the structure of TEMPO-oxidized potato starches with different DO was elucidated through analysis of degradation products after weak and strong acidic hydrolysis and strong acidic methanolysis of the polymers. This provides insight into the distribution of the oxidized groups along the starch backbone and, therefore, also into the mechanism of TEMPO oxidation. Also the vulnerability of the different types of bonds present in the polymer to the breakdown by using acids will be discussed.

Experimental

Materials

Potato starch was obtained from AVEBE (Foxhol, The Netherlands). Sodium hypochlorite was from Breustedt Chemie (Apeldoorn, The Netherlands) and TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) from Degussa (Darmstadt, Germany). Sodium bromide (99%), sodium borodeuteride (98%), trifluoroacetic acid (TFA), D-(+)-glucose (Glc), methyl α-D-glucopyranoside, and D-glucuronic acid (GlcA) were purchased at Sigma-Aldrich (St. Louis, MO, USA). HCl in methanol (3N) was obtained from Supelco (Sigma-Aldrich) and diluted to 2M HCl in methanol by using methanol from a fresh bottle obtained from J. T. Baker (Phillipsburg, NJ, USA). Acetonitril came from Biosolve (Westford, MA, USA), ethanol from Nedalco (Bergen op Zoom, The Netherlands), and 2,5-dihydroxybenzoic acid (DHB) from Bruker Daltonics (Bremen, Germany).

Methods

Procedure for starch oxidation

For oxidation, the procedure used was based on work previously published *(4, 10)*. Twenty-five grams of potato starch (21 g dry matter) was suspended in 460 ml water and gelatinized. The solution was then kept below 8 °C. The solution was brought to pH 10 after which 100 mg of TEMPO and 100 mg of NaBr were added. A solution of NaOCl was then added continuously. During the oxidation process, the degree of oxidation (DO) was monitored by the amount of alkali that was added to keep the pH at 10. DO was defined as: average number of GlcA moieties present per 100 anhydrohexose moieties (Glc and GlcA together) in the starch polymer. When the desired DO had been reached, the influx of NaOCl was stopped and 20 ml of ethanol was added. Remaining aldehydo groups were reduced by introducing 200 mg of $NaBD_4$ and after 1 h the pH was set at 6.5 by using 4M HCl. Products were then precipitated in 66% ethanol, washed, and then redissolved in water at pH 3, after which 20% methanol was added. Borate esters and remaining methanol were removed using a rotating film evaporator, and the remaining solution was freeze-dried. Starches with DO 30, 50, and 70 were obtained in this way. Products were stored at room temperature.

Starch hydrolysis using TFA

For starch hydrolysis, 2 ml of either 0.05M or 2M TFA in MilliQ water was added to 20 mg of starch sample in sealed glass incubation tubes. These samples were subsequently incubated for 2, 4, 8 or 20 hours in a heating block at 100 °C. After cooling down, samples were dried using a stream of dry air. When samples were nearly dry, small amounts of methanol were added to assist the evaporation of TFA. Dried samples were stored at -20 °C until analysis.

Starch methanolysis using HCl

The procedure used for methanolysis is based on a procedure followed by De Ruiter et al. *(11)*. Two milliliter of 2M HCl in dry methanol was added to 5 mg of starch sample in glass incubation tubes. The remaining air in the tube was replaced by dry N_2 and the tubes were closed well to prevent drying out. These samples were subsequently incubated for 2, 4, 8 or 20 hours in a heating block at 80 °C. After cooling down, samples were dried using a stream of dry air and stored at -20 °C until analysis. Twenty per cent of the sample volume of each of the 20 h-incubated samples was transferred to a clean tube and dried, after which 1 ml of 2M TFA in milliQ water was added. These tubes were sealed and subsequently incubated for 1 h at 121 °C in a heating block. Samples were then cooled down, dried under a stream of dry air, and stored at -20 °C until further analysis.

Determination of the molecular size distribution using HPSEC

Before and after incubation, the molecular size distribution of samples was determined by using High Performance Size Exclusion Chromatography (HPSEC). For this analysis, a Dionex Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA, USA) was used. This system was equipped with three TosoHaas TSKgel SuperAW columns in series (4000-3000-2500, 150 mm * 6 mm) preceded by a SuperAW-L guard column (TosoH, Tokyo, Japan). After injection of 20 µl of a 2mg/ml sample solution in 100mM acetate buffer (pH 5.0), elution was performed with 0.2M $NaNO_3$ at 40 °C, using a flow rate of 0.5 ml/min. A Shodex RI-101 RI detector (Showa Denko K.K., Japan) was used. A calibration curve was made by means of a series of pullulans with known molecular weights. Data were processed using Chromeleon software (Dionex Corporation).

Quantification of monomers and analysis of oligomers using HPAEC

After incubation, samples were analyzed using High Performance Anion Exchange Chromatography (HPAEC). A Dionex ICS-3000 HPLC system coupled to a Carbopac PA

1 Guard column (2 mm * 50 mm) and a Carbopac PA 1 column (2*250mm) was used for this analysis (Dionex Corporation). Detection of the eluted compounds was performed by an ED40 EC-detector running in the Pulsed Amperometric Detection (PAD) mode.

To enable quantification of monosaccharides and to get a first impression of the sample composition, 10 µl of a 25 µg/ml sample solution in 100mM Na-acetate buffer pH 5.0 was injected. After injection, compounds were eluted using a flow rate of 0.3 ml/min at 20 °C. Elution was started using a linear gradient from 0.05M NaOAc in 0.1M NaOH to 0.3M NaOAc in 0.1M NaOH in 20 min. This was followed by a linear gradient to 1M NaOAc in 0.1M NaOH in 20 min. After this, the column was washed for 10 min using 1M NaOAc in 0.1M NaOH and equilibrated for 15 min at 0.05M NaOAc in 0.1M NaOH. Calibration curves were obtained by injecting solutions of glucose, methyl α-D-glucopyranoside and glucuronic acid with concentrations varying from 1 to 25 µg/ml.

To get a better view on the less abundant fragments that were below the sensitivity level in the monomer analysis, and to improve the separation of the large and/or acidic oligomers, samples were injected a second time using different conditions. In this case, 10 µl of 2 mg/ml sample solutions in 100mM Na-acetate buffer pH 5.0 were injected. Elution was again performed at a flow rate of 0.3 ml/min, 20 °C, but the gradient was changed. A linear gradient from 0.05M NaOAc in 0.1M NaOH to 0.4M NaOAc in 0.1M NaOH in 35 min was followed by a linear gradient to 1M NaOAc in 0.1M NaOH in 30 min. After this, the column was washed for 5 min using 1M NaOAc in 0.1M NaOH, followed by equilibration for 15 min at 0.05M NaOAc in 0.1M NaOH.
Data were processed using Chromeleon Software (Dionex Corporation).

Fragment structure analysis using MALDI-TOF MS

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to study the exact molecular weight of the fragments released during starch degradation. Prior to analysis, samples were diluted to a concentration of 0.5 mg/ml and subsequently desalted by adding a small spatula of Dowex AG50W-X8 Resin in the hydrogen form (Bio-rad, Hercules, CA, USA) to 100 µl of sample solution. Dowex was then removed immediately by using Ultrafree-MC Durapore PVDF 0.22 µm centrifugal filter devices (Millipore, Bedford, MA, USA). The desalted sample (1 µl) was mixed on a target plate with 1 µl of matrix solution (10 mg DHB in 1 ml milliQ/ACN 70/30 (v/v)) and dried using a stream dry air.

Analysis was performed using an Ultraflex MALDI-TOF MS (Bruker Daltonics) equipped with a 337 nm nitrogen laser. The MS ran in the positive and reflector mode.

After a delayed extraction time of 120 ns was applied, ions were accelerated using a 25 kV kinetic energy. The lowest laser intensity required to obtain an acceptable signal-to-noise ratio was used. Measurements were performed in the *m/z* 500-3000 range. The MS was calibrated using a mixture of maltodextrins (dextrose equivalent 20) with known molecular weights (in the appropriate range). Data were processed using flexAnalysis software (Bruker Daltonics). Based on the relative intensities observed for each oligomer, data were converted into bar graphs. The term "NGO" (Number of Glucuronic acid moieties per Oligomer) was used to indicate the number of glucuronic acid moieties present per oligomer. It was assumed that all molecules were equally ionizable.

Calculation of the number of deuterium atoms present in oligomers

The number of deuterium atoms resulting from aldehyde reduction by using $NaBD_4$, present in oligomers, was calculated based on isotopic distributions in MALDI-TOF mass spectra. The abundance of the natural ^{13}C isotope was assumed to be 1.109%. Furthermore, it was assumed that only non-oxidized Glc moieties could carry a deuterium and that this could only be one deuterium per monomer. The isotopic ratio was subsequently simulated for the several NGOs present, using a binomial distribution. Thereby a random distribution of deuterium in the oligomers was assumed that may be dependent on the DO. The average percentage of Glc carrying a deuterium atom in a certain type of oligomer in the sample followed then from the calculated isotopic ratio that showed the most optimal fit with the measured data. This best fit was based on least square calculations.

Results and Discussion

Analyzable fragments had to be created to study the structure of oxidized potato starches. Starch samples with various degrees of oxidation (DO) were, therefore, hydrolyzed or methanolyzed and subsequently analyzed. The results will be discussed on the basis of the different analysis techniques used.

Molecular weight distribution of samples before and after acidic treatment

Non-oxidized starch and starches with DO 30, 50, and 70 were separately treated with 50mM TFA, 2M TFA, and 2M HCl (in MeOH). Fig. 1 shows the HPSEC results obtained after analysis of the oxidized starches before and after a 20-hour treatment with the acids mentioned.

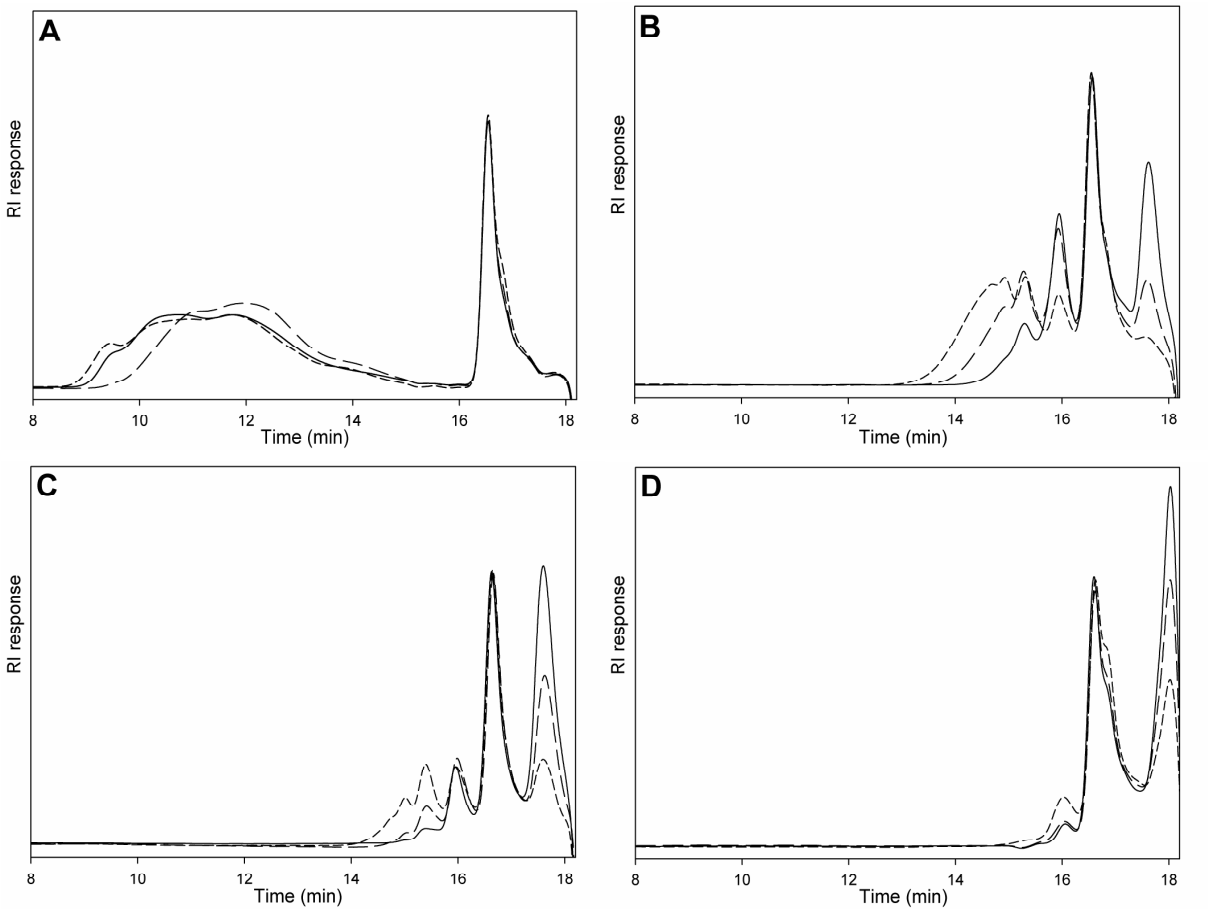

Fig. 1 HPSEC elution patterns of potato starches with DO30 (solid line), DO50 (long dash) and DO70 (short dash) prior to acidic treatment (A), after 20h 50mM TFA hydrolysis (B), after 20h 2M TFA hydrolysis (C) and after 20h 2M HCl methanolysis (D).

The HPSEC profiles of the oxidized starches before acidic treatment (Fig. 1A) show that hydrodynamic volume distributions of the starches with DO30 and DO70 are similar, while the hydrodynamic volume of the starch molecules with DO50 is somewhat lower. Such behavior can be explained in two ways. On the one hand, starch molecules will be partly broken down during the oxidation process because of unstable intermediates *(7)*, causing the hydrodynamic volume to decrease. On the other hand, more charged groups present may cause repulsion within the starch molecule and will, therefore, increase its hydrodynamic volume. In DO30 starch, both effects are relatively small, while the repulsion resulting from the high number of charged groups in DO70 starch compensates for the effect of starch breakdown. The lower number of unstable aldehydes expected in DO70 starch compared to lower DOs makes it, furthermore, less prone to degradation. Therefore, these starches have a similar apparent hydrodynamic volume distribution. In

DO50 starch, the effect of breakdown on the hydrodynamic volume seems to be larger than the effect of charged groups. Therefore, these starch molecules have a lower apparent hydrodynamic volume.

After 50 mM TFA hydrolysis for 20 h, starches have been broken down partly to monomers, but a significant quantity of oligomeric fragments is still present. The higher the DO of the starting material was, the larger the fragments were (Fig. 1B). At a specific temperature, the level of hydrolysis can be expected to be a function of time and acid strength. In 50 mM TFA, the hydrolysis rate was relatively low; the level of hydrolysis was much lower after 8 h of hydrolysis compared to 20 h (data not shown). Non-oxidized starch was broken down completely to monomers and small oligomers under the same conditions. When the concentration of TFA used for hydrolysis is increased to 2M (Fig. 1C), all oxidized starches are broken down to a larger extent than during the 50 mM TFA hydrolysis. Also in this case, a higher DO results in fragments with a larger hydrodynamic volume. After 8 h of hydrolysis, the sample composition was similar to the composition after 20 h (data not shown); the reaction rate is thus higher than during the 50 mM TFA incubations. Non-oxidized starch was broken down completely to monomeric glucose within 2 h. Fig. 1D reveals that oligomers are even present after a harsh acid methanolysis of oxidized starches by using 2M HCl in methanol at 80 °C. It is surprising that oligomers are still present after such a harsh acid treatment at an elevated temperature. This indicates that the linkages in α(1→4) glucuronan are resistant to strong acidic methanolysis. The stability of this type of linkages during acidic lysis was noticed before and can probably for the major part be ascribed to steric factors, as reviewed by BeMiller *(12)*.

De Ruiter et al. *(11)* proposed a method in which 2M HCl in methanol was used for degradation of uronic acid containing water-soluble polysaccharides, while a subsequent 2M TFA hydrolysis was performed to split methylglycosidic bonds prior to HPAEC analysis. Our data imply that this method cannot be applied to α(1→4) glucuronan. Interestingly, the fragments resulting from the incomplete degradation can be used to elucidate the structure of the oxidized starches.

Amounts of monomers released during acidic treatments

To quantify the amounts of monomers that have been released during the acidic treatments, samples were injected on HPAEC in appropriate dilutions. Fifty millimolar TFA was not sufficient to break the native starch down to monomeric glucose (Fig. 2A). Both 2M TFA and 2M HCl lead to a complete breakdown of native starch to monomers (data not shown for HCl). The yield in Glc after 8h/20h 2M TFA hydrolysis was decreasing as a result of

chemical degradation of the monomeric glucose. A release higher than ~90-95% was not reached, which means that about 5-10% of the original sample probably consisted of water or other compounds. Independent of the acid strength, it is clear that the amount of monomers released decreased when DO increased. The higher the acid strength is, the higher the amount of released monomers is, which confirms observations done after HPSEC analysis.

In Fig. 2B-D, an overview of the individual amounts of monomeric Glc and GlcA released during the several treatments of the oxidized starches can be found, expressed as a percentage of the theoretical maximum, which is 100% for each type of monomer.

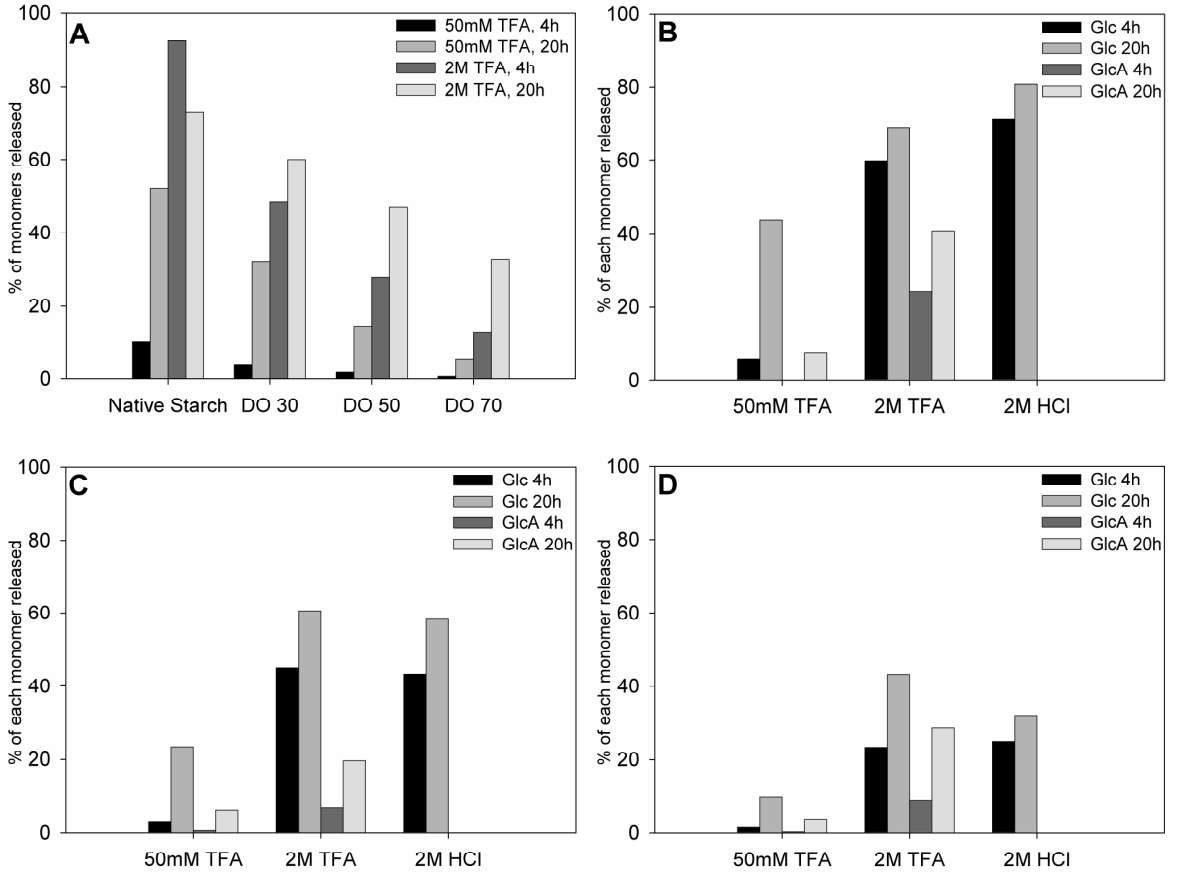

Fig. 2 Overview of the total amount of monomers (Glc and GlcA together) released from the native starch and starch with different DO during hydrolysis (A) and the individual amounts of Glc and GlcA released from the oxidized starches during hydrolysis and methanolysis (B: DO30, C: DO50, D: DO70), all expressed as the percentage of the theoretical maximum. For A, all monomers in the polymer together represent 100%; for B-D, the maximum amount of each type of monomer separately represents 100%. The amount of GlcA O-methylglycoside released during methanolysis was not quantified due to a lacking standard.

The amount of monomers released during 50 mM TFA hydrolysis decreased when DO increased and much more glucose was released compared to glucuronic acid. Only 6% of the DO 70 starch was degraded to monomers. This indicates that α(1→4)GlcA-GlcA bonds are hardly split and that also bonds between Glc and GlcA are hardly split by 50mM TFA. The 2M TFA hydrolysis and the 2M HCl methanolysis lead to a more extensive breakdown of the oxidized starches. During the 2M TFA hydrolysis, more glucose was released compared to glucuronic acid. This could not be quantified for 2M HCl methanolysis, because no standard sample of the methyl glycoside of monoglucuronic acid was available. From HPSEC results it was, however, clear that the 2M HCl methanolysis resulted in fragments with a smaller size than observed after 2M TFA hydrolysis. Furthermore, the peak for monomeric sugars was higher after methanolysis. Especially for DO50 and DO70, the differences in monomeric glucose release are only small. It can, therefore, be concluded that in all cases relatively more monomeric GlcA O-methylglycoside is released during the 2M HCl methanolysis than GlcA during 2M TFA hydrolysis. This altogether means that the α(1→4)GlcA-GlcA bonds are resistant to acidic breakdown depending on acid nature and strength; in descending order: 50mM TFA, 2M TFA, 2M HCl in MeOH. By using 2M TFA, however, more monomeric glucose is released from the DO50 and DO70 starch compared to 2M HCl. This means that the α(1→4)GlcA-Glc bond is more vulnerable to hydrolysis compared to methanolysis under the conditions used. This is probably a result of the O-methylglycosidic glucose unit at the reducing end when methanolysis is applied, which is evidently stabilizing the α(1→4)GlcA-Glc bond. This has to be confirmed by mass spectrometric analysis.

Oligomers released during acidic treatments

Samples were analyzed using HPAEC a second time under changed conditions to get a more detailed view on the oligomeric composition of the starch hydrolysates and methanolysates. Fig. 3 shows elution profiles of acid-treated DO30 starch (4 and 20 h of incubation).

The hydrolysis using 50 mM TFA proceeds relatively slow (Fig. 3A). Some maltodextrins are still present after 4 h of hydrolysis, and between 38 and 55 min a significant amount of large and/or densely charged fragments can be observed. It is interesting that maltodextrins up to degree of polymerization 5 are present after 4 h of hydrolysis using 50 mM TFA, although the quantity seems to be relatively low. This means that blocks of up to at least 5 non-oxidized anhydroglucose units are present within the DO30 starch polymer. After 20 h of hydrolysis, most of the late-eluting fragments have

been degraded (Fig. 3B), only mono- and oligosaccharides can be observed, including some maltose. This is in line with the results of the HPSEC analysis.

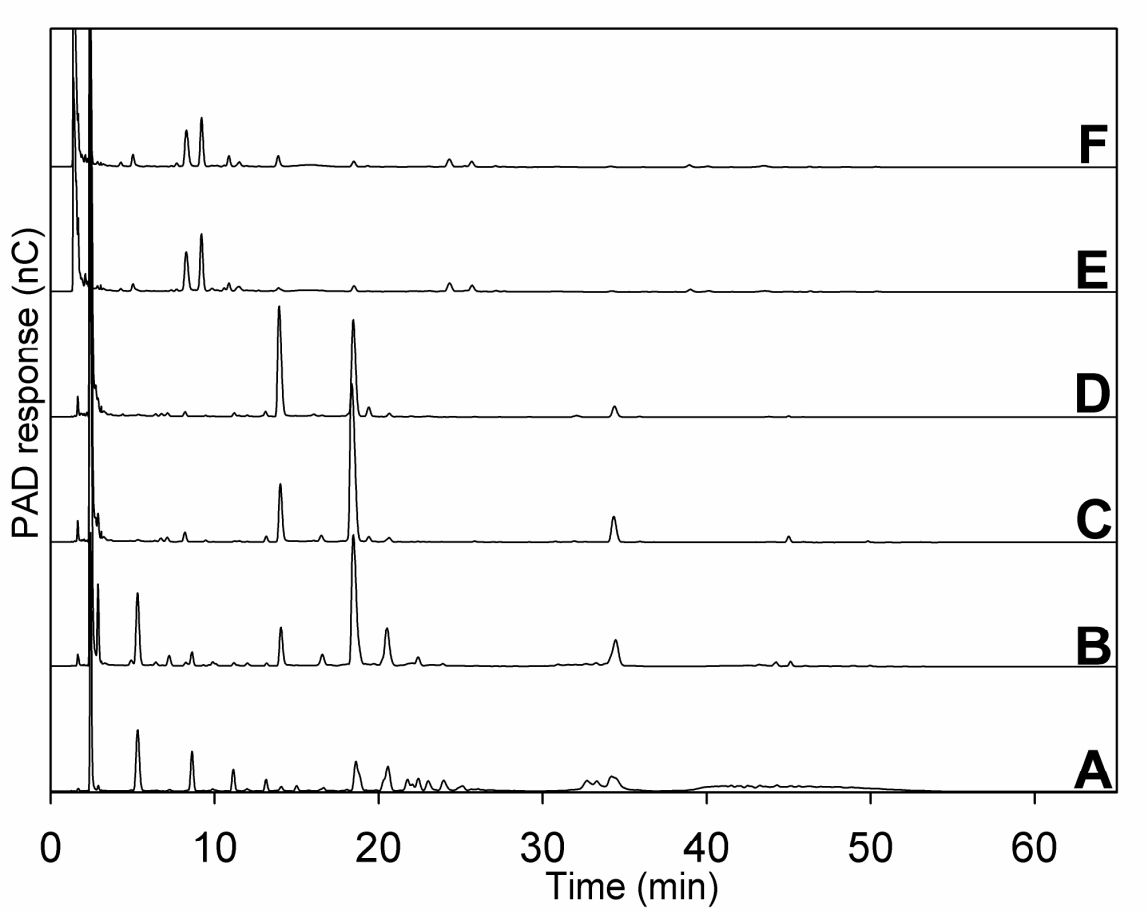

Fig. 3 HPAEC elution profiles of DO30 starch after 50mM TFA hydrolysis (4 h: A, 20h: B), 2M TFA hydrolysis (4 h:C, 20h: D) and 2M HCl methanolysis (4 h: E, 20h: F). Methyl α-D-glucopyranoside is eluted after 1.5 min, glucose after 2.5 min, and glucuronic acid after 13.5 min.

The 2M TFA hydrolysis (Fig. 3C and 3D) is proceeding faster; the only clear difference between 4 and 20 h of incubation is the fact that more monosaccharides have been released after 20 h. The number of peaks is lower after 2M TFA hydrolysis compared to 50 mM TFA hydrolysis. Because of the higher acid concentration, more bonds can be hydrolyzed, which apparently leads to the formation of a less disperse set of products.

The apparent sample composition after methanolysis using 2M HCl (Fig. 3E and 3F) is completely different from the sample composition after hydrolysis. The methyl-glycosidic bonds present in each fragment lead to a shift of the peaks towards shorter elution times, which makes the chromatograms somewhat difficult to compare. However, it can be observed that 2M HCl methanolysis leads to products with a lower size compared to TFA

hydrolysis, as observed before using HPSEC. The similarity between the samples after 4 and 20 h of incubation shows that after a 4-h 2M HCl methanolysis the endpoint of the reaction has already been reached.

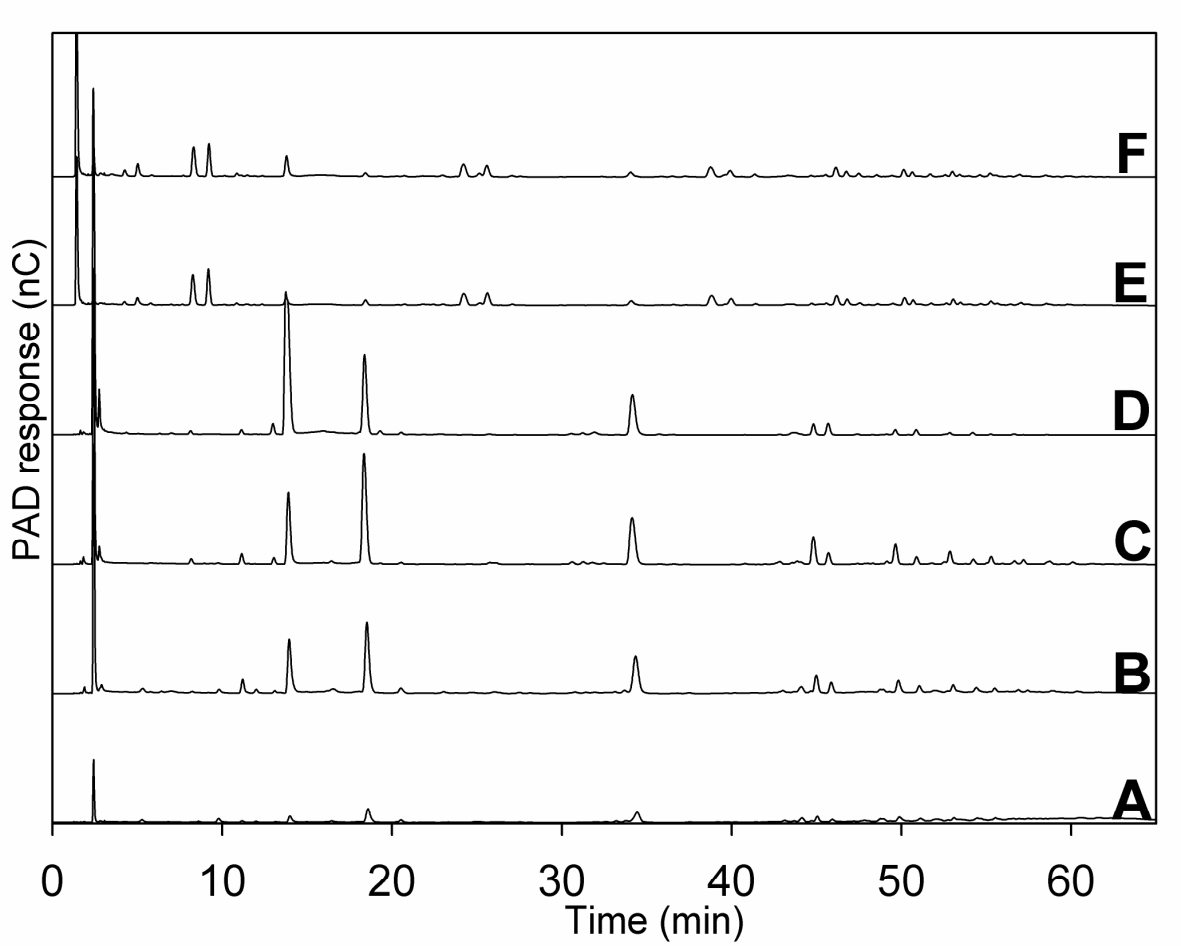

Fig. 4 HPAEC elution profiles of DO70 starch after 50mM TFA hydrolysis (4 h: A, 20h: B), 2M TFA hydrolysis (4 h:C, 20h: D) and 2M HCl methanolysis (4 h: E, 20h: F). Methyl α-D-glucopyranoside is eluted after 1.5 min, glucose after 2.5 min, and glucuronic acid after 13.5 min.

Fig. 4 shows the HPAEC elution profiles of the hydrolyzed/methanolyzed DO70 samples. The most striking differences between Figs. 3 and 4 are within the >35 min elution time region. In the DO70 samples, many late-eluting oligomers are present after hydrolysis/methanolysis, which indicates that they are either large or heavily charged, or both. Apparently, the distribution of the GlcA moieties makes a complete breakdown to small oligomers or monomers impossible. For all DOs, even a one-hour 2M TFA hydrolysis step after 20h of 2M HCl hydrolysis, as proposed by De Ruiter et al. *(11)*, does not affect the backbone of these oligomers (data not shown). This again underlines the conclusion that α(1→4) linked D-glucuronan segments are very resistant to acidic

degradation, even under severe conditions. Compared to results obtained in pectin research, these results are remarkable, as this type of acidic degradations is favorably used for determination of the monomeric composition of uronic acid containing polymers. Very recently *(13)*, a combination of methanolysis and hydrolysis was again recommended for analysis of the sugar composition of, e.g. pectins. However, this treatment appears to be not sufficient for breakdown of oxidized starches.

Polymeric glucuronans have previously been isolated from moulds after a treatment with 2M HCl at 100 °C for 4 hours *(14)*. This is in line with our observations, although these glucuronans consisted of β(1→4) linked uronic acid moieties. The resistance to harsh acidic conditions apparently also accounts for α(1→4) linked uronic acid moieties present in TEMPO-oxidized starch, but not for α(1→4) linked galacturonic acid moieties.

The presence of large oligomers after the acidic treatments furthermore indicates that the distribution of GlcA moieties in starch with a high DO is blockwise. A randomly oxidized starch molecule would at least be degradable to smaller oligomers than observed in these HPAEC spectra. Considering the complete breakdown of native starch, it can be assumed that all Glc-(1→4)-Glc bonds are degraded during the 2M HCl and 2M TFA treatments. The Glc-(1→4)-GlcA bond can also be expected to be degraded, while the GlcA-(1→4)-Glc bond can be expected to be more resistant to the acidic treatment. The same was observed when a polymer containing galacturonic acid (GalA) and galactose (Gal) moieties was degraded by using HF, resulting in GalA-(1→4)-Gal aldobiouronic acids and monomeric Gal *(15)*. This implies that - in our case - glucosyl linkages will be more vulnerable to lysis than glucuronosyl linkages. The more random the distribution of GlcA moieties is, the more uniform the set of degradation products would be. In our case, however, larger oligomers of varying size, which probably mainly consist of GlcA moieties, seem to be present in the polymer. Mass spectrometric techniques will provide more insight into the exact structure of the smaller oligomers (<3000 Da): the results of this analysis will be discussed later. The larger oligomers (>3000 Da) will not be observed by using MS. The fact that these large oligomers are still present after, e.g. the 2M TFA hydrolysis process indicates that these fragments should consist of clustered GlcA moieties, considering the findings with regard to the vulnerabilities of the several bonds present in the polymer, as mentioned above.

Fragment structure determination using MALDI-TOF MS

MALDI-TOF MS analysis was performed to obtain information about the structure of the individual oligomers, and thus about the hydrolysis/methanolysis process, and the structure

of the oxidized starch polymers. Results will be discussed on the basis of the acidic treatment.

Structure of oligomers present after 50 mM TFA hydrolysis

In Fig. 5, the oligomeric composition of the samples after 20h 50mM TFA hydrolysis can be found.

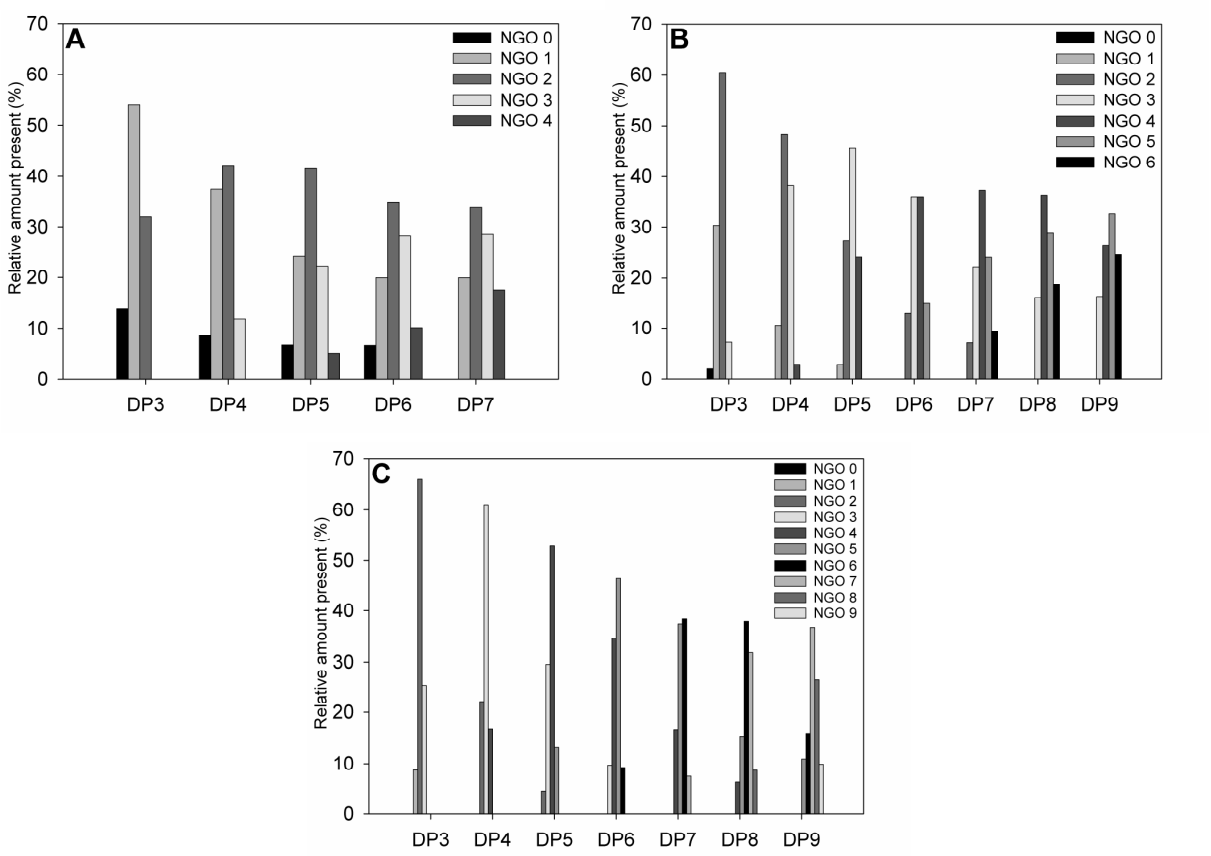

Fig. 5 Sample composition between *m/z* 500-3000 after 20 h hydrolysis using 50mM TFA of DO 30 starch (A), DO 50 starch (B), and DO 70 starch (C), based on MALDI-TOF MS analysis. NGO= number of GlcA moieties per oligomer. The total amount of each DP together represents 100%.

The hydrolysate of DO30 starch contains maltodextrins with DPs up to 6 (Fig. 5A). After 4 h of hydrolysis, even maltodextrins with DP 7 were observed. This indicates the presence of blocks of non-oxidized anhydroglucose units in the polymer. Furthermore, on average not more than half of the sugar moieties present in the oligomers are GlcA moieties. This relative number of GlcA units present in the oligomers decreases when DP increases, while the variety in oligomers present increases. This indicates that the distribution of oxidized groups within the individual oligomers is quite random, assuming

that Glc-Glc bonds are, in general, the most vulnerable to acidic hydrolysis. If the GlcA units were present in blocks, smaller oligomers would have been formed. This would yield a higher average number of GlcA per oligomer.

When polymers with DOs 50 and 70 are hydrolyzed using 50mM TFA (Fig. 5B and C), it appears that the size of the fragments does not increase very much compared to the DO30 samples. The most important trend is that the average number of oxidized groups per oligomer increases with increasing DO. Even fragments solely consisting of GlcA units can be observed in the DO 70 hydrolysate. Furthermore, the relative amount of released monomeric GlcA decreases when DO increases (Fig. 2). This means that the distribution of GlcA units along the polymer has an increasing level of blockiness when DO increases. TEMPO apparently prefers to act in regions were oxidized groups are already present. The fact that the part of the polymer that is degraded to small oligomers decreases when DO increases, as observed in HPSEC analysis, supports this statement.

A restriction of the MALDI-TOF MS analysis is the limited m/z range, which causes the analysis of only a part of the molecules present in each sample. The part of the polymer observed as oligomer in MS analysis can, however, be estimated based on the monomer quantification and on HPSEC results. Depending on the DO, 5-30% of the starch was recovered as monomer (Fig. 2A). Based on HPSEC calibration, it can, furthermore, be calculated that compounds eluting >15 min are observed in the MALDI-TOF MS analysis (data not shown). When these data are combined with HPSEC (Fig. 1B), the oligomers in Fig. 5 can be estimated to represent the following part of the original oxidized starches: DO30: 55%, DO50: 55%, and DO70: 40%. It is hard to draw strong conclusions on the structure of the 15-50% of the polymer that was not observed, since not even all Glc-Glc bonds present in native starch were hydrolyzed in 50 mM TFA. The reduced degradability when DO increases, however, suggests that at least a significant number of clustered GlcA units are present in these relatively large oligomers.

Structure of the oligomers present after 2M TFA hydrolysis

The 2M TFA treatment mainly yields oligomers consisting of GlcA units carrying one Glc unit, the size of these oligomers increases when DO increases. After 20 h, oligomers solely consisting of GlcA units are present, they represent about 30% of the DP 3-7 oligomers present in the DO70 hydrolysate (graphs not shown). From Fig. 2A it is known that 30-60% of the polymer is degraded to monomers (depending on DO). It can then be roughly estimated based on HPSEC results (Fig. 1C) that the oligomers that were actually observed in the MALDI-TOF MS analysis approximately represent the following percentage of the

original oxidized polymers: DO30: 35%, DO50: 40%, and DO70: 40%. This means that these results provide a realistic view on the structure of the original polymer. The part of the oligomers that is not observed consists of structures that are highly resistant to 2M TFA hydrolysis, which are most probably large blocks of GlcA moieties. These results confirm the increasing blockiness with increasing DO.

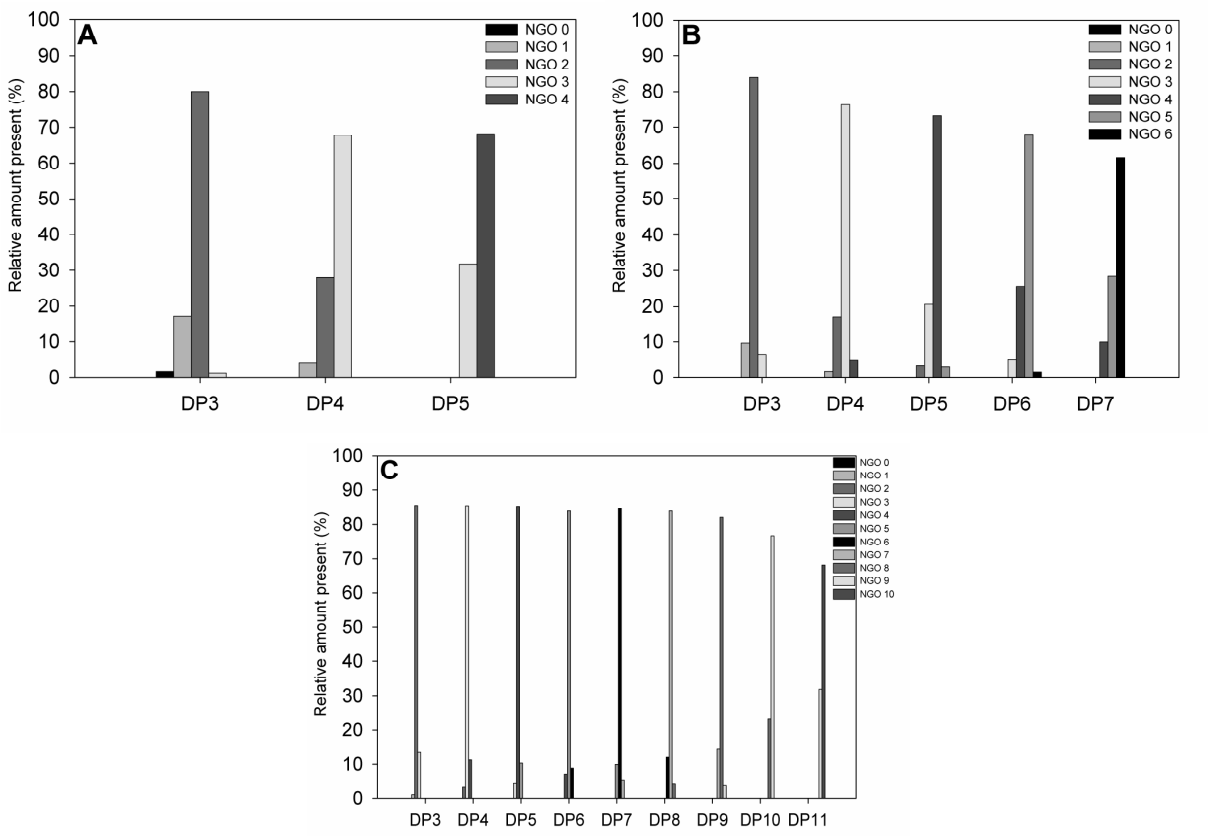

Fig. 6 Sample composition between *m/z* 500-3000 after 4 h methanolysis using 2M HCl of DO 30 starch (A), DO 50 starch (B), and DO 70 starch (C), based on MALDI-TOF MS analysis. NGO= number of GlcA moieties per oligomer. The total amount of each DP together represents 100%.

Structure of oligomers present after 2M HCl methanolysis

In Fig. 6, the composition of the samples after 4 h 2M HCl methanolysis can be found. The time point of 4 h was chosen to minimize the effect of GlcA-GlcA bonds that had been split. Based on HPSEC results, it is known that all fragments are within the m/z range of this MALDI-TOF MS analysis, even after 4 h of incubation (data not shown). Therefore, these fragments, in combination with the monomer analysis, provide a realistic view on the structure of the original polymer. The maximum number of oxidized groups observed per oligomer is about the same as after 50mM TFA hydrolysis, but the proportion of oxidized moieties per oligomer is much higher. Furthermore, the maximum DP of the oligomers

increases when DO increases. The fact that the maximum size of GlcA blocks that can be observed is increasing when DO increases (DO 30: 4, DO 50: 6, and DO 70: 10) indicates that the degree of blockiness is increasing when DO increases. In the very first stage of the oxidation process, TEMPO is probably randomly acting at different places in the polymer. When the oxidation proceeds, TEMPO apparently has a preference to act close to groups that have already been oxidized. This causes the presence of relatively small GlcA blocks in the DO 30 starch, the size of these blocks is then increasing when DO increases, as observed in Fig. 6.

MALDI-TOF MS analysis furthermore suggests that the α(1→4) GlcA-Glc bond is more vulnerable to hydrolysis compared to methanolysis. During 2M HCl methanolysis of DO70 starch, hardly any oligomers solely consisting of GlcA were formed. Only some low DP GlcA oligomers were observed after 20 h of incubation (data not shown), probably as a result of GlcA-GlcA bonds being split. Two moles TFA hydrolysis for 20 h did release relatively more oligomers solely consisting of GlcA from DO70 starch, as discussed before. The O-methylglycosidic glucose moiety apparently increases the resistance of the α(1→4) linkage with the GlcA moiety to acidic breakdown.

Abundance and location of aldehydo groups after starch oxidation

During the oxidation reaction, hydroxyl groups are converted into aldehydes by activated TEMPO. These aldehydes are subsequently converted to carboxyl groups by OCl^-. As determined in a kinetic study using methyl-α-D-glucopyranoside as a substrate *(16)*, the latter is the most rapid reaction. The maximum concentration of aldehydes that can be expected in the reaction medium is, however, still about 10%. The amount of OCl^- used in our experiments indicates that these data are also applicable when starch is oxidized (data not shown). After oxidation, the remaining aldehydo groups, which make the polymer unstable, were reduced to hydroxyl groups. For this, $NaBD_4$ was used instead of the usual $NaBH_4$, $NaBD_4$ causes the incorporation of a deuterium atom instead of a hydrogen atom at the location of the reduced aldehydo groups. These locations are then visible in subsequent mass spectrometric measurements. In Fig. 7A, a zoom-in of a MALDI-TOF mass spectrum after 4 hours of 50mM TFA hydrolysis of DO 30 starch can be found. This sample was chosen because it also contains oligomers which do not contain GlcA moieties. The zoom-in shows the maltohexaose without any oxidized units. Furthermore, oligomers with DP 6

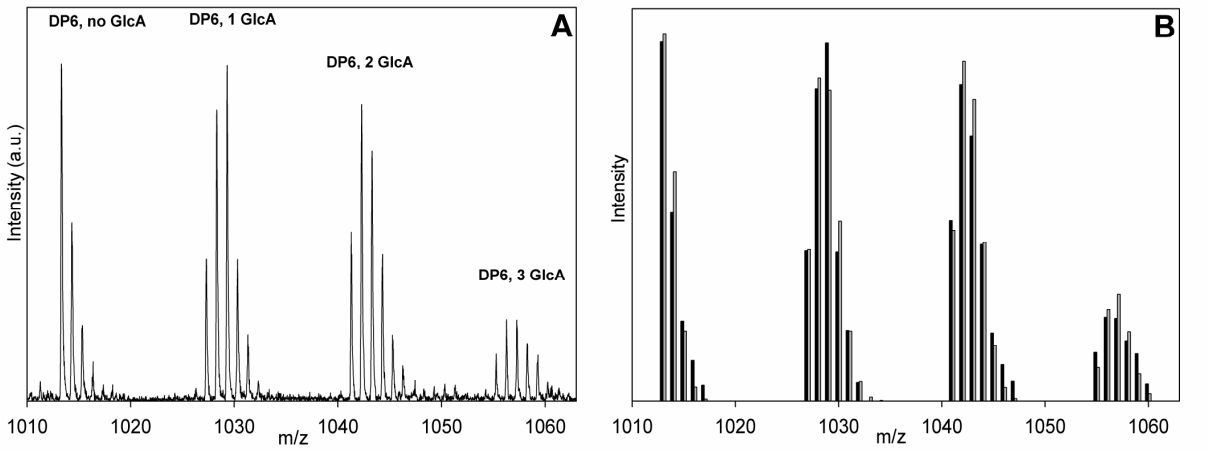

Fig. 7 Zoom-in on the DP6-region of the MALDI-TOF mass spectrum of the 50mM TFA hydrolysate of DO 30 starch after 4 hours of incubation (A) and this measured isotopic distributions (black) combined with the calculated isotopic distributions showing the best fit (gray) in a bar-graph (B). The original maltohexaose has an m/z value of 1013. An increase of this m/z value to 1027, 1041, and 1055 can be observed, which indicates the presence of 1, 2, and 3 GlcA moieties in the oligomer, respectively.

containing 1, 2, and 3 GlcA moieties can be observed in the spectrum. The isotopic distribution of the oligomers without any GlcA moieties seems to be normal, while this distribution changes when one or more oxidized groups are included. A similar trend was observed for other DPs. To determine how many deuterium atoms are on average present in each of the DP 6 oligomers, calculations were made in which the abundance of the natural ^{13}C isotope was taken into account. The percentage of anhydroglucose units carrying a deuterium atom was varied, and the best fit with the measured values was determined. In Fig. 7B, this measured isotopic distributions are combined with calculated isotopic distributions which showed the best fit, in a bar-graph.

In Table 1, the contribution of each type of DP6 oligomer to the total can be found. Furthermore, the average amount of deuterium present in these oligomers can be found, expressed relative to number of Glc units present and relative to the total 6 moieties present in the oligomer. In the last column, the total percentage of moieties converted to either GlcA or an aldehyde can be found.

The oligomers that are not carrying any GlcA unit show a minor amount of deuterium groups (3%), which means that hardly any aldehydo groups have been present in the region where these oligomers result from. The amount of deuterium present in the oligomers, relative to the number of Glc moieties present, substantially increases when one GlcA unit is present, and it further increases when more GlcA units are present. This indicates that a

Table 1 Overview of the contribution of each type of DP6 oligomer to the total amount of DP 6 oligomers in the DO30 sample after 4h of hydrolysis using 50mM TFA. Furthermore, the average amount of deuterium atoms relative to (i) the number of Glc and (ii) the total 6 moieties, in each type of oligomer, are provided. In the final column, the average percentage of moieties in the oligomer converted to either GlcA or and aldehyde are provided for each type of DP6 oligomer. Numbers are based on the data in Fig. 7.

DP6 with NGO:	***% of total DP6 oligomers (amount)***	***% of remaining Glc carrying a deuterium atom***	***% of 6 moieties carrying a deuterium atom***	***% of 6 moieties converted to GlcA/aldehyde***
0	22	3	3	3
1	34	20	16	33
2	33	21	14	47
3	11	32	16	66

relatively large amount of aldehydes has been present in the regions where GlcA units are present. On average, one aldehydo group (~16%) has been present in each DP6 oligomer containing one or more GlcA units. This illustrates that TEMPO prefers to act in a region where GlcA moieties are present already, regardless of the exact number of GlcA present.

These observations can only be explained by a strongly clustered TEMPO oxidation and subsequent conversion to GlcA already in the initial phase of the starch oxidation process. Once TEMPO is active in a certain region of the polymer, it tends to keep on being reactive in this region. The explanation of this probably lies in the fact that the oxidized form of TEMPO, which is the oxidizing agent, is the positively charged nitrosonium ion. Conversely, the carboxyl groups are negatively charged at the pH applied. The attraction between these charges causes the action of the nitrosonium ion close to regions that contain one or more GlcA moieties. This leads to clusters of GlcA units and Glc units carrying an aldehydo group on the one hand, and clusters of non-modified Glc units on the other hand.

Conclusions

The α(1→4) GlcA-GlcA bond present in TEMPO-oxidized potato starch was found to be very resistant to acidic degradation, while the α(1→4) GlcA-Glc bond also showed increased resistance compared to the α(1→4) Glc-Glc bonds. This will on the one hand lead to incorrect results when these methods are used for analysis of the monomeric composition of oxidized starches. On the other hand, the oligomers obtained after acidic fragmentation could be applied to elucidate the structure of these modified biopolymers. It was found that

TEMPO oxidation of starch leads to the formation of blocks of GlcA within the polymers. The size of these blocks increases when the degree of oxidation of the polymer increases. Apparently, the TEMPO radical tends to be active close to groups that have already been converted to GlcA. This can be explained by the negative charge carried by the carboxyl groups at the pH applied during the oxidation process; this attracts the positively charged activated TEMPO. This non-random action of TEMPO was confirmed by the presence of aldehydes clustered around regions were GlcA units were detected, just after the oxidation process.

To date, the distribution of the GlcA units along the backbone of TEMPO-oxidized starch was unknown, while this distribution can be expected to have an impact on the functionality of the polymer. This is also the case, for example, in citrus pectins. The distribution of methylated, uncharged groups along the charged polymer, indicated by the degree of blockiness, had a significant impact on its gelling behavior at different pH and Ca^{2+} levels *(17)*. Our findings may, therefore, lead to an improved functionality of TEMPO-oxidized starch polymers.

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Chapter 3

Synthesis and application of epoxy starch derivatives

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Abstract

Epoxy starch derivatives were synthesized by epoxidation of allylated starch. The reaction was performed with low substituted 1-allyloxy-2-hydroxypropyl-waxy maize starch (AHP-WMS; degree of substitution (DS) of 0.23) using hydrogen peroxide and acetonitrile. Via a two-step spectrophotometric assay, it was determined that epoxy-WMS contained 0.13 ± 0.03 mmol epoxy groups per gram dry allylated starch which corresponds to DS value of 0.025. Enzymatic digestibility, swelling capacity and solubility were significantly reduced after epoxidation. The detailed chemical structure of epoxy-WMS was characterized by enzymatic hydrolysis followed by chromatographic and mass spectrometric techniques. Only a small amount of epoxidized oligomers was found in the enzymatic digested products of epoxy-WMS. Apparently, the epoxidation reaction is highly efficient but subsequent reactions of epoxy groups lead to a considerable amount of cross-links and diol groups. Additionally, epoxy starch derivatives were successfully applied as carrier matrix for immobilization of an enzyme.

Introduction

Because of biocompatibility, degradability and low costs, starch and starch derivatives are used as excipients for tabletting and matrices for delivery systems in pharmaceutical industry *(1, 2)*. There is a strong interest in chemically modified starch derivatives possessing functional groups that allow easy binding of ligands to the surface. Amongst these starch products are derivatives possessing epoxy functions *(3-6)*. These reactive epoxy groups allow easy binding of ligands such as polyamines, peptides and amino acids. Moreover, epoxy groups can advantageously serve as cross-linker in starch. Hence, these starch derivatives can be used in coatings and stabilizers, but they can also serve as carriers for delivery systems and controlled drugs release.

Incorporation of epoxy groups into carbohydrates can be performed directly *(7-9)* and indirectly *(4, 5, 10)*. Direct integration of epoxy groups can be achieved using epichlorohydrin *(7, 8)* or diepoxides *(8, 9)*. Disadvantages of these routes are the side reactions during the synthesis, use of hazardous reagents like butadiene diepoxide as well as harmful solvents such as dimethyl sulfoxide and dichloromethane. Indirect incorporation of epoxy groups can be accomplished by oxidation of double bonds attached to the polysaccharide using peracids and/or hydrogen peroxide *(4, 10)*. Allyl cellulose was efficiently epoxidized using peracids, although this reaction pathway resulted in partial hydrolysis of the cellulose derivative. A major drawback is that the used solvent (dichloromethane) is not applicable for large scale reactions.

In previous studies *(11, 12)*, double bonds were introduced into starch by the reaction of maize starch and allyl glycidyl ether (AGE). Using mild reaction conditions, starch granules reacted with a small amount of AGE, and low etherified starch derivatives (degree of substitution up to 0.23) were obtained. These low allyl substitutions already induced alteration in properties and behaviour of starch *(11, 13)*. The current paper presents the results obtained from the epoxidation of double bonds in these allyl waxy maize starch derivatives. Both, the amount of epoxy groups in the product as well as the structure of epoxy starch derivatives were studied. Furthermore, covalent immobilization of an amine and a protein onto the synthesized epoxy starch products was investigated.

Experimental

Materials

1-Allyloxy-2-hydroxypropyl-waxy maize starch (AHP-WMS; DS_{allyl} = 0.23) was synthesized as described before *(13)*. Acetonitrile (99.5%) and 1,2,5,6-diepoxyhexane (97%) were purchased from Acros Organics (Belgium). Hydrogen peroxide (35% weight solution) was obtained from Chem-Lab NV (Belgium). 1,6-Hexanediamine (HD) (≥99%) and 4-(*para*-nitrobenzyl)pyridine (*p*-NBP) (≥98%) were from Fluka (Switzerland). Allyl glycidyl ether (AGE; ≥ 99%), 1,3-butadiene diepoxide (97%), 1,2,7,8-diepoxyoctane (97%), potassium carbonate (≥99%), phosphate salts, dimethyl sulfoxide (DMSO, 99.5%), ethylene glycol (≥99%), methyl α-D-glucopyranoside (MG), *para*-nitrophenyl-β-D-glucopyranoside (≥99%), α-amylase (EC 3.2.1.1) (from *Bacillus licheniformis*, 408 U mg^{-1}) and amyloglucosidase (EC 3.2.1.3) (from *Rhizopus* sp., 11600 U g^{-1}) were obtained from Sigma–Aldrich Chemie BV. Pullulanase (EC 3.2.1.41) (from *B. licheniformis*, 400 U mL^{-1}) was obtained from Megazyme (Ireland). β-amylase (EC 3.2.1.2) (from barley, 45 U mg^{-1}) was purchased from Merck (Belgium). The thermostable enzyme β-glucosidase from the hyperthermophilic archaea *Pyrococcus furiosus* expressed in *Escherichia coli*, was kindly supplied by the Laboratory of Microbiology of Wageningen University.

15-mL Conical centrifuge tubes (PP) were obtained from Corning Incorporated (United States). 3.5-mL Green capped tubes (PP) were purchased from Greiner Bio-One (Germany). Cuvets (Hellma; 1 mm, 45 × 12.5 × 3.5 mm, 350 μL) were from Elcolab (The Netherlands). Micron Centrifugal Filter Devices with Ultracel YM-10 membrane were purchased from Millipore Corporation (United Kingdom).

Methods

Coupling of methyl α-D-glucopyranoside with diepoxides

Methyl α-D-glucopyranoside (MG) (0.97 g, 5 mmol) was dissolved in 5 mL demineralised water in a 25 mL teflon-sealed bottle. After addition of NaOH (0.10 g, 2.5 mmol) and Na_2SO_4 (0.36 g, 2.5 mmol), the solution was heated to 45 °C in a shaking water bath. Diepoxide (15 mmol; 1.16 mL 1,3-butadiene epoxide, 1.75 mL 1,2,5,6-diepoxyhexane or 2.14 mL 1,2,7,8-diepoxyoctane) was stepwise and slowly mixed to start the reaction. The reaction was followed by thin layer chromatography (eluent: ethyl acetate). After TLC indicated complete conversion of MG, the mixture was cooled in an ice-bath and neutralized by adding 1.0 M HCl-solution (pH = 7). Residual diepoxide was removed by

extraction with diethyl ether (3 × 5 mL). The aqueous layer was dried by lyophilisation. The products were analyzed by HPLC, using a Waters Spherisorb S5-amino column (250 × 10 mm). Elution of the sample components was by an ethyl acetate-methanol gradient (increased from 7:93 to 25:75 by elution of 1 mL min–1). For detection, an Alltech Evaporative Light Scattering Detector (Alltech/Grace, Deerfield, IL, USA) was used. Increasing diepoxide:MG ratios resulted in an increasing number of components in the product. Subsequent LC–MS analysis additionally showed increasing molecular masses of these product components. However, in all cases it was clear that the products did not contain sugars.

Epoxidation of allylated starch

AHP-WMS (10.0 g, 10% w/w H_2O, 0.051 mmol anhydrous glucose unit (AGU), DS_{allyl} = 0.23) was suspended in an aqueous solution of demineralized water (35 mL), Na_2CO_3 (1.75 mg, 0.017 mol) and $NaHCO_3$ (350 mg, 4.17 mmol), and CH_3CN (5 mL, 0.11 mol). The suspension was stirred, while being heated to 30 °C. The reaction was initiated by adding H_2O_2 (35% wt., 9.4 mL, 0.11 mol) in portions of 100 µL during 30 min. After that, the mixture was stirred at 30 °C for 6 h, then cooled down to room temperature, and finally water (25 mL) was added. The product was obtained by filtration on a glass filter (G3), and subsequently washed with water (5 × 75 mL), ethanol (3 × 75 mL) and acetone (3 × 75 mL). The remaining white powder, epoxy-WMS (9.52 g, 9.5% w/w H_2O, yield: 96%), was dried overnight at room temperature and stored at –20 °C.

Coupling of 1,6-hexanediamine to epoxy-starch

Epoxy-WMS (100 mg, 1 equiv.) was suspended in water (0.9 mL), followed by addition of 1 mL of 66 mM 1,6-hexanediamine (HD, 7.67 g mL^{-1} in water) corresponding to five equivalents. The suspension was stirred for one night at 30 °C. The product was isolated at room temperature using an Ultracel YM-10 membrane by centrifugation at 13,400 rpm for 1 h and washing with water (5 × 1 mL). After lyophilisation, a white powder was obtained (HD-WMS; 98 mg, yield: 98%).

Enzymatic degradation

For the enzymatic digestion of the samples, α-amylase, pullulanase, amyloglucosidase and β-amylase were dissolved in deionised water purified by Millipore Milli-Q Gradient A10 (Millipore, United Kingdom), resulting in stock solutions containing 0.44, 0.40, 0.14 and 0.45 U μL^{-1}, respectively.

The procedure for the enzymatic degradation of native starch and its derivatives was as follows: Samples (5 mg, 0.3 mmol) were dissolved in 1 mL 0.05 M sodium acetate buffer (pH 5.0). After pregelatinization of the starch solutions for 10 min at 95 °C, incubation was performed with a combination of pullulanase (5 µL), α-amylase (5 µL) and amyloglucosidase (5 µL) at 40 °C for 18 h. Incubation β-amylase (5 µL) was performed separately at 25 °C for 18 h. Afterwards solutions were boiled for 10 min.

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was carried out using an HPLC (Thermo Scientific, Waltham, MA, USA), with three TSK-gel columns in series (7.5 × 300 mm per column, G4000PWXL, G3000 PWXL and G2500PWXL; TosoHaas, Japan), in combination with a PWXL-guard column (40 × 6 mm; TosoH, Tokyo, Japan). Elution was performed at 30 °C with 0.2 M $NaNO_3$ at a flow rate of 0.8 mL min^{-1}. After injection of a 100-µL sample, the eluting compounds were analyzed with a refractive index detector (Shodex RI-101, Showa Denko K.K., Japan). The data were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

High-performance anion exchange chromatography

High-performance anion exchange chromatography (HPAEC) was performed on an HPLC system (Dionex Corporation). The system was equipped with a quaternary gradient pump, an AS3000 autosampler with a helium degassing unit and an ED40 EC detector in pulsed amperometric detection (PAD) mode. The CarboPac PA1 column (2 × 250 mm; Dionex, United States) with a CarboPac PA1 guard column (2 × 50 mm; Dionex) was operated at a flow rate of 0.3 mL min^{-1} at 20 °C. 20 µL of 10-fold diluted sample was injected and a linear gradient from 0.0 M to 0.5 M NaOAc in 0.1 M NaOH within 40 min was applied, followed by a linear gradient in 5 min to 1 M NaOAc in 0.1 M NaOH. Finally, the column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, and equilibrated for 20 min with 0.1 M NaOH. The data were processed using Chromeleon software (Dionex, United States).

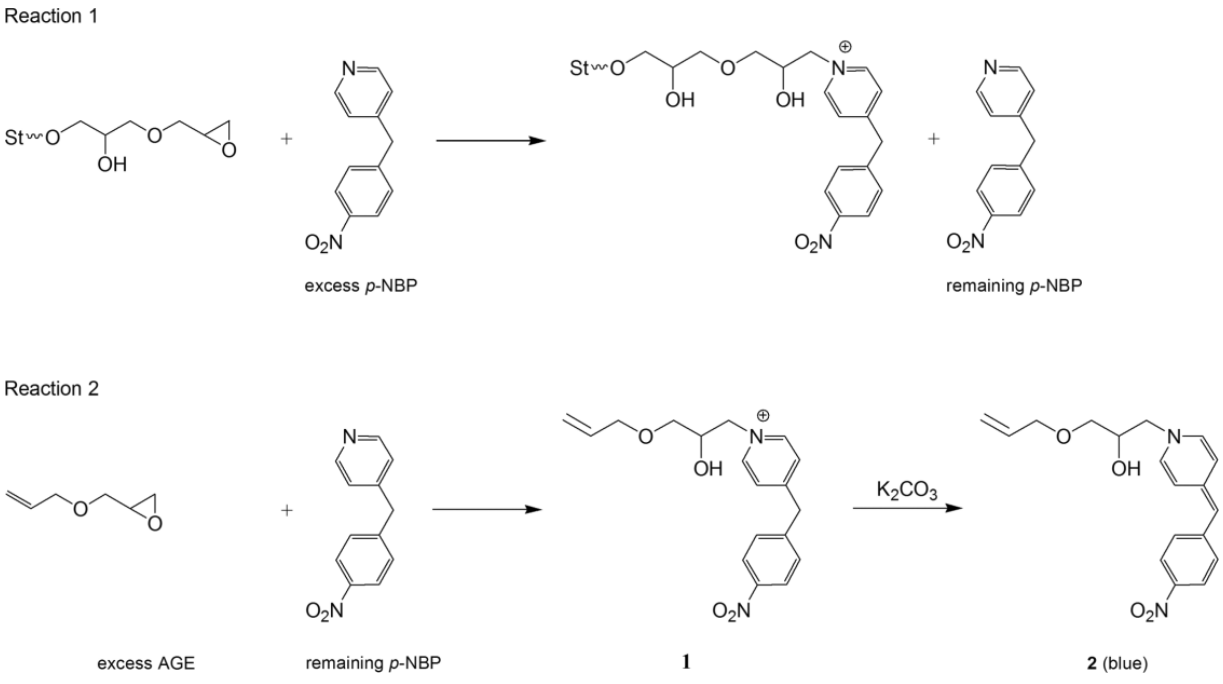
MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight masss (MALDI-TOF MS) was carried out using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. The mass spectrometer was operated in the positive mode and

was calibrated with a mixture of maltodextrins (mass range = 400–3500 Da). After a delayed extraction of 120 ns, the ions were accelerated with a kinetic energy of 25 kV. Hereafter, the ions were detected in the reflector mode. 1-µL 10-fold diluted sample and 1 µL of the matrix solution was mixed and dried on target. The lowest laser power required to obtain good spectra was used. The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in a 1-mL mixture of acetonitrile:water (300 µL:700 µL).

Determination of epoxy groups

The amount of epoxy groups was measured using a spectrophotometric assay adapted from the literature *(14, 15)*. All assays were performed in tubes (3.5 mL or 15 mL). Absorptions at 600 nm were measured using 1-mm cuvets in a Cary 100 UV-vis spectrophotometer, thermostated at 20 °C, and followed with Cary WinUV Kinetic Software. Epoxy-WMS, AHP-WMS as a control, and a calibration curve of 20–100 mM *p*-NBP were used in the two step spectrophotometric assay. The principle of the assay is described in Scheme 1.



Scheme 1 Two-step spectrophotometric assay to quantify epoxy groups in modified starch (St). p-NBP = 4-(para-nitrobenzyl)pyridine; addition of base converts product **1** into the blue chromophore **2**

Reaction 1

In a 15-mL tube, epoxy-WMS (0.1–0.3 g, 9.5% H_2O, 0.35–0.70 mmol AGU) was suspended in 2 mL of 90 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7, containing 10% (v/v)

DMSO. Subsequently, 2 mL of freshly made 50 mM *p*-NBP (100 µmol) in an ethylene glycol/ethanol mixture (80/20 v/v) was added to the suspension. After vortexing, the tube was placed in a heating bath at 80 °C for 2.5 h. The reaction mixture was cooled on ice for 10 min and centrifuged for 15 min at 15,200 rpm.

Reaction 2

The supernatant was analyzed for the amount of remaining (unreacted) *p*-NBP as follows. 1 mL of the supernatant was pipetted into a 3.5-mL tube and mixed with 1 mL freshly made 50 mM AGE (50 µmol) in 90 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7, containing 10% v/v DMSO. Again, the reaction was performed in a heating bath at 80 °C for 2.5 h. The samples were cooled on ice for 10 min and diluted fourfold. Before blue colour development was induced with 100 µL 1 M K_2CO_3, the diluted mixture (200 µL) was mixed with 200 µL ethanol. The sample was rapidly mixed with K_2CO_3 and the absorption at 600 nm (A_{600}) was measured after 1 min.

All assays were performed in triplicate, after which the epoxy content was calculated using the calibration curve and the following equation:

$$n_{\text{epoxy}} = n_{p\text{-NBP}} - n_2 \qquad (1)$$

where n_{epoxy}, $n_{p\text{-NBP}}$ and n_2 are the moles of epoxy groups on the modified starch, the number of moles of *p*-NBP initially (Scheme 1, reaction 1) added and the number of moles of the blue chromophore **2** of *p*-NBP product **1** (Scheme 1, reaction 2), respectively.

Amino substitution

The nitrogen (N%) and carbon contents (C%) of HD-WMS and epoxy-WMS were obtained using a Fisons EA1108 elemental analyzer. In the DS_{HD} calculation, we assumed that only one amino group of hexanediamine (HD) binds to an epoxy group, leaving one amino group free. DS_{HD}, expressed as moles amino groups per mole glucose units, was determined using *n*N (N%/MW; N-atom MW = 14.01), *n*C (C%/MW; C-atom MW = 12.01), and the number of atoms in the sample according to the following equation:

$$\frac{n\mathrm{C}_{\mathrm{HD}}}{n\mathrm{N}_{\mathrm{HD}}} = \frac{\mathrm{C}_{\mathrm{AGU}} + \mathrm{C}_{\mathrm{AGE}} \cdot \mathrm{DS}_{\mathrm{allyl}} + \mathrm{C}_{\mathrm{HD}} \cdot \mathrm{DS}_{\mathrm{HD}}}{\mathrm{N}_{\mathrm{HD}} \cdot \mathrm{DS}_{\mathrm{HD}}} \qquad (2)$$

where $n\mathrm{C}_{\mathrm{HD}}$ is the moles of C-atoms in HD-WMS; $n\mathrm{N}_{\mathrm{HD}}$ is the moles of N-atoms in HD-WMS (nN minus the nN from N-containing compounds of epoxy-WMS proportionally); $\mathrm{C}_{\mathrm{AGU}}$, $\mathrm{C}_{\mathrm{AGE}}$ and C_{HD}-atoms correspond to the number of C-atoms in AGU (in starch), in AGE or HD, respectively; N_{HD} is the number of N-atoms in hexanediamine. $\mathrm{DS}_{\mathrm{allyl}}$ of AHP-WMS is 0.23.

Physicochemical properties

The temperature of gelatinization of the modified starches was measured using a Differential Scanning Calorimeter (DSC) (Perkin Elmer DSC-7, Boston, USA). Sample preparation and analysis were performed as described previously *(13)*. The PE Pyris – DSC-7 software was used for data handling. Onset temperature (T_o), peak temperature (T_p), completion temperature (T_c) and enthalpy of gelatinization (ΔH_{gel}) were calculated from the DSC thermogram. ΔH_{gel} was based on the dry material (J g^{-1} dry starch). The swelling power and solubility index of starches was determined using the previously published method *(13)*. The experiment was repeated three times. The mean values are reported, with standard deviation.

Preparation of immobilized β-glucosidase

For preparation of immobilized β-glucosidase, 50 mg of epoxy-WMS was swollen in 1.0 mL 50 mM sodium citrate buffer pH 5.0 at 80 °C for 1 h. Subsequently, 55 µL β-glucosidase (solution of 0.9 mg/mL enzyme) was added and mixed with swollen starch gel. Incubation at 80 °C was continued for 30 min under frequent mixing. Gel formation was completed by cooling the mixture to room temperature.

Release of unbound β-glucosidase from the starch gel was investigated by repeated washing steps followed by centrifugation. The supernatant obtained was stored for the determination of activity recovery of unbound β-glucosidase. Washing of the starch gel was performed by suspending the gel pellet with 0.5 mL citrate buffer pH 5.0 followed by centrifugation at 12,000 rpm, 4 °C for 10 min. The gel pellet obtained after four washing steps was used for determination of the β-glucosidase activity immobilized in epoxy-WMS gel.

Assay of β-glucosidase activity

Activities of free and immobilized β-glucosidase were assayed using *para*-nitrophenyl-β-D-glucopyranoside as substrate *(16)*. Hydrolysis of *para*-nitrophenyl-β-D-glucopyranoside was followed by spectrophotometric measurements at 50 °C *via* continuous determination of the increase of absorbance at λ = 405 nm due to the liberation of *para*-nitrophenol. The reaction mixture contained 1.0 mL 50 mM sodium citrate buffer pH 5.0, with 5 mM *para*-nitrophenyl substrate (final concentration 4.0 mM), and 0.2 mL free enzyme or starch gel. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the liberation of 1.0 μmol of *para*-nitrophenol per minute at 50 °C under these conditions, using a molar extinction coefficient of 290 $M^{-1}cm^{-1}$ at 405 nm for *para*-nitrophenol. Activity recovery was related to the activity of free β-glucosidase, obtained after treatment of the enzyme solution in a similar way as in the immobilization procedure, at 80 °C.

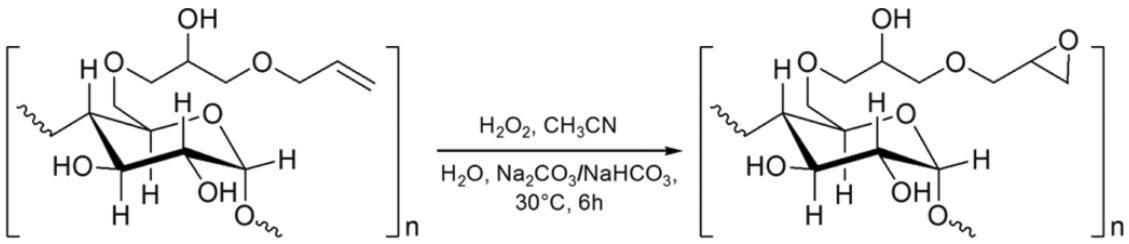
Results and discussion

Direct introduction of epoxide moieties

A one-step route to synthesize epoxy starch derivatives is the use of diepoxides *(8, 9)*. This option was explored in a system consisting of methyl α-glucopyranoside (MG) as a model compound, and 1,3-butadiene diepoxide, 1,2,5,6-diepoxyhexane and 1,2,7,8-diepoxyoctane as epoxide donors. The slightly yellow-coloured clear reaction mixtures were worked up and the products of each reaction were isolated. The transparent foamy products appeared to be a very complex mixture of mainly ring-opened and cyclised diepoxides *(17)* and unreacted MG, as concluded from HPLC and LC–MS analysis (not shown). Reference compounds for HPLC analysis were prepared by reaction of diepoxides under similar conditions, but without MG. Comparing HPLC analysis of products isolated from reactions with and without MG showed that reactions with MG yielded mainly compounds derived from epoxide cross-linking, and unreacted MG (not initially detected by TLC before work-up due to possible entrapment into polymerised epoxides). No epoxy MG derivatives were detected. This indicated that diepoxides rather react with themselves than with another compounds. Therefore, substitution of starch with diepoxides is not an option for the synthesis of epoxy starch derivatives under the conditions applied.

Epoxidation of allyl starch

A more viable route for synthesizing epoxy starch derivatives is the oxygenation of allylated starch *(5)*. The double bonds of granular AHP-WMS were epoxidized using H_2O_2 and CH_3CN in slightly alkaline suspension at 30 °C (Scheme 2). The combination of hydrogen peroxide and acetonitrile is a well-known reagent for the epoxidation of carbon double bonds *(18)*. A two-step colorimetric assay was developed to determine the amount of epoxy groups in the product. The obtained epoxy-WMS was subjected to enzymatic digestion to characterize the structure of the starch derivative.



Scheme 2 Synthesis of epoxy starch derivatives *via* epoxidation of allylated starch.

Quantitative analysis of epoxy groups

The amount of epoxy groups was determined using a quantitative spectrophotometric assay *(14)*. In this method, 4-(*para*-nitrobenzyl)pyridine (*p*-NBP) was used to assay epoxides through the formation of a blue chromophore. This assay was tested with epoxy-WMS and *p*-NBP, but the colorimetric analysis of the starch suspension was impossible due to the insoluble nature of the starch. Therefore, a new two-step spectrophotometric assay was developed (Scheme 1). In the first step, an excess of *p*-NBP was used to quantitatively convert all epoxide groups on the starch (Scheme 1, reaction 1). A dark blue/green starch derivative was obtained. Subsequently, a small part of the supernatant containing the remaining *p*-NBP was transferred to a tube with a high concentration of allyl glycidyl ether (AGE). In this tube, the remaining *p*-NBP was converted in the *p*-NBP product **1** (Scheme 1, reaction 2). The *p*-NBP product is deprotonated in an alkaline medium using K_2CO_3, resulting in the blue chromophore **2**. The concentration of chromophore **2** was determined by its absorption at 600 nm. The amount of *p*-NBP initially added (reaction 1) minus the amount of **2** formed (reaction 2) equals the amount of epoxy groups on the starch. With this assay, DS_{epoxy} of 0.025 was determined corresponding to 0.13 ± 0.03 mmol epoxy groups

per g dry allylated starch (Table 1). This suggests that 11% of the allyl groups were converted into epoxy groups. The controls showed hardly any differences in A_{600}.

Amine substitution

Epoxy groups on a surface are able to react with nucleophiles such as NH_2- and OH-groups. Using hexanediamine (HD), the binding of NH_2-groups to epoxy groups was investigated. In this reaction, we assume that one amino group of HD reacts with an epoxy group since there is a 10-fold excess of amino groups. The amount of NH_2-groups in HD-WMS was determined using an elemental analyzer (Table 1). Via equation (2), a DS_{HD} of 0.026 was obtained. This result corresponds to DS_{epoxy} obtained by the colorimetric assay, which means that every epoxy group in epoxy-WMS has reacted with HD.

Table 1 Substitution levels of epoxy-WMS and hexanediamine (HD) treated epoxy-WMS

Sample	***Epoxy groups (mmol g^{-1})***	***DS***	***DS/DS_{allyl}***	***C (%)[b]***	***N (%)[b]***
Epoxy-WMS	0.13 ± 0.03	0.025[c]	11[a]	43.08	0.24[d]
HD-WMS	n.d.[e]	0.026[b]	11	39.09	0.53

[a] $DS_{allyl} = 0.23$.

[b] Determined using elemental analysis.

[c] Determined using 4-(*para*-nitrobenzyl)pyridine titration.

[d] Derived from proteins which are present in WMS.

[e] Not determined.

Physicochemical properties

The structural changes upon epoxidation of AHP-starch may affect the physicochemical properties such as gelatinization, swelling and solubility of the granules. Hardly any differences in the gelatinization temperatures between AHP-WMS and epoxy-WMS were observed (Table 2). However, both the swelling power and solubility index decreased after epoxidation of the allyl groups. This suggests that cross-links may have been formed in the starch, although no significant increase of gelatinization temperatures was obtained. In previous research, cross-linked starches exhibited reduced swelling and solubility of the granules, but also increased gelatinization temperatures *(19, 20)*.

Table 2 Thermal properties[a], swelling power, and solubility index of native starch and its derivatives.

Sample	T_o [a]	T_p [a]	T_c [a]	ΔH_{gel} [a]	*Swelling power (g g^{-1})*	*Solubility index (%)*
WMS	57.3	69.4	75.7	13.8	4.5 ± 0.2	0.2 ± 0.0
AHP-WMS	49.1	56.6	65.5	5.5	14.7 ± 1.0	7.8 ± 1.0
Epoxy-WMS	50.3	56.5	64.7	4.9	12.9 ± 0.2	5.4 ± 0.2

[a] Measured by Differential Scanning Calorimetry; T_o, T_p and T_c are the onset, peak and completion temperature respectively, and ΔH_{gel} is the enthalpy of gelatinization.

Structural characterization of epoxy-starch

The results of the spectrophotometric assay of epoxy groups, and the elemental analysis of the HD reaction, imply that only a small amount of epoxy groups is available for binding to nucleophilic groups. Furthermore, the reduced swelling and solubility of epoxy-WMS suggest that subsequent reactions may have taken place, such as the formation of internal cross-links in the starch granule. To study the structure of epoxy-WMS, the modified starch was enzymatically hydrolyzed. The enzymatically degraded products will give information about the differences in structure between AHP-WMS and epoxy-WMS.

Enzymatic digestion with β-amylase

The extent of β-amylase hydrolysis of native starches and its derivatives was studied using HPAEC and HPSEC elution profiles. These profiles showed that hydrolysis of the AHP-WMS and epoxy-WMS liberated less maltose than the native starch hydrolysis (results not shown). Furthermore, the relative amount of liberated maltose was determined at 89% of native release for AHP-WMS and at 73% for epoxy-WMS. This suggests that enzymatic degradation of epoxy-WMS was more sterically hindered due to the presence of intra- or intermolecular ether cross-links. Derivatization, especially at surface, leaves quite some native-like molecules. According to other studies, cross-linked starch derivatives are less accessible for enzymatic hydrolysis than their non-cross-linked precursors *(19, 21)*.

Enzymatic digestion with pullulanase, α-amylase and amyloglucosidase

To study the structure of epoxidized WMS in more detail, epoxy-WMS, AHP-WMS and non-modified starch were also subjected to simultaneous enzymatic digestion using pullulanase, α-amylase and amyloglucosidase. HPSEC elution profiles of both WMS derivatives show remaining high molecular weight enzymatic resistant fragments that are

not presented in the native starch digest (results not shown), i.e. the enzymes were sterically hindered *(11)* in both WMS derivatives.

Likewise, the HPAEC elution profiles of both AHP-WMS and epoxy-WMS (Fig. 1) show high molecular weight oligomers which are resistant to further enzymatic digestion. All fragments were eluted within 20 min. Different molecular weight patterns of the enzymatically generated oligomeric fragments were obtained for AHP-WMS and epoxy-WMS. The oligomeric fragments of AHP-WMS eluting at 9.3 min, between 10.3–10.4 min and at 12.2 min were not presented in the profile of digested epoxy-WMS, whereas other enzyme-mediated degradation products appeared at 10.1, 10.8–11.4 and 12.7 min.

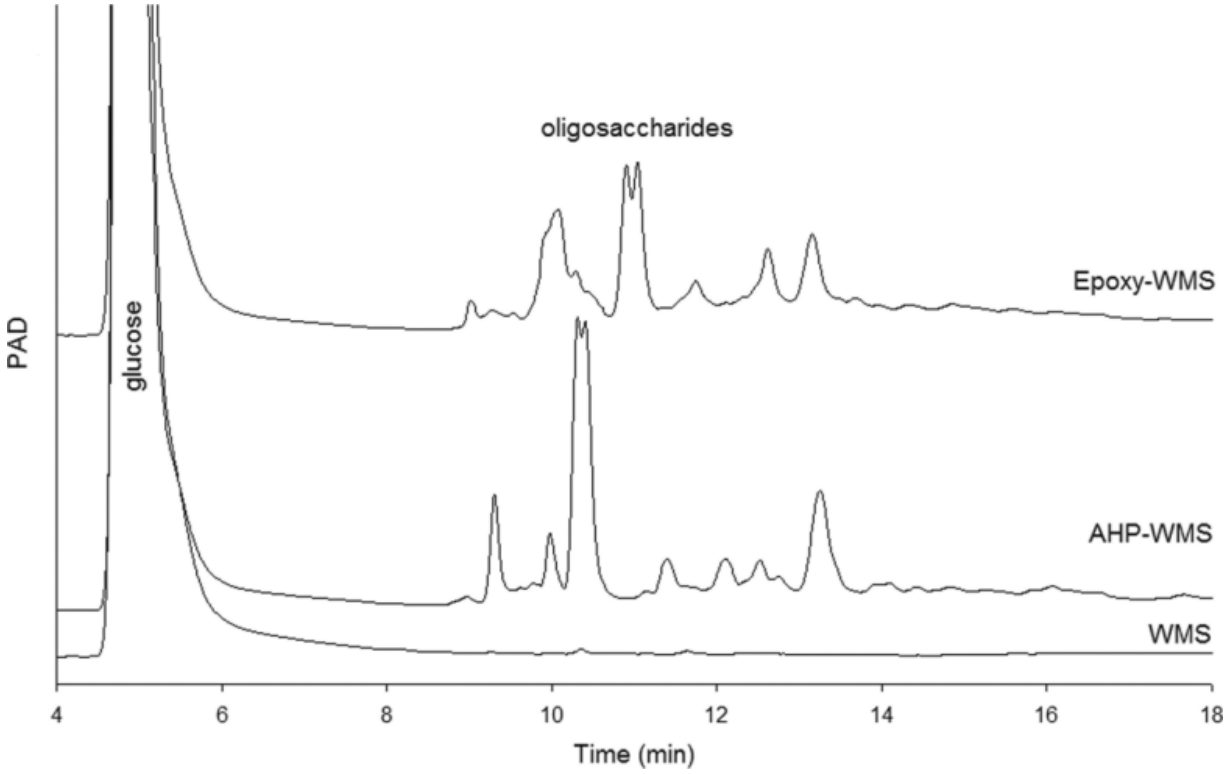


Fig. 1 HPAEC elution profiles of oligomers mixture of WMS, AHP-WMS and epoxy-WMS obtained after enzymatic degradation by pullulanase, α-amylase and amyloglucosidase.

The differences in the oligomeric fragments released from epoxy-WMS and AHP-WMS after enzymatic digestion was visualized in more detail by MALDI-TOF MS (Fig. 2). In the MALDI-TOF mass spectrum of AHP-WMS digest, enzyme-resistant substituted oligosaccharides with various degrees of polymerization (DP) were identified, ranging from two to nine DP with one to five allylhydroxypropyl groups (AHP). The mass spectrum of epoxy-WMS showed a regular pattern with more and diverse oligomers (DP ranging from

two to seven). The mass distribution of the epoxy-WMS digest contained several different enzyme-resistant oligomers having epoxy groups (Ox) and unreacted AHP groups. In addition, oligomeric fragments containing diol groups (DL) were found. Furthermore, there were fragments possessing internal cross-links (Cr_{intra}) or cross-links between two different fragments (Cr_{inter}). These oligomers with diol groups and cross-links were a result of subsequent reactions of epoxy groups, such as hydrolysis of the epoxy moieties or the formation of intra- and intermolecular bridges with free hydroxyl groups in starch.

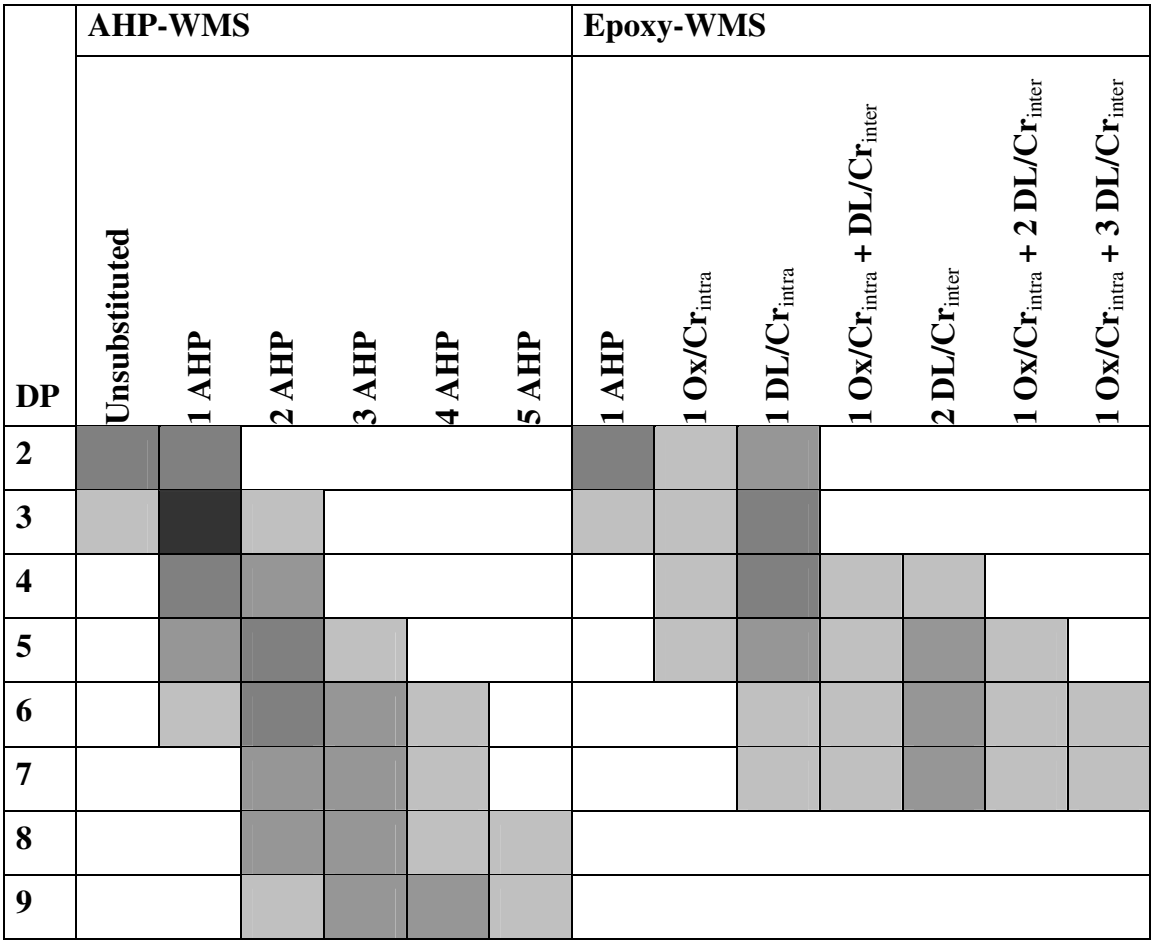


Fig. 2 Mass distribution of the oligomeric fragments of AHP-WMS and epoxy-WMS. Enzyme-resistant oligomeric fragments of AHP-WMS contain allyl groups (AHP), whereas fragments of epoxy-WMS contain AHP or epoxy groups (Ox) as well as diol groups (DL) and cross-links within the oligomer (Cr_{intra}) or between two fragments (Cr_{inter}). DP: degree of polymerization. The total signal intensities of the oligomers in a certain DP are normalized to 100%.

Relative intensities: 0% 100%

Some enzyme-resistant oligomeric DP3 fragments that can theoretically be obtained after enzymatic hydrolysis of epoxy-WMS are illustrated in Fig. 3. Fragments containing epoxy groups (Ox) and cross-links within the oligomer (Cr_{intra}) have the same mass over charge ratios, as is illustrated in Fig. 3. Thus, they are indistinguishable in the MS analysis. Similarly, the oligomers having diol groups (DL) and cross-links between two fragments (Cr_{inter}) give the same mass over charge ratios in the mass spectrum of epoxy-WMS. The diversity of possible enzymatically degraded fragments becomes larger for oligomers containing more than three glucose units. The fragments at higher m/z values (Fig. 2) could not be unambiguously assigned due to their large diversity, low relative intensities and overlap of signals.

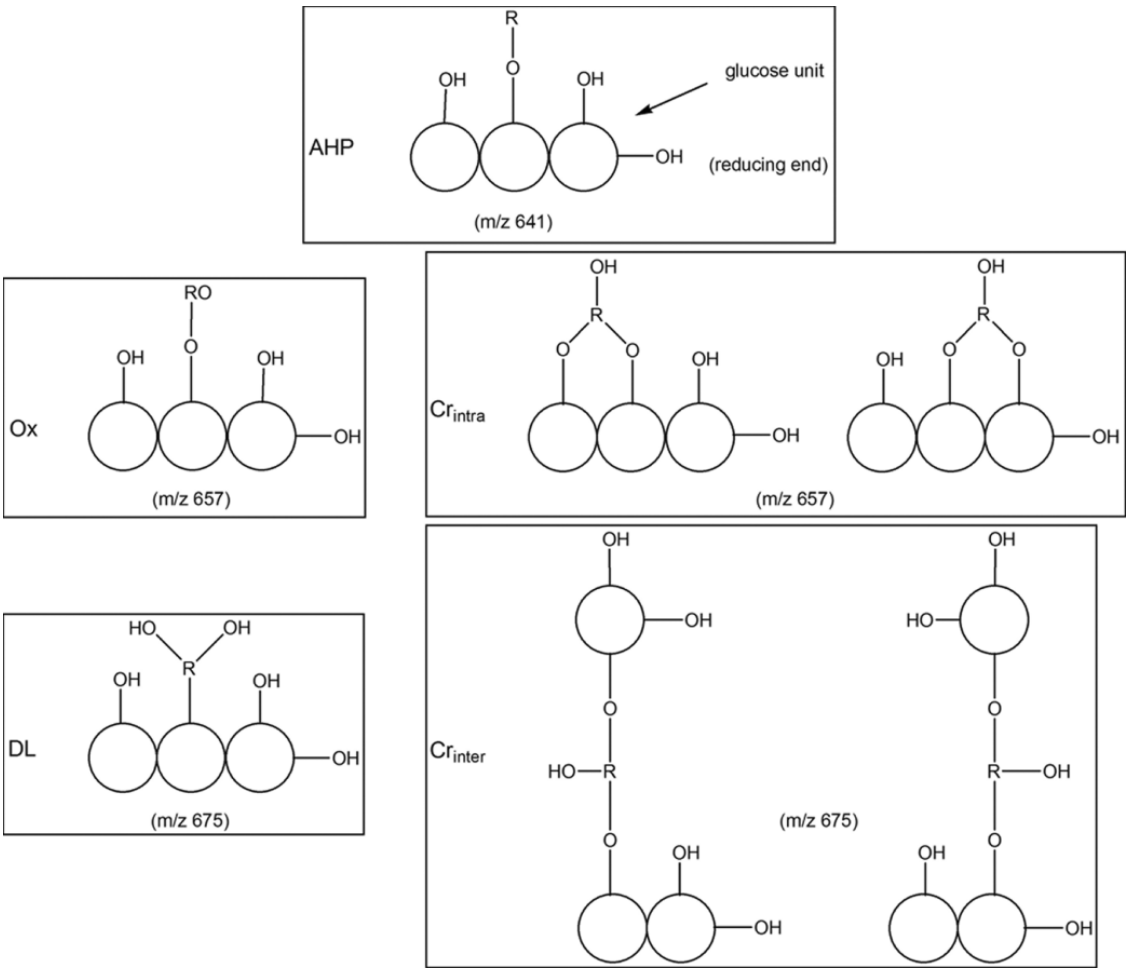


Fig. 3 Simplified scheme of some possible different enzyme-resistant DP3 fragments of the pullulanase, α-amylase and amyloglucosidase digest of epoxy-WMS. Substituted DP3 fragments may contain one allylhydroxypropyl group (AHP, *m/z* 641), an epoxy group (Ox, *m/z* 657), a diol group (DL, *m/z* 675), a cross-link within the oligomer (Cr_{intra}, *m/z* 657) or a cross-link between two fragments (Cr_{inter}, *m/z* 675). For reasons of clarity, only fragments with a substituent at the central glucose unit are drawn. DP: degree of polymerization; R: AHP group; RO: epoxidized AHP group.

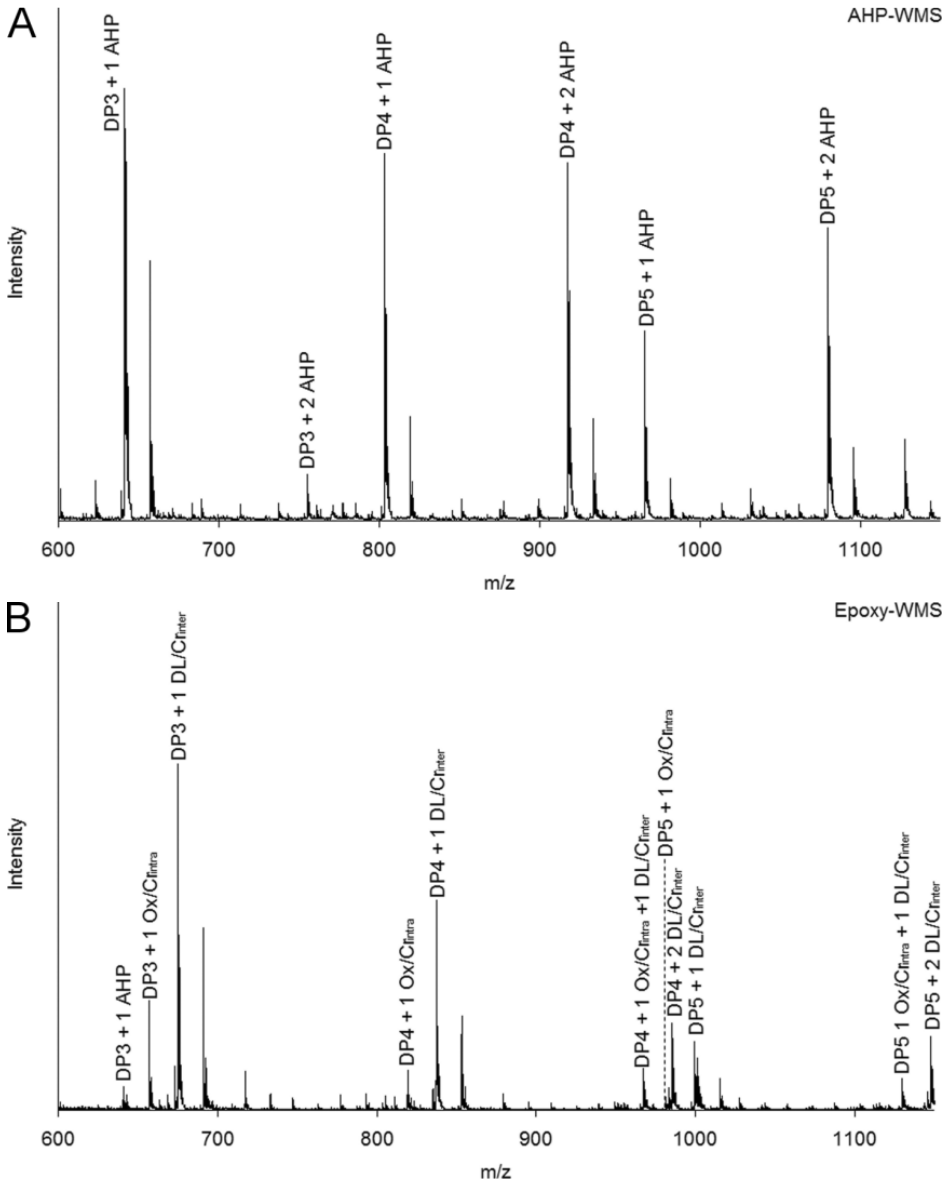


Fig. 4 MALDI-TOF mass spectra ranging from *m/z* 1600 to 2000 of the pullulanase, α-amylase and amyloglucosidase digests of AHP-WMS (A) and epoxy-WMS (B). Enzyme-resistant oligomeric fragments of AHP-WMS contain allylhydroxypropyl groups (AHP), whereas fragments of epoxy-WMS contain AHP or epoxy groups (Ox) as well as cross-links within the oligomer (Cr_{intra}) and between two fragments (Cr_{inter}), or diol groups (DL). DP: degree of polymerization.

In the enlargements of the MALDI-TOF mass spectra (Fig. 4), differences in the oligomeric structure and distribution after enzymatic degradation of AHP-WMS and epoxy-WMS, respectively, are clearly shown. The maltriose containing one AHP group is still present after epoxidation. Next to this oligomer, its epoxidized fragment or trioligosaccharide with Cr_{intra} is identified at the signal of *m/z* 657, followed by DP3 with Cr_{inter} or DL (*m/z* 675).

These differences in oligomeric fragments are also observed for fragments containing four or five glucose residues. Moreover, tetra- and pentaoligosaccharide with two Cr_{inter} or DL, and with one epoxy group or Cr_{intra}, and one cross-link between two fragments or a diol group are found for epoxy-WMS. Furthermore, DP5 with one Ox group or Cr_{intra} and two Cr_{inter} or DL are identified in the MS analysis.

These results show significant differences in the oligomeric fragment patterns of AHP-WMS and epoxy-WMS after enzymatic degradation. Oligomeric fragments resistant to further enzymatic digestion with several allyl groups were obtained for AHP-WMS, whereas a regular pattern of more and diverse oligomers was found for epoxy-WMS. A small number of oligomers carrying unmodified AHP groups and epoxy groups were found in the MALDI-TOF MS of epoxy-WMS. Most fragments were identified as fragments decorated with diol groups and/or with ether cross-links generated, respectively, by hydrolysis of the epoxy moieties or by the formation of covalent bonds within the oligomer or between two different fragments. These subsequent fragments may have been formed during the epoxidation or storage of the compound, but it is also possible that these fragments were generated during the enzymatic digestion. This suggests that a larger amount of allyl groups in AHP-WMS was converted into epoxy groups than determined with *p*-NBP test, but a significant amount of the epoxy groups reacted further with nucleophilic OH-groups in glucose units or water to generate cross-links or diol groups, respectively.

Enzyme immobilization in epoxy starch gel

A small amount of epoxy groups in the synthesized epoxy starch derivatives is available for covalent binding with biomolecules. Therefore, epoxy-WMS was employed as carrier matrix for entrapment of the thermostable β-glucosidase from *Pyrococcus furiosis*. The immobilization of β-glucosidase was most efficiently done during the swelling process of the epoxy starch derivative. After pregelatinization of the epoxy-WMS, β-glucosidase was immobilized by mixing the thermostable enzyme with the swollen starch gel. The enzymatic activity of covalently immobilized β-glucosidase was determined by the activity recovery assays of β-glucosidase after washing the carrier matrix several times (Table 3). The enzymatic activity in the epoxy starch gel and the supernatant of the washing steps was compared with the activity recovery of free β-glucosidase under the immobilization conditions. The immobilization procedure is very mild, since free enzyme has still 92% activity under the conditions used during the immobilization. However, most of the enzyme appears to be loosely bound, since only 13% of immobilized enzyme activity remains after

four rounds of washing. The total recovered activity of the immobilized enzyme plus the washings is 96%, again showing the mildness of the procedure. To conclude, β-glucosidase was predominantly immobilized onto epoxy starch by weak adsorption. A small part of the enzyme molecules are firmly bound by covalent linkages. The latter is undoubtedly due to the epoxy groups in the starch matrix.

Table 3 Enzymatic activity recovery of β-glucosidase by immobilization in pregelatinized epoxy-WMS

Enzyme localization	***Enzyme activity (U)***[a]	***Recovery (%)***
Free β-glucosidase	25.0 ± 0.5	107
Free β-glucosidase, immobilization conditions[b]	23.0 ± 0.4	100
Immobilized β-glucosidase[c]	2.9 ± 0.5	13
Washed from the starch gel 1st step[d]	15.4 ± 0.6	67
Washed from the starch gel 2nd step[d]	2.7 ± 0.6	12
Washed from the starch gel 3rd step[d]	0.83 ± 0.6	3.6
Washed from the starch gel 4th step[d]	0.16 ± 0.01	0.7

[a] Measured in an enzyme activity assay for β-glucosidase

[b] Immobilization conditions: incubation of β-glucosidase (0.05 mg, 0.9 mg mL^{-1}) in 1 mL citrate buffer pH 5.0 at 80 °C for 30 min.

[c] Immobilization of 0.05 mg β-glucosidase in 50 mg pregelatinized epoxy-WMS in 1 mL citrate buffer.

[d] Enzymatic activity of β-glucosidase in the supernatant after washing the epoxy starch gel.

Conclusions

We have studied two ways to make epoxy derivatives of (poly)saccharides. The first method involved direct coupling of a sugar derivative to diepoxides. This approach did not lead to the desired products, since only epoxide-derived cyclisation products were recovered. Much better results were obtained with an indirect approach, in which allylated starch was epoxidized using hydrogen peroxide and acetonitrile. A two step spectrophotometric assay was developed to determine the amount of epoxy groups in starch. Subsequently, a DS_{epoxy} of 0.025 was determined corresponding to a quantity of epoxy groups of 0.13 mmol g^{-1} per dry allylated starch. Epoxy starch derivatives exhibited reduced enzymatic susceptibility, swelling capacity and solubility compared with AHP-WMS. Enzymatic hydrolysis elucidated that a larger amount of allyl groups of AHP-WMS

was converted into epoxy groups than determined in the colorimetric assay. However, only a small amount of epoxidized fragments were found, due to secondary reactions of the reactive epoxy groups, like intra- and intermolecular cross-linking with free hydroxyl groups of polysaccharide chains, and hydrolysis. In addition, epoxy starch derivatives were employed as carrier matrix for a biomolecule, more particular, enzyme. β-Glucosidase was covalently immobilized to pregelatinized epoxidized starch, 13% enzymatic activity remained after extensive washing of the epoxy starch gel. This study provides a method to functionalize nongelatinized starch with a reactive group suitable for the development of delivery systems and as carrier for entrapment of biomolecules.

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Chapter 4

Molecular sieves provoke multiple substitutions in the enzymatic synthesis of fructose oligosaccharide-lauryl esters

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Abstract

The cause of discrepancies in the literature regarding the specificity of immobilized *Candida antarctica* lipase B in the acylation of oligosaccharides was examined. Molecular sieves, generally used to control the water content during acylation reactions, turned out to have an important role in this. It was proven that molecular sieves alone can catalyze the acylation of fructose oligomers using vinyl laurate, leading to multiple substitution of the oligomers. This effect was the most profound at conditions unfavorable for the enzyme, because this resulted in a relatively high concentration of the chemically produced adducts. The enzyme alone catalyzed the formation of monosubstituted oligomers. It was proven that even solvent pre-drying by molecular sieves already causes the release of catalyzing compounds to the liquid, leading to subsequent catalysis. These findings should be taken into account when applying molecular sieves in this type of reactions in the future. Molecular sieves could, moreover, be used as a catalyst when multiple substitution is desired.

Introduction

There is an increasing interest for the synthesis and application of modified oligo- and polysaccharides. An example of this is the production of block co-polymers or co-oligomers. Cellulose, for example, can be esterified using fatty acid chlorides. In this way, building blocks for biodegradable 'plastics' have been produced from poorly valorized agricultural waste streams *(1)*. A drawback of chemical reactions is their low specificity. To overcome this problem, enzymes can be used for catalyzing and controlling esterification reactions of oligo- and polysaccharides. This leads to increased substitution specificity, which is considerably improving the functionality compared to the products of chemical substitutions *(2)*. Cellulose has been acetylated using Lipase A12 from *Aspergillus niger* in an organic medium. The cellulase side-activity of this enzyme and particularly the optimized water activity of the system turned out to enhance the cellulose acetylation *(3)*. The reduced molecular weight resulting from the cellulase activity and the subsequent improved solubility of this substrate apparently leads to an increased reaction rate. Furthermore, starch could be esterified by using fatty acids from recovered coconut oil using a lipase from *Thermomyces lanuginosa (4)*. The regio-selectivity of immobilized *Candida antarctica* lipase B for the primary hydroxyl group at the C6-position of a sugar was evident when starch nanoparticles incorporated in reverse micelles coated with Aerosol-OT (bis(2-ethylhexyl)sodium sulfosuccinate) were esterified using vinyl stearate in toluene *(5)*.

The modification of several oligosaccharides by lipases in organic media has also been described. Trehalose, maltose and saccharose have been acylated with ethyl butanoate and ethyl dodecanoate. This reaction was catalyzed by immobilized lipase from *C. antarctica* in refluxing Bu^tOH. The conversion to mono- or di-esters appeared to be dependent on the structure of the disaccharide and the reaction rates turned out to be directly related to the solubility of the disaccharides *(6)*. In previous research within our groups *(2)* with the same *C. antarctica* lipase, lauryl esters of fructose oligomers were produced. The fructose oligosaccharides (FOS) used in these researches were products of partial inulin hydrolysis. Inulin is a water soluble, prebiotic fiber obtained from the chicory root (*Cichorium intybus*). Native inulin has a linear structure with the formula GF_n (G, glucose and F, fructose), having the fructose units β(2→1) linked. After partial hydrolysis, a mixture of inulin oligomers is obtained with the formulas GF_m and F_x *(7)*. The conjugates obtained after acylation of these oligomers showed to have functional properties that are comparable to those of synthetic block co-polymers. On average one lauric acid was coupled per FOS

oligomer which had an average degree of polymerization (DP) of 10 *(2)*. A schematic illustration of a proposed structure of these products can be found in Fig. 1.

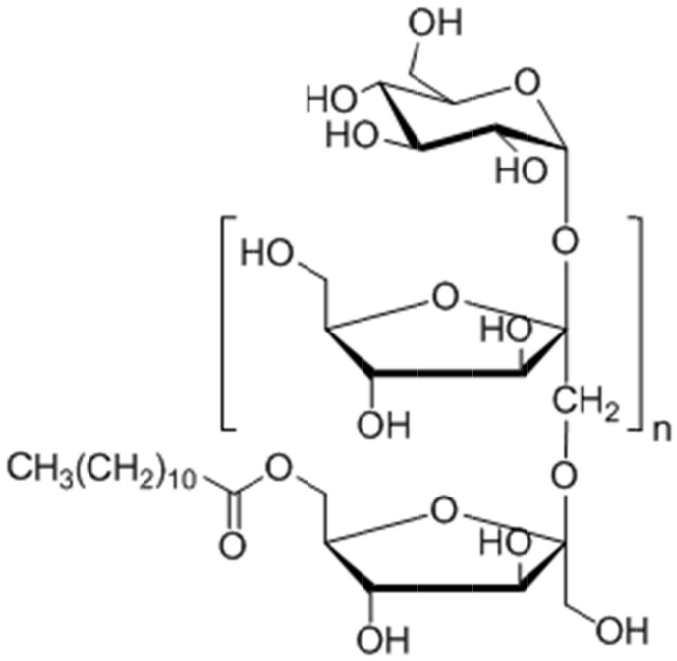


Fig. 1 Schematic structure of a lauryl mono-ester of FOS.

When evaluating literature concerning oligosaccharide (trans-)esterification, it appears that many research groups are observing strong regio-selective actions of this immobilized lipase B from *C. antarctica (2, 5, 6, 8-10)*. The presence of mainly one substituent per oligomer has been described *(2, 8, 9)*. Other results, however, imply that the enzyme is far less selective with regard to its substitution action *(11, 12)*. In the present research, the cause of this discrepancy was studied. Based on initial experiments, molecular sieves were suspected to have a role in the appearance of side reactions. The effect seemed to be dependent on the DMSO concentration. To investigate this thoroughly, a series of FOS-laurate synthesis experiments with and without the presence of immobilized *C. antarctica* lipase B was performed at 20% as well as 40% (v/v) DMSO in Bu^{t}OH. Three different applications of molecular sieves were studied: (a) without using any molecular sieves, (b) in solvents only pre-dried using molecular sieves, and (c) with molecular sieves present during the reaction. The consequences of the results of these experiments will be discussed.

Experimental

Materials

The FOS mixture Raftiline LS was a kind gift from BENEO-Orafti (Tienen, Belgium), having DP from 3 to at least 17 (data not shown) and a water content of 0.68% (w/w after drying under vacuum, as determined using the Karl-Fisher method *(13)*). Dimethyl sulfoxide (DMSO), vinyl laurate and butyl laurate were obtained from Fluka Biochemica (Buchs, Switzerland). N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS) was from Regis Technologies (Morton Grove, IL, USA). *Tert*-Butanol (Bu^tOH) and molecular sieves (type 4Å) were from Sigma-Aldrich (St. Louis, MO, USA). 1-Butanol, hexadecane and lauric acid (99%) were from Merck (Darmstadt, Germany) and pyridine from Acros Organics (Geel, Belgium). Acetonitrile was purchased from Biosolve (Westford, MA, USA) and 2,5-dihydroxy benzoic acid was from Bruker Daltonics (Germany). Immobilized *C. antarctica* lipase B (Novozym 435), was received from Novozymes (Bagsværd, Denmark).

Methods

Determination of the FOS solubility

The solubility of FOS was determined in different concentrations of DMSO in Bu^tOH at 30 °C. Five milliliter of each solvent mixture was transferred to a glass vessel and small quantities of FOS were added, under continuous stirring. When no more material could be dissolved, indicated by the appearance of turbidity, the experiment was stopped. The quantity added up to this point, was considered as being the maximum solubility at a certain solvent-ratio and was expressed as milligram of FOS dissolved per 100 ml of solvent.

Determination of the enzyme activity

The transesterification activity of immobilized *C. antarctica* lipase B was determined at different concentrations of DMSO in Bu^tOH. Two milliliter of each solvent mixture was transferred to a reaction tube and thermostated at 40 °C. Three millimole of vinyl laurate and 1 mmole of 1-butanol were transferred to each tube. Thirty microliter of hexadecane was added as an internal standard. After subsequent addition of 50 mg immobilized *C. antarctica* lipase B, samples were incubated for 24 hours at 40 °C under continuous stirring by magnetic bars.

For determination of the esterification activity, 0.2 mmole of lauric acid and 0.4 mmole of 1-butanol were incubated with 40 mg immobilized *C. antarctica* lipase B under the same conditions as mentioned for the transesterification activity.
After incubation, the immobilized *C. antarctica* lipase B was removed by centrifugation (4 min, 44.000 x *g*). One microliter of each sample was subsequently injected on a GC-system (Varian Star, Palo Alto, CA, USA) connected to a SGE BPX5 GC column (30m x 0.25mm x 0.25µm). The separation was performed at an initial temperature of 80 °C followed by a linear temperature gradient of 7.5 °C/min up to 300 °C. The temperature was subsequently kept constant at this maximum value for 5 minutes. Helium was the carrier gas with a pressure of 150 kPa. Eluting compounds were monitored by Flame Ionisation Detection (FID) at 300 °C. Quantification of the lauryl ester of 1-butanol was hindered by co-elution of disturbing unreacted compounds in the reaction mixture. Therefore, these compounds were silylated prior to analysis by adding 200 µl of BSTFA +1% TMCS and 500 µl pyridine to 500 µl of reaction mixture and subsequent incubation for 1 h at 70 °C.
The amounts of ester formed were calculated by using a calibration curve, based on the response measured after injection of different concentrations of butyl laurate in hexane. The average product yield was expressed as micromole of product formed per milligram of enzyme (including carrier material) per hour.

Synthesis of the oligosaccharide-fatty acid esters

Synthesis experiments were performed in a Carrousel 12-place Reaction Station RR98030 (Radleys Discovery Technologies, Saffron Walden, UK). To 10 ml of either 20% or 40% (v/v) DMSO in Bu^tOH 135 mg Raftiline LS (~0.83 mmole anhydrohexose units) , 135 mg immobilized *C. antarctica* lipase B, 259 µl vinyl laurate (1 mmole) and 500 mg (5% w/v) of molecular sieves were added (in various combinations). These ratios were based on work published previously *(2)*. In Table 1, an overview of the composition of the samples is shown. Incubation was performed under continuous stirring (magnetic bars) at 60 °C for 5 days.

Table 1 Overview of the incubated samples. FOS and vinyl laurate were present during each reaction

Sample	***DMSO (%, v/v)***	***Bu^{t}OH (%, v/v)***	***Enzyme present***	***Molecular sieves' role***
1	20	80	-	None
2	40	60	-	None
3	20	80	+	None
4	40	60	+	None
5	20	80	-	*For solvent drying prior to use*
6	40	60	-	*For solvent drying prior to use*
7	20	80	+	*For solvent drying prior to use*
8	40	60	+	*For solvent drying prior to use*
9	20	80	-	Present during synthesis
10	40	60	-	Present during synthesis
11	20	80	+	Present during synthesis
12	40	60	+	Present during synthesis

Analysis of the reaction products by MALDI-TOF MS

After incubation, the composition of the reaction mixture was determined by Matrix-Assisted Laser Desorption-Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS). After removal of the solid components, 5 µl of the reaction mixture was mixed with 5 µl of matrix solution (10 mg 2,5-dihydroxy benzoic acid in acetonitrile:water 300 µl:700 µl). Two microliter of this mixture was subsequently transferred to a target plate and dried under a stream of dry air. Measurements were performed on an Ultraflex Workstation (Bruker Daltonics, Bremen, Germany), running in the positive mode and equipped with a 337 nm laser. Ions were accelerated with a 25 kV voltage after a delayed extraction time of 200 ns. Detection was performed in the reflector mode. The lowest laser intensity needed to obtain a good quality spectrum was applied. The machine was calibrated using a mixture of maltodextrins (Avebe, Veendam, The Netherlands) with known molecular masses. FlexControl and FlexAnalysis software packages (Bruker Daltonics, Bremen, Germany) were used for acquisition and processing of the data.

Spectra were recalculated and for a correct interpretation, we would like to introduce the term "Degree of Substitution per Oligomer (DSO)" to indicate the number of lauryl esters present per oligomer. Within one MALDI-TOF mass spectrum, the intensities of all signals caused by the sodium-adducts of oligomers with the same DP, while having a different DSO's were added up and together set at 100%. The relative contribution of each DSO was subsequently determined. It was assumed that there were no differences between the

response factor ratios of the molecules. A similar procedure has been used before when digests of acetylated starch were characterized *(14)*.

Results and discussion

Sugar solubility / enzyme product yield in different solvent-ratio mixtures

FOS solubility and the product yield after using immobilized *C. antarctica* lipase B in several DMSO/Bu^{t}OH solvent mixtures were examined at first. The esterification as well as the transesterification yield was determined in order to decide on the optimal acyl donor to be applied. Vinyl laurate, lauric acid and 1-butanol were assumed to be well soluble under all conditions. The results of these experiments can be found in Fig. 2.

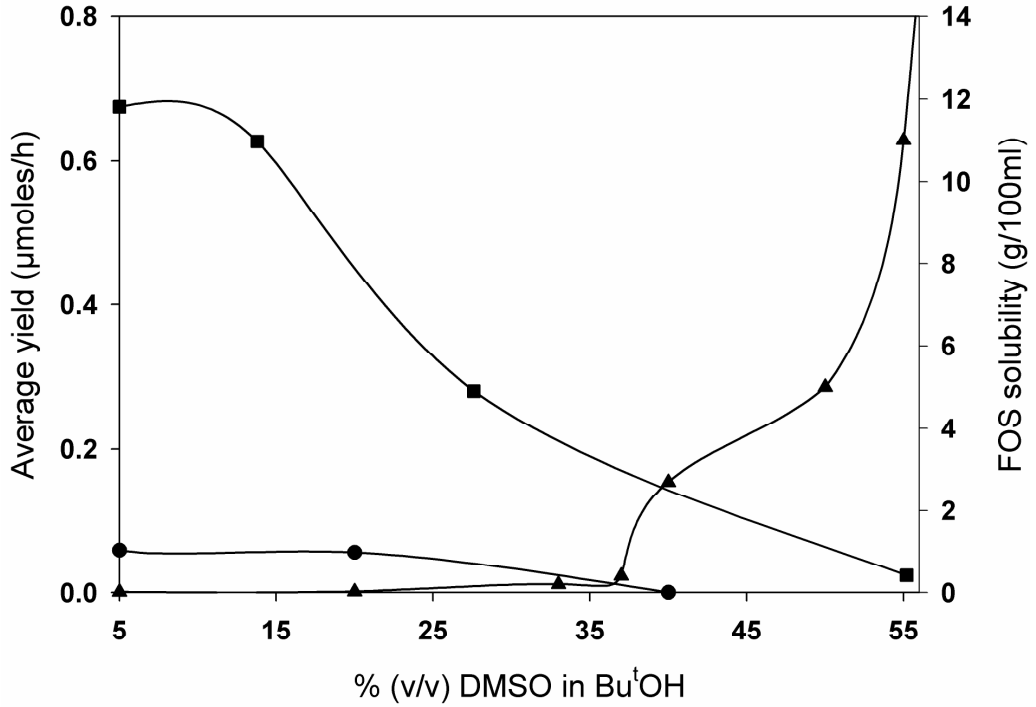
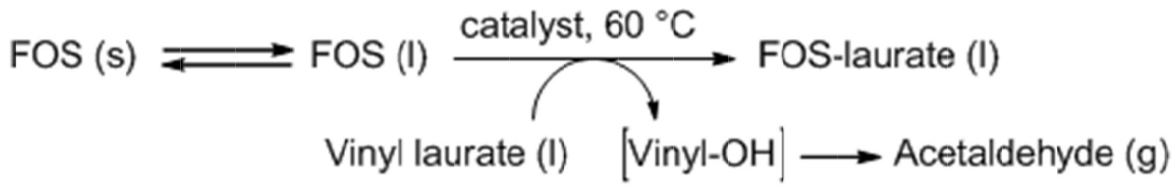


Fig. 2 FOS solubility (▲) and the average product yield after esterification (●) / transesterification (■) using immobilized *C. antarctica* lipase B at different concentrations of DMSO in Bu^{t}OH for 24h at 40 °C. The average product yield is defined as micromole of butyl laurate formed per milligram of enzyme (including carrier) per hour.

The average product yield of the esterification reaction is much lower than the yield of the transesterification reaction under all conditions tested. In addition, both of them are decreasing when the DMSO concentration in Bu^{t}OH is increasing. Because of this higher yield after transesterification, it was decided to use vinyl laurate as the acyl donor in further experiments. An increased FOS solubility can be observed when the DMSO concentration

is increasing. This could be expected based on the more hydrophilic character of DMSO compared to Bu^tOH. Especially from 35-40% (v/v) DMSO onwards, there is a clear increasing trend in the amount of FOS that can be dissolved. Summarizing these results, it can be concluded that at 20% (v/v) DMSO (as applied before *(2)*) the average product yield is relatively high, but the FOS have a relatively low solubility. Reaction products can be expected to have a good solubility. Therefore, extra FOS will dissolve when part of the material is converted to fatty acid esters. The reaction can therefore continue and leads to acceptable yields *(2)*. This is visualized in Scheme 1.



Scheme 1 Acylation of FOS with vinyl laurate. S, in solid state; L, in dissolved state; and G, in gas state

At 40% (v/v) DMSO, the average product yield is still acceptable, while the FOS solubility has increased >100 times. This higher FOS solubility can be expected to lead to an increased reaction rate. This was observed before using maltose and saccharose *(6)*. A lower enzyme activity at this higher DMSO concentration can, however, be expected to diminish this effect. Both DMSO concentrations were applied in the further research to be able to evaluate the consequences of each compromise between FOS solubility and enzyme activity for the eventual product composition.

Synthesis of FOS-lauric acid esters at different DMSO concentrations, studying the role of molecular sieves

The synthesis of FOS-lauric acid esters was performed with and without using immobilized *C. antarctica* lipase B . Furthermore, molecular sieves were applied in several ways. This was already outlined in Table 1. The results of these experiments will be shown and discussed in the next paragraphs.

Synthesis without using molecular sieves

Synthesis experiments without using molecular sieves in any preparation or reaction step were performed initially to measure the baseline values. A zoom-in of the MALDI-TOF mass spectra of the reactions at both DMSO concentrations catalyzed by *C. antarctica*

lipase B, after 5 days of incubation at 60 °C (samples 3 and 4 in Table 1), can be found in Fig. 3. Processed data of the complete spectra can be found in Fig. 4. The blanks, containing no enzyme (samples 1 and 2 in Table 1), did not contain any detectable acylated oligosaccharides after incubation (data not shown).

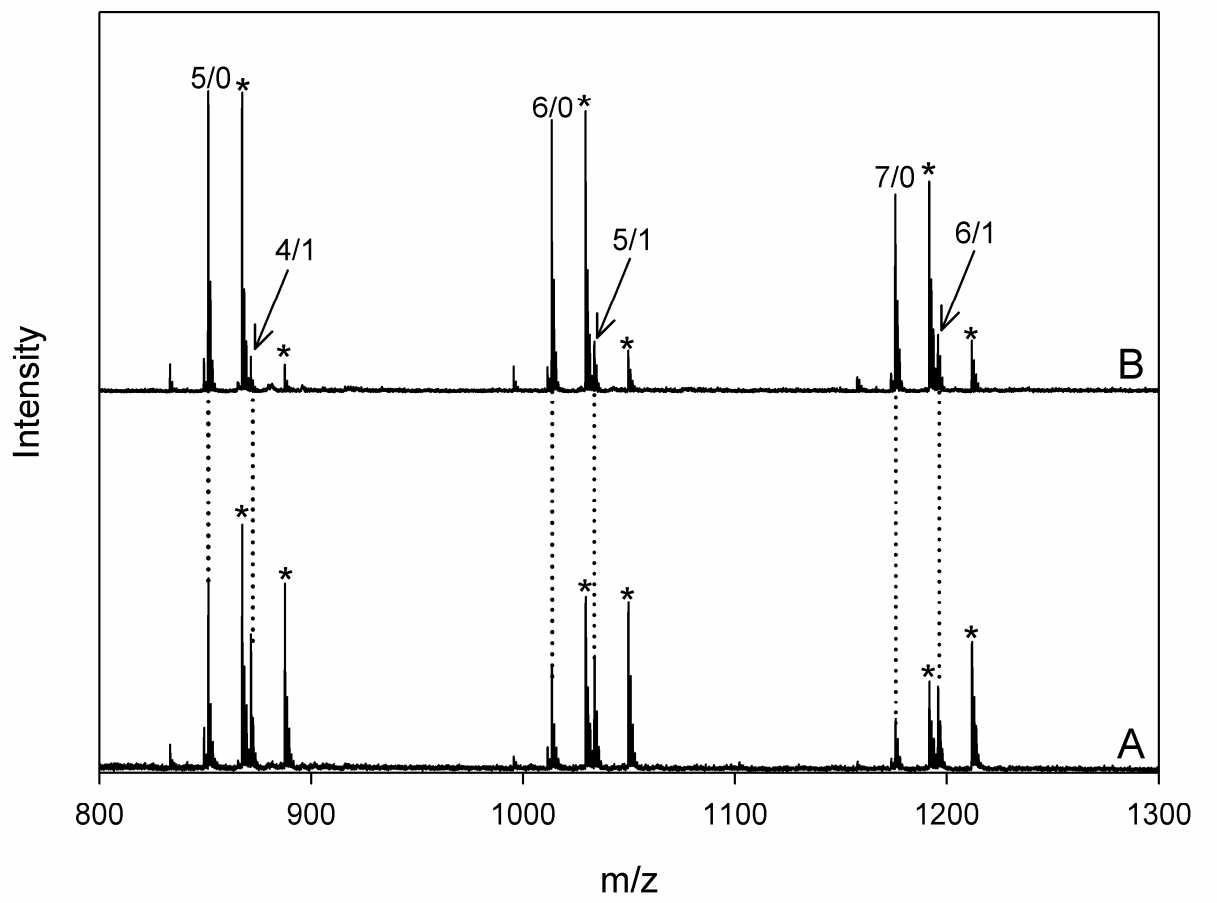


Fig. 3 Zoom-in of MALDI-TOF mass spectra after incubation of FOS with vinyl laurate, in the presence of immobilized *C. antarctica* lipase B in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH for 5 days at 60 °C, no molecular sieves were used. E.g. 5/1 stands for a fructose oligomer with a degree of polymerization 5 and one lauric acid moiety attached (Δm/z=182). Only sodium-adducts (Δm/z=23) are annotated, potassium adducts (Δm/z=39) are indicated by an asterisk. Dashed lines indicate the presence of the same type of molecule (sodium adduct) in the lower spectrum.

The most important observation in these figures is that only monosubstitution per oligomer occurs after incubation. The fact that the blanks did not contain any of these products proves that the reaction is indeed catalyzed by the enzyme. The uniformity in the reaction products is probably a result of the regio-selectivity of the enzyme. It was shown recently that immobilized *C. antarctica* lipase B is very regio-selective in case of acylation of polyhydroxylated iridoid glycosides; only the primary hydroxyl group at the sugar moiety

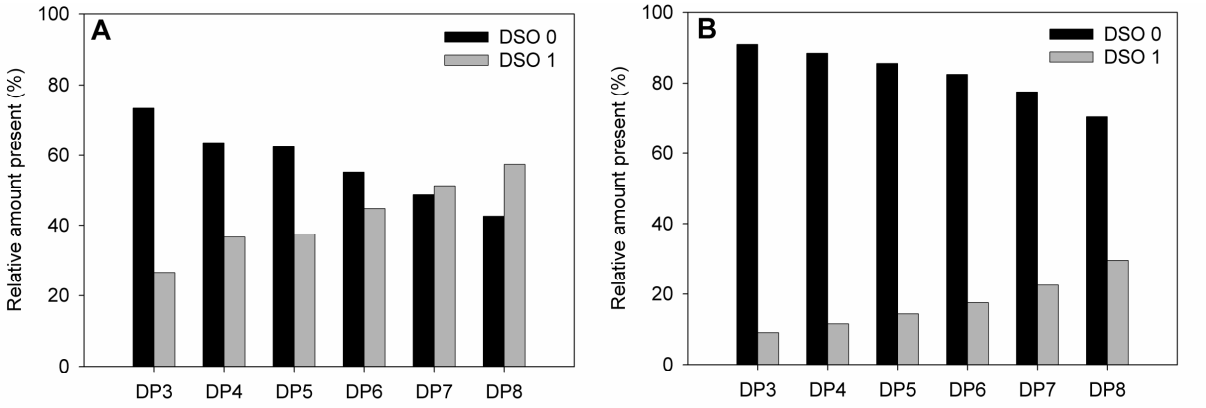


Fig. 4 Relative composition of reaction mixtures after incubations of FOS with vinyl laurate catalyzed by immobilized *C. antarctica* lipase B in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH at 60°C for 5 days, derived from MALDI-TOF mass spectra. No molecular sieves were used. DP stands for degree of polymerization of the fructose oligomer and DSO stands for degree of substitution per oligomer.

and secondary hydroxyl groups at the aglycon moiety turned out to be prone to acylation by this enzyme *(15)*. This is in line with our observations, and again shows the power of enzymes as a tool for selective modifications. The fact that only one primary hydroxyl group is modified per oligomer by *C. antarctica* lipase B, could be a result of steric hindrance and has been described before. When raffinose, melezitose and 1-kestose were acylated using vinyl laurate, only a small proportion of di-ester could be observed after TLC separation of the reaction mixture *(9)*. In our previous research, the presence of one lauric acid moiety per fructose oligomer was also indicated, although not evidenced by mass spectrometry *(2)*. This altogether proves that immobilized *C. antarctica* lipase B will only catalyze mono-ester synthesis of FOS with DP 3-8 at both 20% and 40% (v/v) DMSO in Bu^{t}OH.

When the results are evaluated quantitatively, it appears that the efficiency of the synthesis reaction is higher in 20% (v/v) DMSO compared to 40% (v/v) DMSO. If the enzyme would have the same activity at both DMSO concentrations, the eventual ratio between DSO 0 and DSO 1 should be the same at both DMSO concentrations. In absolute terms, more FOS are dissolved at 40% DMSO than at 20% DMSO, but this should also lead to a higher reaction rate, because sugar solubility is an important factor for this reaction rate *(6)*. The relative amount of DSO 1 compared to DSO 0 is, however, much lower at 40% DMSO than at 20% DMSO for all DPs (Fig. 4). This means that the activity of the immobilized *C. antarctica* lipase B is indeed lower at 40% (v/v) DMSO compared to 20% (v/v) DMSO, as could be expected based on the observations shown in Fig. 2. The

effect of the enzyme inactivation by the solvent on the reaction rate is apparently stronger than the effect of improved sugar solubility.

Synthesis in the presence of molecular sieves

The same experiments as described above were performed in the presence of molecular sieves, a general way to make and keep solvents as dry as possible. First the results of the blanks, not containing enzyme, will be shown (samples 9 and 10 in Table 1). Samples were taken again after 5 days of incubation and were analyzed by using MALDI-TOF MS. The relative composition of the samples after incubation can be found in Fig. 5.

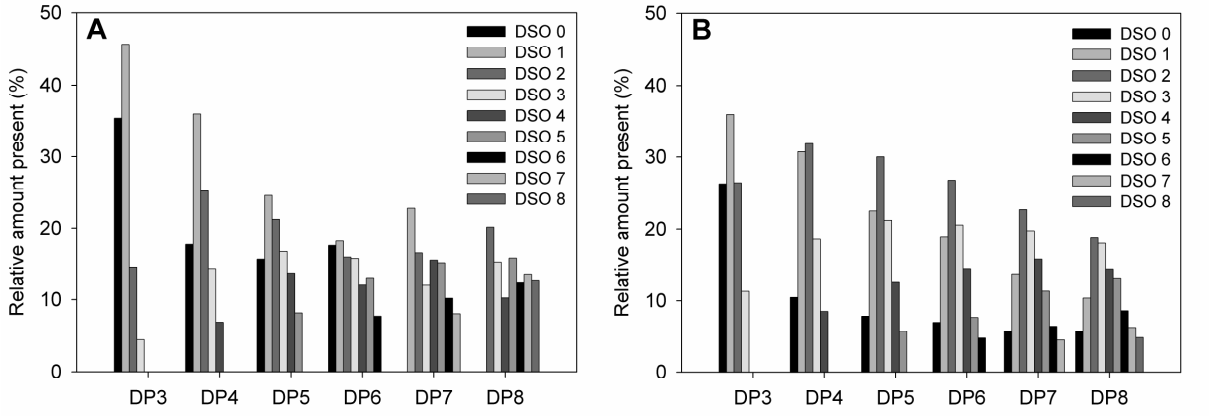


Fig. 5 Relative composition of reaction mixtures after incubations of FOS with vinyl laurate in the presence of molecular sieves and in the absence of enzyme, at 20% (A) and 40% (B) DMSO in Bu^{t}OH at 60°C for 5 days, derived from MALDI-TOF mass spectra. Molecular sieves were present during the incubation. DP stands for degree of polymerization of the fructose oligomer and DSO stands for degree of substitution per oligomer.

In contrast to the blanks not containing molecular sieves, a variety of reaction products is formed when molecular sieves are present in the reaction mixture. It is striking that a large number of products with multiple substitution is formed in the absence of any enzyme. Fig. 5 shows that there is a difference between the distributions at the different DMSO concentrations. At 40% (v/v) DMSO, the +1, 2 and 3 substitutions per oligomer are generally prevailing, while the distribution of substituted oligomers at 20% (v/v) DMSO is more evenly. The fraction of highly substituted oligomers is slightly higher at the latter concentration, while the lower substituted oligomers are relatively less abundant, this especially accounts for DP ≥ 5. The explanation of this phenomenon can probably be found in the lower solubility of the high-DP oligomers at the lower DMSO concentration compared to the lower DP oligomers. When less non-substituted oligomers are dissolved,

the relatively better soluble acylated oligomers are more likely to be modified to a higher extent by a catalyst, because their relative concentration is higher. The lower solubility at lower DMSO concentrations mainly accounts for higher DP values, this explains that the differences between the reaction products at different DMSO concentrations are mainly found in the higher DP range.

By these observations, the catalysis itself is not explained yet. It is known that multiple substitutions can result from chemical catalysis. Saccharose acylation using methyl palmitate in DMSO (molar ratio saccharose : methyl palmitate 1:7), catalyzed by the alkaline catalyst K_2CO_3, resulted for example in a non-random distribution with an average of 5.8 esters per saccharose molecule *(16)*. The only explanation for the effect observed in our experiments is the presence of molecular sieves in the reaction mixture. They consist of a macromolecular network of aluminosilicate. According to the product description of the supplier, this network is composed of "1 Na_2O : 1 Al_2O_3 : 2.0 ∀. 0.1 SiO_2 : x H_2O" *(17)*. These structures, actually being zeolites, have cations trapped in tunnels or in highly regular cages, the latter being of a precise size *(18)*. Corma and co-workers *(19)* showed in 1989 that these types of structures are able to catalyze esterification reactions via strong acidic sites present. In their studies, HY zeolites, which are also aluminosilicates, were used for the esterification of carboxylic acids, the catalyzing capacity turned out to be improved by increasing the Si/Al ratio via dealumination. Also recent publications show the power of aluminosilicates in esterification catalysis. Al-MCM-41 aluminosilicates with a Si/Al ratio of 30 appeared to be successful in catalyzing the esterification of acetic acid with *n*-propanol and isopropanol and it is believed that this reaction mainly occured within the pores *(20)*. The same type of aluminosilicates with a Si/Al ratio of 8 was active as a catalyst in the esterification of palmitic acid with methanol, ethanol and isopropanol *(21)*. Next to these esterification reactions, also transesterification reactions have been catalyzed by high silica, large pore aluminosilicates. Transesterification of a number of alcohols in dry toluene was the most successful with aliphatic β-keto esters, while other substrate esters gave less promising results *(22)*.

The catalytic action of the molecular sieves that we used has, however, not been described yet. Despite of the less optimal Si/Al ratio found in the molecular sieve structure used in the experiments, it is very likely that the multiple transesterification reactions observed in the blanks are caused by the presence of these molecular sieves, especially because elevated temperatures and long reaction times are applied. This should be taken into account when these sieves are used to keep solvents dry in esterification and transesterification reactions in the future.

The same experiments were performed in solvents that were pre-dried using molecular sieves prior to use (samples 5 and 6 in Table 1). Molecular sieves and enzyme were not present during these reactions. After incubation, monoacylated oligomers (DP 3-8) could be detected, the ratio DSO 0 : DSO 1 was generally 80-90% : 20-10% and was not distinctly dependent on the DMSO-concentration (no further data given). This indicates that even pre-drying of the organic reaction medium using molecular sieves causes catalysis of the subsequent transesterification reaction, probably by compounds released from the molecular sieves' matrix to the solvent. The observation that the presence of molecular sieves during the reaction has a bigger effect on the catalysis than solvent pre-drying only, indicates that the elevated temperatures during the reaction may increase the release of catalyzing components from the sieves' structure.

Immobilized *C. antarctica* lipase B was also incubated together with molecular sieves at both DMSO concentrations, in this way the combined effect was studied (samples 11 and 12 in Table 1). The relative composition of the samples after 5 days of incubation at 60 °C can be found in Fig. 6.

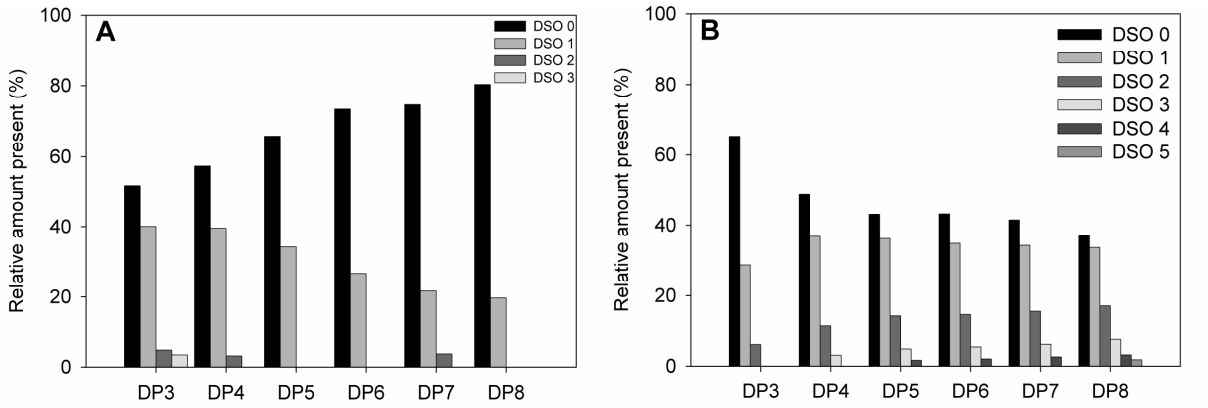


Fig. 6 Relative composition of reaction mixtures after incubations of FOS with vinyl laurate in the presence of molecular sieves and immobilized *C. antarctica* lipase B, in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH at 60°C for 5 days, derived from MALDI-TOF mass spectra. DP stands for degree of polymerization of the oligomer and DSO stands for degree of substitution per oligomer.

After the reaction performed at 20% (v/v) DMSO, mainly monosubstituted oligomers are observed (next to unsubstituted oligomers). Due to the relatively high enzyme activity at these conditions, the enzyme is overruling the chemical catalysis induced by molecular sieves and, therefore, mainly monosubstituted oligosaccharides are detected. At 40% (v/v) DMSO, again multiple substitution appears, mainly in the higher DP region. Under these conditions, the lower enzyme activity makes the molecular sieve material relatively more

active, leading to the detection of more multiple substituted oligosaccharides. Competition for substrates may also play a role in these effects.

When the results of the enzyme reactions with, and without molecular sieves at 20% (v/v) DMSO are compared, a difference in the ratio DSO 0 : DSO 1 can be observed for each DP. Without sieves (Fig. 4), the relative amount of DSO 1 present (compared to DSO 0) is increasing when the DP increases, this is the other way around when molecular sieves are present during the reaction (Fig. 6). This could be a result of the slightly reduced water content of the samples due to the drying effect of molecular sieves, making the oligomers with the higher DP's somewhat less soluble and therefore less reactive. It could also be that the reduced water content makes the enzyme more rigid, this could reduce the conversion of especially longer oligosaccharides.

Experiments including enzymes were also performed in solvents that were only pre-dried using molecular sieves (samples 7 and 8 in Table 1). The results showed only minor differences with those presented in Fig. 6, but multiple substituted oligosaccharides were not observed. The activity of catalysts released during drying is apparently too low compared to the enzyme activity to have a pronounced effect.

These results substantiate the warning published by Plou *et al.* to be cautious when selecting adjuvants present during carbohydrate acylations. This warning was based on the acylation of sucrose, catalyzed by several additives such as Celite, Eupergit-C or Na_2HPO_4. This implicated that chemical acylations must be taken into account when hydroxyl-containing compounds are acylated with with enol esters in polar solvents, using immobilized enzymes *(23)*. Based on our findings, molecular sieves should be added to this list of additives with catalytic properties. This might even have implications for several results that were published previously. Multiple substitutions that have been ascribed to enzyme activity before *(6, 11)*, may have been caused by the molecular sieves present. Very recently, side products were reported in work published by Walsh et al. *(12)* addressing the production of lactose monolaurate by immobilized *C. antarctica* lipase B. These authors already mentioned that the side products could be multiple substituted lactose oligomers; our work shows that the molecular sieves present during the synthesis are probably responsible for the formation of these products. The fact that Walsh et al. did not observe the presence of these multiple substituted products to the same extent in all cases means that the catalytic action of molecular sieves strongly depends on the reaction conditions. This explains the fact that the side reactions provoked by molecular sieves have been overlooked for a long time.

Conclusion

The presence of molecular sieves in the immobilized *C. antarctica* lipase B-catalyzed acylation of fructose oligosaccharides, using vinyl laurate, turned out to lead to multiple substituted products. Even the pre-drying of solvents using molecular sieves caused the release of catalyzing components to the solvents. These observations should be kept in mind when using molecular sieves in carbohydrate acylations in the future, especially since the catalytic action of molecular sieves seems to apply for several acyldonors and -acceptors. Conclusions drawn in the past about enzyme specificity should in some cases be re-evaluated. Conversely, the catalytic action of molecular sieves could be used when multiple substitution is desired.

Acknowledgement

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Chapter 5

Proof of principle for the synthesis of hydroxy-aryl esters of glycosidic polyols and non-reducing oligosaccharides with subsequent enzymatic coupling to a tyrosine-containing tripeptide

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Abstract

To enable enzymatic coupling of saccharides to proteins, several di- and trisaccharides were hydroxy-arylated using an anhydrous transesterification with methyl 3-(4-hydroxyphenyl)propionate, catalyzed by potassium carbonate. This transesterification resulted in the attachment of up to 3 hydroxy-aryl units per oligosaccharide molecule, with the monosubstituted product being by far the most abundant. The alkaline reaction conditions, however, resulted in a partial breakdown of reducing sugars. This breakdown could easily be bypassed by a preceding sugar reduction step converting them to polyols. Hydroxy-arylated products were purified by using solid phase extraction, based on the number of hydroxy-aryl moieties attached. Monohydroxy-arylated saccharose was subsequently linked to a tyrosine-containing tripeptide using horseradish peroxidase, as monitored by LC-MS^n. This proof of principle for peptide and protein glycation with a range of possible saccharides and polyols can lead to products with unique new properties.

Introduction

Proteins and (poly)saccharides are widely used in the food and non-food industry. Covalent hetero-coupling of these two classes of compounds results in the formation of new macro-molecular compounds, which may have techno-functional properties different to their non-modified analogues *(1)*. An example of this is the use of sodium caseinate-dextran conjugates in double emulsions *(2)*, which leads to smaller oil droplets and a narrower particle size distribution compared to systems containing the non-modified protein. In addition, improved resistance against aggregation and coalescence has been observed under acidic conditions. Improved emulsifying properties have also been reported when whey protein isolate (WPI) was conjugated with dextran by applying the Maillard reaction. The improved properties of the conjugate have been attributed to enhanced sterical stabilization caused by the attached hydrophilic polysaccharide moiety *(3)*. Positive effects on emulsifying properties were also observed after WPI conjugation with maltodextrins. These positive effects were mainly caused by the increased solubility of the conjugates at low pH. This type of conjugates has, therefore, been proposed as an alternative to gum Arabic *(4)*.

Because of the promising properties of protein-(poly)saccharide conjugates, the development of non-toxic procedures for their synthesis is of major interest for the food industry *(3)*. The covalent coupling of tyrosine-containing peptides to ferulic acid has been established using a kinetically controlled horseradish peroxidase-mediated reaction *(5)*. Based on this procedure, ferulic acid-containing arabinoxylans have been covalently linked to β-casein *(1)*. The natural availability of these oligo- and polysaccharides containing phenolic moieties for peroxidase-mediated linkage to proteins, however, is limited. This restricts the synthesis of a wide range of products. For this reason, a procedure for introduction of functional ferulic acid-type linkers to oligo- and polysaccharides is desired.

There are several examples of successful coupling of functional groups to oligo- and polysaccharides using both enzymatic and chemical transesterification reactions in anhydrous media. Recent research *(6)* has revealed that molecular sieves can catalyze the transesterification of vinyl laurate with inulin oligomers in mixtures of Bu^tOH and dimethylsulfoxide (DMSO). Earlier, saccharose-stearate and –palmitate have been produced in solvents like dimethylformamide (DMF) and DMSO based on methyl esters of the fatty acids, while using alkaline catalysts, like sodium methoxide and potassium carbonate *(7, 8)*. Polysaccharides have been modified using the same synthetic methodology. Starch has, for example, been transesterified with methyl palmitate to yield

thermoplastic polymers *(9)*. The coupling of a functional ferulic acid-like linker to oligo- and polysaccharides has, however, not been reported yet.

In this paper, the attachment of a functional phenolic moiety to oligosaccharides and polyols by anhydrous transesterification is described, which will be referred to as 'hydroxyarylation'. Attention is directed toward the synthesis, analysis, and purification of the products synthesized. As a *proof-of-principle* for enzymatic protein glycation, the peroxidase-mediated coupling of the products to small tyrosine-containing peptides, acting as protein mimics, is addressed. The opportunities created by the synthesis of the hydroxyarylated oligosaccharides are discussed.

Experimental

Materials

Ammonia (25% v/v), granular $NaBH_4$, H_2O_2 (30% v/v), K_2CO_3, DMSO (Seccosolv), glacial acetic acid (100%), saccharose, and maltose (monohydrate) were obtained from Merck (Darmstadt, Germany). Methyl 3-(4-hydroxyphenyl)propionate (hydroxyphenylpropionate methylester, HPPME), dichloromethane (Chromasolv), raffinose (pentahydrate), trehalose, maltitol, horseradish peroxidase Type VI (P-8375, EC 1.11.1.7), catalase (C-3155, EC 1.11.1.6), and D_2O (99.9 atom%) were from Sigma Aldrich Corporation (St. Louis, MO, USA). MeOH was from J.T. Baker (Phillipsburg, NJ, USA) and the tripeptide H-Gly-Tyr-Gly-OH (GYG, H-3670) from Bachem (Bubendorf, Switzerland). Met-Arg-Phe-Ala (MRFA) was bought from Research Plus, Inc. (Barnegat, NJ, USA). Water was purified by using a Milli-Q Gradient A10 system (Millipore Corporation, Billerica, MA, USA).

Methods

Reduction of oligosaccharides and subsequent purification

Reduction of oligosaccharides was performed using a protocol based on a previously published method *(10)*. One gram of each oligosaccharide was dissolved in 20 mL water in a plastic reaction tube, the pH of this solution was then adjusted to ~10.0 using ammonia. Granular $NaBH_4$ was subsequently added in a molar ratio reducing ends:$NaBH_4$ = 1:1.33 after which the tubes were capped accurately. Next, samples were incubated in a shaking incubator (120 rpm) at 40 °C for 90 min. Subsequently, acetic acid was added till the pH

remained below 5.0 and an excess of MeOH was added. Samples were dried under a stream of dry air and the addition of MeOH followed by drying was repeated twice.
Solid phase extraction (SPE) was applied for sample purification. Alltech Carbograph SPE columns (Alltech, Deerfield, IL, USA) were first activated by rinsing with 3 CV MeOH (CV = Column Volume: 3mL) and 4 CV water, respectively. Up to 500 mg of reduced sugar product was dissolved in 1 CV of water and the column was subsequently loaded with this sample. Salts were eluted with 3 CV of water, after which reduced sugars were eluted with 3 CV of 10% (v/v) MeOH in water and 3 CV of 40% (v/v) MeOH in water, respectively. Fractions were dried using a stream of dry air and stored at -20 °C until further analysis or application.

Verification of oligosaccharide reduction products

After reduction and purification, oligosaccharides were analyzed using High Performance Anion Exchange Chromatography (HPAEC) using equipment and columns as described previously *(11)*. Ten microliters of a 1 mg/mL sample solution was injected. Initial eluent composition (0.3 mL/min) was 0.1M NaOH, which was linearly changed to 0.4M NaOAc in 0.1M NaOH in 30 min. After this, the column was rinsed with 1M NaOAc in 0.1M NaOH for 5 min followed by an equilibration at starting conditions for 18 min. Data were processed using Chromeleon software (Dionex Corporation).

Synthesis of oligosaccharide-hydroxy-aryl esters

Oligosaccharides were transesterified with methyl 3-(4-hydroxyphenyl)propionate (HPPME) by dissolving 0.7 mmole of oligosaccharide together with 1.7 mmole of HPPME (ratio 1:2.3) and 25 mg of K_2CO_3 in 5 mL of DMSO in a 15 mL capped plastic reaction tube. Reaction mixtures were subsequently incubated in a shaking incubator (80 rpm) at 40 °C for seven days. To study the influence of the reagents on product yield and composition, a range of samples with different reagent ratios was incubated under the same conditions for four days. The reaction was stopped by cooling the samples to -20 °C until further analysis or purification.

Purification of the oligosaccharide-hydroxy-aryl esters

The hydroxy-arylated oligosaccharides in the reaction mixture were precipitated by mixing 1 mL of the reaction mixture with 20 mL of dichloromethane. After centrifugation (5 min; 2540 x *g*, room temperature), the supernatant was decanted and the precipitate was washed with 10 mL of dichloromethane. The precipitate was air-dried and subsequently dissolved

in 8 mL of water (0 °C). This solution was used for purification by means of Solid Phase Extraction (SPE). Sep-Pak Vac 20cc (5g) C18 Cartridges (Waters Corporation, Milford, MA, USA) with a CV of 8 mL were activated by rinsing with 3 CV of MeOH and 3 CV of water, respectively. The 8 mL of sample solution was then loaded. After this, compounds were eluted using successively: Water (6 CV), 10% (v/v) MeOH in water (6 CV), 20% (v/v) MeOH in water (3 CV), 30% (v/v) MeOH in water (3 CV), 40% (v/v) MeOH in water (3 CV), and 60% (v/v) MeOH in water (3 CV). Fractions were collected per 3 CV, dried using a stream of dry air, and weighted. Fractions were subsequently dissolved in water to a concentration of 5 mg/mL and analyzed by using RP-UPLC-MS. Fractions were stored at -20 °C until further use.

U-HPLC-MS analysis of oligosaccharide-hydroxy-aryl esters

The compositions of the reaction mixtures from oligosaccharide hydroxy-arylation and the SPE fractions were determined using U-HPLC-MS equipment and columns as described elsewhere *(12)*. For the reaction mixture analysis, 3 µL of a 200x-diluted sample in DMSO was injected. For the SPE fractions, 5 µL of a 5 mg/mL solution in water was injected. For elution (30 °C), a gradient was applied at 300 µL/min using the following 2 eluents: A: water containing 1% (v/v) acetonitril and 1% (v/v) HOAc; B: acetonitril containing 1% (v/v) HOAc. Gradient: 0-2 min: 95-80% A; 2-9 min: 80-70% A; 9-11 min: 70-60% A; 11-15 min: 60-20% A; 15-16 min: 20-0% A; 16-18 min: 0% A isocratic; 18-22 min: 0-95% A; 22-26 min: 95% A isocratic. Linear gradients were used in all non-isocratic steps. Detection was performed by the PDA detector scanning between 200 and 400 nm. Half of the eluent was led to the ESI-MS system running in the negative mode. Helium was used as a sheath gas and nitrogen as auxiliary gas. The capillary temperature was set at 250 °C and the spray voltage at 4.7 kV. Settings of the MS were tuned using a purified saccharose-HPP mono-ester sample. After each first scan event, being a full scan between *m/z* 300-2000, MS/MS analysis was performed in a second scan event for the most intense ion in the full MS spectrum, using normalized collision energy of 35.0 units. During the first 3.5 min of each run, eluent was directed to the waste in order to protect the MS from DMSO influx. Data were processed using XCalibur software (Thermo Scientific).

NMR analysis of hydroxyl-arylated saccharose

Purified saccharose-HPP mono-ester (14mg) was dissolved in 0.5 mL D_2O containing 0.07% 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt (TMSP). NMR spectra were recorded at a probe temperature of 300K on a Bruker Avance-III-600 spectrometer (Bruker

Daltonics, Bremen, Germany), equipped with a cryo-probe located at Biqualys (Wageningen, The Netherlands). Chemical shifts are expressed in ppm relative to internal TMSP at 0.00 ppm. 1D and 2D COSY, TOCSY, HMBC, and HMQC spectra were acquired using standard pulse sequences delivered by Bruker. For the ^{1}H-COSY and -TOCSY spectra, 400 experiments of 2 scans and 4 scans were recorded, resulting in measuring times of 0.5 h and 1 h, respectively. The mixing time for the TOCSY spectra was 100 ms. For the [^{1}H,^{13}C]-HMBC and -HMQC spectra 800 experiments of 24 scans and 512 experiments of 4 scans were recorded, resulting in measuring times of 6.2 h and 1.2 h, respectively.

Peroxidase-mediated coupling of hydroxy-arylated oligosaccharides to tyrosine-containing peptides

The SPE-purified hydroxy-arylated oligosaccharides were linked to the peptide GYG by using a method based on the work published before (5). GYG (0.5 mg) was dissolved in 1 mL 0.1 M sodium phosphate buffer pH 7.0 in a capped reaction tube. Then, 35 µL of a 1 mg/mL horseradish peroxidase solution was added. This was followed by the addition of 8 x 3 µL of 0.5M H_2O_2 and 8 x 15 µL of HPPME (3 mg/mL) or oligosaccharide-HPP (5mg/mL). Between the 8 sets of additions, intervals of 20 min were applied. Samples were incubated at 30 °C at 400 rpm in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany). Thirty minutes after the final substrate addition, the reaction was stopped by adding 4 µL of a 1% (w/v) catalase solution. Samples were then stored at -20 °C until analysis.

Analysis of glycopeptides

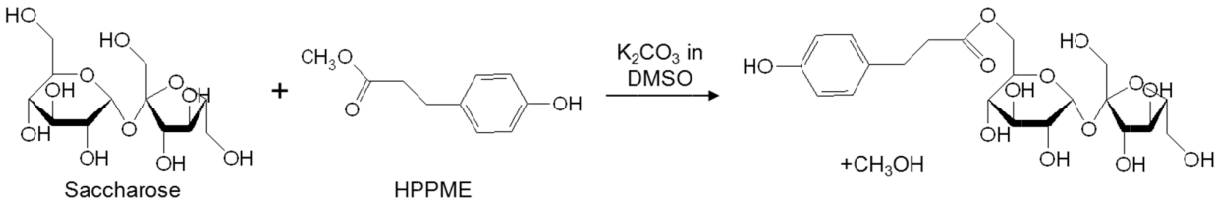
Products of the peroxidase-catalyzed coupling of GYG to HPPME or oligosaccharide-HPP compounds were also analyzed using RP-UHPLC-PDA/MS. The system, column (30 °C), flow rate, and eluents used for analysis were the same as outlined above. Ten µL of the sample solutions, as obtained in the previous section, was injected. The following linear gradients were applied: 0-9 min: 98-80% A; 9-11 min: 80-65% A; 11-15 min: 65-20% A; 15-16 min: 20-0% A; 16-18 min: 0% A isocratic; 18-22 min: 0-98% A; 22-26 min: 98% A isocratic. ESI-MS was run in the positive mode. Helium was used as the sheath gas and nitrogen as the auxiliary gas. The capillary temperature was set at 350 °C and the source voltage was 3.5 kV. Settings of the MS were tuned using a solution of the peptide MRFA. After each first scan event, being a full scan between *m/z* 100-2000, MS/MS analysis was performed in a second scan event on the most intense ion in the full MS spectrum, using

normalized collision energy of 35.0 units. Data were processed using XCalibur software (Thermo Scientific).

Results and discussion

Hydroxy-arylation of various oligosaccharides

Since enzymatic linkage of oligosaccharides to proteins requires an activated molecule, a compound containing a hydroxyl-aryl side group was first covalently bound to various oligosaccharides. In line with results obtained before with feruloylated arabinoxylans and β-casein *(1)*, this would enable peroxidase-mediated covalent oligosaccharide-protein coupling. Methyl 3-(4-hydroxyphenyl)propionate (HPPME) was selected as an activator based on availability and similarity to ferulic acid. Various oligosaccharides were subjected to a transesterification with this compound catalyzed by K_2CO_3 in DMSO at 40 °C for several days, yielding hydroxy-arylated products. This reaction is illustrated in Scheme 1.



Scheme 1 Schematic representation of the hydroxyl-arylation of saccharides, in this case saccharose. The reaction can theoretically occur at any free hydroxyl group of the saccharide.

In Fig. 1, RP-UHPLC elution profiles of hydroxy-arylated saccharose, trehalose, and raffinose (non-reducing sugars) after seven days of incubation are shown. The compounds, visible using PDA, that have been annotated based on MS spectra are indicated in this figure. An overview of these compounds including masses of the mother ions and their fragments is given in Table 1.

As can be observed in Fig. 1 and Table 1, a wide range of products with one or more HPP (hydroxyphenylpropionyl) substituents has been formed during the hydroxy-arylation of saccharose, trehalose, and raffinose. This illustrates the non-selective character of the modification. The fragmentation data confirm the nature of the compounds. Mono- and di-substituted saccharoses are, for example, represented by mother ions with *m/z* 489 and 637, respectively. For the mono-substituted saccharose, loss of the complete hydroxyl-aryl moiety occurs, resulting in a fragment with *m/z* 341, representing the saccharose unit.

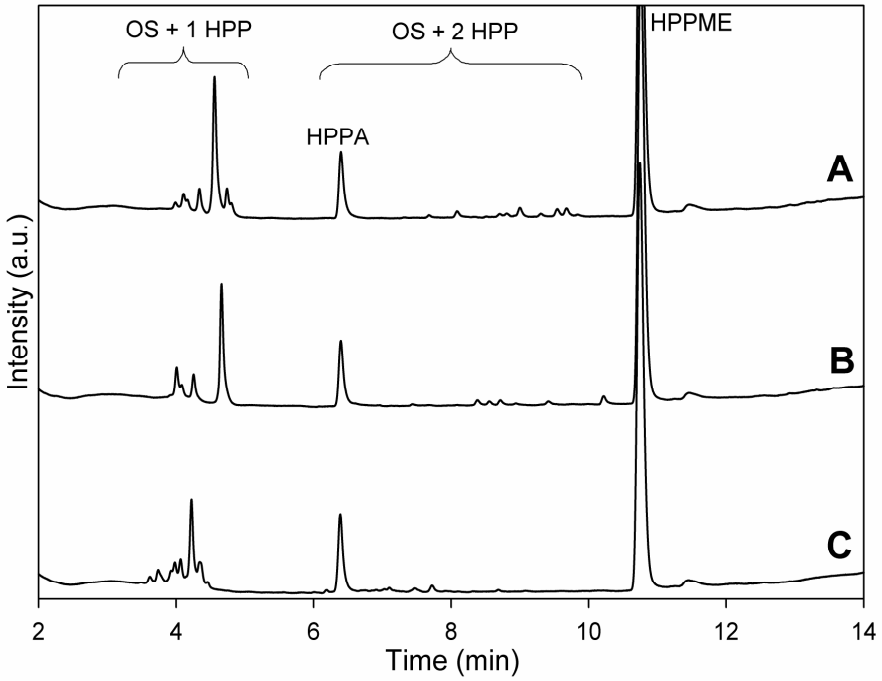


Fig. 1 RP-UHPLC-PDA 200-400nm elution profiles of (A) saccharose, (B) trehalose, and (C) raffinose after a 7-day incubation with HPPME and K_2CO_3 in DMSO at 40 °C, molar ratio oligosaccharide : HPPME 1:2.3. The number of HPP (hydroxyphenyl propionyl) substituents per saccharide molecule is indicated. OS=oligosaccharide, HPPA=hydroxyphenyl propionic acid.

Table 1 Overview of the products identified using ESI-MS and ESI-MS/MS after a 7-day K_2CO_3-catalyzed transesterification of HPPME and various oligosaccharides in DMSO.

Compound	***Elution time (min)***	***m/z expected (neg. mode)***	***m/z observed (neg. mode)***	***m/z fragment ions observed (neg mode)***
Saccharose-$(HPP)_1$	3.9-5.0	489.46	489.32	323.14; 341.16; 407.09
Saccharose-$(HPP)_2$	7.6-10.0	637.62	637.27	341.15; 471.27; 489.23
Saccharose-$(HPP)_3$	13.2-13.8	785.78	785.32	471.29; 489.33; 619.36; 637.29
Trehalose-$(HPP)_1$	3.9-4.9	489.46	489.30	164.94; 323.20
Trehalose-$(HPP)_2$	7.4-9.6	637.62	637.26	323.19; 471.24; 489.36
Trehalose-$(HPP)_3$	13.4-13.5	785.78	785.32	-
Raffinose-$(HPP)_1$	3.5-4.6	651.57	651.34	485.31; 503.32
Raffinose-$(HPP)_2$	5.8-7.9	799.73	799.30	485.31; 633.26; 651.36
Raffinose-$(HPP)_3$	12.1-12.2	947.89	947.40	633.32; 651.42; 781.40; 799.35
HPPME	10.7	179.20	357.26	-
HPPA	6.4	165.17	165.02; 329.19	-

MS/MS on the di-substituted saccharose results in a loss of one hydroxy-aryl unit (*m/z* 489) or both hydroxy-aryl units (*m/z* 341). Non-reacted HPPME and hydrolyzed HPPME, the latter referred to as hydroxyphenylpropionic acid (HPPA), are also present in the samples after incubation. The formation of HPPA is the result of the presence of traces of water, either from the environment, solvents, or substrates used, leading to hydrolysis of HPPME. Furthermore, the HPPA was found already as an impurity in the substrate used.

The presence of oligosaccharides containing up to 3 hydroxylphenylpropionyl (HPP) units was confirmed after seven days of incubation, with oligosaccharides containing one HPP substituent as the most abundant product. Compounds with equal *m/z* ratios generally elute in a cluster of peaks. The time window of such a cluster is generally wider and the number of peaks is higher when the number of HPP units is higher. Esterification using alkaline catalysts preferably leads to derivatization at the primary positions, although subsequent migrations to other hydroxyl groups have also been reported *(13)*. It is clear from Fig. 1 that there is indeed a difference in the abundance of the several isomers. Saccharose contains 8 hydroxyl groups, of which 3 are primary. In theory, there are 8 possible isomers of the mono-ester. At least 7 of them can be observed in the U-HPLC chromatogram, of which one is the most abundant. NMR analysis was performed on SEP-PAK purified saccharose-HPP mono-ester, being the 30% MeOH fraction in Fig. 5 (*vide infra*). The combination of various 2D techniques afforded full assignment of both proton and carbon spectra of the two main HPP-esters of saccharose. With a total product area of 1.24 (Fig. 2), and areas for glucose O-6 and fructose O-6 of 0.70 and 0.32, respectively, 0.70/1.24 → 56% of HPP was substituted the O-6 position of the glucose moiety and 0.32/1.24 → 26% to the O-6 position of the fructose moiety. Besides cross peaks in the HMBC spectrum between the HPP carboxyl and H-6 protons, the esterification at the 6 positions is also evident from the downfield shifts of 3.0 or 3.4 ppm for the C-6 and upfield shifts of 2.1 or 3.0 ppm for the C-5 of glucose and fructose, respectively. Three minor signals (area 0.22) are visible indicating that the remaining 0.22/1.24 → 18% HPP is linked to at least three different other positions. These structures could not be assigned, but they comprise probably esterification of the other possible positions of saccharose. The preferred substitution at the O-6 position of the glucose moiety is in line with previous observations, as it was shown to be the most stable ester in the case of acylation *(14)*. Trehalose and raffinose show a similar trend regarding the preference for a particular isomer, but were not analyzed in more detail. The abundance of the two major esters in NMR appears not to be in line with the distribution observed in Fig. 1A, as only one major peak is observed in this trace. This changed distribution might be caused by the purification step, as will be discussed later.

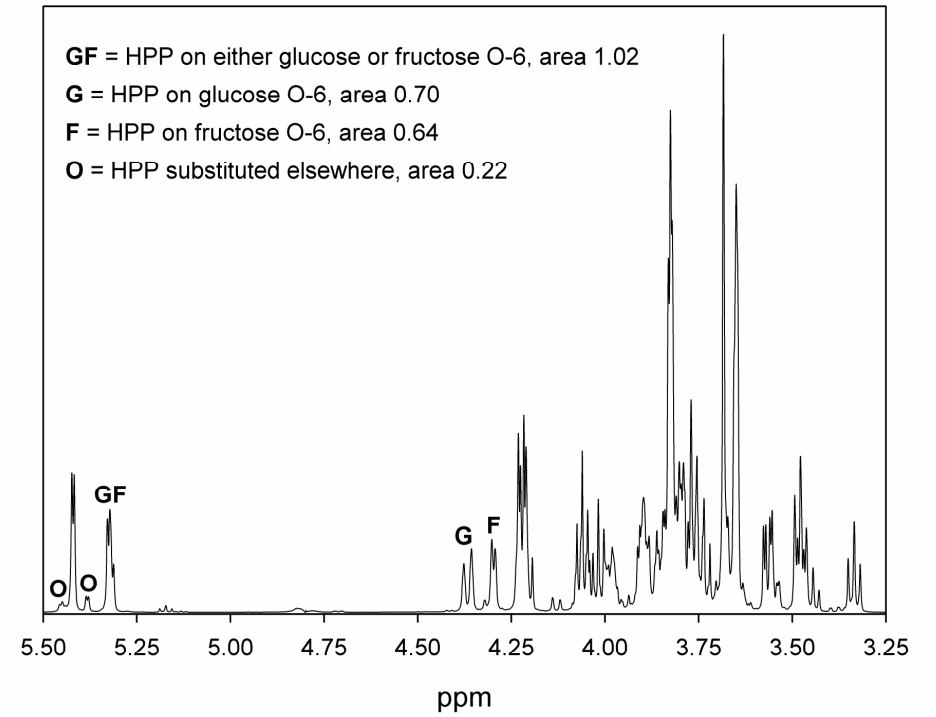


Fig. 2 ^{1}H NMR spectrum of saccharose-HPP mono-ester. Relevant peaks are indicated. For calculations, the area of HPP at fructose O-6 (**F**) was halved because of two protons coinciding.

To increase the transesterification reaction rate, samples were incubated at increased temperature (80 °C) and reduced pressure (3 kPa) for a shorter time (12 h) compared to our base case (40 °C, atmospheric pressure, seven days). Based on UHPLC elution profiles, this led to similar results with respect to reaction yield (data not shown). This illustrates that a faster production of the hydroxy-arylated saccharides is made feasible by adjustment of the reaction conditions. In this study, however, this was not regarded as a main goal.

When the results after hydroxy-arylation of maltitol and maltose are compared, some interesting observations can be made. After incubation, hydroxy-arylated maltitol (a polyol) in DMSO showed a slight off-white coloring compared to the solvent alone, while the reaction mixture containing maltose (reducing sugar), HPPME, and K_2CO_3 showed a distinct brown color. When solely maltose was incubated with K_2CO_3 in DMSO, the same brown color appeared. In addition, it was demonstrated that HPPME does not contribute to this brown color. This indicates that reducing sugars are apparently degraded due to the alkaline conditions caused by the K_2CO_3. These alkaline decomposition reactions related to the reducing ends of saccharides have been observed before *(15)*.

One of the goals of the hydroxy-arylation of oligosaccharides is their subsequent conjugation with proteins. Oligosaccharide properties, such as charge, conformation,

linkage flexibility, and chain length are expected to have a major effect on the properties of the glycated proteins. In order to apply a wide range of sugar properties, it is required to be able to hydroxyl-arylate reducing as well as non-reducing sugars. Therefore, reducing sugars were reduced to polyols to prevent degradation during hydroxy-arylation.

Reduction and hydroxyl-arylation of reducing oligosaccharides

Trace A in Fig. 3 shows that the hydroxy-arylation of maltose (trace A) is not as successful as observed for its non-reducing equivalent maltitol (trace B). This illustrates that, apart from the brown color formation, also lower yields are observed as a result from alkaline decomposition reactions of reducing sugars.

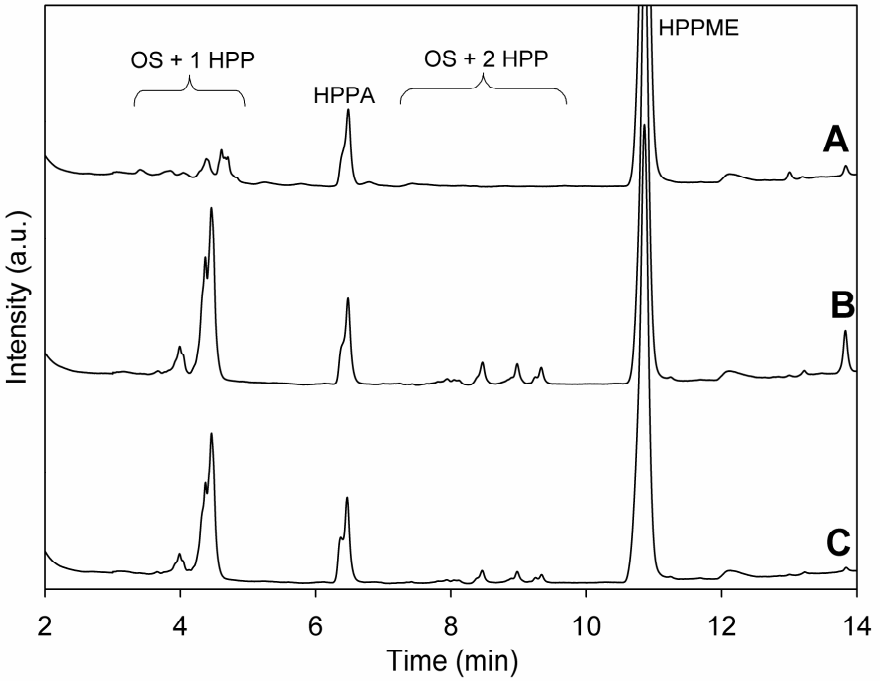


Fig. 3 RP-UPLC-PDA 200-400nm elution profiles of (A) hydroxy-arylated maltose, (B) hydroxy-arylated commercial maltitol, and (C) hydroxy-arylated purified reduced maltose, after 3 days of incubation with HPPME and K_2CO_3, molar ratio oligosaccharide : HPPME 1:1 on a 150µl incubation scale. Equal amounts were injected for analysis. The number of HPP substituents per saccharide has been indicated in the figure. OS=oligosaccharide.

The modification using maltitol is more successful, while the number of isomers present is lower than that observed after a three-day incubation of saccharose with HPPME in a 1:1 ratio, catalyzed by K_2CO_3 (data not shown). The lower number of isomers observed for maltitol might be related to a strong preference for specific hydroxyl groups present in maltitol. The hydroxyl groups in the open chain D-glucitol moiety present in maltitol might

be more prone to modification than those in the α-D-glucose moiety because of better accessibility, as mentioned previously for enzymatic modification *(16)*.

Although maltitol is commercially available, maltose was subsequently used as a model compound to design a generic reduction and clean-up procedure to enable the use of reducing sugar-based compounds as starting materials for the hydroxy-arylation. After reduction of maltose using $NaBH_4$, the effectiveness of this treatment was verified by using HPAEC analysis. Reduced maltose showed retention times similar to the retention time of commercial maltitol. No signal was observed at the retention time of maltose, indicating that the reduction of maltose was complete. While performing subsequent hydroxy-arylation experiments using the reduced maltose samples, salts originating from the reduction treatment inhibited the transesterification reaction and no products were observed (data not shown). For this reason, reduced maltose was purified by using carbo SPE columns. Salts could successfully be eluted using water, after which the reduced maltose could be eluted using 10% (v/v) MeOH in water, as verified using HPAEC. The yield after hydroxy-arylation of purified reduced maltose (trace C) is much higher than for maltose, and comparable to the yield observed after transesterification of commercial maltitol. Furthermore, no formation of a brown color was observed after hydroxy-arylation of the reduced maltose. The latter indicates that the reduction to a polyol using $NaBH_4$, followed by accurate purification, is a suitable method to prevent degradation reducing sugars during hydroxy-arylation in DMSO catalyzed by K_2CO_3. This means that a range of oligosaccharides can be activated for subsequent enzymatic coupling to protein, possibly after conversion to a polyol. An additional advantage of the application of non-reducing sugars or glycosidic polyols for protein glycation is that, in contrast to reducing sugars, they cannot take part in undesired Maillard reactions.

Effect of substrate concentration and ratio on oligosaccharide hydroxy-arylation

For future applications, an understanding of the effects of substrate ratios and concentrations on yield and composition of hydroxy-arylated oligosaccharides is necessary. To obtain a first indication of these effects, two types of experiments were performed in DMSO using saccharose and HPPME. In the first experiment, the oligosaccharide concentration was varied at a fixed concentration of HPPME. In the second experiment, the HPPME concentration was varied while the oligosaccharide concentration was fixed. The concentrations used during the synthesis experiments as discussed above were used as a basis, and the catalyst concentration was kept constant. The amount of mono-substituted

oligosaccharide was determined after four days of incubation, using RP-UPLC-PDA at a wavelength of 274nm.

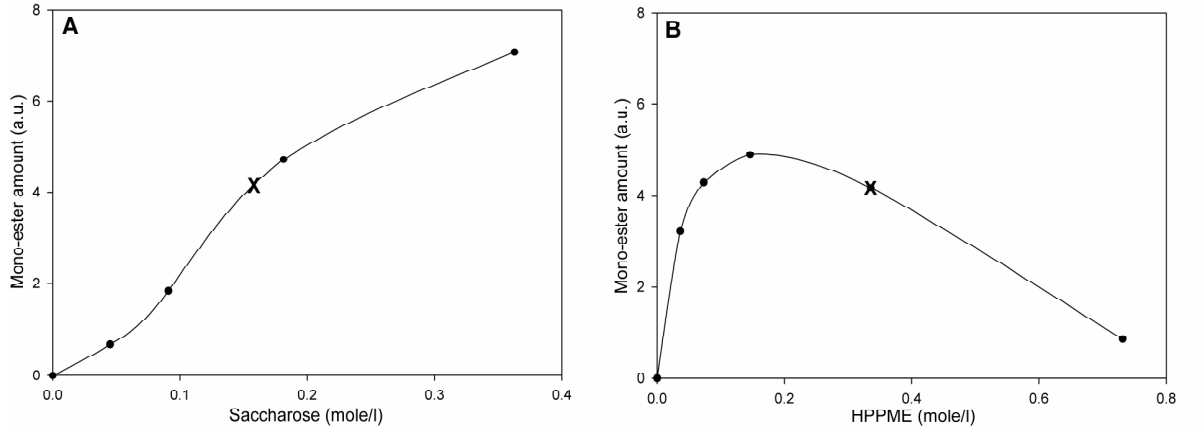


Fig. 4 Relative amounts of mono-esters formed after hydroxy-arylation of saccharose in DMSO (40 °C, 80 rpm, 4 days) using (A) a fixed HPPME concentration of 0.34 mole/l at various saccharose concentrations and (B) a fixed saccharose concentration of 0.15 mole/l at various HPPME concentrations. The crosses indicate the point where concentrations in both figures are equal, being the conditions used for synthesis as described in Fig. 1 (molar ratio saccharose: HPPME = 1:2.3).

The amount of mono-ester formed after incubation is almost linearly increasing with the amount of saccharose present (Fig. 4A). Apparently, saccharose concentration is the limiting factor. At the maximum saccharose concentration used, the molar ratios of the substrates are about 1:1. A further increase of the saccharose concentration may lead to a higher absolute yield, but in this case not enough HPPME would be available to modify all saccharose molecules present. Interestingly, the amount of multiple substituted saccharose molecules is negligible under all conditions after four days of incubation, even when the saccharose amount is relatively low (data not shown). This indicates that multiple substitution is relatively hard to reach, probably as a result of sterical factors. Thus, the non-selectivity of the reaction is mainly expressed in the variety of isomers observed, and to a much lower extent in multiple substitution of the saccharides.

When the HPPME concentration is varied while using a fixed saccharose concentration (Fig. 4B) it appears that an excess of HPPME is not beneficial for the reaction rate, as the maximum yield is obtained at a 1:1 substrate ratio. A further increase of the HPPME concentration leads to a decrease in yield. Amounts of multiple esters are relatively low in all cases, and their amounts are proportionally increasing with the amount of mono-ester (data not shown). Addition of an excess of HPPME does, under these conditions, not promote a multiple substitution of the saccharose. The lower reaction rate at higher HPPME

concentrations may be a result of the presence of increased levels of HPPA in the reaction mixture, having an influence on the alkaline conditions needed for catalysis. Use of a higher purity of HPPME or a higher concentration of catalyst may help to overcome this problem. These results indicate that adjustment of substrate and catalyst ratios and concentrations can be used to increase the reaction efficiency and possibly to steer the product composition.

Fractionation of hydroxy-arylated oligosaccharides

Hydroxy-arylated oligosaccharides offer the possibilities for glycation of proteins using a horseradish peroxidase-mediated reaction. The mono-substituted oligosaccharides would be the most suitable to start with, since the multiple substituted oligosaccharides might lead to protein cross-linking. Separation of oligosaccharides by their number of substituents is, therefore, important for a successful application. Products as described in Fig. 1 were first precipitated in, and rinsed with, dichloromethane to get rid of DMSO and non-reacted HPPME. The precipitate was then dissolved in water and fractionated using reversed phase SPE. Compounds were eluted using different water/MeOH ratios, the composition of the fractions was determined using RP-UHPLC. In Fig. 5, the composition of (1) fractions of interest resulting from hydroxy-arylated saccharose, (2) the initial product mixture, and (3) the material removed using dichloromethane can be found.

While precipitating the products using dichloromethane, most of the non-reacted HPPME remained in solution and was, therefore, removed from the reaction mixture. Furthermore, the separation using SPE appears to be successful. Especially the 20% (v/v) and 30% (v/v) MeOH fractions contain rather pure mono-substituted oligosaccharides. In the 40% (v/v) and 60% (v/v) MeOH fractions, most of the higher substituted oligomers can be found. Separation of the isomers has not been established by SPE, but could possibly be performed in the future by applying RP-HPLC on a preparative scale. Based on product weights, about 40% of the saccharose has been converted to products containing one or more HPP moieties. By far, the major part of those consists of the mono-substituted saccharose, desired for protein glycation. The isomeric distribution of the mono-ester in the fractions appears to be somewhat different from the distribution in Fig. 1A although fully in agreement with earlier presented NMR-data. This might be a result of the slightly preferred elution of the relatively apolar fructose O-6 ester with 30% MeOH, or intramolecular migration of the substituted groups during dissolution in water or MeOH due to unstable esters, as observed before *(13, 14)*. Hence, the abundance on the glucose O-6 just after synthesis might be slightly underestimated, and the abundance on the fructose O-6 slightly

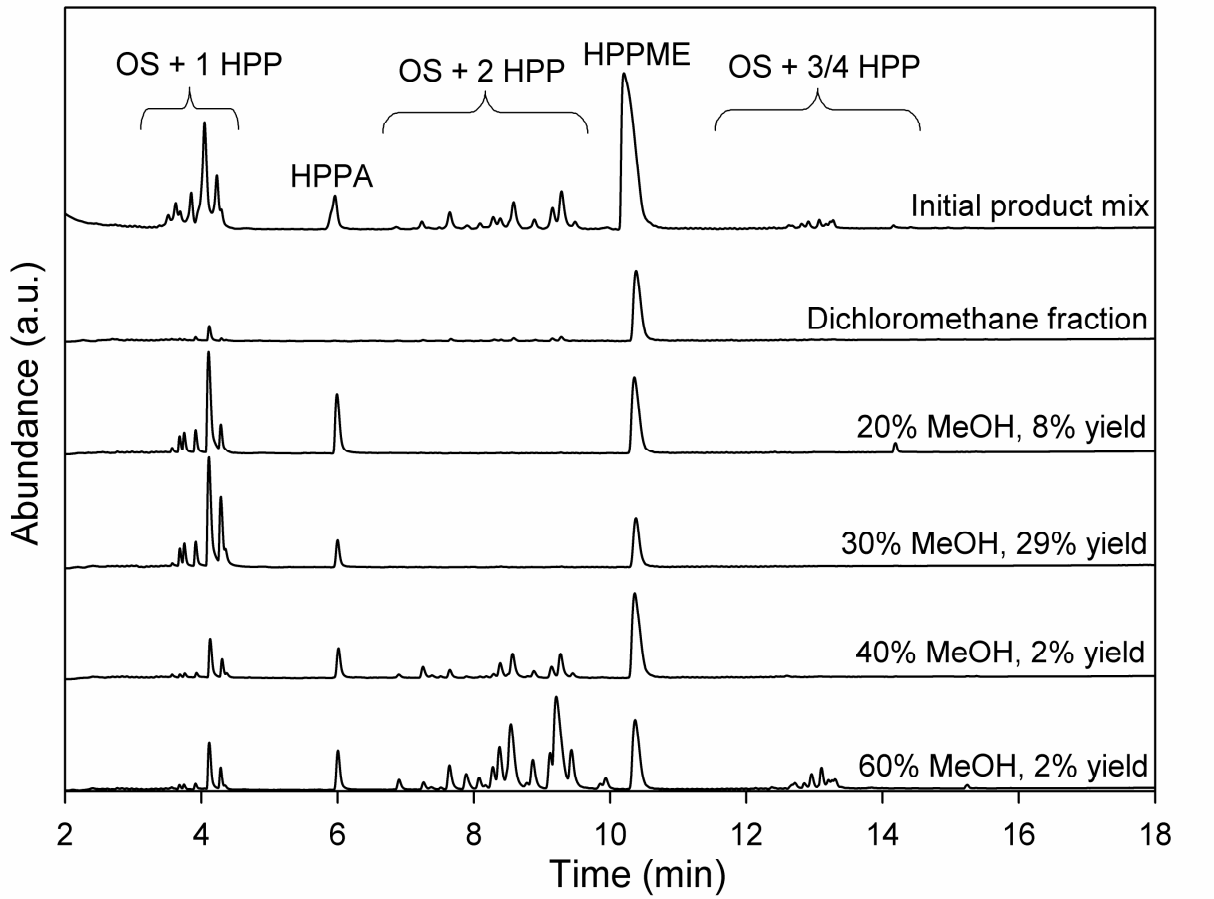
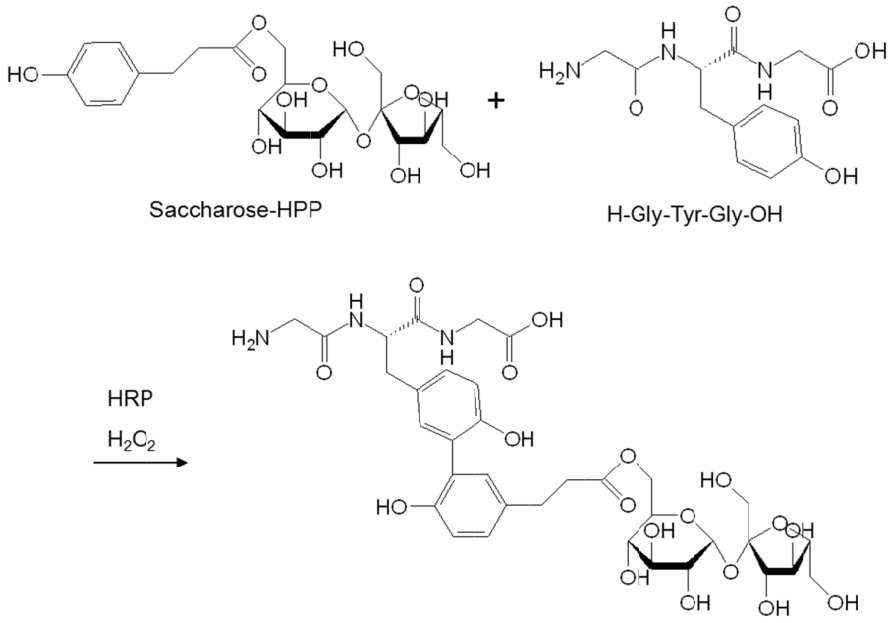


Fig. 5 RP-UHPLC-PDA 200-400nm elution profiles of reversed phase SPE fractions of the HPPME-saccharose reaction mixture, the initial mixture, and the dichloromethane fraction. Product yields are expressed as weight % of initial saccharose. The number of HPP substituents has been indicated. OS=oligosaccharide.

overestimated when analyzing isolated fractions. The presence of HPPME and HPPA in the fractions is probably the result of hydrolysis/methanolysis of part of the product due to remaining traces of catalyst.

3.5 Attachment of hydroxy-arylated oligosaccharides to GYG

After synthesis and fractionation of the hydroxy-aryl mono-esters of saccharose, the peroxidase-mediated attachment of this compound to tyrosine-containing peptides was explored. For this, the tripeptide Gly-Tyr-Gly (GYG) was used. Next to the purified mono-ester of saccharose, also the HPPME itself was used as a substrate to be linked to the GYG peptide. An illustration of this reaction can be found in Scheme 2.



Scheme 2 Schematic representation of the peroxidase-mediated linkage of hydroxy-arylated saccharose to the tripeptide GYG. There are several possibilities for the position of the link between the aromatic rings. HRP stands for horseradish peroxidase

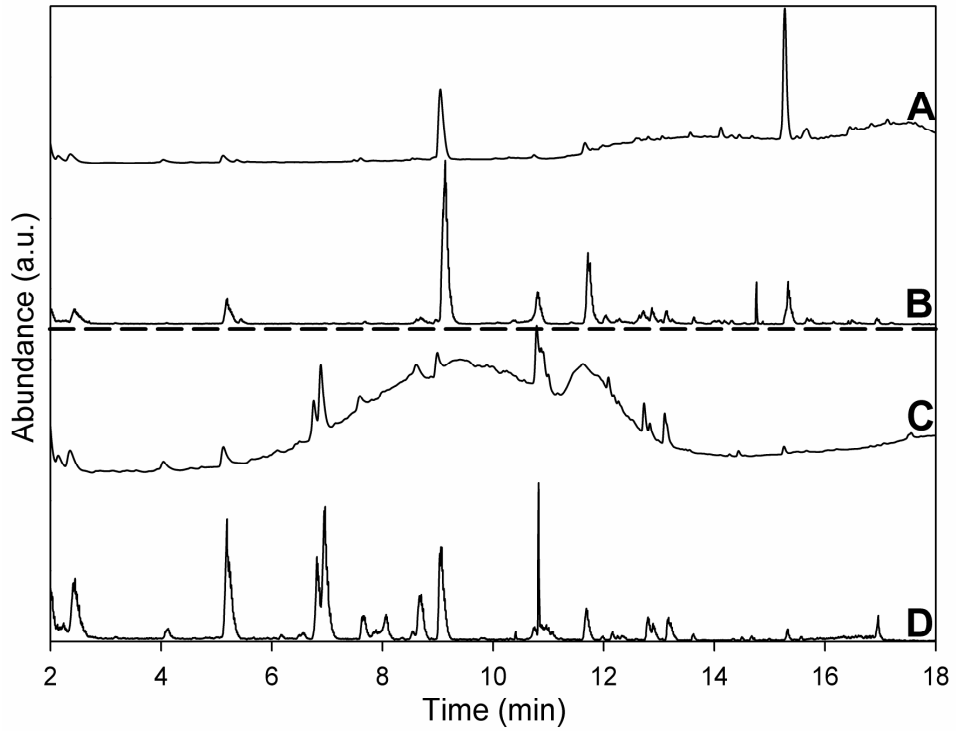


Fig. 6 Elution profiles of reaction mixtures after incubation of: GYG with HPPME and peroxidase on RP-UPLC-PDA (A) and RP-UPLC-MS (B), and after incubation of: GYG with saccharose-HPP and peroxidase on RP-UPLC-PDA (C) and RP-UPLC-MS (D). PDA was scanning between 200-400 nm, base peak MS profiles are shown. Profiles were scaled to their highest peak.

Table 2 Compounds identified using ESI-MS (positive mode) during the peroxidase-mediated attachment of GYG to HPPME and to saccharose-HPP, including their elution times (Fig. 6).

GYG-HPPME (fig 6 A/B)			
Elution time / window (min)	***m/z expected (pos mode)***	***m/z observed (pos mode)***	***Compound***
5.1-5.6	-	514.38	Not identified
8.5-8.9	-	384.49	Not identified
9.0-9.4	474.49	474.38	GYG-HPPME
10.5-11.0	474.49	474.40	GYG-HPPME
11.6-11.9	474.49	474.43	GYG-HPPME
12.0-15.0	652.68 830.86 1009.05	652.46 830.50 1008.54	GYG-$HPPME_2$ GYG-$HPPME_3$ GYG-$HPPME_4$
15.2-15.5	359.39	359.38	$HPPME_2$
15.5-17.5	537.58 715.76 893.94	537.41 715.38 893.38	$HPPME_3$ $HPPME_4$ $HPPME_5$
Saccharose-HPP-GYG (fig 6 C/D)			
Elution time / window (min)	***m/z expected (pos mode)***	***m/z observed (pos mode)***	***Compound***
5.1-5.4	-	514.40	Not identified
6.1-6.7	784.75	784.38	GYG-Saccharose-HPP
6.7-7.2	784.75	784.41	GYG-Saccharose-HPP
7.6-7.8	460.47	460.39	GYG-HPPA
7.8-8.9	784.75	784.44	GYG-Saccharose-HPP
9.0-9.3	474.49	474.39	GYG-HPPME
10.7-11.2	979.91	996.37	$(Saccharose\text{-}HPP)_2$ (possibly +OH)
11.6-11.9	474.49	474.44	GYG-HPPME
12.7-13.4	-	686.33	Not identified

From the RP-UHPLC-PDA/MS results in Fig. 6 and the overview in Table 2, it can be concluded that the coupling of HPPME to GYG by horseradish peroxidase (HRP) has been successful. The two major peaks observed in the chromatogram (traces A and B) represent GYG-HPPME and HPPME-HPPME products. This indicates that, as expected, both GYG and HPPME are substrates for HRP. It also means that, despite the gradual addition of HPPME, still a major amount of HPPME homoconjugates is formed. Furthermore, a low amount of GYG homoconjugates is formed. Apparently, HPPME is a more favorable substrate for HRP than GYG. This means that kinetic control is of major importance when a maximum amount of heteroconjugates is desired. This is in line with observations done when ferulic acid (FA) was incubated with HRP *(5)*. FA appeared to be far more reactive than GYG, which led to the conclusion that GYG had to be present in a large excess compared to FA to yield similar radical concentrations for each compound *(5)*. As can be seen in Table 2, not all GYG-HPPME products elute in the same peak.

For the coupling of saccharose-HPP to GYG, a similar trend can be observed (Fig. 6, traces C and D). The major peaks at 6.7-7.2 min and 10.7-11.2 min represent saccharose-HPP-GYG and (saccharose-HPP)$_2$ respectively. This means that the HRP reactivity of HPPME is not majorly affected by its coupling to saccharose. Two more peaks for saccharose-HPP-GYG have been observed in the analysis, representing isomers. This also accounts for the GYG-HPPME products; next to the major peak around 9.0 min, another 2 relatively small peaks are observed. This could be a result of coupling of the phenolic ring of HPP(ME) at different positions on the phenolic ring of tyrosine. The appearance of GYG-HPP and GYG-HPPA is related to the presence of HPPME and HPPA in the fractionated saccharose-HPP product, as mentioned before. These compounds of course also take part in the reaction.

Despite the side-reactions observed, it is clear that the coupling of saccharose-arylesters to GYG is successful. This successful enzymatic synthesis of glycopeptides, as described above, confirms that the pathway for controlled enzymatic protein glycation is realistic.

Conclusions

Several hydroxy-arylated non-reducing oligosaccharides and polyols were synthesized using a transesterification reaction under anhydrous conditions. The saccharose mono-ester was mainly substituted at the O-6 position of the glucose moiety, and was successfully linked to a tyrosine-containing tripeptide using peroxidase. The latter can be regarded as a model for enzymatic protein glycation. The possibility to hydroxy-arylate a range of oligosaccharides and glycosidic polyols tackles the dependence on naturally occurring

hydroxy-arylated compounds for protein modifications catalyzed by oxidative enzymes. This may open the synthesis of products with unique new functional properties.

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Chapter 6

Effect of saccharide structure and size on the degree of substitution and product dispersity of α-lactalbumin glycated via the Maillard reaction

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Abstract

The course of the Maillard reaction between α-lactalbumin and various mono- and oligosaccharides in the solid state has been studied using UPLC-ESI-TOF MS. Individual reaction products were monitored for their degree of substitution per protein molecule (DSP) and for subsequent dehydration steps. The Maillard reaction rate appeared to depend on saccharide type and decreased when the size of the reacting saccharide increased. Interestingly, the conjugation with charged saccharides was clearly hindered when a specific average DSP had been reached, probably as a result of electrostatic repulsion. The DSP varied between 0 and 15, and the standard deviation of the average DSP increased up to 1.9 and was hardly dependent on saccharide type. This standard deviation illustrates the dispersity of the Maillard reaction products. In order to eliminate the influence of the protein properties on the Maillard reaction rate, similar experiments were performed with a lysine-containing model peptide and a series of maltooligosaccharides. Also in this case, the relative Maillard reaction rate quickly decreased with increasing size of the saccharide, with these relative rates being 1 for glucose, 0.28 for maltose, and 0.16 for maltotriose. Comparison of the results obtained using α-lactalbumin and the dipeptide made clear that the Maillard reaction rate is determined by a number of factors, including saccharide reactivity, and the accessibility of the lysine residues.

Introduction

The Maillard reaction, also referred to as non-enzymatic browning, is an important reaction in relation to food quality, because color, flavor, and nutritional value of the food product are affected *(1)*. The reaction is initiated by heating a dry mixture or a solution of proteins and reducing saccharides. Amadori rearrangements cause the amino groups in the protein to link to the reducing end of the saccharide *(2)*. The reactivity of different types of saccharides and the properties of the resulting glycated proteins have been the subject of a number of studies. A generally accepted view states that the reaction rate under given conditions is related to the proportion of the reducing sugar present in the open chain form: the higher this proportion, the faster and more intense the browning *(1)*. The open-chain proportion of a saccharide in water depends on type of saccharide and temperature, as estimated for a series of monomeric saccharides *(3)*. The reactivity of a number of monomers in the Maillard reaction has been tested in solution using a shrimp protein hydrolysate, and resulted in the following reactivity order: fructose ~ glucose < arabinose < xylose < ribose *(4)*, which is in line with data obtained in solution using β-lactoglobulin *(5)*. This confirms that pentoses are, in general, more reactive than hexoses, as supported by a number of studies *(4, and references therein)*. Furthermore, the influence of the chain length on oligosaccharide reactivity using ovalbumin was examined in the dry state using a range of maltooligosaccharides, where the reactivity decreased when chain length increased *(6)*. For galacturonic acid-containing saccharides, oligomers also showed a lower reactivity in the Maillard reaction compared to monomers *(6)*.

The degree of glycation of proteins after the Maillard reaction is often estimated by using techniques like gel electrophoresis, analysis of the number of free amino groups through colorimetric essays, and MALDI-TOF Mass Spectrometry *(2, 7, 8)*. These techniques can be laborious and/or may include time-consuming necessary purification steps. Furthermore, they only provide information on the average level of modification, and no information about the product dispersity is obtained. In the past years, the application of ESI- and MALDI-based MS techniques for the analysis of glycated proteins has become feasible *(9)*. The detailed structure information obtained by a number of these techniques will lead to insight in the relation between incubation time and conditions on the one hand and product composition and functionality on the other hand. In this paper, the application of a fast ESI-TOF MS-based protocol for intact glycated protein analysis is discussed by using glycated α-lactalbumin. This technique is applied to determine the influence of the type and size of the saccharide on the Maillard reaction rate. A small lysine containing

peptide is applied to determine the reactivity of both the protein/peptide and different saccharides in the Maillard reaction. The relevance of detailed product analysis for controlling and understanding the Maillard reaction is discussed.

Experimental

Materials

α-Lactalbumin was obtained as a commercial powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA), containing 95% (w/w) protein of which 90% (w/w) was α-lactalbumin *(10)*. Trigalacturonic acid (T7407), glucose (G7528), maltotriose (M8378), maltopentaose (M8128), maltoheptaose (M7753), sodium iodide, and cesium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Maltose (monohydrate) was from Merck (Darmstadt, Germany), maltooctaose from Carbosynth Ltd. (Compton, UK), galacturonic acid from Fluka Biochemica (Buchs, Switzerland), and 3-(2-furyl)acryloyl-Ala-Lys-OH (FAAL, M-1350) from Bachem (Bubendorf, Switzerland). The water used was in all cases purified by using a Milli-Q Gradient A10 system (Millipore Corporation, Billerica, MA, USA). UPLC grade eluents were bought from Biosolve B.V. (Valkenswaard, The Netherlands).

Methods

Synthesis of peptide-saccharide conjugates

For coupling of various saccharides to the peptide 3-(2-furyl)acryloyl-Ala-Lys-OH (FAAL) via the Maillard reaction in the dry state, 1 ml of a 3 mg/mL ($8.9*10^{-6}$ mole) FAAL solution was added to $1.78*10^{-5}$ moles of (solid) saccharide (molar ratio FAAL:saccharide 1:2) in a plastic reaction tube. Prior to this, the pH of the FAAL solution had been set to 8.0 using 0.1% ammonia. The mixtures were subsequently freeze-dried. Samples were incubated in a dessicator at 60 °C for given time intervals varying from 1 to 192 h depending on the saccharide nature, at a relative humidity of 65%. The latter was reached by equilibrating the dessicator with a saturated $NaNO_2$ solution, as described previously *(7, 11)*. Per time interval, a separate sample was incubated. Samples were then stored at -20 °C.

Analysis of remaining FAAL and FAAL conjugates

For analysis, 12 mL of water was added to FAAL samples after incubation, leading to a concentration of 0.25 mg FAAL/mL (based on the initial quantity present). Two µL of

these solutions were injected to a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) consisting of an autosampler, pump, column oven (40 °C), and PDA detector. The system was equipped with a Waters Acquity BEH 130 C18 PST column (2.1*150 mm) preceded by a Waters Vanguard BEH 300 C18 guard column, both with a particle size of 1.7 µm. Compounds were eluted at a flow rate of 250 µL/min by using a gradient obtained by mixing eluent A: water containing 1% (v/v) acetonitrile and 0.03% (v/v) TFA, with eluent B: acetonitrile containing 0.03% (v/v) TFA. Gradient description (steps are linear unless stated otherwise): 0-2 min: 97-90% A, 2-4 min: 90-75% A, 4-9 min: 75-0% A, 9-11 min: isocratic 0% A, 11-13 min: 0-97% A, 13-16 min: isocratic 97% A. Eluting compounds were detected using the PDA detector, which was constantly scanning between 200-400nm. The signal at 304 nm was used for quantification of non-conjugated FAAL by means of a calibration curve (0-0.3 mg/mL). In the linear part of the time vs. % FAAL modified-curve, the reaction rate for each saccharide was determined, which was defined as percentage of the initial amount of FAAL reacting per hour.

For FAAL conjugate identification, eluting compounds were subsequently directed to an in-line Waters Synapt ESI-TOF Mass Spectrometer, equipped with a Z-spray electrospray ionisation (ESI) source. The capillary voltage was set at 3.0 kV, and the source temperature at 120 °C. Nitrogen was used as desolvation gas (350 °C, 400 L/h) and cone gas (50 L/h). MS data were acquired in the positive V-mode between m/z 50-2000 in the continuum mode at 0.5 s/scan. The TOF detector was calibrated daily using a solution prepared by mixing sodium iodide (0.4 g) and cesium iodide (23 mg) in 200 ml water/isopropanol 50/50 (v/v). PDA and MS data were acquired and processed using MassLynx software (Waters Corporation).

Synthesis of α-lactalbumin-saccharide conjugates

Mono- and oligosaccharides were covalently linked to α-lactalbumin using the Maillard reaction in the dry state. Ten mg of α-lactalbumin ($8.47*10^{-6}$ mole lysine residues) was mixed with $1.69*10^{-5}$ mole reducing saccharide in 2 mL of water, to reach a molar ratio Lys : reducing end groups of 1:2. The pH of the protein and saccharide solutions was set to 8.0 using 0.1% ammonia (if necessary). Solutions were subsequently freeze-dried. Samples were incubated and stored using the conditions mentioned for peptide-saccharide synthesis.

Analysis of α-lactalbumin-saccharide conjugates

Glycated proteins were analyzed using the UPLC-Synapt ESI-TOF MS system mentioned for FAAL (-conjugate) analysis. To facilitate protein analysis, the UPLC system was

equipped with a MassPREP Micro desalting column and pre-column tubing from a UPLC Intact Mass Analysis Application Kit (Waters Corporation). A column temperature of 80 °C was applied. Two µL of a 1 mg protein/mL solution was injected. Conditions as mentioned in the instruction sheet belonging to this kit were used for compound elution and column regeneration (>2 min.). Because of their complexity, the gradient and flow rates used are provided in Table 1.

Table 1 Eluents, gradient, and flow rates applied for elution of intact proteins and regeneration of the column. All gradient steps were performed linear.

Time (min)	***%A***	***%B***	***Flow rate (mL/min)***
0.0	95	5	0.5
0.5	95	5	0.5
0.51	95	5	0.2
2.0	10	90	0.2
2.1	95	5	0.5
2.7	10	90	0.5
2.8	95	5	0.5
3.4	10	90	0.5
3.5	95	5	0.5
4.0	95	5	0.5
A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile			

After elution, compounds were directed to the in-line Synapt ESI-TOF MS. In each run, eluent from the first 0.5 min was directed to waste, in order to prevent the MS from influx of disturbing compounds. The Synapt ESI-TOF MS was running in the positive V-mode at a capillary voltage of 3 kV with a source temperature of 120 °C. Nitrogen was used as desolvation gas (800 L/h, 350 °C) and cone gas (50 L/h). Data were acquired between m/z 500-4000 in the continuum mode with a scan time of 0.5 s. In the elution time range of the (modified) protein, about 70 MS scans were combined to one m/z spectrum. The MaxEnt1 option within the MassLynx software (Waters Corporation) was used for subsequent deconvolution of these spectra. From the intensities of the peaks for the various products present in a spectrum, the weighted average of the degree of substitution per protein molecule (DSP) and the accompanying standard deviation were calculated. The latter was used as a value for product dispersity, and was indicated as “product dispersity index”. In

the DSP range indicated, the DSP's representing less than 2% of the total product spectrum were not included. In the linear part of the time vs. average DSP curve, the reaction rate for each saccharide was determined, which was defined as moles saccharide linked per mole protein per hour.

Results and discussion

Maillard reaction rate of saccharides towards α-lactalbumin

To determine the course of the Maillard reaction, α-lactalbumin was incubated with saccharides of various types and sizes. After incubation for various time intervals, samples were analyzed using UPLC-ESI-TOF MS. In Figs. 1 and 2, the original and deconvoluted spectra of α-lactalbumin, modified using glucose and maltotriose, are shown.

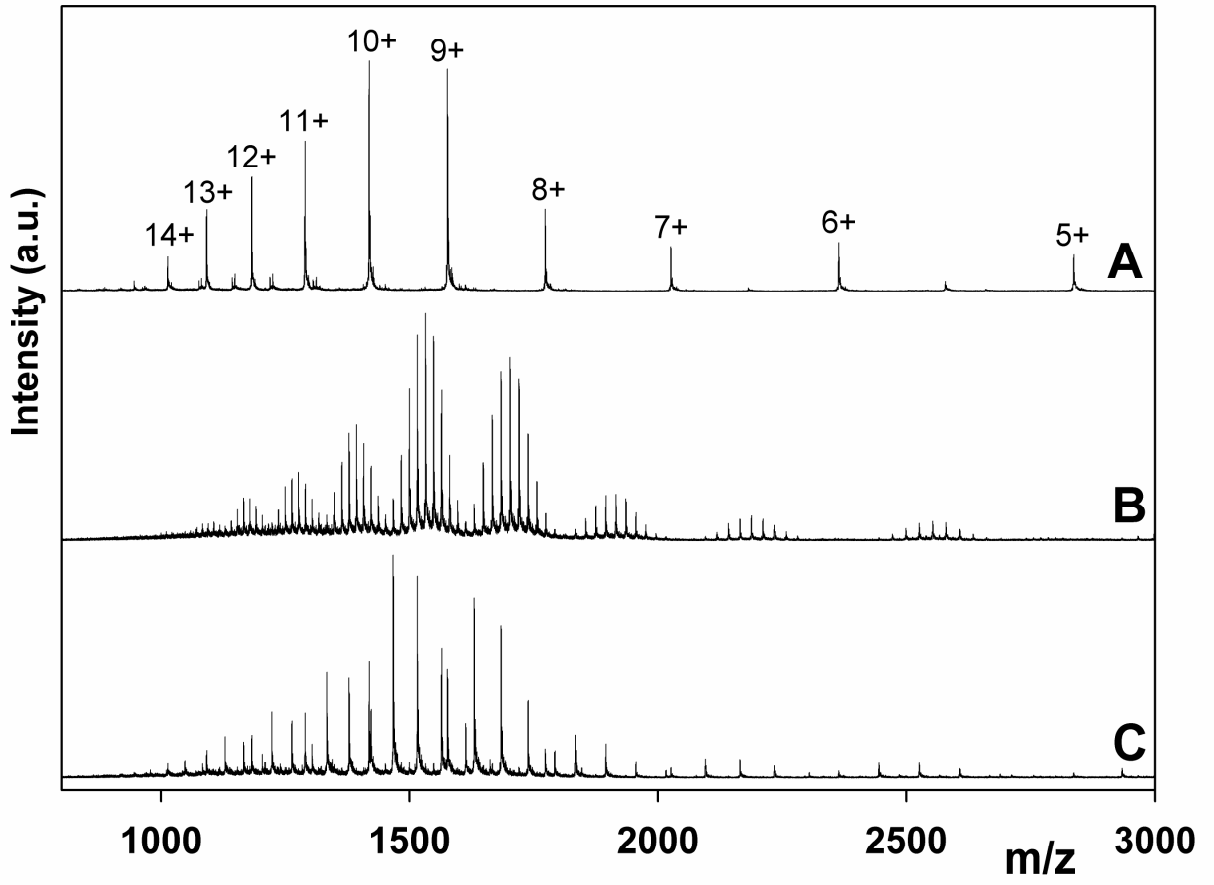

Fig. 1 Combined m/z spectra (± 70 scans/spectrum) after UPLC-ESI-TOF MS analysis of α-lactalbumin incubated A) without sugar, B) with glucose for 4h, and C) with maltotriose for 4h. Charge states are indicated in spectrum A. Intensities are scaled to the highest peak.

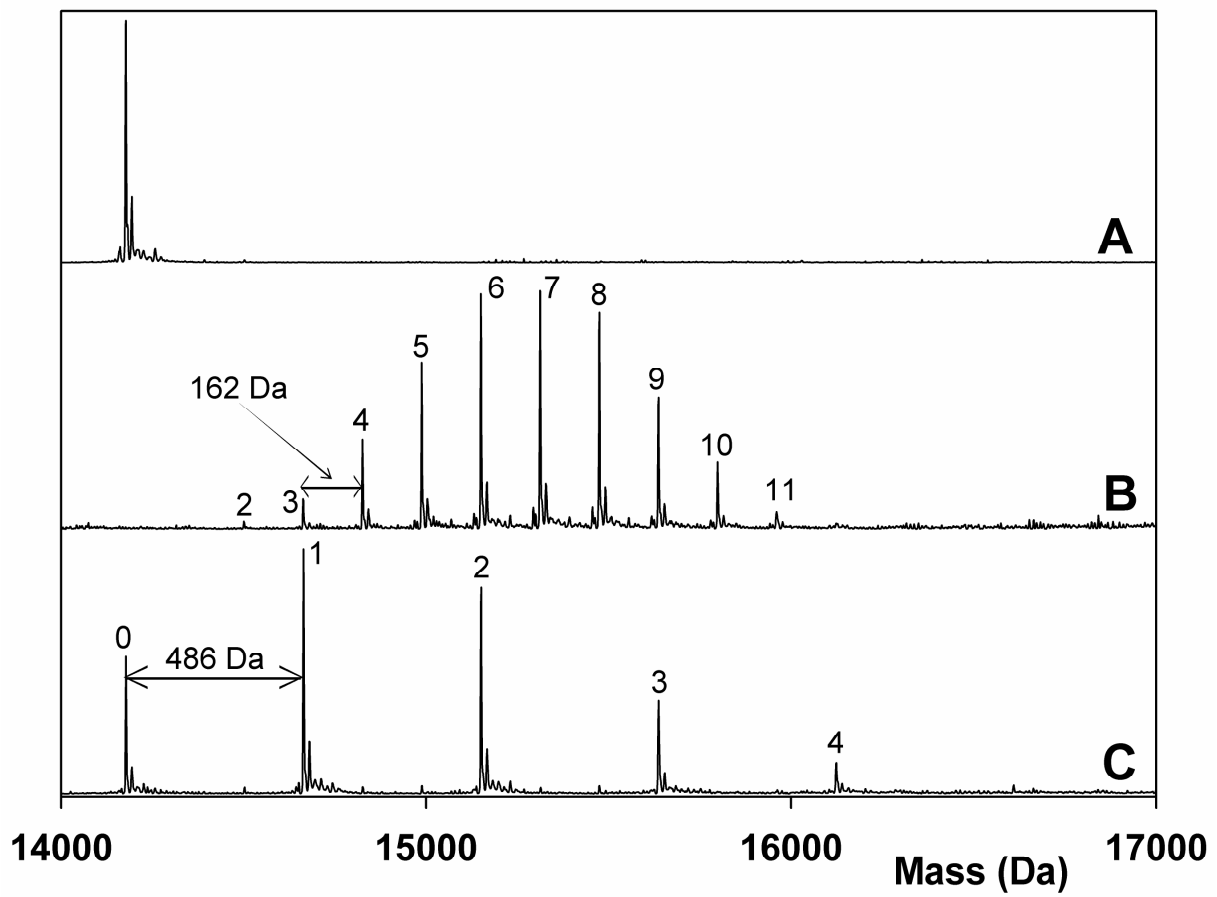

Fig. 2 Deconvoluted UPLC-ESI-TOF mass spectra of α-lactalbumin incubated A) without saccharide, B) with glucose for 4h, and C) with maltotriose for 4h. Spectra are based on the *m/z* spectra presented in Fig. 1 and are scaled to the highest peak. The degree of substitution per protein molecule (DSP) has been indicated.

Fig. 1 shows the combined m/z spectra as acquired. Charge states of the protein ranging from 5+ to 15+ were generated during the ionization, resulting in a range of peaks visible in spectrum A for the same compound. The clear spectra indicate that the in-line protein separation has been successful, because co-eluting salts or saccharide molecules would have disturbed protein ionization. After software-assisted deconvolution of the spectrum in Fig. 1A, the mass spectrum depicted in Fig. 2A is obtained. A major peak appears at 14178 Da, which corresponds with the expected mass of α-lactalbumin. Spectra B in Figs. 1 and 2 represent α-lactalbumin after a 4-hour incubation with glucose. Again, several charge states can be observed in the *m/z* spectrum and multiple peaks are observed per charge state. This indicates the presence of a range of products, as visible in the deconvoluted mass spectrum. In Fig. 2, this product dispersity is clearly illustrated. Covalent coupling of a glucose moiety to the protein via the Maillard reaction, accompanied by loss of a water molecule,

results in a mass increase of 162 Da per anhydroglucose moiety incorporated. α-Lactalbumin molecules with 2 to 11 glucose molecules attached are present after 4 hours of incubation, with the main products carrying 6 and 7 glucose moieties. By summing up the intensities of all of the products within one spectrum, the weighted average of the degree of substitution per protein molecule (DSP) can be calculated to be 6.8. The same average DSP could have been determined by free $-NH_2$ analysis, but this method does not provide information about product dispersity. Furthermore, analysis of free $-NH_2$ groups may be hampered by changes in the protein conformation, as reported for glycated β-lactoglobulin analysis *(12)*. For α-lactalbumin incubated with maltotriose (spectrum C in Fig. 2) the average DSP after 4 hours of incubation was 1.5 and a range of products with different DSP was again present. Generation of this mass spectrum (Fig. 2C) shows that the software is able to deconvolute an m/z spectrum with overlapping clusters of multiply charged peaks (Fig. 1C). The method presented is, altogether, a fast and accurate high-throughput tool for glycated protein analysis. Similar procedures have been developed based on LC-ESI-iontrap MS, where detailed views on the glycation of β-lactoglobulin with glucose, lactose, and galactose were obtained *(13-15)*. Since both saccharide type and size are known to influence Maillard reaction rate, DSP calculations were made for a range of saccharides and incubation times. The results are presented in Fig. 3. Additionally, the DSP range, the product dispersity index, and the reaction rate were determined. All data are summarized in Table 2.

Reaction rates in the initial phase

In Table 2, initial reaction rates are provided, which have been calculated from the linear parts of the curves. When the glucose mono- and oligomers are compared, a clear decrease of the initial reaction rate is observed with increasing oligomer size (Table 2). This trend is in line with results obtained using ovalbumin *(6)*, based on free $-NH_2$ analysis. The reaction rate observed using α-lactalbumin was, however, much higher compared to ovalbumin. This is probably a result of the difference in protein type, and the difference in incubation conditions used by Aoki *et al.* (pH 7.5, 50 °C, 65% RH) compared to our conditions (pH 8.0, 60 °C, 65% RH). Especially the temperature can be expected to have a major influence, as the open-ring proportion tends to increase when temperature increases *(3)*. The relatively high saccharide concentration used by Aoki *et al.* (Lys : reducing end groups ~ 1:6) did not counterbalance the effect of the lower temperature.

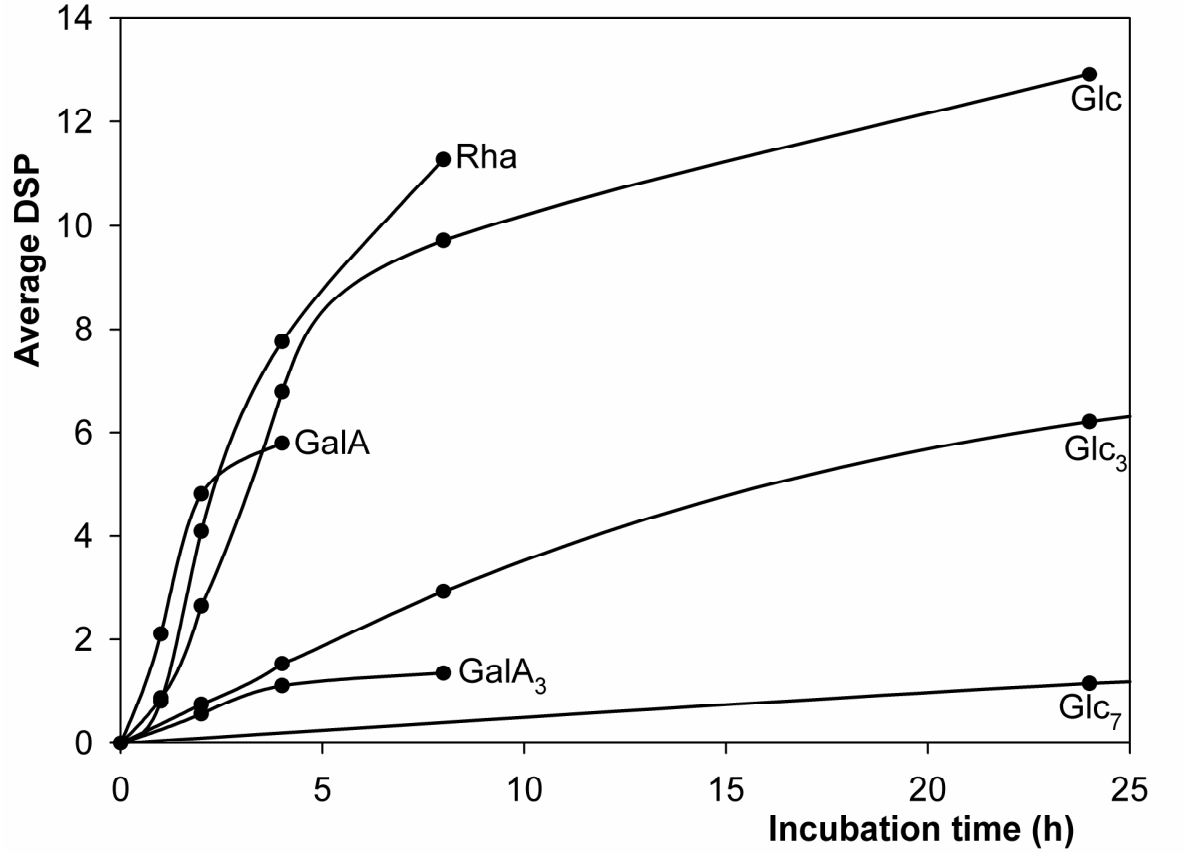

Fig. 3 Overview of average degree of substitution per protein molecule (DSP) resulting from the Maillard reaction between α-lactalbumin and different saccharides and incubation times. Data are based on UPLC-ESI-TOF MS analysis. $GalA_3$ = trigalacturonic acid, Glc_3 = maltotriose, Glc_7 = maltoheptaose.

As shown in Table 2, the initial reaction rate observed for rhamnose is about 65% higher than observed for glucose. This result, obtained in solid state conditions, differs from results obtained with β-lactoglobulin in solution, where similar reaction rates for glucose and rhamnose were found *(5, 12)*. An explanation for the higher reaction rate for rhamnose compared to glucose may be the proportion of rhamnose present in the open-ring form, which is 3 times higher than for glucose as studied earlier in solution *(3)*. It is, however, not clear to which extent the data concerning open-ring proportions apply to the solid-state conditions used in this research. Arabinose was also incubated and showed an initial reaction rate more than twice as high than that of glucose in the first two hours of incubation. The high reaction rate can be explained based on previous observations, where arabinose was found to be in the open-ring form to a >10 times higher extent than glucose *(3)* thus increasing the reaction rate. This is in line with other comparative studies, of which some were performed in the liquid state *(4, 5, 12, 16)*.

Table 2 Overview of the weighted average DSP, the product dispersity index, and the DSP range after incubation of α-lactalbumin with several saccharides for several time intervals, together with the reaction rate. Product dispersity index is defined as the weighted standard deviation of the DSP. The initial reaction rate is defined as the average number of moles saccharide linked per mole protein molecules per hour in the linear part of the curve in Fig. 3.

Saccharide type	***Incubation time***	***DSP range (2% lower limit)***	***Weighted average DSP***	***Product dispersity index***	***Initial reaction rate (moles saccharide/mole protein/h)***
Glucose	*1h*	0-3	0.9	0.9	
	2h	0-6	2.7	1.4	
	4h	3-10	6.8	1.8	2.0
	8h	6-13	9.7	1.7	
	24h	10-15	12.9	1.3	
Maltotriose	*2h*	0-3	0.8	0.8	
	4h	0-4	1.5	1.1	
	8h	0-6	2.9	1.4	0.4
	24h	3-9	6.2	1.6	
	57h	5-11	8.0	1.7	
Maltoheptaose	*24h*	0-4	1.2	1.0	
	33h	0-4	1.3	1.1	
	57h	0-5	1.9	1.2	0.05
	120h	0-5	2.9	1.4	
	192h	1-7	3.7	1.7	
Rhamnose	*1h*	0-3	0.8	0.8	
	2h	1-7	4.1	1.5	3.3
	4h	4-11	7.8	1.8	
	8h	8-14	11.3	1.7	
Arabinose	*1h*	0-4	1.4	1.1	
	2h	2-9	6.0	1.7	4.6
Galacturonic acid	*1h*	0-5	2.1	1.2	
	2h	2-8	4.8	1.7	2.7
	4h	2-9	5.8	1.9	
Trigalacturonic acid	*2h*	0-2	0.6	0.7	
	4h	0-4	1.1	1.0	0.3
	8h	0-4	1.4	1.1	

Galacturonic acid is about 35% more reactive than glucose and a fast development of a brown color was observed. However, galactose and glucose showed a similar reaction rate in previous research when β-lactoglobulin was used *(12)*. The higher reactivity of galacturonic acid compared to glucose may be explained by an effect of the incorporation of the carboxyl group on the open-ring proportion. Trigalacturonic acid shows a reaction rate that is similar to or even slightly lower than the rate observed for maltotriose, which is contradictory to galacturonic acid being more reactive than glucose. The high charge load of trigalacturonic acid may play a role in this, because it may lead to electrostatic repulsion, as will be discussed later.

Reaction rates after the initial phase

Depending on the type of saccharide, changes in de reaction rate can be observed in Fig. 3 when a specific average DSP has been reached. For rhamnose and glucose, the flattening of the curves is probably caused by the decreasing number of free amino groups present. The reaction rates for maltotriose and maltoheptaose also decrease when the reaction proceeds, as can be deduced from the values in Table 2. This could be related to the decreasing availability of easily accessible lysine moieties, which might become an issue for more bulky oligosaccharides. A flattening of the galacturonic and trigalacturonic acid curves at an average DSP of ~6 and ~1.5, respectively, is observed and this occurs at lower average DSP values than observed for e.g. glucose and maltotriose. This 'early' flattening is probably caused by electrostatic repulsion as a result of the negatively charged groups present in galacturonic acid and/or in the protein. The coupling of a (tri-)galacturonic acid molecule to a given lysine moiety may disturb the diffusion of (tri-)galacturonic acid to other lysine moieties in the vicinity. The 3D structure of the protein *(17)* shows that at least 5 lysine moieties are located in clusters. Other amino acids which can carry a negative charge are aspartic acid and glutamic acid. When these amino acids are considered within the 3D structure, only two lysine moieties are clearly free from possible disturbance of charged groups. The flattening of the trigalacturonic acid curve at a lower average DSP compared to galacturonic acid can be explained by the incorporation of three negative charges per trigalacturonic acid molecule, in contrast to one charge per galacturonic acid molecule. The reaction rate decrease with increasing DP, and the curve flattening have also been observed before to some extent, when the Maillard reaction between ovalbumin and glucuronic acid, galacturonic acid or pectic hydrolysates was monitored using free $-NH_2$ analysis *(6, 18)*.

Dispersity of the reaction products

In Table 2, a generic trend can be observed regarding the product dispersity index. It increases when the reaction proceeds and slowly decreases when the maximum number of substitutions has been reached (12 + 1, based on Lys residues and terminal amino group). This index, being the standard deviation of the DSP, indicates how close the different DSP values within one sample are clustered around the average DSP. The product dispersity observed indicates that not each individual protein molecule reacts equally. The maximum dispersity is not dependent on the type of saccharide, except for trigalacturonic acid, as a result of its low average DSP. A higher amount of reducing ends present did not increase the product dispersity, but slightly increased the reaction rate, as was tested for glucose. A reaction rate increase of ~17% was observed when the molar ratio Lys : glucose was increased from 1:2 to 1:5, and a reaction rate increase of ~14% from 1:5 to 1:10. A further change to 1:16 did not lead to an extra increase of the reaction rate (based on 50mg α-lactalbumin per sample, after 2 hours of incubation).

By means of the detailed product composition data provided by ESI-TOF MS, including the proportion of each glycated (DSP) species in the product mixture, a relative reaction rate of each individual DSP species within one reaction mixture can be calculated per time interval (rate DSP 0→1, rate DSP 1→2, etc.). The relative reaction rates provide more insight in the factors determining the course of the Maillard reaction. Calculations were made for the incubation between α-lactalbumin and glucose. After a start-up phase, the relative reaction rates of protein molecules tended to decrease when the number of saccharide units attached to it decreased (rate DSP 1→2 > rate DSP 6→7 > rate DSP 10→11), but rates for the same reaction also decreased when incubation time increased. This led to the conclusion that reducing ends availability may have become a limiting factor during the reaction, which was performed in the solid state. This limiting factor would make it impossible to draw mechanistic conclusions. Generation of data with a wider range of saccharides and a higher excess of reducing ends is needed to provide more details.

Advantages of MS-techniques generating multiple charged ions

Limited protein glycation has, under dilute conditions, little or no effect on the protein ionization potential *(19)*. In our research, the α-lactalbumin sample incubated for 8 hours with glucose, resulting in an average DSP of 9.7, was examined for decrease in ionization potential because it is highly substituted and still shows clear peaks. The results after deconvolution of the highly charged section of the spectrum and the lowly charged part of the spectrum were compared. In the lowly charged part, the abundance of the highly

substituted protein molecules was relatively higher, resulting in an average DSP for this section of 10.0. The highly charged part contained a relatively low amount of substituted protein molecules, resulting in an average DSP of 9.4 for this section. This illustrates the slight decrease in ionization potential when DSP increases. Hence, for calculations of the average DSP of the samples, as shown in Table 2, the complete spectrum was taken into account, meaning that a correct view on the sample composition was obtained.
As indicated by these observations, the generation of molecular ions with multiple charges by ESI-TOF MS represents an advantage compared to MS techniques generating mainly monovalent molecular ions, like MALDI-TOF MS. A possible decrease in ionization potential due to structure modifications would in the case of monovalent ions lead to an incorrect view on the sample composition, because part of the molecules would not be ionized at all. In case of the generation of multiple charged molecular ions, molecules that are less sensitive to ionization might indeed carry fewer charges as a result of structure modifications, but will still be taken into account in a correct way.

Analysis of intermediate Maillard reaction products

For a number of samples, the DSP could not be completely determined using UPLC-ESI-TOF MS due to degradation reactions, resulting in a product mixture which was too disperse for accurate analysis. This was the case for α-lactalbumin incubated with arabinose (>2h), rhamnose (>8h), galacturonic acid (>4h), and trigalacturonic acid (>8h). Apparently, instability is saccharide structure-dependent and is not automatically related to initial Maillard reactivity. This is illustrated by the relatively high stability of the products substituted with glucose and glucose oligomers. Mass spectra of dehydrated Amadori products are provided in Fig. 4. Spectrum A represents the α-lactalbumin sample incubated for 8 hours in the presence of rhamnose. Apart from the main peaks representing the protein carrying 6-14 anhydro-rhamnose moieties, a number of single or multiple additional 18 Da mass losses compared to these main peaks can be observed. The number of additional 18 Da mass losses increases with increasing DSP. Degradation via 2,3 enolization is favored by a high pH, but this pathway does not include dehydration of saccharides linked to the protein molecule. Therefore, the observed dehydration of the rhamnose moieties linked to α-lactalbumin is probably a result of degradation via the 1,2 enolization pathway, although this would only be expected at low pH values *(20, 21)*. A maximum of one water loss per rhamnose moiety can occur before hydrolysis and amino group release occurs *(21)*. The number of dehydrations observed per protein molecule logically increases when the number of rhamnose moieties attached increases. The average DSP of this sample can still be

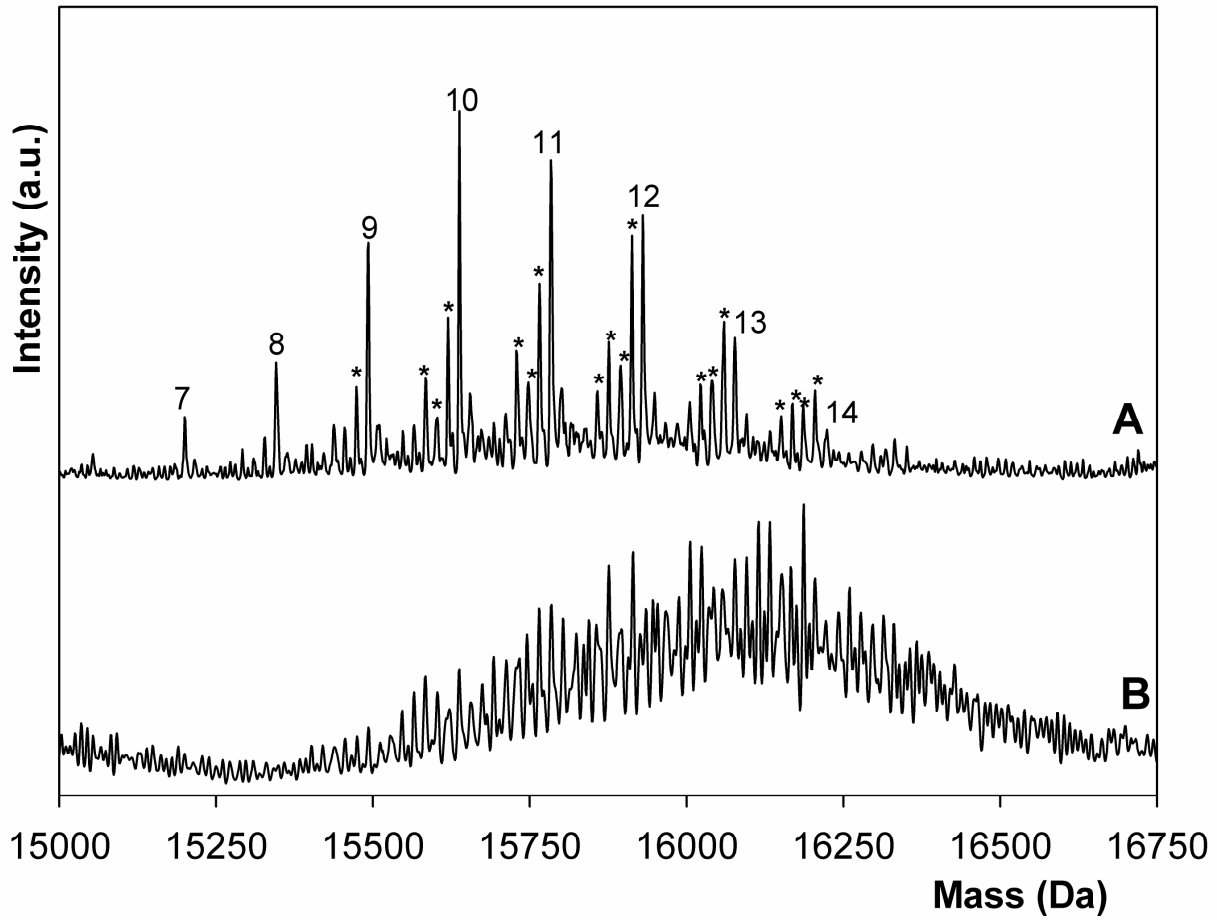

Fig. 4 Deconvoluted UPLC-ESI-TOF mass spectra of α-lactalbumin after incubation with rhamnose for A) 8 hours, and B) 24 hours. Dehydrated products in spectrum A are indicated by *, the number of rhamnose moieties attached is also provided. Intensities are scaled to the heighest peak.

calculated, with or without taking the dehydration products into account (average DSP: 11.3 and 10.5, respectively). Hydrolysis and release of amino groups, as mentioned before, makes the amino groups available for attachment of new saccharide moieties or other reactive compounds. This explains the high product dispersity even after a relatively long incubation time. The dehydration effect has been observed before at a lower resolution using ESI Iontrap MS after the reaction of ovalbumin with glucose, and the dehydrated products were referred to as deoxyosone derivatives *(22)*.

Analysis of the α-lactalbumin/rhamnose mixture after 24 hours of incubation yields the mass spectrum as shown in Fig. 4B. Due to degradation reactions, the sample composition has become too disperse for an adequate interpretation. In general, the extent of degradation was found to be dependent on both incubation time and the stability of the saccharide involved. The highly reactive products formed as a result of dehydration, deamination, and

fission of Amadori products may have reacted with free amino groups via their carbonyl groups *(23)*, yielding an even more complex set of products.

The detailed view on the early stage of product dehydration obtained by UPLC-ESI-TOF MS, as shown in Fig. 4A, could lead to a better understanding of these reactions. In addition, detailed monitoring enables a better control of the Maillard reaction in e.g. the production of food ingredients, making this pathway less "notoriously difficult to control" *(23)* and thus more exploitable than generally assumed.

Maillard reaction of saccharides with a model peptide

As mentioned before, the reaction rate observed can be expected to be a function of more factors than the relative open-ring proportion, like lysine accessibility and substrate mobility. To exclude the accessibility and mobility factors, additional experiments were performed with a lysine-containing model peptide instead of α-lactalbumin. For this, the dipeptide 3-(2-furyl)acryloyl-Ala-Lys-OH (FAAL) was selected because of its blocked terminal amino group, good accessibility of Lys due to the compact Ala moiety, and the possibility to quantify the peptide using RP-UPLC-PDA analysis (304nm). The reactivity of a series of glucose and pure maltooligosaccharides with increasing DP towards the peptide was investigated. The series of maltooligosaccharides was chosen to study solely the effect of DP increase on the Maillard reaction rate, without the influence of other factors like saccharide type. The course of the reaction between these saccharides and FAAL can be found in Fig. 5. As observed earlier in the incubation using α-lactalbumin, there is a clear correlation between the DP of the glucose oligosaccharides and the Maillard reaction rate with the FAAL peptide. The higher the DP is, the lower the reaction rate. The trends of the curves indicate that, when incubation time suffices, 100% of FAAL modification will be reached for all saccharides.

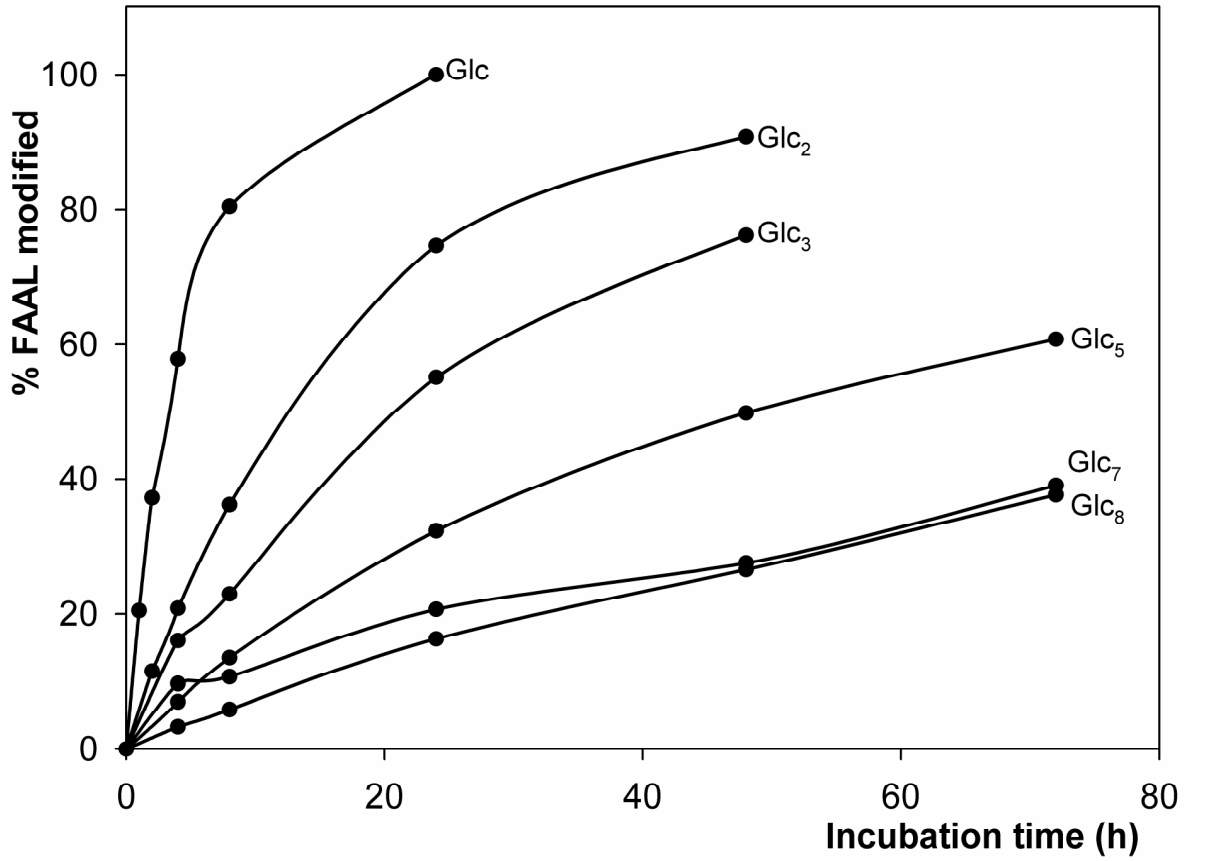

Fig. 5 Course of the Maillard reaction of a series of glucose and maltooligosaccharides with FAAL, expressed as the percentage of FAAL in the sample having reacted with a saccharide.

Comparison of the initial reaction rates in the linear parts of the curves is facilitated by Fig. 6. The effect of a DP increase on the Maillard reaction rate tends to decrease when DP increases, so the largest difference in reaction rate is found between glucose and maltose, the smallest difference between maltoheptaose and maltooctaose. This difference is a result of two factors: saccharide reactivity and substrate mobility. Saccharide reactivity is mainly a result of the relative proportion present in the open-chain form *(1)*. The solid-state conditions used in this research make it difficult to draw conclusions on substrate mobility, but it most probably decreases when the size of the oligomer increases. On the basis of these data, it cannot be concluded what the main factor for the decrease in reaction rate is. The fast reaction rate decrease with increasing saccharide DP and the expected high mobility of FAAL, however, suggest that the saccharide reactivity is the major factor responsible for the effects observed. More data about the relative open-chain proportion are needed to confirm this.

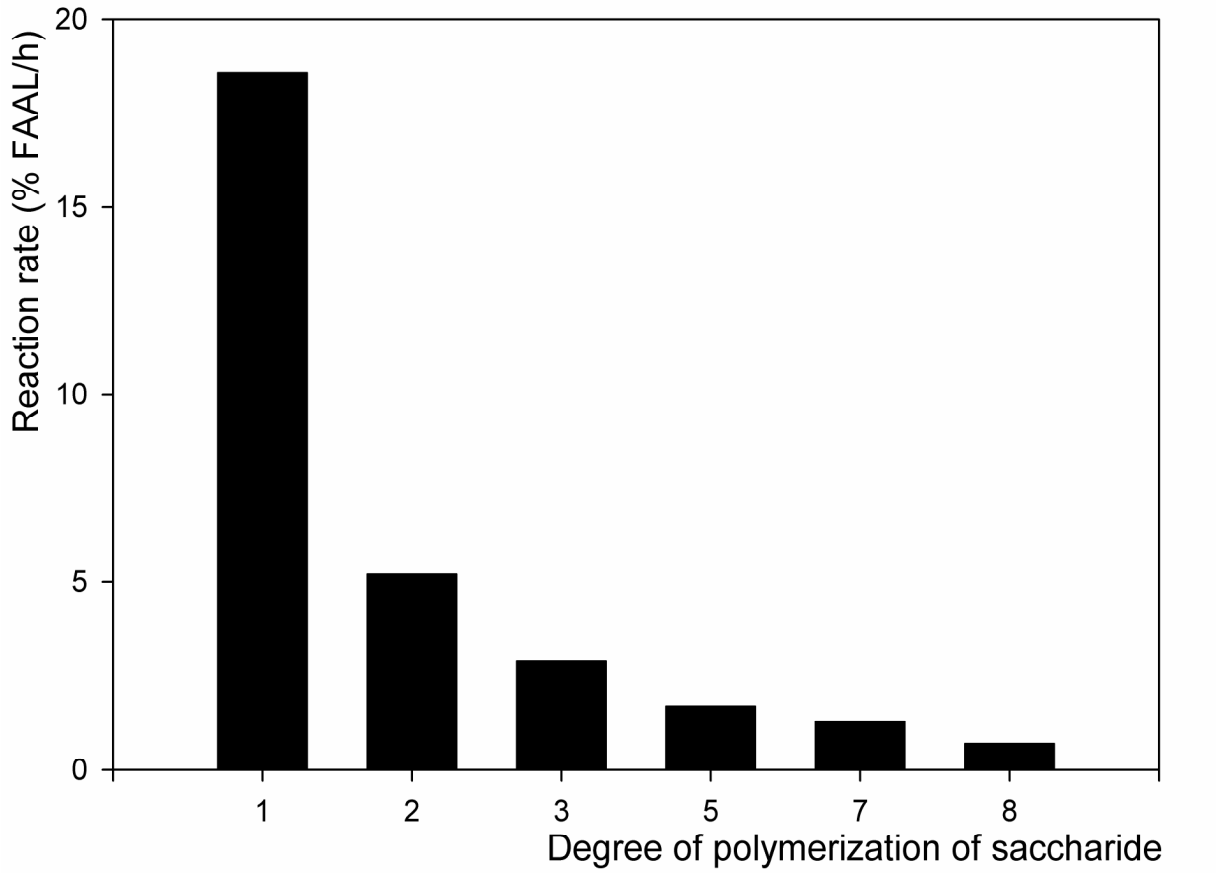

Fig. 6 Maillard reactivition rate of glucose and maltooligosaccharides with different degrees of polymerization with model peptide FAAL. Data are deduced from the linear part of the curves in Fig. 5, and the reaction rate is expressed as % FAAL reacted per hour.

Comparison of saccharide reactivity towards FAAL and α-lactalbumin

To be able to draw conclusions concerning the influence of the protein characteristics on the rate of its modification by the Maillard reaction, a comparison was made between the results obtained using the α-lactalbumin protein as well as the FAAL peptide. To do this, the theoretical substitution of all 12 lysine residues present in α-lactalbumin was set at 100%, and the average number of lysines substituted with glucose, maltotriose, and maltoheptaose at the several time points (Fig. 3) was expressed as a percentage of this. Overlay graphs were then made in combination with the data presented in Fig. 5 to facilitate comparison between the saccharide reactivity towards α-lactalbumin and FAAL. These graphs are shown in Fig. 7.

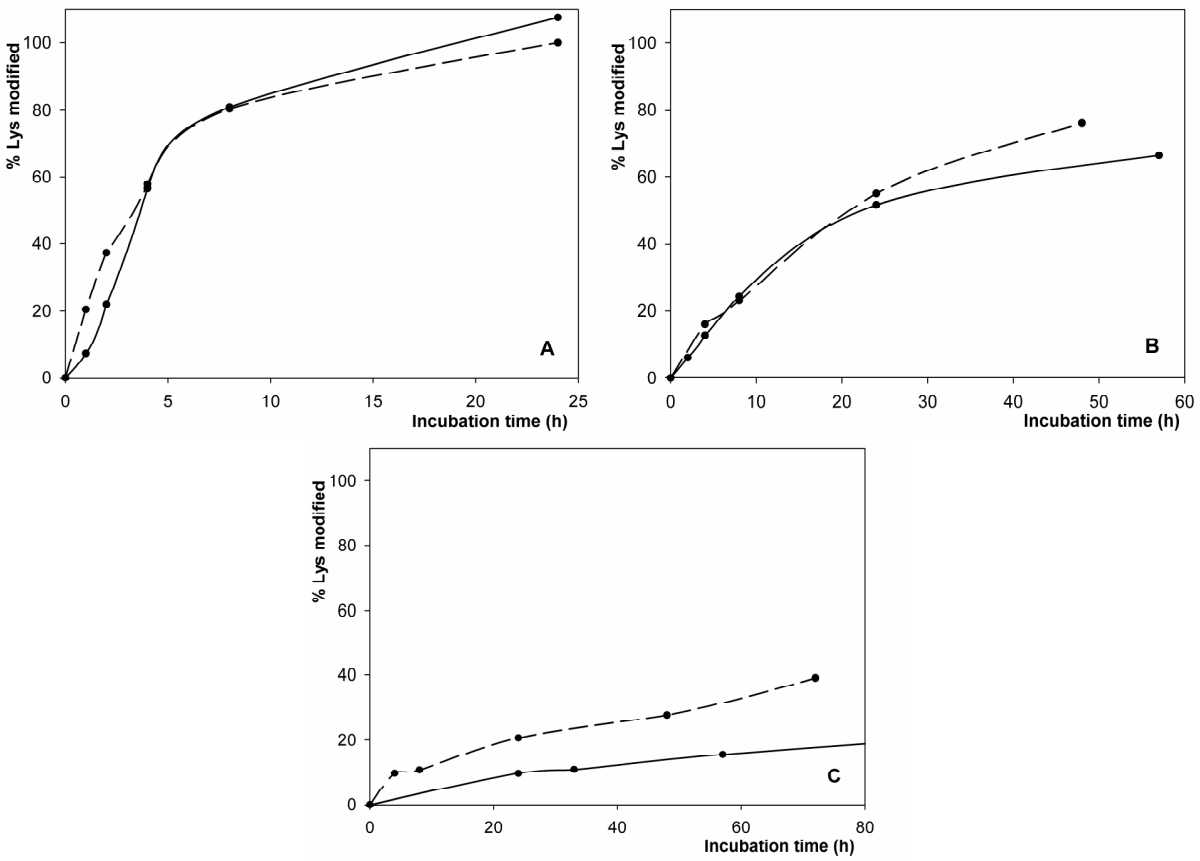

Fig. 7 Comparison of Maillard reactivity of A) glucose, B) maltotriose, and C) maltoheptaose towards α-lactalbumin (solid line) and 3-(2-furyl)acryloyl-Ala-Lys-OH (FAAL, dashed line). Reactivity is expressed as the percentage of lysine moieties in the substrate being modified.

According to the data in Fig. 7A, the course of the Maillard reaction using glucose as the saccharide is similar for FAAL and α-lactalbumin. This means that the α-lactalbumin structure does not have an influence on the lysine accessibility for glucose. After a 24h incubation between glucose and α-lactalbumin, more than the theoretical number of lysine moieties has reacted, which means that also a small number of other groups has taken part in the reaction. The terminal α-amino group of the peptidic chain could be one of these and the guanidyl group of arginine and the thiol group of cysteine may also participate *(23, 24)*. This is also clear from the DSP range as shown in Table 2, which is exceeding the number of lysines present (12) in a number of cases.

When the reactivities of maltotriose and maltoheptaose in the Maillard reaction with FAAL and α-lactalbumin are compared (Figs. 7 B and C), clear differences compared to glucose are observed. For maltotriose, the curves for FAAL and α-lactalbumin are in the first 24 hours of incubation following a similar trend, but the reaction rate when using α-

lactalbumin is lower afterwards. This would mean that about 50% of the lysine residues present in α-lactalbumin is easily available for maltotriose and that, as a result of the protein structure, the reaction rate from this point onwards is influenced by the reduced lysine accessibility. This was not observed for glucose, as a result of its smaller molecular size. For maltoheptaose, the reaction rate using α-lactalbumin is lower than the rate when using FAAL already from the start of the reaction. The protein structure is, apparently, of major influence on the course of the reaction. This can be explained by the molecular size of maltoheptaose, which is reducing its mobility and apparently hinders its diffusion to or reaction with the lysine moieties.

It has been discussed before that, apart from lysine, other groups present in the protein structure may also participate in the Maillard reaction. When this effect would be taken into account, the curves for α-lactalbumin in Fig. 7 would display a slight downshift. This would even strengthen the conclusions about the influence of lysine availability and substrate mobility on the Maillard reaction rate.

To conclude, it can be stated that a high throughput UPLC-ESI-TOF MS assisted technique can be applied to reveal the dispersity of the Maillard reaction product mixture as well as the average degree of substitution per protein molecule (DSP). Furthermore, it visualizes dehydration steps in the more advanced Maillard reaction stage. The product dispersity was mostly independent of the type of saccharide used. The Maillard reaction rate varies between saccharides types and is for charged saccharides influenced by electrostatic repulsion. The decreasing reaction rate with increasing oligomer size is a result of both decreased saccharide reactivity and reduced lysine accessibility in the protein.

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Chapter 7

Cross-linking behavior and foaming properties of bovine α-lactalbumin after glycation with various saccharides

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Henk A. Schols, Harry Gruppen
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Abstract

α-Lactalbumin was glycated via the Maillard reaction in the dry state using various mono- and oligosaccharides. The reaction resulted in coupling of the saccharides to α-lactalbumin, but also in cross-linked proteins. The glycation rate as well as the extent of cross link formation was highly dependent on the saccharide used. Glycation by arabinose and xylose led to a very fast protein cross-link formation, while glucose showed a relatively low protein cross-linking ability. The stability of foams, created using the various glycated protein samples, was analyzed, and turned out to be dependent on the type of saccharide used, the extent of glycation, and possibly on the amount of cross-linked protein present. Compared to non-modified α-lactalbumin, glycation with rhamnose and fucose improved the foam stability. Application of glucose, galacturonic acid and their oligosaccharides for glycation of α-lactalbumin did not exert a clear effect on the foam stability. Mass spectrometric analysis of the glycated proteins revealed that dehydration of the Amadori products is an indicator the formation of protein cross links.

Introduction

The Maillard reaction is a reaction between an amino group, e.g. an amino acid or a protein, and a carbonyl group, mostly a reducing saccharide *(1)*. Via several reaction steps, Amadori products are formed, which are vulnerable to further degradation reactions. No extraneous chemicals are needed to induce the Maillard reaction *(2)*. The reaction occurs spontaneously during food processing and is also suitable for directed protein glycation. The Maillard reaction is known to result in cross-linked proteins after degradation of the Amadori compounds. Based on the first coherent mechanism proposed *(3)*, Amadori compound degradation mainly occurs via 1,2-, or 2,3-enolization, depending on the pH. Via various hydrolysis, fission, and dehydration steps, highly reactive species, like keto-aldehydes, dicarbonyls, and reductones are formed *(4)*. These eventually react with one or more amino groups to form melanoidins. The latter have been defined as "brown nitrogenous polymers and co-polymers" *(5)*. The mechanism of protein cross-linking via the Maillard reaction *in vivo* has been the subject of a large number of literature studies *(6, and references therein*). Recent research has revealed that Amadori compounds may degrade to dideoxyosones, leading to the major *in vivo* lysine-arginine cross-links glucosepane and pentosidine. These pathways involve several dehydration steps, but do not include deamination *(7, 8)*. It is, however, not clear to which extent these findings are applicable to food systems. The complexity of food products and the wide range of conditions applied in food processing makes the elucidation of the chemical pathways and the resulting cross links a challenge.

From a protein functionality point of view, a number of studies on the modification of food protein properties by using the Maillard reaction has been published, as reviewed by Oliver *et al. (9)*. The emulsification properties and the solubility at low pH of caseinate improved after conjugation with maltodextrins *(10)*. Another study showed that the change in the functional properties of β-lactoglobulin is related to the nature of the saccharide used for modification *(11)*. Conjugation of caseins with dextrans improved the properties of the emulsions created using this protein under acidic conditions *(12)*. Cross-linking of food proteins was also found to have a profound effect on their functional properties *(6, 13)*.

This paper provides an initial study of the relation between the extent of glycation of the protein, protein cross-linking, and glycated protein foam stability after the Maillard reaction between α-lactalbumin and various saccharides. While additional research will be needed to elucidate precise mechanistic details, the results illustrate the importance of saccharide selection and control of the glycation process for successful protein modification.

Experimental

Materials

Bovine α-lactalbumin was obtained as a commercial powder (BioPURE, Davisco Foods International Inc., Le Sueur, MN, USA), containing 95% (w/w) protein of which 90% (w/w) was α-lactalbumin, according to the manufacturer's specifications. Arabinose (A6085), glucose (G7528), rhamnose monohydrate (R3875), maltotriose (M8378), maltoheptaose (M7753), and trigalacturonic acid (T7407) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Galacturonic acid was from Fluka Biochemica (Buchs, Switzerland), xylose from Merck (Darmstadt, Germany), fucose from Alfa Aesar (Ward Hill, MA, USA), and D-methionine from Acros Organics (Geel, Belgium). The water used was purified by using a Milli-Q Gradient A10 system (Millipore Corporation, Billerica, MA, USA).

Methods

Synthesis and purification of glycated α-lactalbumin

To glycate α-lactalbumin, 50 mg of α-lactalbumin (~$4.2 * 10^{-5}$ moles lysine residues) was mixed with $8.5 * 10^{-5}$ moles of saccharide in 10 mL of water (molar ratio lysine : reducing end groups 1:2). The pH of this solution was set to 8.0 using 0.1% ammonia. Samples were subsequently freeze dried. Incubation was performed for different time intervals at 60 °C in a dessicator. The relative humidity was kept at 65% by equilibrating the atmosphere in the dessicator with a saturated $NaNO_2$ solution. To remove non-reacted saccharides, samples were subsequently dissolved in 5 mL water and dialyzed for 24h against demineralized water, using dialysis tubes with a molecular mass membrane of 12-14 kDa, followed by freeze-drying. Per time interval, two samples were incubated. These samples were pooled prior to dialysis.

Determination of the average degree of substitution of α-lactalbumin

Calculation of the average degree of substitution per protein molecule (DSP) and the product dispersity index of the glycated proteins was performed after applying UPLC ESI-TOF MS for sample analysis, as described previously *(14)*. When part of the product was insoluble, analysis was performed on the soluble part.

Molecular mass distribution

(Glycated) protein samples were analyzed by High Performance Size Exclusion Chromatography using a Superdex 75 10/300 GL column (GE Healthcare, Uppsala, Sweden). Procedure and equipment have been described previously *(15)*. In our case, 10 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl and 6 M urea was used as eluent. Urea was added to exclude non-covalent protein aggregation. When part of the product was insoluble, analysis was performed on the soluble part. Eluting compounds were monitored using UV absorbance at 280nm. The proportion of cross-linked protein was determined by comparing the area of the peak of the monomeric protein with the area of the peaks representing polymeric protein (complexes of >1 protein molecules). UV responses for mono- and polymeric material were assumed to be similar. Bovine serum albumin (67000 Da), β-lactoglobulin (36300 Da, dimer in solution), carbonic anhydrase (29400 Da), and ribonuclease A (13700 Da) were analyzed used for column calibration.

Protein content determination

After dialysis and freeze-drying of the glycated α-lactalbumin samples, the nitrogen content of samples used for functionality tests was determined using a Thermo Flash EA 1112 Element Analyzer (Thermo Fisher Scientific Inc. Waltham, MA, USA), according to the manufacturer's instructions. A calibration curve was made using D-methionine. For each measurement, about 2-4 mg of sample was used. Analyses were performed in duplicate. For conversion of nitrogen content to protein content, a factor of 6.26 was used, as calculated for α-lactalbumin based on its amino acid sequence *(16)*.

Protein foam stability testing

Foam stability was investigated based on a procedure applied previously *(17)*, using a foam beaker with a radius of 3.1 cm. Soluble samples were dissolved in 10 mM sodium phosphate buffer pH 7.0 to 0.4 mg protein/mL (based on the protein content analysis as described above). Thirty mL of this solution was added to the foam beaker. Nitrogen gas was sparged through the solution at a flow rate of about 0.85 L/min, until the resulting foam reached a height of 8 cm. The foam height was subsequently recorded as a function of time. The time point at which the foam height had decreased to 5 cm was defined as the foam half life time. This number was used for foam stability comparisons. Samples were analyzed at least in duplicate, using a fresh protein solution for each measurement.

Results and Discussion

Modification extent after α-lactalbumin glycation

Via the Maillard reaction, α-lactalbumin was glycated using a selection of neutral and charged mono- and oligosaccharides. Samples with a different extent of glycation, defined as the weighted average degree of substitution per protein molecule (DSP), were created to study the relation between average DSP, protein cross-linking, and protein foam stability. Experiments were based on the results from previous research *(14)*, in which the average DSP was determined as a function of incubation time using mass spectrometry. In the present research, it was attempted to reach a DSP of around 3-4 for the different mono-and oligosaccharides applied by adjusting the incubation time. Samples subjected to a longer incubation were included to create highly modified and, possibly, cross-linked proteins. After incubation, the average DSP of the samples was determined using UPLC-ESI-TOF MS. In Fig. 1, mass spectra obtained after deconvolution of *m/z* spectra of α-lactalbumin modified using various monosaccharides are given. In Table 1, incubation times, the calculated average DSP, the product dispersity index (standard deviation) *(14)*, the protein content, and the extent of cross-linking (*vide infra*) of the various modified α-lactalbumin samples are provided.

Even though the DSP's aimed for were not always achieved as a result of the high monosaccharide reactivities, the DSP's after the short incubations are in the same order of magnitude for glucose, rhamnose and fucose. The Amadori products of these saccharides can be clearly observed in Fig. 1. For example for glucose (Fig. 1A), a series of 162 Da mass increase steps can be observed compared to the original non-modified α-lactalbumin signal (14177 Da). The 162 Da mass increase can be explained by the addition of glucose (mass 180 Da) to an amino group of α-lactalbumin, which is followed by loss of a water molecule (18 Da) under formation of a Schiff base. This compound will react further to the Amadori product *(1)*. The same accounts for the other saccharides, where a similar series of [saccharide Mw-18Da] mass increases is observed.

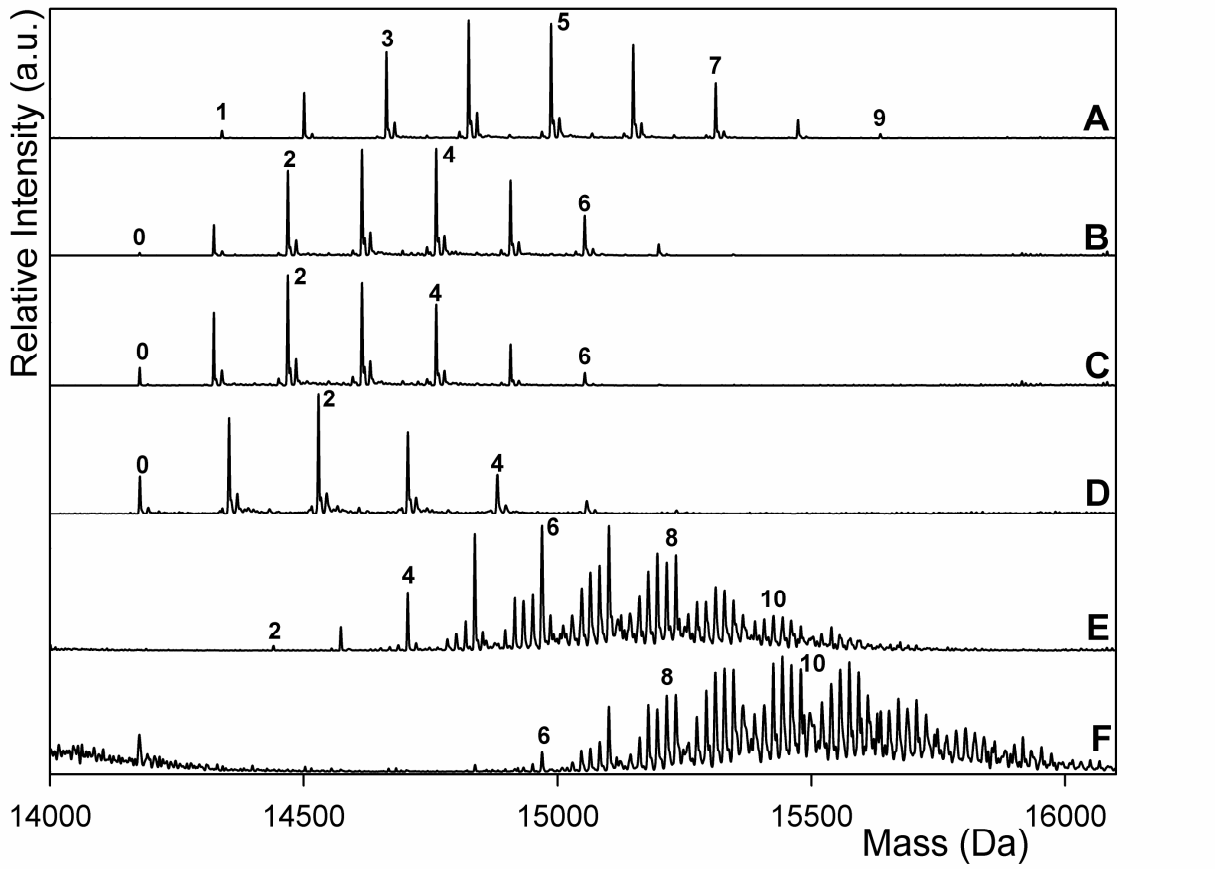

Fig. 1 Deconvoluted UPLC-ESI-TOF mass spectra of α-lactalbumin after Maillard-mediated glycation with A] glucose (2h), B] rhamnose (1.5h), C] fucose (1.5h), D] galacturonic acid (1h), E] arabinose (1.5h), and F] xylose (1.5h). Degrees of substitution per protein molecule are indicated.

In the monosaccharide-containing samples subjected to a 1.5-2h incubation, the DSP is in some cases higher than expected based on previous data *(14)*. The order of reactivity of the different saccharides, nevertheless, is the same. The lower reaction rates observed in previous research could be a result of the frequent sample-taking involved in those experiments and the consequent destabilization of the relative humidity of the reaction mixture *(14)*.

For the most reactive saccharides, arabinose and xylose, the DSP is hard to control. The dehydration of the Amadori products is proceeding fast (Fig. 1 E/F), as discussed before for arabinose *(14)*. The dehydration steps are visible as a series of 18 Da mass decrease steps compared to the peaks representing the Amadori products. Due to the high extent of dehydration and possible other degradation products present in the xylose-containing sample after 1.5 hours of incubation, the average DSP was estimated from the mass spectrum and a dispersity index could not be calculated. The same accounts for samples

incubated with rhamnose and fucose for 24 hours (data not shown). DSP-analysis of the arabinose and xylose-containing samples after 24 hours of incubation could not be performed due to sample insolubility and complexity.

Glycation with oligosaccharides was also successful (Table 1). As expected, high levels of modification were not reached due to the relatively low reactivity, electrostatic repulsion for the charged oligomers and due to steric factors, as reported previously *(14)*. The protein content, in general, inversely correlates with the average DSP and the size of the saccharides attached. Some of the values are deviating from the expected levels, probably caused by the hampered removal of non-reacted saccharides, especially trigalacturonic acid.

Table 1 Average DSP, product dispersity index (standard deviation), % cross-linked protein, and protein content after dialysis of glycated proteins.

Type of saccharide	***Incubation time***	***Average DSP[1]***	***Dispersity index***	***%Cross-linked protein***	***Protein content***
None (α-lactal-bumin only)	2h	-	-	0%	92.6%
	24h	-	-	0%	96.1%
Glucose	2h	4.6	1.7	0%	87.7%
	24h	12.7	1.3	34%	84.7%
Rhamnose	1.5h	3.6	1.5	0%	86.5%
	24h	~ 13	NA	62%	84.6%[2]
Fucose	1.5h	2.7	1.4	0%	89.4%
	24h	~ 13	NA	64%	83.3%[2]
Arabinose	1.5h	7.4	1.6	~40%	87.9%[2]
	24h	NA	NA	>90%	75.7%[2]
Xylose	1.5h	~10.5	NA	~60%	87.0%
	24h	NA	NA	>90%	86.7%
Galacturonic acid	1h	2.1	1.6	0	92.6%
	24h*	NA	NA	68%	-
Trigalacturonic acid (GalA3)	15h	1.4	1.0	0	67.4%
	55h*	NA	NA	57%	-
Maltotriose (Glc3)	8h	2.4	1.3	0	86.6%
	57h*	8.0	1.7	18%	-
Maltoheptaose (Glc7)	96h	2.1	1.3	0	75.3%
	192h*	3.7	1.7	0	-

[1]Degree of substitution per protein molecule. For partly insoluble products analysis was performed on the soluble part [2] Only indicative due to product inhomogeneity

** These samples were only included to study the extent of cross-linking after long incubation times*

NA: could not be analyzed due to sample complexity or (nearly) complete insolubility

Molecular size distribution after protein glycation

The development of brown color in a number of samples during synthesis indicated the progress of the Maillard reaction into more advanced stages. Furthermore, insolubility of some of the products was observed. For these reasons, the development of protein cross-linking or complex formation was suspected. To examine differences between saccharides in cross link inducing tendency, samples were analyzed using size exclusion chromatography under denaturing conditions. α-Lactalbumin incubated in the absence of any saccharide was also included in the analysis. In Fig. 2, a selection of the resulting elution profiles is given. Table 1 provides information on the extent of cross link formation for all samples analyzed, based on comparison of the areas of the monomeric and polymeric (>1 protein molecule) protein peaks.

In the α-lactalbumin blank, incubated in the absence of saccharide, the major compound eluting around 11.2 mL is α-lactalbumin. No conjugates are observed in this sample, also not after 2 and 24 hours of incubation (Table 1). The elution volume for α-lactalbumin is lower than expected based on the column calibration. The α-lactalbumin elution volume of 11.2 mL would correspond to a molecular mass of ~22.5 kDa. The reason for this deviation from the actual value (14.2 kDa) is unknown, but similar results were obtained in previous research using this column *(18)*. Nevertheless, conclusions can be drawn based on these data, since all samples were analyzed within the same run.

When saccharides are present during incubation, the elution pattern observed is different compared to the pattern of the α-lactalbumin blank. As can be seen in Fig. 2A, polymeric protein molecules elute between 7.5 mL (void) and 10-10.5 mL. The monomeric protein molecules that are glycated show a slightly lower elution volume compared to the α-lactalbumin blank, as a result of their mass increase due to conjugation with the saccharides (Fig. 2A). This effect seems to be relatively strong for the charged saccharides in Fig. 2B. Although the charged groups were aimed to be shielded by 150 mM NaCl present in the eluent, they still seem to have an increased effect on the apparent molecular weight compared to neutral saccharides.

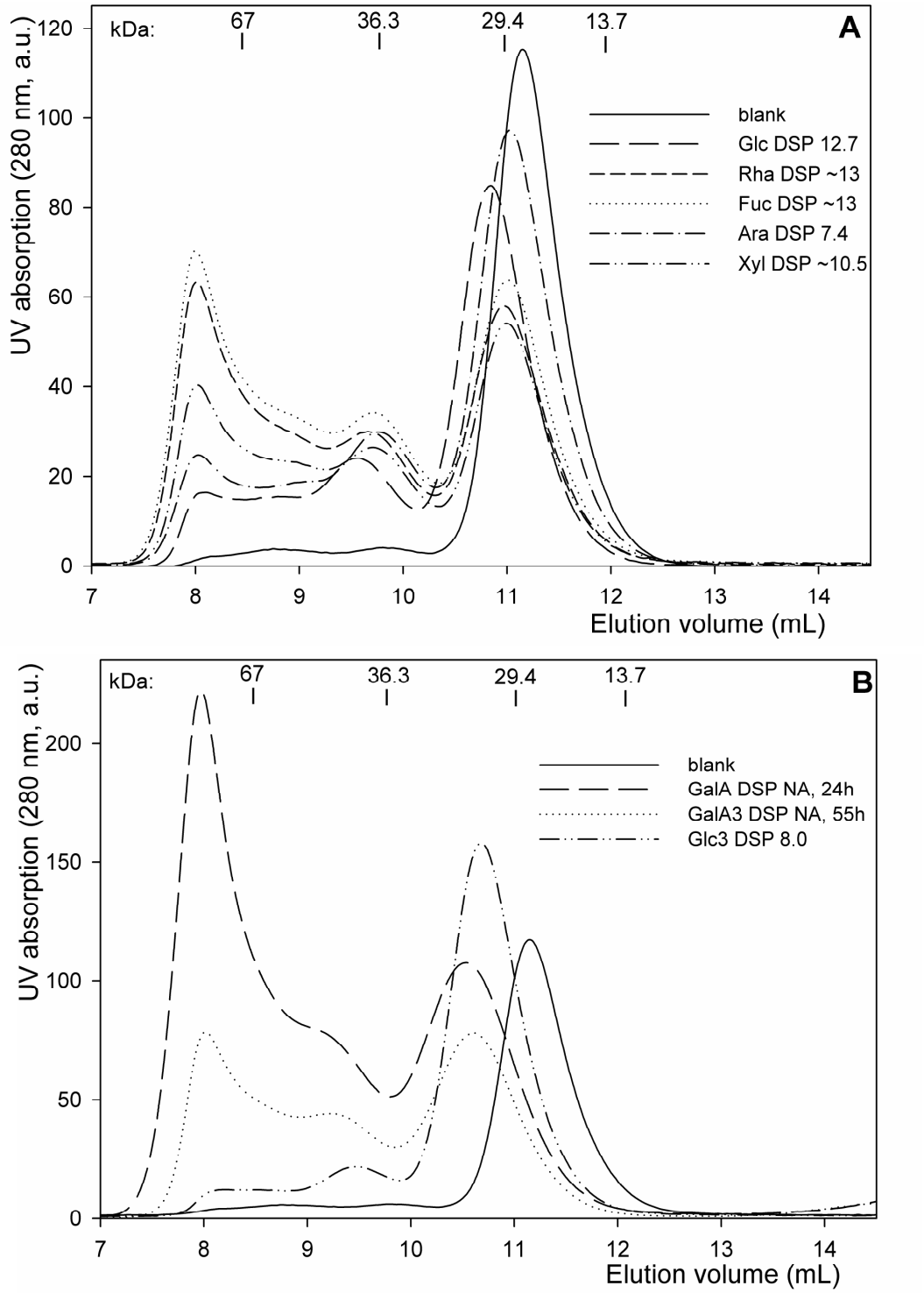

Fig. 2 Size exclusion chromatography elution profiles under denaturing conditions of α-lactalbumin samples glycated using neutral monosaccharides (A) and others (B). The average degree of substitution per protein molecule of the samples is indicated. Elution volumes of proteins used for column calibration are included. DSP NA: sample too complex to determine extent of glycation.

When the behavior of the monosaccharides is compared more precisely, based on the elution profiles in Fig. 2 and the data in Table 1, some differences can be observed. Arabinose and xylose show a very fast conjugation with α-lactalbumin and cross-linked protein is observed already in an early stage of the reaction. After 24 hours of incubation, the cross-linking of proteins induced by these monosaccharides lead to mostly insoluble products. The other monosaccharides generate cross-linked proteins after longer incubation times. Glucose clearly has the lowest tendency to generate cross-linked proteins. Oligosaccharides generate less cross-linked protein formation compared to their corresponding monosaccharides (Fig. 2B). The oligosaccharide with the highest DP used in this study, maltoheptaose, generates no cross-linked protein at all within the incubation times applied (Table 1).

When the appearance of cross-linked protein is compared to the obtained mass spectra, a parallel can be noted. The appearance of cross-linked protein is related to the appearance of dehydrated Amadori products. This is visualized in Figs. 1 and 3 by the presence of peaks for dehydrated Amadori products, depending on the saccharide type and incubation time applied.

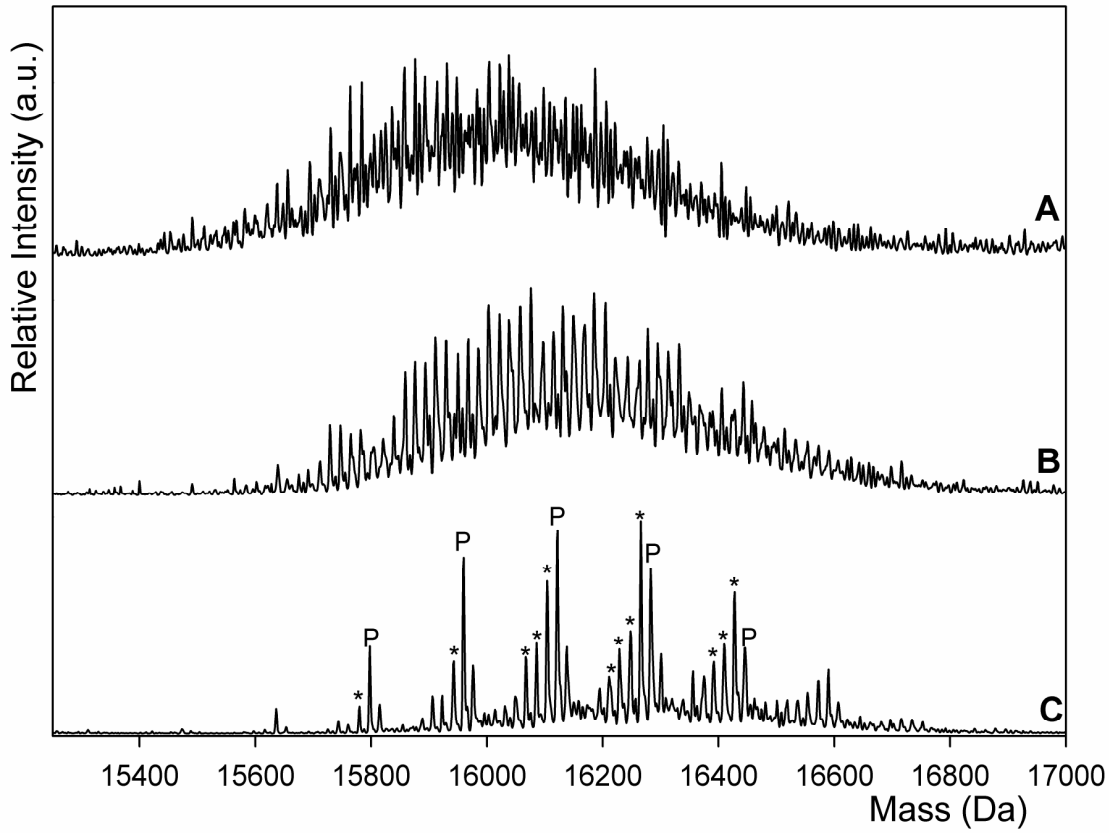

Fig. 3 ESI-TOF MS spectra of α-lactalbumin incubated for 24h with A] fucose, B] rhamnose, and C] glucose. Amadori products (P), and a number of dehydrated Amadori products (*) are indicated.

The only two saccharides inducing cross-linking in an early reaction stage were arabinose and xylose (Table 2). As can be seen in Figs. 1 E and F, only α-lactalbumin molecules carrying Amadori products of these two saccharides show dehydration already in this stage of the reaction. This already indicates that dehydration and protein cross-linking may be connected. Furthermore, Fig. 3 shows that the Amadori product of glucose is clearly less susceptible to dehydration than the Amadori product of rhamnose and fucose. The latter two yield a very complex product mixture, while α-lactalbumin conjugated with glucose clearly shows the main Amadori product peaks accompanied by a number of dehydrated conjugates. As mentioned, glucose also showed the lowest cross-linked protein inducing tendency. The formation of cross links is, apparently, strongly related to the instability of the Amadori compounds involved. The relatively low reactivity of glucose in the Maillard reaction due to its stable ring structure has been observed before *(19)*.

The fact that cross-linked protein is only observed during or after dehydration steps of the Amadori products can be explained by the fact that reactive compounds have to be formed from the Amadori products to induce this cross-linking. A number of proposed mechanisms, including cross-linking after 1,2 enolization *(1, 3)*, and via dideoxysones to glucosepane and pentosidine *(7, 8)*, involve dehydration of the original Amadori compound. A high Maillard reactivity of a saccharide, resulting in a fast average DSP increase, strengthens this effect, because it leads to a fast formation of these Amadori compounds, which can subsequently dehydrate. The statement that the often doubted importance of Amadori compounds in protein cross-linking should be reconsidered *(8)*, is underlined by these observations.

The similar behavior of a number of the saccharides used in this research in terms of glycation rate and cross link formation seems to be related to their molecular structure. Arabinose and xylose are both aldopentoses, which could for example lead to cross-link formation via pentosidine *(8)*. Rhamnose and fucose are both deoxy-hexoses, and this is also resulting in similar behavior. High Maillard reactivity of a saccharide and fast formation of cross links are, however, not automatically related to each other. Arabinose and xylose show the highest glycation rate, and indeed show the highest extent of cross-linking. Fucose, rhamnose, and glucose, however, are also similar to each other in terms of glycation rate, but glucose is clearly less inclined to cross link formation.

Cross-linked protein formation was also induced by trigalacturonic acid and to a lower extent by maltotriose (Fig. 2B, Table 1) after conjugation to α-lactalbumin. This corresponds with the higher cross link inducing capacity of galacturonic acid compared to glucose. An increase in the DP of the saccharides results in a decrease in the cross-linking

behavior, which is underlined by the behavior of maltoheptaose, inducing no cross-links. This effect of the DP is a result of two factors. On the one hand, α-lactalbumin samples glycated using neutral oligosaccharides have a lower average DSP than the samples conjugated with monomers. A lower DSP automatically results in a lower probability of the formation of reactive species, which decreases the probability of cross link formation. On the other hand, the cross-linking induced by conjugated oligosaccharides may take place by a different mechanism than the cross-linking induced by conjugated monosaccharides. This is indicated by the MS-spectrum of maltotriose with an average DSP of 8.0, where no dehydration of the conjugated saccharides is visible (data not shown), while cross-linking has been observed for this sample (Fig. 2B, Table 1). A stabilizing effect of e.g. methyl groups or saccharide moieties coupled to the Amadori product of glucose has been observed before, when the development of the Maillard reaction between ovalbumin and glucose, methylglucose, and lactose was compared *(20)*. It also been concluded that mainly 1,4-dideoxyhexulose is formed from oligo- or polysaccharides during the Maillard reaction, but the assignment of all compounds and mechanisms involved remains difficult *(21)*. It was, however, not the goal of this inital study to examine the detailed mechanism of formation of cross-linked proteins in these specific systems.

Foam stability of glycated and cross-linked proteins

With a number of samples mentioned in Table 1, foams were created. These foams were evaluated for their stability by measuring their half-life time. Samples that were (partly) insoluble due to complex formation were not tested. Non-modified α-lactalbumin was used as a reference, which means that the half-life time of its foam was set at 1. Half-life times of other foams are expressed relative to this. Results can be found in Fig. 4.

The stability of foams created with α-lactalbumin is greatly reduced after protein incubation for 2h in the absence of any saccharide. Additional experiments, however, indicate that mostly the preceding freeze-drying and dialysis steps are responsible for this effect, and not the incubation at 60 °C (data not shown). After 24 hours of incubation, a slight increase of the foam stability is observed compared to the 2-hours incubated sample. This may be the result of an additional change in the conformation of the protein as a result of the heating step. When the stability of the foams created with various glycated protein samples is evaluated, it appears that this stability depends on the extent of glycation of the protein as well as the type of saccharide used for protein glycation.

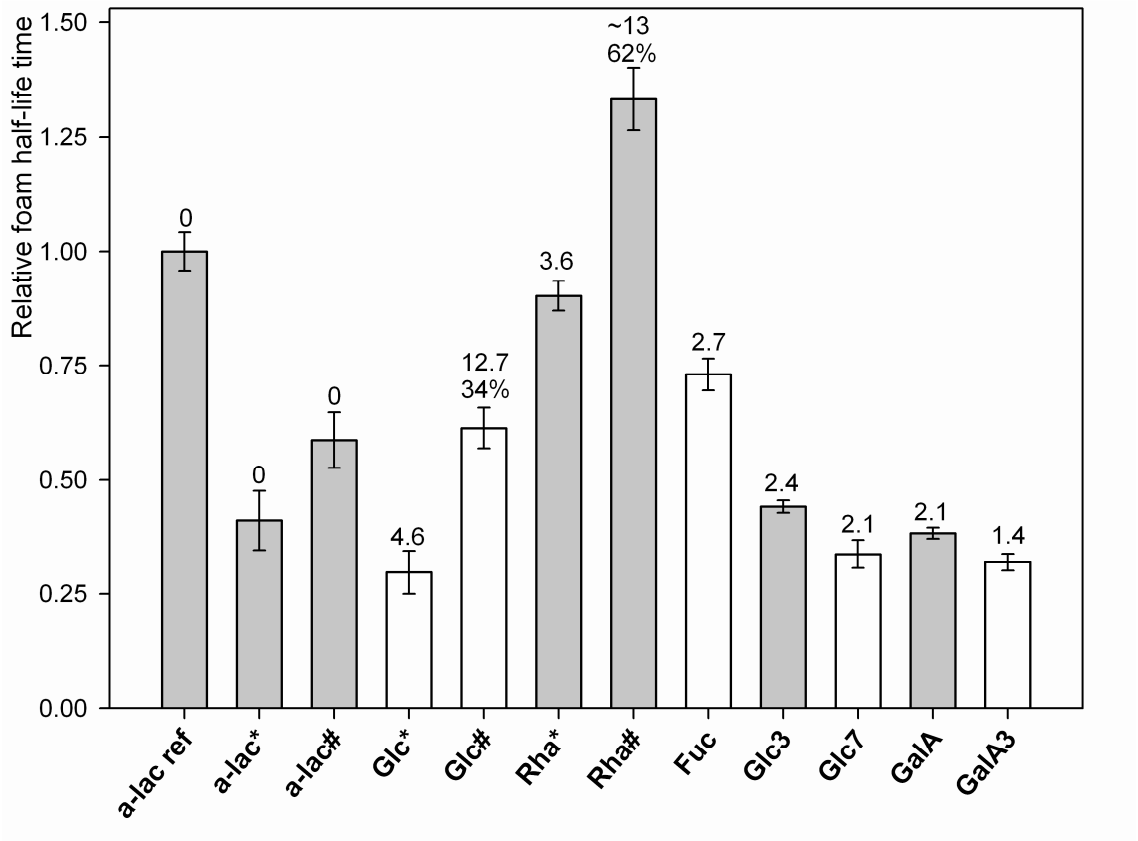

Fig. 4 Relative half-life times of foams created with various (modified) α-lactalbumin samples. Data are presented relative to the half-life time of foam created using untreated α-lactalbumin (ref). The average degree of substitution per protein molecule of the glycated α-lactalbumin samples is provided, together with the percentage of cross-linked protein (if applicable). *:1.5-2h incubation, #: 24h incubation (Table 1).

Attachment of glucose to α-lactalbumin does not have a major influence on the stability of its foams. A slight increase in stability is observed when the DSP increases to 12.7, but this corresponds to an incubation time of 24h and about 34% of the protein is cross-linked. Compared to the sample incubated for 24h without any saccharide present, there is no difference in foam stability. This is a remarkable finding, as conjugation with glucose has been reported to improve the foam stability of other proteins like β-lactoglobulin *(11, 22)*, and lysozyme *(17)*. The cross-linking observed for the 24h incubated sample containing glucose may be an explanation for its low foam stability, as enzymatically cross-linked α-lactalbumin was found to have anti-foam properties *(23)*. This may mask a positive effect of the glucose units attached. Also conjugation with charged saccharides and maltooligosaccharides does not induce a very clear effect on the foam stability. The degree of substitution may be too low to have an influence. The low stability of the Amadori

products and the presence of charges make it hard to create monomeric proteins with a high degree of substitution using (tri-)galacturonic acid *(14)*. Furthermore, long incubation times are needed to reach a high extent of glycation with maltooligosaccharides. These glycation difficulties may make the application of these saccharides in protein property modification through the Maillard reaction difficult. A different synthesis route for saccharide-protein coupling, as proposed previously *(24)*, may be more applicable in this case, but was not included in the present study. A clear positive influence on the foam stability is observed after conjugation of α-lactalbumin with rhamnose, and to some extent also with fucose. For α-lactalbumin glycated using rhamnose, an increase of the average DSP resulted in a higher foam stability. The stability of the foam created with α-lactalbumin with an average DSP of ~13 exceeds the foam stability of the original α-lactalbumin sample. The latter might even be an underestimation due to the high amount of cross-linked protein in this sample, which was in previous research involving enzymatic cross-linking found to reduce the foam stability of α-lactalbumin *(23)*. These findings are in contrast to results obtained using glycated β-lactoglobulin, where conjugation with rhamnose did not exert a clear effect on the foam stability *(11)*. The incorporation of fucose in the α-lactalbumin structure also increased its foam stability already at a low DSP (Fig. 4). The similarity in the structures of rhamnose and fucose, and the expected similarity in their glycation mechanisms and sites, is reflected in their effect on the foam stability of α-lactalbumin glycated using these saccharides.

Conclusions

The amount of cross-linked protein observed after glycation of α-lactalbumin by the Maillard reaction is, apart from reaction time and conditions, highly dependent on the type of saccharide applied. The dependency on the saccharide type also accounts for the effect on the foam stability of the glycated protein. Dehydrated Amadori products in the more advanced stages of the Maillard reaction, as monitored by UPLC ESI-TOF MS, were a clear indicator for the formation of cross-linked protein.

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Chapter 8

General Discussion

Table 1 Overview of the analytical techniques applied in this thesis for analysis of modified biomolecules, with the corresponding findings and conclusions

Thesis chapter	***Technique applied***	***Observation / Finding***	***Conclusions based on the finding***
2 TEMPO-oxidized starch (1)	HPSEC/ HPAEC	* (Charged) oligomers present after harsh acid treatment of oxidized starch * Monomer quantification after acid hydrolysis	* Acid-resistant glycosidic linkages * Oligomer structures can be used to model polymer structure
	MALDI-TOF MS	* Blocks of glucuronic acid moieties present in TEMPO-oxidized starch * Intermediate aldehydo groups only present close to glucuronic acid moieties	* TEMPO-oxidation proceeds blockwise * More intermediate aldehyde groups are formed, but not oxidized to carboxyl groups
3 Epoxy starch derivatives (2)	HPSEC/ HPAEC	* β-amylase hydrolysis slightly hindered after allylation, and more hindered after epoxidation * Enzymatic starch hydrolysis hindered by allylation/epoxidation * Difference in oligomeric profiles of enzymatic digests of allylated/epoxidized starch	* Modification not random over the starch molecules * Cross-linking after epoxidation * Amylolytic enzymes sterically hindered by the modifications
	MALDI-TOF MS	* Wide range of structures observed in enzymatic digests: allylhydroxypropyl groups, epoxy groups, diols, several cross-links	* Epoxy groups proceed in secondary reactions, leading to diols and cross-links * More allylhydroxypropyl groups modified than concluded based on a spectrophotometric assay
4 Molecular sieves provoke side reactions (3)	MALDI-TOF MS	* Detailed view on product composition after FOS acylation obtained * Multiple substitution only observed when molecular sieves were involved	* Immobilized *Candida antarctica* lipase only produces mono-lauryl esters of FOS * Molecular sieves may catalyze acylation reactions, even if only applied for solvent pre-drying

5 Oligosaccharide hydroxy-aryl esters (4)	Solid phase extraction (SPE)	* Saccharide esters purified and separated based on substitution degree * Yield saccharose mono-ester: ~ 37%, di/triesters: ~ 4%	* Pure mono-ester fraction available for glycosylation experiments * Chemically catalyzed reaction mostly leads to mono-ester formation, depending on substrate ratio
	RP-UPLC ESI-Iontrap MS	* Successful monitoring of the hydroxy-arylation reaction, desired products obtained * Characterization SPE-fractions: SPE separation based on degree of substitution * Monitoring of peptide glycosylation: a.o. GYG-HPP-saccharose, (saccharose-HPP)$_2$	* Hydroxy-aryl esters synthesized successfully * Primarily mono-substitution of saccharides * SPE suitable for product purification/separation * Glycosylated peptides formed * Quite some homoconjugate formation * Kinetic control important
	NMR	* Substitution saccharose mono-ester mainly on the glucose O-6 and the fructose O-6	* Chemical catalysis quite regioselective
6 Glycated α-lactalbumin	UPLC-ESI-TOF MS	* Determination of the average degree of substitution per protein molecule (DSP) after glycation via the Maillard reaction * Exact view on the Maillard product dispersity obtained * Dehydration products of the Amadori compounds observed *Reactivity of maltooligosaccharides with model peptide FAAL decreases when saccharide DP increases	* Maillard reaction rate highly dependent on saccharide type and size * Maillard products are disperse * Calculation of reaction rate of individual product species possible. * Reaction rate decreases when DSP increases * Visualization of the seamless change of initial to intermediate reaction stage * Decreasing reaction rate with increasing saccharide DP results from decreasing saccharide reactivity as well as increasing influence of protein structure
7 Cross-linking and foaming of glycated proteins	Size Exclusion Chromato-graphy	* Cross-linked proteins observed	* Cross-linking of proteins can result from the Maillard reaction, the extent depends on the saccharide structure and size
	UPLC-ESI-TOF MS	* Determination of average DSP of samples * Dehydration of Amadori products observed	* Reaching a desired DSP requires controlled reaction conditions * Dehydrated Amadori products are an indicator for protein cross-link formation
	Foaming experiments	* Foam half life time depends on the type of saccharide used for protein glycation and on the average DSP	* The optimal saccharide-type and reaction conditions used for protein glycation depend on the type of protein used and on the desired properties of the glycated protein

General Discussion

Introduction

The development of routes for the in vitro synthesis of novel or modified biomolecular structures in combination with an understanding of these structures and their properties cannot be performed successfully without a proper structure analysis. This is the common theme throughout this thesis. The research presented has a dual relevance. On the one hand, the importance of analysis and analytical strategies is stressed. On the other hand, the novel findings on reactions, mechanisms, and molecular structures are of relevance. In this chapter, this dual relevance will be further discussed. In Table 1, an overview of the analytical techniques applied in this thesis is provided, including the findings done using these techniques, and the conclusions that were based thereon.

Starch modification, structure characterization, and functionality

Structure of TEMPO-oxidized starch

As can been seen in Table 1, several analytical techniques are applied in **chapter 2** to study acid hydrolysates and methanolysates of TEMPO-oxidized potato starch. HPSEC and HPAEC were important for the first notification of the fact that oligomers still existed after harsh acidic hydrolysis of TEMPO-oxidized starch. These techniques also showed that the size of the remaining oligomers increases when de degree of oxidation (DO) increases. Furthermore, HPAEC enabled quantification of the mono- and oligosaccharides formed during hydrolysis. This made clear that the quantity of oligosaccharides obtained was high enough to provide a realistic view on the structure of the initial polymer. The oligosaccharides were analyzed in detail for their composition by MALDI-TOF MS. Also, the exclusive presence of intermediate aldehydo groups close to oxidized groups was identified by MALDI-TOF MS. Altogether, the blockwise oxidation of gelatinized starch by TEMPO was confirmed, and conclusions about the underlying mechanism could be drawn. Literature published so far only involved analysis of an average degree of oxidation without taking this distribution into account. This was done by either direct analysis of the number of oxidized C6-groups, by titration, or by control of the influx of reagents *(5-8)*. The functionality of the oxidized starches is subsequently discussed based on these averages. It is, however, known that the distribution of charged groups along a polysaccharide backbone has consequences for its functionality, as is the case for e.g.

pectins *(9, 10)*. This underlines the importance of a detailed analysis of the starch structure, as described in **chapter 2**.

As published recently *(11)*, TEMPO-oxidized starches may find their application in nanoparticles for controlled drug delivery to cancer cells. The incorporated carboxyl groups in this case serve as points for attachment of the specific ligand to target for cancer cells. Next to that, the carboxyl groups have interaction with cationic compounds present in the nanoparticle for structure stabilization *(11)*. It can be expected that the distribution of the functional carboxyl groups along the starch backbone, and hence the distribution of the ligand, has an influence on the stability and functionality of the nanoparticles as a whole. Control and adjustment of the synthesis conditions, together with careful monitoring of the oxidized starch fine structure and subsequent evaluation of its functionality, may make these applications for controlled drug delivery even more successful.

TEMPO-oxidized starch as a dietary fiber

Another conclusion from the research on TEMPO-oxidized starch is the high acid-resistance of the α(1→4) GlcA-GlcA and α(1→4) GlcA-Glc linkages compared to the low acid resistance of the α(1→4) Glc-Glc linkage. In other experiments, indications for resistance to digestion by α-amylase were obtained (data not shown). From a nutritional perspective, this resistance to degradation may lower the caloric value of the oxidized starch, and makes it partly a food fiber. Research on maize starch oxidized by using hypochlorite has already revealed that starch oxidation increases the amount of resistant starch *(12)*. TEMPO-oxidized starch samples need to be subjected to a simulation of the human digestive system, including fermentation steps, to be able to draw more profound conclusions on this subject. Detailed analysis of the oxidized starch used as well as the breakdown products formed during digestion will lead to insight in the possible low-caloric and prebiotic properties of TEMPO-oxidized starch. Based on these results, the oxidation conditions can be tailored for production of a polymer with optimal food fiber properties.

Structure and functionality of epoxy starch derivatives

In **chapter 3**, it can be seen that enzymatic hydrolysis followed by chromatographic and mass spectrometric analysis has led to a number of benefits in the characterization of epoxy starch derivatives. The slightly limited β-amylase digestion of allylhydroxypropyl (AHP) starch compared to non-modified starch indicated already that the substituted groups are apparently present in clusters, possibly only in specific parts of the granule. The increased resistance to β-amylase digestion after epoxidation indicated that additional reactions had

taken place, possibly cross-linking. The latter was also suspected based on the change in swelling power and solubility index of the starch after epoxidation. For this reason, it could already be assumed that conversion of only 11% of the AHP groups to epoxy groups, as concluded on the basis of spectrophotometric assay, is an underestimation. To get a comprehensive view, however, a more detailed study of the polymer had to be performed. The formation of epoxy groups, several cross-links, and/or diols was confirmed by chromatographic and mass spectrometric analysis after hydrolysis of the polymer by amylolytic enzymes. Identical *m/z* values of epoxy groups and intramolecular cross-links on the one hand, and diol groups and intermolecular cross-links on the other hand, hampered the unambiguous assignment of some of the structures. It was not clear if all of these structures were formed before enzymatic hydrolysis. It can, nevertheless, be stated that the application of the advanced analytical techniques provided a much better view on the epoxy starch derivative than the application of only the spectrophotometric method, as the latter does not take subsequent reactions of the epoxy groups into account.

An unambiguous assignment of structures present in the enzymatic hydrolysates may be reached by applying different mass spectrometric techniques. Due to the complexity of the samples, separation prior to analysis is considered beneficial. For this, the recently published method involving porous graphitized carbon LC coupled to ESI- Iontrap MS may be employed *(13)*. ESI-Ion Mobility-TOF MS might also be used to separate and analyze the compounds, because the ion mobility cell is able to separate compounds that have the same *m/z* value, but differ in their structure. The latter represents a major advantage compared to MALDI-TOF(-TOF) MS. In addition, more research on the distribution of the substituents in the starch granule, which was only indicated by the extent of digestion reached by the exo-enzyme β-amylase, could promote an increased understanding of the modification mechanism. Peeling of the starch granules by chemical gelatinization followed by analysis of the structures present in each of the fractions, as proposed by Jane and Shen *(14)*, and for example applied on acetylated starch *(15, 16)*, would be the most obvious strategy for this.

Transesterification as a strategy for creating specific saccharide functionalities

Chemical vs. enzymatic (trans-)esterification

In **chapters 4 and 5** transesterification is used to increase the functionality of various oligosaccharides. Chemical as well as enzymatic catalysis of these reactions is applied. Enzymes have gained a prominent position within synthetic chemistry because of their

regioselective and stereoselective action, in combination with the possibility to tailor the properties of the enzyme. The fact that techniques have become available for low cost enzyme production also contributed to their wide application *(17)*. In **chapter 4**, we showed that the employment of enzymes is, however, not always as straightforward as assumed. By detailed analysis of the product composition using MALDI-TOF MS, it became clear that molecular sieves, under certain conditions, catalyzed side reactions (Table 1). Careful control of the conditions during enzyme-catalyzed reactions and monitoring of the reaction products is thus of primary importance, especially when the specificity of the enzymes is important. The catalyzing role of the adjuvant molecular sieves in acylation reactions, either by their presence during synthesis or by solvent pre-drying, was especially noticed under conditions in which the enzyme showed a relatively low activity, for example at a high DMSO concentration in tertiary butanol. In **chapter 4**, several examples in literature of possible side reactions catalyzed by molecular sieves have been mentioned already. Since then, some more examples of the application of molecular sieves in synthesis procedures have been published. Kakasi-Zsurka et al. *(18)* applied immobilized *Candida antarctica* lipase in organic medium for the synthesis of copolymers of D-glucono-lactone and 3-hydroxybutyric acid in the presence of molecular sieves, in a 7-day synthesis (80 °C) involving esterification. It cannot be excluded that part of the activity attributed to the enzyme may actually be provoked by the molecular sieves present, although we only proved this for a transesterification reaction (**chapter 4**). The same may account for the recently reported synthesis of metoprolol-saccharide conjugates in solvents dried using molecular sieves. Nevertheless, it has to be noted that high regioselectivity was reported in this case. This means that enzymes have probably been the most active catalyst *(19)*. In another recent publication, no problems with catalysis by molecular sieves are reported in the transesterification between various sugar alcohols and a phenolic ester (HPPME) *(20)*. The catalysis of side reactions by molecular sieves is apparently highly dependent on the substrates and conditions used and on the enzyme activity under these conditions.

In the research described in **chapter 5**, it was aimed to obtain a mono-ester of various oligosaccharides, using the phenolic compound HPPME as the substituent. This would enable subsequent peroxidase-mediated protein glycosylation. Several chromatographic techniques, mass spectrometric techniques, and NMR were used. These techniques were useful for reaction monitoring, product purification, and determination of the selectivity of reactions (Table 1). The lipase applied in **chapter 4** was evaluated to synthesize the mono-esters. This did not lead to formation of the desired product. As monitored by RP-UPLC-ESI-MS, monosaccharides like methyl α-D glucopyranoside could to some extent be

substituted with HPPME by the immobilized *Candida antarctica* lipase, but this approach was not successful for oligosaccharides. After modeling of the three dimensional structure of the enzyme, it was concluded that the catalytic cleft of the enzyme is deep and narrow. This led to the hypothesis that it was impossible to fit both the bulky phenolic group and oligo- or polysaccharides together in this cleft. The latter was also concluded recently *(20)*, when the lipase-catalyzed transesterification of HPPME or vinyl laurate with 1-octanol was monitored. In this case, the reaction involving the bulky HPPME showed a much lower reaction rate than the reaction where less bulky vinyl laurate was involved.

Based on the hypothesis involving the catalytic cleft, it was decided to perform the transesterification of saccharides with HPPME using a chemical catalyst, as described in **chapter 5**. As mentioned, mono-esters were desired for subsequent enzyme-catalyzed coupling to peptides or proteins, since application of multiple substituted esters might lead to unwanted protein cross-linking. The specific hydroxyl group in the mono-ester used for substitution was of minor importance for enzymatic protein glycation, especially in the *proof-of-principle* stage. The product composition as obtained for the transesterification of saccharose with HPPME was analyzed by RP-UPLC-ESI-MS. It showed a surprisingly high proportion of mono-esters, being about 90%. A relatively simple solid phase extraction-based purification procedure delivered the pure mono-ester, and this procedure also enabled gravimetric quantification of the yield. According to NMR-data, the purified saccharose mono-ester was for about 82% composed of esters on the O-6 positions of the glucose (56%) or fructose (26%) moiety. The quite selective course of the chemically catalyzed reaction is most probably related to steric factors. This is possibly a result of the bulky and rigid phenolic group attached. As mentioned in **chapter 5**, the mono-ester yield may even be increased by a further optimization of substrate ratios and/or incubations conditions.

Altogether, it can be stated that the choice between a chemical and an enzymatic catalyst depends on the substrates and on the type of products desired. It is clear that the application of enzymes requires a well-controlled reaction environment. Also chemical catalysis may lead to a selective reaction.

Application of saccharide-HPP conjugates

In **chapter 5**, the application of saccharide-HPP conjugates in the peroxidase-mediated glycosylation of proteins was evaluated by using a model peptide instead of a protein. As monitored by RP-UPLC-MS, the synthesis of glycopeptides out of these substrates by using peroxidase was successful. However, apart from the formation of glycopeptides, a number

of other conjugates was formed, mostly homoconjugates of HPP-saccharose. This can be explained by the properties of peroxidase. GYG is a much less favorable substrate for peroxidase than phenolic compounds like ferulic acid *(21)* and hence also the phenolic linker used in this research. In fact, the reaction using peroxidase is a radical reaction initiated by the enzyme with no clear specificity. A more efficient production of the desired glycopeptide will be a challenge that can only be fulfilled by careful kinetic control. The use of a higher excess of GYG may lead to lower amounts of saccharose-HPP homoconjugates. However, on the other hand it may induce higher amounts of GYG homoconjugates.

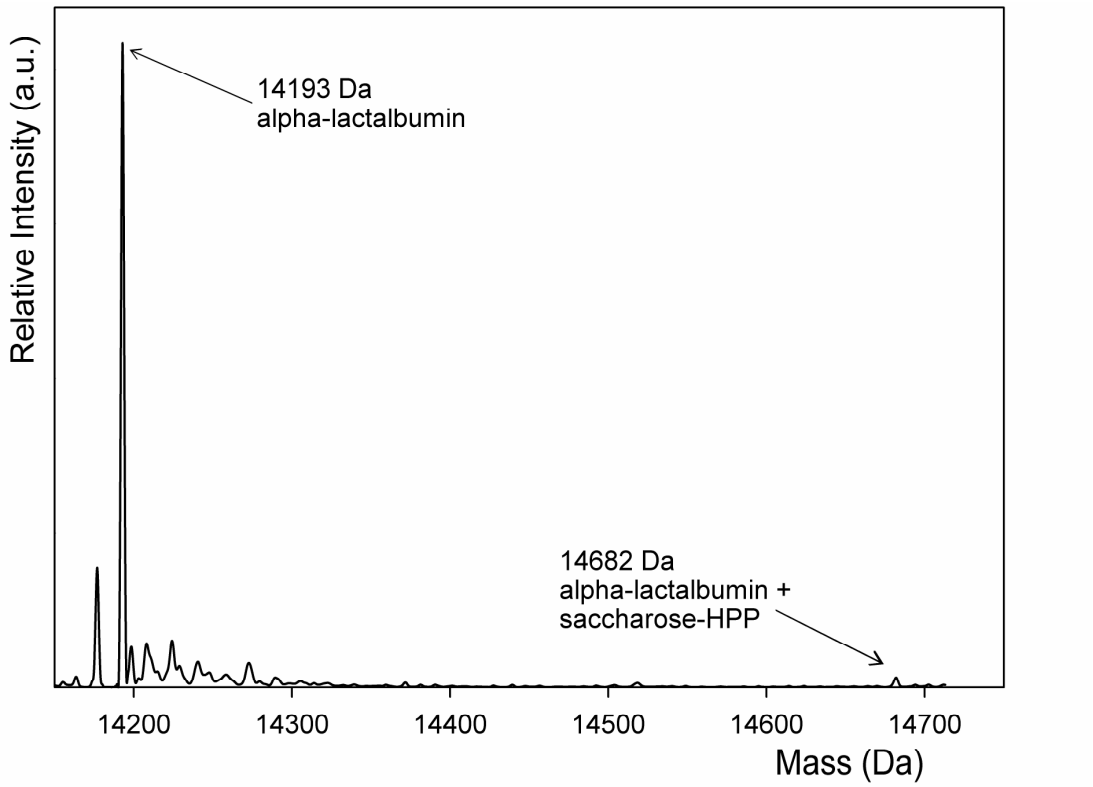

Fig. 1 UPLC-ESI-TOF mass spectrum of α-lactalbumin incubated with horseradish peroxidase under gradual addition of saccharose-HPP and hydrogen peroxide.

To reach the eventually desired peroxidase-catalyzed protein glycosylation, the kinetic control will be even more important. The tyrosine moieties present in a protein may be even a less favorite substrate for peroxidase compared to GYG, because they are enclosed in the protein structure. Indications for this have already been obtained when preliminary experiments were performed using α-lactalbumin and saccharose-HPP as the substrates and horseradish peroxidase as the catalyst. After sample separation and analysis using UPLC-ESI-TOF MS, it was concluded that only a minor amount of α-lactalbumin-HPP-saccharose

had been formed (Fig. 1). The peak at 14193 Da represents (oxidized) α-lactalbumin, the peak at 14682 Da may represent α-lactalbumin with one saccharose-HPP molecule covalently linked to it. Denaturation of proteins, which might make the tyrosine moieties somewhat more accessible, may help to create glycoproteins using oxidizing enzymes.

The application of the Maillard reaction for protein glycation

Novel approaches to Maillard reaction monitoring

In **chapters 6 and 7**, the Maillard reaction rates of a number of saccharides with α-lactalbumin are compared. Variations depending on the type and size of the saccharide are observed. This was already known based on a number of studies published. As shown in Table 1, the novelty of our work lies in detailed monitoring of the degree of substitution per individual protein molecule, enabled by applying UPLC-ESI-TOF MS. First studies using a similar approach have been published in 2003, when LC-ESI-quadrupole MS was applied for the analysis of β-lactoglobulin glycated with glucose, galactose, and lactose *(22, 23)*. The UPLC-ESI-TOF MS method, as applied in **chapter 6 and 7,** was used for separation and analysis of the glycated proteins. It was about 3-4 times faster than the methods published earlier *(22, 23)*. Apart from the speed and the accuracy of the ESI-TOF MS-based method compared to techniques based on gel electrophoresis or spectrophotometric approaches, a major advantage is found in the visualization of the precise composition of the products. Fig. 2 shows the exact composition of solid state mixtures of α-lactalbumin and glucose after 1, 2, 4, 8, and 24h of incubation, as obtained by UPLC-ESI-TOF MS followed by deconvolution of the *m/z* spectra to mass spectra. For each individual glycated protein species with a given degree of substitution per protein molecule (DSP 0, DSP 1, etc.), an individual peak with an individual intensity can be observed, as indicated in Fig. 2.

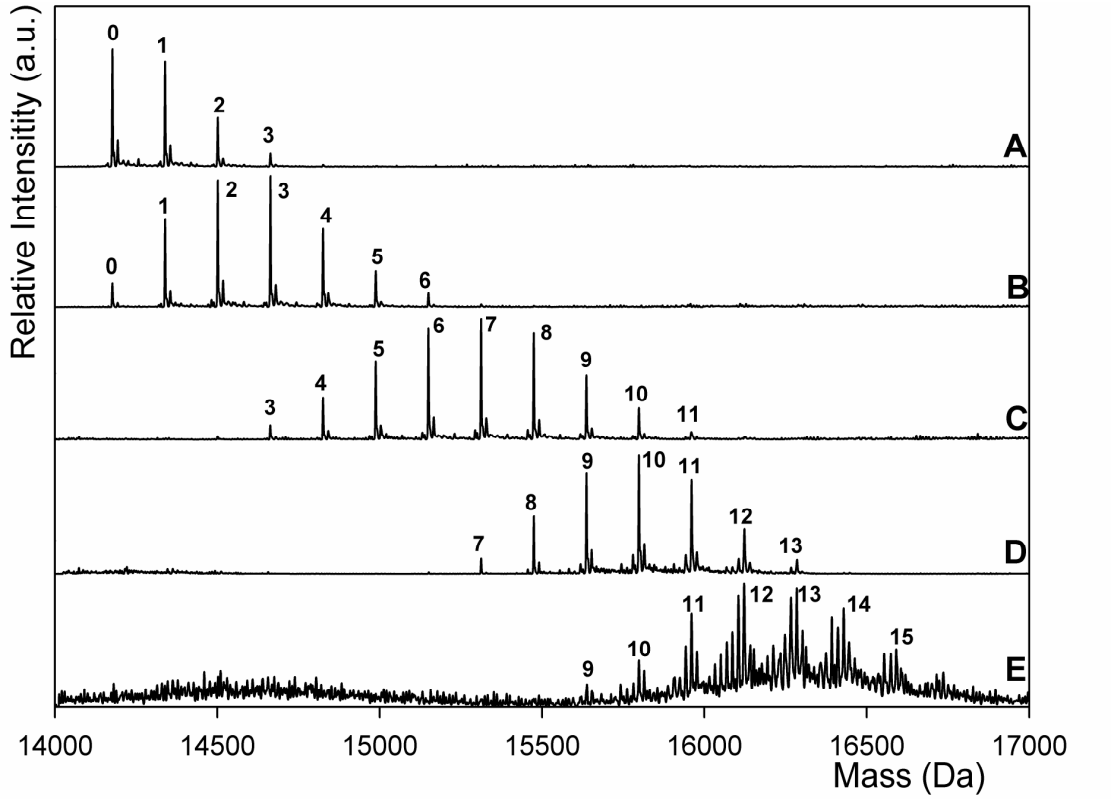

Fig. 2 Mass spectra of solid state α-lactalbumin-glucose samples after incubation (60 °C, 65% RH) for A] 1h, B] 2h, C] 4h, D] 8h, and E] 24h. Mass spectra were obtained by deconvolution of their UPLC-ESI-TOF MS spectra. Degree of substitution per protein molecule (DSP) is indicated.

Because the exact product composition is known at several time points, including the proportion of each DSP (degree of substitution per protein molecule) species, a relative reaction rate for each individual DSP species during a certain time interval can be calculated. To do this, data within each spectrum were first normalized by calculating the proportion of each DSP in the total product mixture. Subsequently, relative reaction rates for were calculated for each time interval based on the decrease in proportion of a certain DSP, starting with the lowest DSP present at that time and finishing with the highest DSP. For example, the reaction rate for non-modified α-lactalbumin to α-lactalbumin with one glucose moiety attached (DSP 0→1) was, for each time interval where DSP 0 was initially present, based on the decrease in the proportion of DSP 0 in the product mixture in that time interval. Then, the rate for DSP 1→2 was present based on the decrease in the proportion DSP 1, etc.. Calculated rates are listed in Table 2. Because these values were calculated based on relative product compositions, and not on measured amounts (e.g. moles), the values should be considered as being relative. Hence, comparisons should only be made within the same set of data (Table 2).

Table 2 Relative reaction rates in the Maillard reaction between α-lactalbumin and glucose, calculated based on the product composition analyzed by UPLC-ESI-TOF MS.

Time interval → / ***Relative reaction rate ↓***	***0h - 1h***	***1h – 2h***	***2h – 4h***	***4h - 8h***	***8h - 24h***
DSP 0-1	2	4.4	5	5*	-
DSP 1-2	2.35#	4.15	6	6*	-
DSP 2-3	1.8#	4.25	7	7*	-
DSP 3-4		3.8#	5.9	5.9*	-
DSP 4-5		4#	5	5*	-
DSP 5-6		3.7#	5	5	5*
DSP 6-7			4.7#	1.85	2*
DSP 7-8			4.7#	2	2*
DSP 8-9			3.8#	1.8	1.8*
DSP 9 -10			3.5#	1.6	1.6
DSP 10-11			2.4#	1.4	0.47
DSP 11-12				1.2#	0.56
DSP 12-13					0.35#
DSP 13-14					0.34#
DSP 14-15					0.2#

 # Minimum value, could not be determined exactly, because time point of first appearance is not precisely known

** Could not be determined exactly, because time point of disappearance is not precisely known*

The calculated rates provide insight in a number of interesting items. The low reaction rates in the first hour of incubation show that the reaction has a short lag phase, as the same reaction goes relatively faster in the following hours. Furthermore, it can be observed that the reaction rates of DSP species are decreasing when the DSP is exceeding the value of 6, and that rates for the same reaction (e.g. DSP 10→11) decrease when incubation time increases. This may lead to the conclusion that the initial reacting lysine moieties have a higher exposure than the lysine moieties reacting in a later stage. From research using the lysine containing model peptide FAAL, however, we know that the reaction between glucose and FAAL developed similar to the reaction between α-lactalbumin and glucose (**chapter 6**). The decreasing rates observed in Table 2 are, apparently, not a result of influence of the protein structure, but of the decreasing number of free amino groups present in combination with a relatively low amount of reducing ends available for the reaction. The use of a larger excess of reducing ends during the reaction may be evaluated

to reduce the influence of this factor. Furthermore, a more frequent sampling would be beneficial, because this would result in an even more accurate representation of the relative reaction rates.

Additional research on Maillard reaction products

By applying a combination of the methods in **chapter 6** for a wider range of saccharides and proteins than described, and also in other substrate ratios, more information about the factors determining the course of the Maillard reaction can be obtained. This especially counts when the calculation of the relative reaction rates of the individual DSP species, as described above, would also be included in such research.

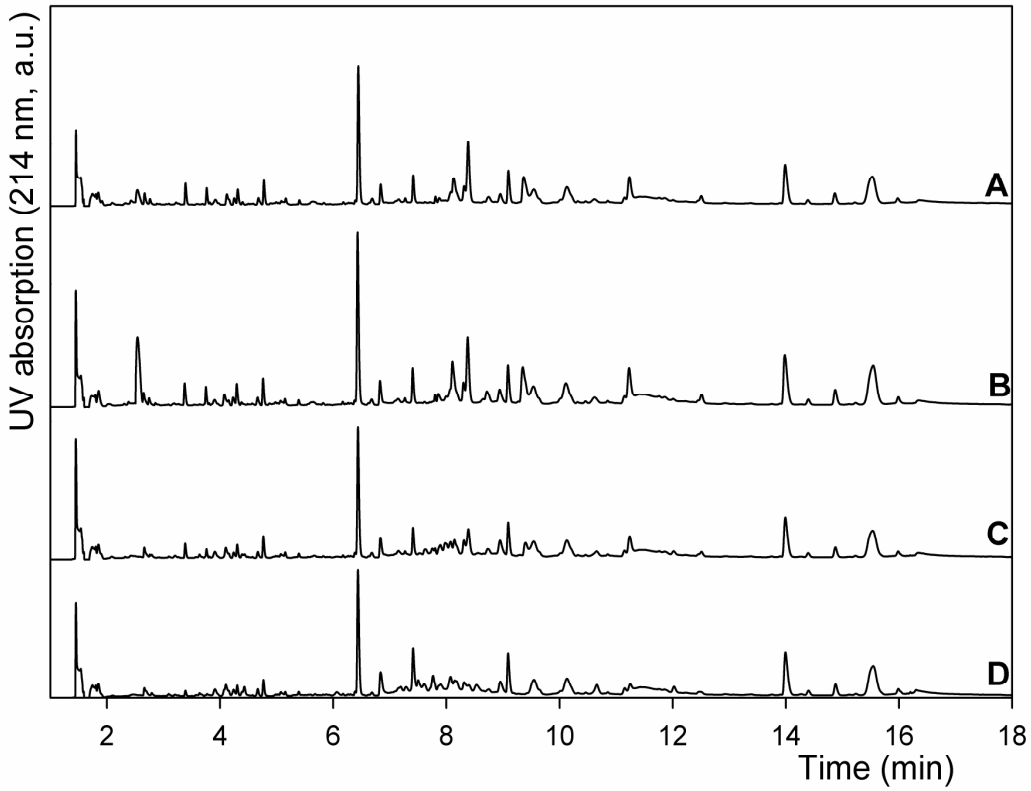

Fig. 3 RP-UPLC-UV (214nm) elution patterns of chymotryptic hydrolysates of β-casein glycated via the Maillard reaction. Glycation extent (before hydrolysis): A] no glycation, B] 0-1 maltotriose moieties, C] 0-5 maltotriose moieties, D] 4-9 maltotriose moieties.

The determination of the individual lysine moieties that are modified during the Maillard reaction by studying glycoprotein hydrolysates has already been discussed in **chapter 1**. β-Casein, glycated with maltotriose using a procedure similar to the glycation of α-lactalbumin in **chapter 6**, was subjected to hydrolysis using chymotrypsin and was analyzed using RP-UPLC-UV (Fig. 3). Chymotryptic degradation of glycated β-casein

obviously results in a different hydrolysate composition compared to non-modified β-casein. This difference increases when the extent of glycation of the protein increases (Fig. 3). A number of peptides present in the hydrolysate of the non-glycated β-casein disappears when the extent of glycation increases. At the same time new (glyco-)peptides are present in the digests of the glycated protein. These changes are the most profound at elution times between 7.5 and 12 min. A small number of peaks is hardly affected (e.g. around 6.5, 9.1, and 14.0 min.). These results indicate that the exact sites of modification within in the glycated β-casein may be determined by two approaches. It may be done either by mass spectrometric analysis of the glycopeptides present in the hydrolysates of the glycated proteins. Another option is found in the analysis of the peptides that are not present in glycoprotein hydrolysates, but are present in the hydrolysate of the non-glycated protein. When the information about the sites of glycation is combined with the results of a determination of relative reaction rates of DSP species, as mentioned before (Table 2), new insights in the influence of lysine accessibility in the protein molecule may be obtained. At the time point where low exposed lysine moieties show the first modifications, observed in hydrolysates of the glycoprotein, a decreased reaction rate for a certain glycated species may be observed.

Controlling the Maillard reaction through analysis

In **chapters 6 and 7,** dehydration of Amadori products has been made visible in deconvoluted UPLC-ESI-TOF mass spectra (Table 1). Clearly, this technique makes the seamless transition from the initial to the intermediate Maillard reaction stage visible. This indicates that the reaction stages, as defined more than half a century ago and still applied *(24, 25)*, can all occur at the same time. From the results, it is clear that some Amadori products can still be formed while other Amadori products are subject to degradation already. Furthermore, the detection of dehydrated Amadori products indicates that reactive compounds, able to induce protein cross-linking in the final reaction stage, are about to be formed. This altogether shows that detailed analysis of the Maillard reaction products creates an improved understanding of the Maillard reaction progress. Hence, the Maillard reaction might be controlled and the product composition can be directed. The advances in analytical chemistry, therefore, may assist the application of the Maillard reaction in controlled food ingredient production.

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Summary

Summary

Nature offers a wide variety in biopolymers applied in food and non-food applications. Modification of these polymers can be performed to improve their functionality. This can be done by the addition, removal, or modification of functional groups, and by cross-linking. Tailored modification of the structure of oligo- and polymers is necessarily accompanied by a detailed analysis of the structure of the resulting products. Such a structure characterization provides information on the relation between modification procedure, the chemical fine structure of the modified molecules, and the product functionality. The aim of the present PhD thesis is the detailed molecular characterization of products obtained after structure modification of oligosaccharides, starch, a model peptide, and bovine α-lactalbumin. The methods theirselves as well as the information on structure characteristics obtained by applying these methods are of relevance.

In **chapter 1**, a general introduction into the biomolecules involved in this research as well as the relevance of being able to monitor their modification is given. Background information about *in vitro* enzymatic protein glycosylation and Maillard-mediated protein glycation is provided. The mechanism of the Maillard reaction, the factors determining the course of the Maillard reaction, and the structure and functionality of the glycated proteins are reviewed. Subsequently, the conjugation of oligosaccharides with functional groups via transesterification is discussed. The chemical as well as enzymatic catalysis of this reaction is described. The final section of chapter 1 deals with chemical modifications of starch and structure elucidation of the resulting products.

In **chapter 2**, the elucidation of the fine structure of gelatinized starch oxidized via the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated reaction is described. Starches with several degrees of oxidation (DO) were degraded by using weak and strong acid hydrolysis and methanolysis. These degraded starches were subsequently analyzed by using chromatographic and mass spectrometric techniques. A remarkable resistance of the α(1→4) glucuronic acid-glucuronic acid linkage and to a lesser extent of the α(1→4) glucuronic acid-glucose linkage against harsh acid treatments was observed. Mass spectrometric analysis of the fragments revealed the presence of blocks of glucuronic acid moieties in TEMPO-oxidized starch, whose size increased when the DO increased. Furthermore, the presence of intermediate aldehydo groups adjacent to these blocks of glucuronic acid moieties was confirmed. Based on these observations, it was concluded that the TEMPO-oxidation proceeds block wise. It is hypothesized that this is a result of the

interaction between the positively charged active nitrosonium-ion of TEMPO and the negatively charged carboxyl groups in the glucuronic acid moieties.

In **chapter 3**, the structure of derivatives obtained by the epoxidation of 1-allyloxy-2-hydroxypropyl-waxy (AHP) maize starch is subjected to analysis. Enzymatic digestibility, swelling capacity and solubility were significantly reduced after epoxidation. Via a spectrophotometric assay, it was determined that ~11% of the allyl groups had been modified during the epoxidation reaction. The molecular structure of epoxidized starch was revealed by using chromatographic and mass spectrometric techniques after enzymatic hydrolysis. It was concluded that a major part of the reactive epoxy groups formed had proceeded in subsequent reactions. The presence of a considerable amount of cross-links and/or diol groups was confirmed, which were not identified by the spectrophotometric assay. This result shows the benefit of advanced molecular analysis compared to indirect analysis by, e.g., spectrophotometric techniques. Additionally, epoxy starch derivatives were successfully applied as carrier matrix for the immobilization of a thermostable β-glucosidase.

In **chapter 4**, the cause of discrepancies in literature regarding the specificity of immobilized *Candida antarctica* lipase B in the acylation of oligosaccharides were explained. Molecular sieves are widely used to remove free water from solvents prior to and during this type of reactions. By MALDI-TOF MS analysis of the product composition after acylation of fructooligosaccharides under different conditions, the role of molecular sieves in the catalysis of side reactions was made clear. It was proven that molecular sieves alone can catalyze the acylation of fructose oligomers using vinyl laurate, leading to multiple substitution of the oligomers. This effect was most visible at conditions unfavorable for the enzyme as this resulted in a relatively high concentration of the chemically produced adducts. The enzyme alone selectively catalyzed the formation of monosubstituted oligomers.

In **chapter 5**, the hydroxy-arylation of oligosaccharides to enable their subsequent peroxidase-mediated coupling to proteins is decribed. Hydroxy-arylation was performed by a chemically catalyzed transesterification between 3-(4-hydroxyphenyl)propionate and a number of oligosaccharides. Esterified saccharides resulting from the transesterification reaction were analyzed by UPLC-ESI MS. Oligosaccharides with up to three hydroxy-aryl units were detected. NMR-analysis on purified mono-esters revealed that saccharose was mainly substituted at the O-6 position of the glucose or fructose moiety. Solid phase extraction enabled a product separation based on the degree of substitution of the saccharides. The purified mono-ester of saccharose was subsequently coupled to the model

peptide glycine-tyrosine-glycine via a peroxidase-mediated reaction, as monitored by UPLC-ESI MS. By doing this, a *proof-of-principle* for protein and peptide glycosylation with a wide range of saccharides was provided.

Chapters 6 and 7 were dedicated to a detailed study of bovine α-lactalbumin glycated via the Maillard reaction in the solid state. By applying UPLC-ESI-TOF MS, individual reaction products could be monitored and analyzed for their degree of substitution per protein molecule (DSP). In this way, not only an average DSP of the glycated protein mixture was determined, but also information on the dispersity of the products was obtained. A number of mono- and oligosaccharides was applied in the Maillard reaction with α-lactalbumin, leading to DSP values ranging between 0 and 15. The standard deviation of the average DSP, referred to as the product dispersity index, increased up to 1.9. Clear differences in Maillard reaction rates were found, depending on saccharide type and size. The results of incubation of a series of pure maltooligosaccharides with increasing degree of polymerization with a lysine-containing peptide were compared with the results obtained by incubation of these saccharides with α-lactalbumin. The decrease of the Maillard reaction rate with increasing DP turned out to result from a decreasing saccharide reactivity, but was also related to an increasing influence of substrate mobility and lysine accessibility. Cross-linking of α-lactalbumin as a result of the Maillard reaction was also evaluated, using size exclusion chromatography. The amount of cross-linked protein turned out to depend on both saccharide type and reaction time. Dehydration of Amadori-products could clearly be recognized by UPLC-ESI-TOF MS and was a clear indicator of protein cross-linking. The amount of cross-linked protein, as measured by using size exclusion chromatography, was lower when the DP of the saccharide used was higher. The stability of foams created using the glycated α-lactalbumin was evaluated and depended on the saccharide applied for glycation and the average DSP. It was concluded that glycation of a protein for functionality improvement should incorporate control of synthesis conditions and a careful selection of the saccharide type used for glycation.

In chapter 8, the relevance of the methods presented in this thesis and the information obtained by applying these methods are further discussed. Additionally, the calculation of the reaction rates of individual glycated species in a Maillard product mixture, based on results of detailed UPLC-ESI-TOF MS analysis, is further elaborated.

Samenvatting

Samenvatting

De natuur biedt een grote variëteit in biopolymeren die kunnen worden gebruikt in voedsel- en niet-voedseltoepassingen. Deze polymeren kunnen worden gemodificeerd om hun functionaliteit te vergroten. Dit kan worden gedaan door middel van toevoeging, verwijdering of modificatie van functionele groepen en door cross-linking. Bij gerichte modificatie van de structuur van oligo- en polymeren is een gedetailleerde analyse van de structuur van de verkregen producten essentieel. Zo'n analyse levert informatie op over de relaties tussen modificatieprocedure, de chemische fijnstructuur van de gemodificeerde moleculen en de functionaliteit van de producten. Het doel van dit proefschrift is gedetailleerde moleculaire karakterisering van producten verkregen na structuurmodificatie van oligosacchariden, zetmeel, een modelpeptide, en α-lactalbumine uit koemelk. Zowel de methoden als de resultaten verkregen door toepassing van deze methoden zijn relevant.

In **hoofdstuk 1** wordt een algemene introductie gegeven over de biomoleculen gebruikt in dit onderzoek en over het belang van de mogelijkheid om hun modificaties te monitoren. Achtergrondinformatie over enzymatische eiwitglycosylering *in vitro* en eiwitglycering via de Maillardreactie wordt gegeven. Het mechanisme van de Maillardreactie, de factoren die het verloop van die reactie bepalen en structuur en functionaliteit van glyco-eiwitten worden uiteengezet. Vervolgens wordt de conjugatie van oligosacchariden met functionele groepen via transesterificatie besproken. Zowel chemische als enzymatische katalyse van deze reactie wordt beschreven. Het laatste deel van hoofdstuk 1 gaat over chemische modificaties van zetmeel en over structuuropheldering van de resulterende producten.

In **hoofdstuk 2** wordt de opheldering van de fijnstructuur van verstijfseld zetmeel geoxideerd via een 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) gemedieerde reactie beschreven. Zetmelen met verschillende oxidatiegraden zijn afgebroken met behulp van zwak en sterk zure hydrolyse en methanolyse. De afgebroken zetmelen zijn vervolgens geanalyseerd met behulp van chromatografische en massaspectrometrische technieken. Een opvallende resistentie tegen behandeling met sterk zuur van de α(1→4) glucuronzuur-glucuronzuur binding en in mindere mate de α(1→4) glucuronzuur-glucose binding werd geobserveerd in deze experimenten. Massaspectrometrische analyse van de fragmenten liet zien dat clusters van glucuronzuur-eenheden aanwezig waren in het TEMPO-geoxideerde zetmeel, en dat de grootte van deze clusters toenam met toenemende oxidatiegraad. Bovendien werd de aanwezigheid van intermediaire aldehydegroepen in de buurt van de clusters van glucuronzuur-eenheden aangetoond. Op basis van deze observaties werd geconcludeerd dat de TEMPO-oxidatie geclusterd verloopt. Als hypothese is gesteld dat dit

een gevolg is van interacties tussen het positief geladen nitrosonium-ion van TEMPO en de negatief geladen carboxylgroepen in de glucuronzuur-eenheden.

In **hoofdstuk 3** wordt de structuur van geëpoxideerd 1-allyloxy-2-hydroxypropyl-waxy (AHP) maiszetmeel geanalyseerd. Enzymatische afbreekbaarheid, zwellingscapaciteit en oplosbaarheid werden aanmerkelijk gereduceerd door epoxidatie. Door toepassing van een spectrofotometrische techniek kon worden vastgesteld dat ~11% van de aanwezige allylgroepen was gemodificeerd tijdens de epoxidatie reactie. De moleculaire structuur van geëpoxideerd zetmeel werd opgehelderd door gebruik van chromatografische en massaspectrometrische technieken, na enzymatische afbraak van het zetmeel. Op basis daarvan kon worden geconcludeerd dat een aanzienlijk deel van de gevormde reactieve epoxygroepen had deelgenomen aan verdere reacties. De aanwezigheid van een grote hoeveelheid cross-links en diolgroepen werd bevestigd, terwijl deze niet werden opgemerkt bij toepassing van de spectrofotometrische techniek. Dit resultaat laat de voordelen van geavanceerde moleculaire analyse ten opzichte van analyse met indirecte technieken, zoals spectrofotometrische technieken, duidelijk zien. Ook zijn epoxy zetmeelderivaten toegepast voor de immobilisatie van een thermostabiele β-glucosidase.

In **hoofdstuk 4** worden discrepanties in de literatuur omtrent de specificiteit van *Candida antarctica* lipase B in de acylering van oligosacchariden verklaard. Moleculaire zeven worden veel gebruikt om voorafgaand aan en tijdens dit soort reacties vrij water uit de oplosmiddelen te halen. Producten verkregen na acylering van fructo-oligosacchariden onder verschillende condities werden geanalyseerd met behulp van MALDI-TOF MS. Deze analyse liet de rol van moleculaire zeven in de katalyse van ongewenste reacties zien. Er is bewezen dat door aanwezigheid van alleen moleculaire zeven de acylering van fructose oligomeren met vinyllauraat wordt gekatalyseerd en dat dit leidt tot meervoudige substitutie van de oligomeren. Dit effect was het sterkst onder condities die niet ideaal waren voor het enzym omdat dit resulteerde in een relatief hoge concentratie van de producten van chemische katalyse. Het enzym katalyseerde alleen de vorming van enkelvoudig geacyleerde oligomeren.

In **hoofdstuk 5** wordt het hydroxyl-aryleren van oligosacchariden beschreven, met als doel om deze geschikt te maken voor koppeling aan eiwitten met behulp van peroxidase. Hydroxy-arylering werd uitgevoerd middels een chemisch gekatalyseerde transesterificatie tussen 3-(4-hydroxyphenyl)propionaat en een aantal oligosacchariden. Veresterde oligosacchariden werden vervolgens geanalyseerd met UPLC-ESI-MS. Oligosacchariden met maximaal 3 hydroxy-aryl groepen werden gedetecteerd. NMR-analyse van mono-esters liet zien dat saccharose vooral was gemodificeerd op de O-6 positie van de glucose of the

fructose eenheid. Solid phase extractie maakte de scheiding van producten op basis van substitutiegraad mogelijk. De gezuiverde mono-ester van saccharose werd vervolgens gekoppeld aan het modelpeptide glycine-tyrosine-glycine met behulp van peroxidase. Deze reactie werd gemonitord met UPLC-ESI-MS. Op deze manier is een bewijs geleverd voor de werking van het principe van enzymatische glycosylering van eiwitten en peptiden.

Hoofdstuk 6 en 7 zijn gewijd aan een gedetailleerde analyse van runder- α-lactalbumine geglyceerd via de Maillard reactie als vaste stof. Door toepassing van UPLC-ESI-TOF MS konden individuele reactieproducten worden gemonitord en geanalyseerd op hun substitutiegraad per eiwitmolecuul. Op deze manier werd niet alleen een gemiddelde substitutiegraad verkregen, maar ook informatie over de dispersiteit van de producten. Een aantal mono- en oligosacchariden werd toegepast in the Maillardreactie met α-lactalbumine, resulterend in substitutiegraden per eiwitmolecuul van 0-15. De standaarddeviatie van deze substitutiegraad, de productdispersiteitsindex, liet waarden oplopend tot 1.9 zien. Duidelijke verschillen tussen de snelheid van de Maillardreactie werden geobserveerd, afhankelijk van het type en de grootte van het saccharide dat werd gebruikt. Resultaten van incubatie van een serie maltooligosacchariden met oplopende polymerisatiegraad met een lysine-bevattend peptide werden vergeleken met de resultaten verkregen na incubatie van deze serie sacchariden met α-lactalbumine. Op basis daarvan bleek de afname van de snelheid van de Maillardreactie met oplopende polymerisatiegraad enerzijds het resultaat te zijn van afnemende reactiviteit van de saccharide, en anderzijds van de toenemende invloed van substraatmobiliteit en bereikbaarheid van de lysines. Cross-linking van α-lactalbumine als gevolg van de Maillardreactie werd onderzocht met behulp van size exclusion chomatografie. The hoeveelheid cross-linked eiwit hing af van zowel het type saccharide als van de incubatietijd. Dehydratie van Amadori-producten werd zichtbaar gemaakt met UPLC-ESI-TOF MS en was een duidelijk indicator van eiwit cross-linking. De hoeveelheid cross-linked eiwit, geanalyseerd met size exclusion chromatografie, was lager wanneer de polymerisatiegraad van de saccharide hoger was. De stabiliteit van schuimen gecreëerd met geglyceerd α-lactalbumine werd geëvalueerd en bleek afhankelijk van het type saccharide en van de substitutiegraad. Er is geconcludeerd dat glycering van een eiwit voor verbetering van de functionaliteit gepaard moet gaan met beheersing van de condities gebruikt voor synthese en met zorgvuldige selectie van het type saccharide.

In **hoofdstuk 8** worden de relevantie van de methoden gepresenteerd in dit proefschrift en de informatie verkregen door hun toepassing verder bediscussieerd. Ook wordt de berekening van reactiesnelheden van individuele populaties in een Maillard-reactiemengsel, gebaseerd op gedetailleerde UPLC-ESI-TOF MS analyse, verder uitgewerkt.

Dankwoord

Dankwoord

…En zo zit je op een benauwde vrijdagmiddag in augustus 2011 de eerste woorden van het dankwoord op het scherm te zetten. De afgelopen 4 jaar in de rol van AIO zijn omgevlogen. In het begin leek het een enorm lange tijd waarin ik toch vooral m'n best zou doen om alle wegen die in het projectvoorstel stonden te volgen en alle daaraan gerelateerde vragen te beantwoorden. Gaandeweg het project kwam het besef dat voortschrijdend inzicht veel kan veranderen in de loop van een project, dat 4 jaren helemaal niet zo lang zijn vanuit een onderzoeksperspectief en dat onderzoek doen meer vragen oproept dan het beantwoordt. Ik had het werk in de afgelopen jaren niet uit kunnen voeren zonder alle mensen om me heen, en daar wil ik er een aantal uitlichten.

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About the author

Curriculum vitae

Ruud ter Haar was born on July 29th, 1983 in Winterswijk, the Netherlands. After finishing his primary education at C.B.S. Groen van Prinsterer in Aalten in 1995, he started his pre-University education at Chr. College Schaersvoorde in Aalten. In June 2001, he obtained his Dutch VWO-diploma. In September of the same year, he started his Bachelor Food Technology at Wageningen University, followed by an MSc in Food Technology at the same University from 2004 onwards. The BSc and MSc education programmes included a thesis about foodborne viruses at the Laboratory of Food Microbiology, a thesis involving bioactive peptides at the Laboratory of Food Chemistry and DMV International, and an internship about polysaccharides from flax seed carried out at Massey University in Palmerston North, New Zealand. Ruud obtained his MSc diploma in June 2007. In September 2007, he started his Ph.D. research at the Laboratory of Food Chemistry of Wageningen University. The results of this research are discussed in this thesis. Currently, he is temporarily employed as a post-doc researcher at the Laboratory of Food Chemistry, while looking for a job in an industrial setting.

List of publications

***Ruud ter Haar**, Henk A. Schols, Lambertus A.M. van den Broek, Dilek Saglam, August E. Frissen, Carmen G. Boeriu, Harry Gruppen*
Molecular sieves provoke multiple substitutions in the enzymatic synthesis of fructose oligosaccharide–lauryl esters
Journal of Molecular Catalysis B: Enzymatic 62 (2010), 183–189

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Synthesis and application of epoxy starch derivatives
Carbohydrate Polymers 79 (2010), 858–866

***Ruud ter Haar**, Johan W. Timmermans, Ted M. Slaghek, Francisca E.M. van Dongen, Henk A. Schols, Harry Gruppen*
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Carbohydrate Research 346 (2011), 1005-1012

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Effect of saccharide structure and size on the degree of substitution and product dispersity of α-lactalbumin glycated via the Maillard reaction
Journal of Agricultural and Food Chemistry 59 (2011), 9378-9385

***Ruud ter Haar,** Yvonne Westphal, Peter A Wierenga, Henk A. Schols, Harry Gruppen*
Cross-linking behavior and foaming properties of bovine α-lactalbumin after glycation with various saccharides
Journal of Agricultural and Food Chemistry (2011), submitted

Overview of completed training activities

Discipline specific activities

Food Enzymology (VLAG), *Wageningen,* 2008
Pectins and Pectinases (VLAG), *Wageningen,* 2008
Summer Course Glycosciences (VLAG), *Wageningen,* 2008
EPNOE meeting, *Utrecht,* 2009
Polysaccharides as a source of advanced materials, *Turku, Finland,* 2009
61. Starch Convention, *Detmold, Germany,* 2010
CCC Scientific Days, *Groningen,* 2010
Advanced Food Analysis (VLAG), *Wageningen,* 2010
First Dutch Starch Round Table, *Groningen,* 2011
CCC Scientific Days, *Groningen,* 2011

General courses

Organizing and supervising MSc thesis work, *Wageningen,* 2007
Philosophy and Ethics of Food Science & Technology, *Wageningen,* 2008
Ph.D. Competence Assessment, *Wageningen,* 2008
Techniques for Writing and Presenting Scientific Papers, *Wageningen,* 2009
Project and Time Management, *Wageningen,* 2009
Scientific Writing, *Wageningen,* 2009
Waters course Mass Spectrometry, *Wageningen,* 2010

Optionals

Preparing Ph.D. Research Proposal
Food Chemistry Ph.D. Trip, *Beijing and Shanghai area, China,* 2008
Food Chemistry study trip, *Ghent,* 2009
Organization Ph.D. Trip Switzerland/Italy, *Wageningen,* 2009/2010
Food Chemistry Ph.D. Trip, *Switzerland and Italy,* 2010
Food Chemistry Seminars, 2007-2011
Food Chemistry Colloquia, 2007-2011

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