Journal of Molecular Catalysis B: Enzymatic 62 (2010) 183–189

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

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

Ruud ter Haar[a], Henk A. Schols[a], Lambertus A.M. van den Broek[b], Dilek Sağlam[a], August E. Frissen[b], Carmen G. Boeriu[b,c], Harry Gruppen[a,*]

[a] Wageningen University, Laboratory of Food Chemistry, Bomenweg 2, 6703 HD Wageningen, The Netherlands
[b] Wageningen University and Research Centre, Agrotechnology and Food Sciences Group, Division of Biobased Products, Wageningen, The Netherlands
[c] University "Aurel Vlaicu", Department of Chemical & Biological Sciences, Arad, Romania

ARTICLE INFO

Article history:
Received 8 September 2009
Received in revised form 21 October 2009
Accepted 26 October 2009
Available online 1 November 2009

Keywords:
Fructose oligosaccharides
Transesterification
Immobilized *Candida antarctica* lipase B
Side reactions
Molecular sieves

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.

1. 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 $\beta(2\rightarrow1)$ 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

* Corresponding author. Tel.: +31 317 483211; fax: +31 317 484893.
E-mail address: Harry.Gruppen@wur.nl (H. Gruppen).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.10.008

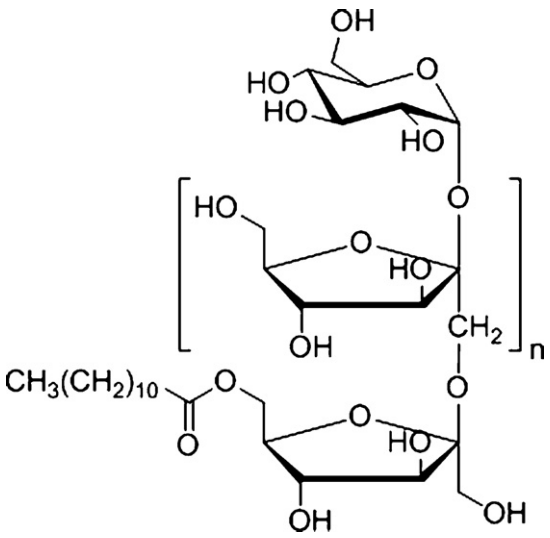


Fig. 1. Schematic structure of a lauryl mono-ester of FOS.

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.

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.

2. Experimental

2.1. 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^{t}OH) and molecular sieves (type 4 Å) 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).

2.2. Methods

2.2.1. Determination of the FOS solubility

The solubility of FOS was determined in different concentrations of DMSO in Bu^{t}OH at 30 °C. Five millilitre 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.

2.2.2. Determination of the enzyme activity

The transesterification activity of immobilized *C. antarctica* lipase B was determined at different concentrations of DMSO in Bu^{t}OH. Two millilitre 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 microlitre of hexadecane was added as an internal standard. After subsequent addition of 50 mg immobilized *C. antarctica* lipase B, samples were incubated for 24 h 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 × *g*). One microlitre of each sample was subsequently injected on a GC-system (Varian Star, Palo Alto, CA, USA) connected to a SGE BPX5 GC column (30 m × 0.25 mm × 0.25 μm). 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 min. 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.

2.2.3. 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^{t}OH 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.

2.2.4. 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

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

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 microlitre 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].

3. Results and discussion

3.1. 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.

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^{t}OH. 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

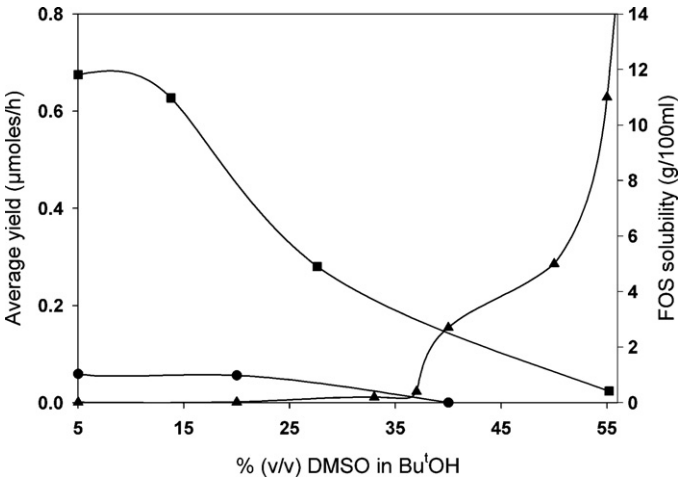


Fig. 2. FOS solubility (▲) and the average product yield after esterification (●)/transesterification (■) using immobilized *C. antarctica* lipase B at different concentrations of DMSO in Bu^{t}OH for 24 h at 40 °C. The average product yield is defined as micromole of butyl laurate formed per milligram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

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.

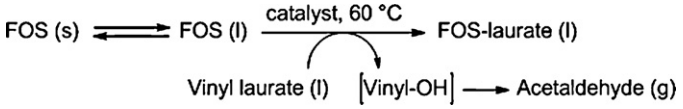
At 40% (v/v) DMSO, the average product yield is still acceptable, while the FOS solubility has increased >100 times. This higher FOS solubility can be expected to lead to an increased reaction rate. This was observed before using maltose and saccharose [6]. A lower enzyme efficiency at this higher DMSO 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 efficiency for the eventual product composition.

3.2. 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 as outlined in Table 1. The results of these experiments will be shown and discussed in the next paragraphs.

3.2.1. 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).



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

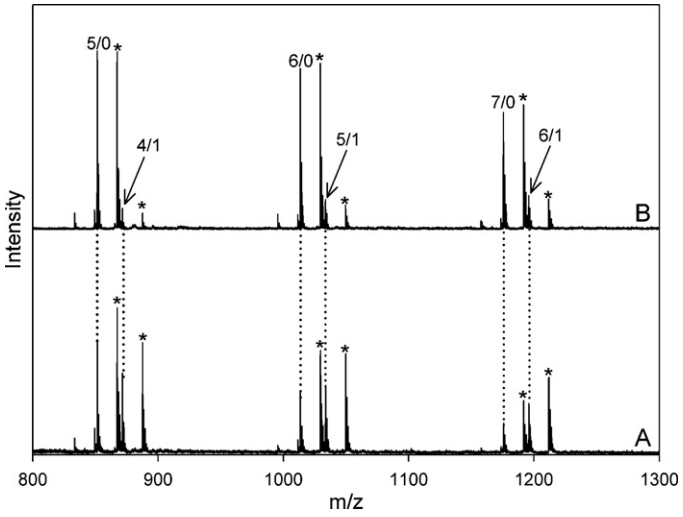


Fig. 3. Zoom-in of MALDI-TOF mass spectra after incubation of FOS with vinyl laurate, in the presence of immobilized *C. antarctica* lipase B in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH for 5 days at 60 °C, no molecular sieves were used. E.g. 5/1 stands for a fructose oligomer with a degree of polymerization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

3.2.2. 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, without 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.

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 $\geq$ 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

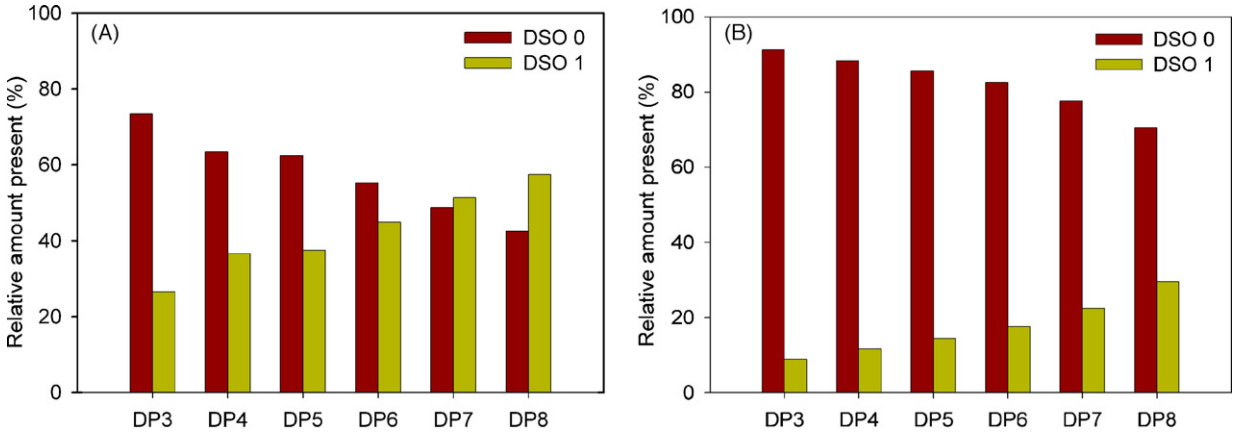


Fig. 4. Relative composition of reaction mixtures after incubations of FOS with vinyl laurate catalyzed by immobilized *C. antarctica* lipase B in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH at 60 °C for 5 days, derived from MALDI-TOF mass spectra. No molecular sieves were used. DP stands for degree of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

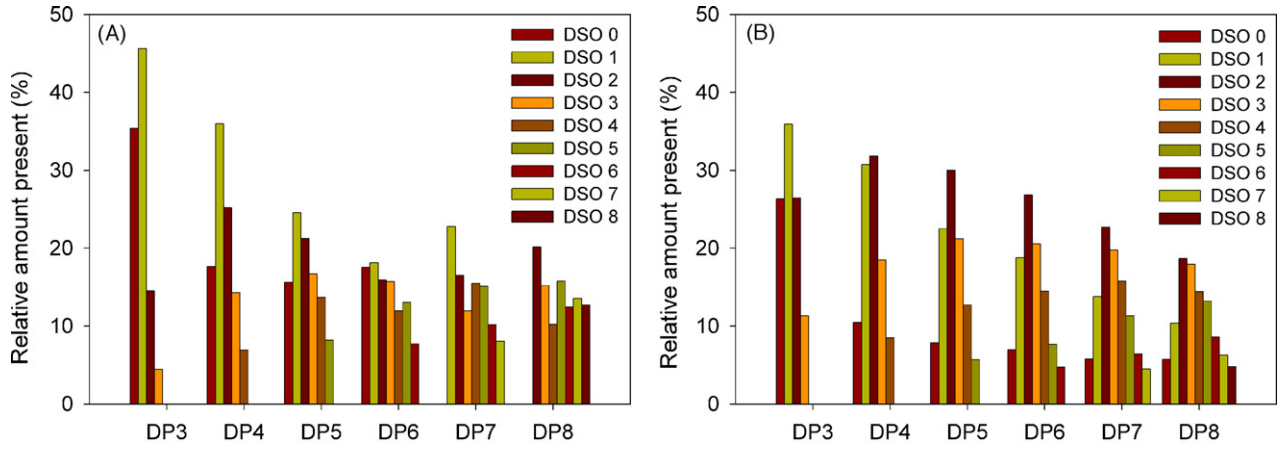


Fig. 5. Relative composition of reaction mixtures after incubations of FOS with vinyl laurate in the presence of molecular sieves and in the absence of enzyme, at 20% (A) and 40% (B) DMSO in Bu^{t}OH at 60 °C for 5 days, derived from MALDI-TOF mass spectra. Molecular sieves were present during the incubation.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

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 et al. [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 occurred 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.

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.

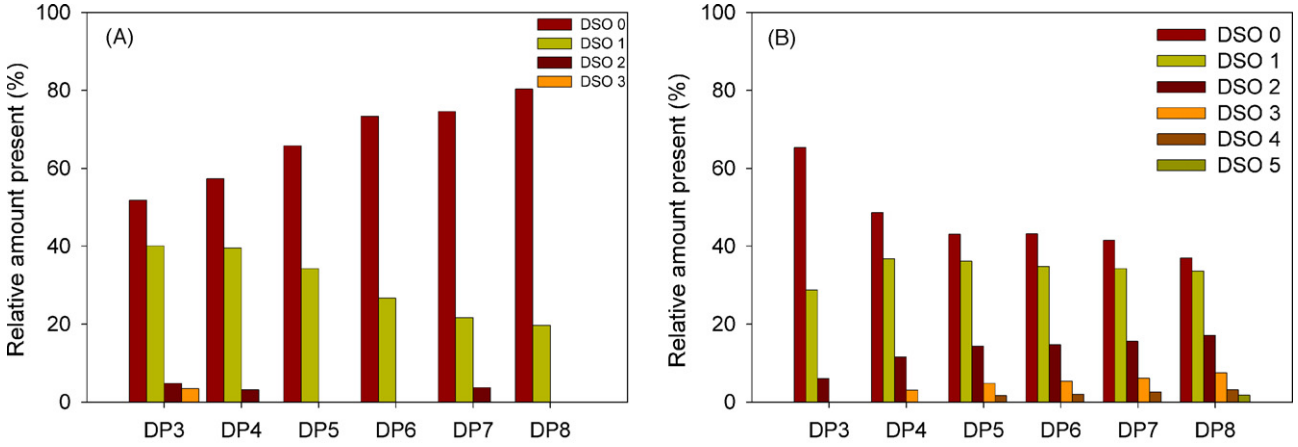


Fig. 6. Relative composition of reaction mixtures after incubations of FOS with vinyl laurate in the presence of molecular sieves and immobilized *C. antarctica* lipase B, in 20% (A) and 40% (B) (v/v) DMSO in Bu^{t}OH at 60 °C for 5 days, derived from MALDI-TOF mass spectra. DP stands for degree of polymerization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

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 [23]. This warning was based on the acylation of sucrose, catalyzed by several additives such as Celite, EupergitC or Na_2HPO_4 and implicated that chemical acylations must be taken into account when hydroxyl-containing compounds are acylated 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.

4. 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

The authors would like to thank Kim Kreuzen for her contribution to the experimental work. Within the framework of the Carbohydrate Competence Center, this research has been financially supported by the European Union, the European Regional Development Fund and The Northern Netherlands Provinces (Samenwerkingsverband Noord-Nederland), KOERS NOORD. Part of the work was performed within the framework of the IP-project Bioproduction (NMP-2CF-2007-026515), funded by the European Commission.

References

[1] A. Memmi, R. Granet, M.A. Gahbiche, A. Fekih, A. Bakhrouf, P. Krausz, Journal of Applied Polymer Science 101 (2006) 751–755.
[2] L.M.C. Sagis, C.G. Boeriu, G.E. Frissen, H.A. Schols, P.A. Wierenga, Langmuir 24 (2008) 359–361.
[3] K. Yang, Y.-J. Wang, M.-I. Kuo, Biotechnology Progress 20 (2004) 1053–1061.
[4] A. Rajan, V.S. Prasad, T.E. Abraham, International Journal of Biological Macromolecules 39 (2006) 265–272.
[5] S. Chakraborty, B. Sahoo, I. Teraoka, L.M. Miller, R.A. Gross, Macromolecules 38 (2005) 61–68.
[6] M.W. van Oosterom, F. van Rantwijk, R.A. Sheldon, Biotechnology and Bioengineering 49 (1996) 328–333.
[7] D. Bosscher, J. Van Loo, A. Franck, International Dairy Journal 16 (2006) 1092–1097.
[8] M. Ferrer, M.A. Cruces, M. Bernabé, A. Ballesteros, F.J. Plou, Biotechnology and Bioengineering 65 (1999) 10–16.
[9] I. Pérez-Victoria, J.C. Morales, Tetrahedron 62 (2006) 878–886.
[10] Z.-G. Chen, M.-H. Zong, G.-J. Li, Process Biochemistry 41 (2006) 1514–1520.
[11] J. Tsukamoto, S. Haebel, G.P. Valença, M.G. Peter, T.T. Franco, Journal of Chemical Technology & Biotechnology 83 (2008) 1486–1492.
[12] M.K. Walsh, R.A. Bombyk, A. Wagh, A. Bingham, L.M. Berreau, Journal of Molecular Catalysis B: Enzymatic 60 (2009) 171–177.
[13] H.-D. Isengard, P. Heinze, Food Chemistry 82 (2003) 169–172.
[14] J. Huang, H.A. Schols, R. Klaver, Z. Jin, A.G.J. Voragen, Carbohydrate Polymers 67 (2007) 542–550.
[15] P.P. Singh, N.A. Qazi, S. Shafi, D.M. Reddy, A.H. Banday, P.B. Reddy, K.A. Suri, B.D. Gupta, N.K. Satti, B.P. Wakhloo, H.M.S. Kumar, G.N. Qazi, Journal of Molecular Catalysis B: Enzymatic 56 (2009) 46–54.
[16] T. Weiss, M. Brown, H. Zeringue, R. Feuge, Journal of the American Oil Chemists' Society 49 (1972) 524–526.
[17] Sigma–Aldrich-Chemie Product Description Molecular Sieves, 1999.
[18] D.F. Shriver, P.W. Atkins, Inorganic Chemistry, 3rd ed., Oxford University Press, Oxford, 1999.
[19] A. Corma, H. Garcia, S. Iborra, J. Primo, Journal of Catalysis 120 (1989) 78–87.
[20] N. Gokulakrishnan, A. Pandurangan, P.K. Sinha, Journal of Molecular Catalysis A: Chemical 263 (2007) 55–61.
[21] A.C. Carmo Jr., L.K.C. de Souza, C.E.F. da Costa, E. Longo, J.R. Zamian, G.N. da Rocha Filho, Fuel 88 (2009) 461–468.
[22] M. Sasidharan, R. Kumar, Journal of Molecular Catalysis A: Chemical 210 (2004) 93–98.
[23] F.J. Plou, M.A. Cruces, E. Pastor, M. Ferrer, M. Bernabé, A. Ballesterose, Biotechnology Letters 21 (1999) 635–639.

Ruud ter Haar (1983) received his M.Sc. degree in Food Technology from Wageningen University, The Netherlands in 2007. The master thesis focused on the development of quick methods for the detection of ACE-inhibiting peptides in protein hydrolysates. His internship at Massey University in New Zealand was related to polysaccharides from flaxseed. Currently, he is a Ph.D. student at the Laboratory of Food Chemistry of Wageningen University. His Ph.D.-project, which is part of the Carbohydrate Competence Center (CCC), is related to the controlled glycosylation of proteins.

Henk Schols did his Ph.D. research in the Laboratory of Food Chemistry at Wageningen University, The Netherlands on "complex pectins from fruit and vegetables" (1995). Afterwards, he became responsible for carbohydrate research as assistant professor with emphasis on the structure–function relationship of the various classes of carbohydrates. The main area of research deals with plant cell wall polysaccharides, their chemical fine structure and changes during ripening, storage and processing of fruit, vegetables and cereals. During the last years, bioactive carbohydrates became a subject of interest as expressed in research performed in the field of prebiotica, exo-polysaccharides, etc. Being associate professor at the moment, Dr. Schols participates in many European and National research programmes.

Lambertus (Ben) van den Broek is researcher at WUR-AFSG Biobased Products, The Netherlands. He started his career in 1985 at the Physiology Department of the Catholic University Nijmegen. After 4 years he started to work at the Laboratory of Food Chemistry of the Wageningen University. Since 1997 he was active in the field of molecular biology with emphasis on carbohydrate degrading enzymes and plant proteins. He did his Ph.D. on glycosyl hydrolases from *Bifidobacterium adolescentis*. Subsequently he started in 2005 at WUR-AFSG BbP. His topic is biocatalysis with main focus on enzymatic modification of carbohydrates/proteins and enzymatic conversion of biomass.

Dilek Sağlam received her masters degree from Wageningen University in 2008 in the field of Food Science and Technology. She studied characterization and functionality of fatty acid esters of oligosaccharides in her thesis. After obtaining her bachelor degree in food engineering in 2004, she has worked as product development specialist for 2 years. Currently she is a Ph.D. student in a project supported by TI F&N (Top Institute Food and Nutrition) and Wageningen University and Research Center. Her work focuses on the stability of protein based building blocks in liquid and semi-solid model systems.

August (Guus) Frissen graduated in 1984 in Chemistry at the Faculty of Science of the University of Nijmegen, the Netherlands. He started his career in 1985 at Wageningen University in the research group of Prof. van der Plas, investigating the intramolecular inverse electron demand Diels-Alder reaction of pyrimidines. He obtained his Ph.D. degree in Natural Sciences in 1990. From 1990 to 1996 he has been working as an associated university teacher at the Laboratory of Organic Chemistry of Wageningen University and for RIKILT—Institute of Food Safety on the synthesis of reference compounds for quality control. From 1996 on he is working at the Agrotechnology and Food Sciences Group, Division of Biobased Products, of Wageningen University and Research Centre, on research topics related to environmental friendly additives for polymers and application of biocatalysts in organic synthesis.

Carmen Boeriu graduated "Cum Laudae" at University "Politehnica" of Timisoara, Romania (1978), with a major in organic chemistry, and she obtained her Ph.D. at the same university (1989). In 1981, after 3 years with industry, she started a career in research and academia in Romania. In 1986, holding an UNDP-grant she worked in the research group of Prof. A.M. Klibanov at MIT, USA. She was a visiting scientist at ATO (Wageningen, Netherlands, 1991–1995). Currently, she is senior scientist at WUR-AFSG, Department of Biobased Products, Wageningen (NL) and professor at University "A. Vlaicu" Arad, Romania. Recent research interests are in the field of biocatalysis in peptide and protein chemistry, controlled biosynthesis of GAGs, production of biobased building blocks and modification of oligo- and polysaccharides.

Harry Gruppen (1960) received his M.Sc. in Food Technology in 1985 from Wageningen University, The Netherlands. Subsequently, he joined food industry as product development manager starch derivatives. Next, he did his Ph.D. (cereal cell wall carbohydrates; 1992) at Wageningen University. After his Ph.D. he was subsequently appointed as assistant/associate professor at the Laboratory of Food Chemistry of Wageningen University. In 2008, he was appointed as Chair of Food Chemistry. The Laboratory focuses on the molecular properties, biochemical modification and interactions of non-starch polysaccharides, protein and phytonutrients during industrial processing of agricultural raw materials and digestion of foods.