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Comparative analysis of non-natural acceptor glucosylation with sucrase enzymes of family GH 70

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Abstract

Mutan- and alternansucrase were analyzed for their non-natural glucosylation potential with catecholic compounds caffeic acid and nordihydroguaiaretic acid (NDGA) as well as with non-catecolic p-coumaric acid and umbellic acid. Mutansucrase accepted both catecholic substrates and high glucosylation yields of 92 % with caffeic acid and 81 % with NDGA were obtained. The enzyme showed a clear regio-preference for the catechol 4-OH, which corresponds to findings from our previous work with *Leuconostoc* and *Weissella* derived glucansucrases. The substrate spectrum of the alternansucrase was broader and all substrates were successfully glucosylated with a preference for the catechols. Interestingly alternansucrase possessed a different regio-specificity. With caffeic acid the 3-O-α-D-glucoside was the major product. A similar substrate spectrum and regioselectivity pattern was observed in previous glucansucrase screenings only with glucansucrase from strain *Weissella beninensis* DSM 22752. Therefore it may be concluded that the *W. beninensis* enzyme is an alternansucrase type enzyme as well.

1. Introduction

Glucansucrases of family GH70 are found in lactic acid bacteria. They are divided into different groups according to the type of glucan they synthesize (Fig. 1). Dextransucrases generate polymers with mainly α-1,6-glycosidic bonds, mutansucrases, isolated from *Streptococcus*, produce preferentially α-1,3-glycosidic bonds and reuteransucrases synthesize α-1,4-linked glucans. While the former three groups are classified into enzyme class E.C. 2.4.1.5, alternansucrases belong to enzyme class E.C. 2.4.1.140. They are found in *Leuconostoc* species and produce polymers with alternating α-1,3- and α-1,6-glycosidic bonds. Typical enzymes from the different groups are e.g. dextransucrase DSR-S from *Leuconostoc mesenteroides* B-512 [1], mutansucrase GTF-B from *Streptococcus mutans* S5 [2] and alternansucrase ASR from *Leuconostoc mesenteroides* B-1355 [3]. Compilations of

sequenced glucansucrases and their specificities are found in reviews by *Andre et al.* [4] and *Leemhuis et al.* [5].

The non-natural acceptor side reaction of glucansucrases was originally shown with a *Streptococcus* glucansucrase and (+)-catechin as substrate by *Nakahara et al.* [6]. Besides flavonoids like catechin some other polyphenols were accepted by glucansucrases. The glycosylation of epigallocatechin gallate yielded nine different products with mono-, di- and triglucosylated products [7]. Glucose was either attached to the catechol motifs in 4-O- and 5-O-position or to the 7-O-position of the dihydroxylated non-catecholic aromatic ring structurally comparable to the 7-O-glycosylation of flavonoids [8,9]. From the glycosylation of catecholic polyphenols caffeic acid [10] and L-DOPA [11] it became obvious that the 4-O-glycoside is the preferred product species in all cases. Also the transformation of methylcatechol led to a preference for the 4-methyl- over the 3-methylcatechol [12]. A detailed study on the glycosylation of catechol revealed the tendency to form multiple glycosylations with glucose moieties attached to each other whereas a glycosylation of both adjacent catechol hydroxyl groups could not be observed [13]. Though exhibiting low activity, a few non-catecholic phenols could be glycosylated with glucansucrases. Glucosides of resveratrol [14], hydroquinone [15] and salicyl alcohol [16] were isolated and structurally identified.

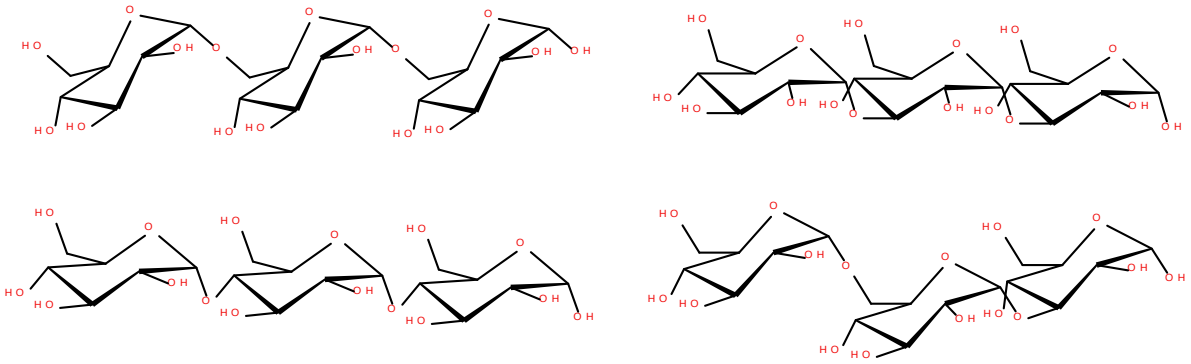

Fig.1. Major glucose repeating units of A) dextran (upper left), B) mutan (upper right), C) reuteran (lower left) and D) alternan (lower right)

In previous studies by our group *Leuconostoc* and *Weissella* glucansucrases were analyzed for their non-natural acceptor glucosylation potential [17,18]. Both genera are good sources of glucansucrases, the majority of them being dextransucrase type enzymes [19]. Caffeic acid and several structurally related catecholic and non-catecholic substrates were successfully glucosylated with the newly isolated glucansucrases. The enzyme from *Leuconostoc citreum* DSM 5577 was the best enzyme for caffeic acid glucosylation [17]. The biologically active nordihydroguaiaretic acid (NDGA) was glucosylated less efficiently by most enzymes. Upon reaction optimization by statistical design the successful glucosylation with > 90 % yield was achieved with glucansucrase from *Leuconostoc pseudomesenteroides* DSM 20193 [18].

The goal of this study was the comparative analysis of the glucosylation potential of mutan- and alternansucrase. Caffeic acid and structurally related non-catecholic p-coumaric acid and

umbellic acid as well as the bicatecholic NDGA were investigated and compared to our previously identified glucansucrases from *Leuconostoc* and *Weissella* species.

2. Materials and Methods

2.1 Enzymes and chemicals

Mutansucrase and alternansucrase were obtained from exoxx Technologies GmbH. Caffeic acid, 4-coumaric acid and umbellic acid were purchased from Sigma Aldrich and NDGA was obtained from Alfa Aesar.

2.2 Glucansucrase activity

Glucansucrase activity was determined spectrophotometrically in microtiter plate format with 3,5-dinitrosalicylic acid (DNS method) using a SpectraMax 190 plate reader at 540 nm as described before [17,18].

2.3 Biocatalytical glucosylation and glycoside purification

Glucosylation mixtures contained 200 mM sucrose, 40 mM polyphenol in 20 mM sodium acetate, pH 5.4 supplemented with 0.45 mM $CaCl_2$ and 15 % (v/v) dimethyl sulfoxide (DMSO). Reactions were started by addition of 0.375 U/ml glucansucrase and incubated statically for up to 24 hours at 30 °C. Samples were taken periodically and the reaction was stopped by addition of 9 vol. of ethanol (-20 °C). Samples were vortexed for 20 s and precipitated glucans were removed by centrifugation at 3,300 x g for 20 min at 4 °C according to the method of Overwin et al. [20]. Ethanol was evaporated from the glycoside containing samples under reduced pressure. The samples were lyophilized and dissolved in 20 % (v/v) acetonitrile in water. Preparative HPLC was carried out with an Interchim puriflash 4250/250 system as described before [17,18].

2.4 Chromatographic and spectroscopic analysis of glucosides

LC-UV was carried out with a Thermo Scientific Accela system equipped with an Accela 80 Hz PDA detector and a Hitachi LaChrom II C18 reversed phase column (4.6 x 250 mm, 5 µm) as described before [17,18]. Mass spectrometric analyses were performed using a Shimadzu LC-30AD with a SPD M20A diode array detector and a LCMS-2020 single quadrupole mass spectrometer with electrospray ionization (ESI-MS) equipped with the same column. 1H- und 13C-NMR spectra including 2D-NMR experiments were recorded with a 400 MHz Bruker Ascend™ 400 as described before [17,18].

3. Results & Discussion

3.1 Glucosylation with mutansucrase

Mutansucrase from *Streptococcus* species was already successfully utilized for the glucosylation of flavonoids like catechin [6,9,20], methylcatechol [12] and other phenolic compounds [14] before. The mutansucrase, which was obtained from recombinant

expression in *E. coli*, showed good glucosylation efficacy with the catecholic compounds caffeic acid and NDGA (Fig. 2). The high turnover of the non-natural acceptor substrates suggests that the parallel formation of mutan polymers is relatively slow and that enough sucrose is available for catechol glucosylation. Mutansucrase exhibits a clear regiopreference for the 4-OH-position with both substrates as was judged from LC retention times with our previously synthesized glucosides [17,18]. The regioselectivity pattern is in accordance with data obtained for methylcatechol by *Meulenbeld et al.* [12]. The caffeic acid-3-*O*-α-D-glucoside is visible in LC-UV analysis only as a minor peak at a retention time of 8 – 8.3 min. With both substrates the formation of some di- and oligoglucosides was observed, whereas the 4-*O*-α-D-monoglucoside was the major product with both substrates. The non-catecholic substrates p-coumaric acid and umbellic acid were not glucosylated by mutansucrase under the chosen glucosylation conditions.

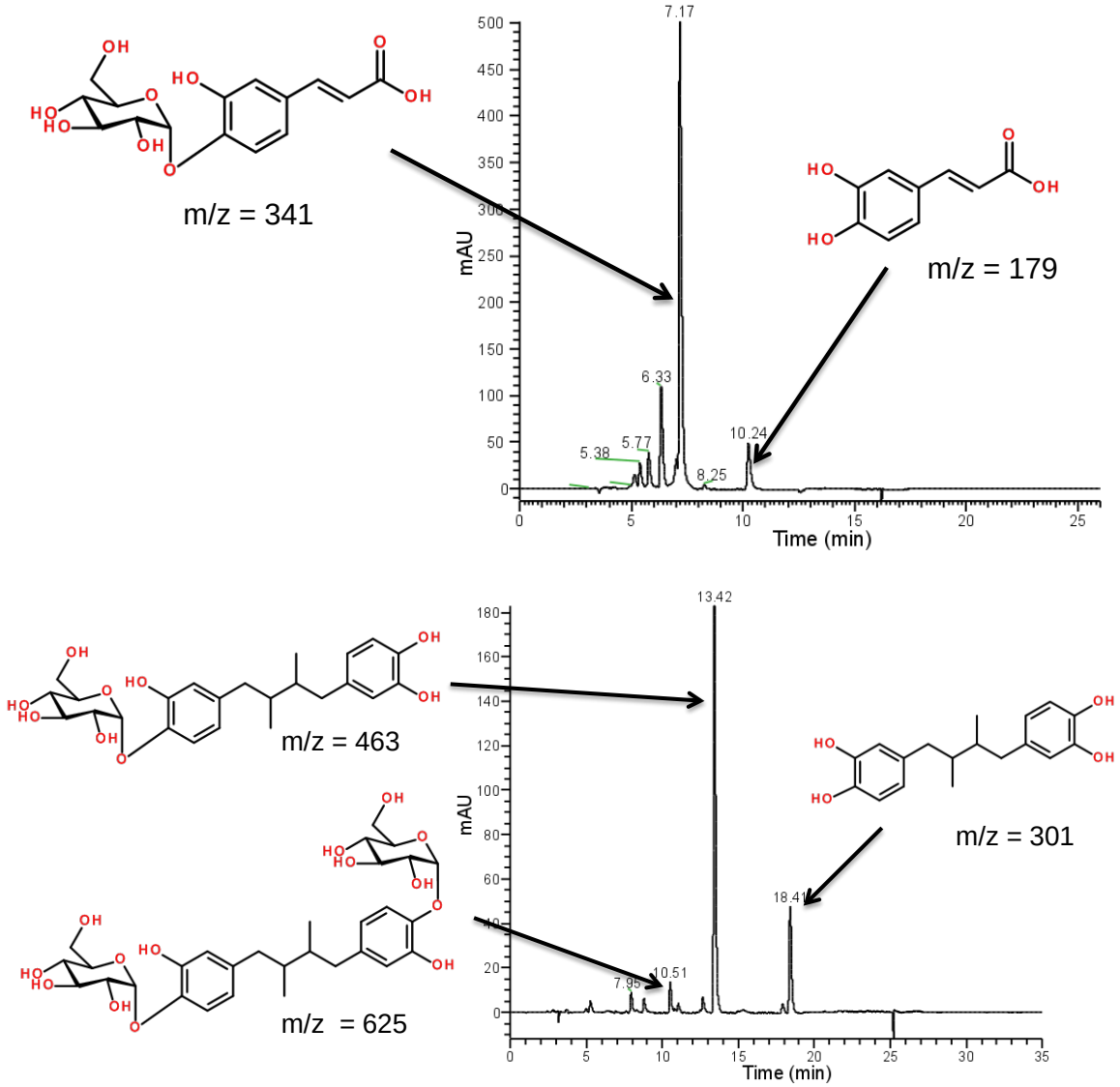

Fig. 2. Mutansucrase catalyzed transformation of caffeic acid (top) and NDGA (bottom), LC-UV analyses with mass and structure assignments, caffeic acid di- and oligoglucosides elute at 5 - 7 min and NDGA di- and oligoglucosides elute in the range of 7 – 13 min.

3.2 Glucosylation with alternansucrase

Alternansucrases have so far been seldom used for non-natural acceptor glucosylations. The successful transformation of flavonoids [8] as well as the glucosylation of stevioside [21] was shown with this class of enzymes. In contrast to mutansucrase the alternansucrase accepted the catecholic as well as the non-catecholic substrates (Fig. 3). With caffeic acid a divergent regioselectivity was detected. The peak at retention time of 8.1 min was the major reaction product, which was purified by preparative HPLC and spectroscopically analyzed by NMR. In agreement to our previously published data [17], the structure was identified as the caffeic acid-3-*O*-α-D-glucoside. Besides that also the 4-*O*-α-D-glucoside was synthesized and some di- and oligoglucosides were observed as well. The transformation of NDGA led to one major product, which was identified with good probability as the 4-*O*-α-D-glucoside by comparison of the LC retention times of the mutansucrase biotransformation and our previous results [18]. As the product was not isolated for NMR structure elucidation it might be possible that the 3-*O*-α-D- and 4-*O*-α-D-glucosides possess identical retention times however. The transformation of the non-catecholic substrates umbellic acid and p-coumaric acid were verified by mass spectrometric assignment of the product peaks. With umbellic acid mono- and oligoglucosides were detected and in the case of p-coumaric acid a weak diglucoside peak was found together with the monoglucoside. The non-catecholic substrates were less efficiently glucosylated than the catechols however.

3.3 Comparison of mutan- and alternansucrase glucosylation to glucansucrases obtained from *Leuconostoc* and *Weissella* screening

Comparison to the best glucosylation catalysts from *Leuconostoc citreum* DSM 5577 and *Leuconostoc pseudomesenteroides* DSM 20193 identified in our lactic acid bacteria screening revealed, that mutansucrase is an efficient glucosylation catalyst (Table 1). The highest initial caffeic acid glucosylation yield was obtained with mutansucrase and also transformation of the more hydrophobic and sterically hindered NDGA was comparable to that of DSM 20913. Alternansucrase displayed a different behavior regarding regioselectivity and acceptor substrate acceptance. In our previous screening only glucansucrase from *Weissella beninensis* DSM 22752 exhibited a similar glucosylation pattern (Table 1), which suggests that the *Weissella* enzyme belongs to the group of alternansucrases.

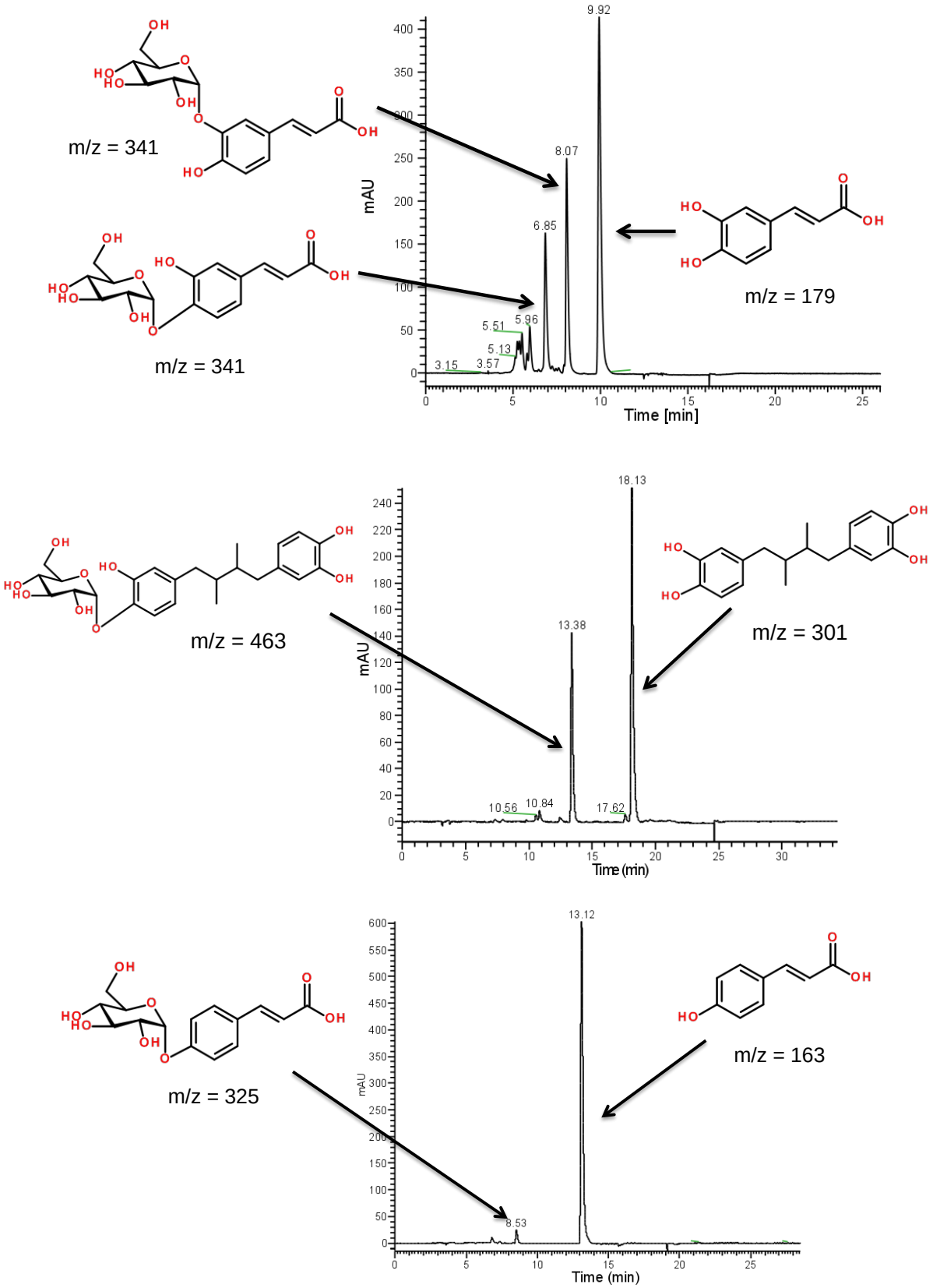

Fig. 3. Alternansucrase catalyzed transformation of caffeic acid (top) NDGA (middle) and p-coumaric acid (bottom), LC-UV analyses with mass and structure assignments, caffeic acid di- and oligoglucosides elute at 5 - 7 min and NDGA di- and oligoglucosides elute in the range of

7 – 13 min, glucoside structures of NDGA and p-coumaric acid were not analyzed by NMR and represent the most probable glucosides.

Table 1: Analysis of alternan- and mutansucrase transformations in comparison to glucansucrases from *Leuconostoc* and *Weissella* strains (data taken from [17,18]), nd = not detected

	Mutansucrase	**DSM 5577**	**DSM 20913**	**Alternansucrase**	**DSM 22752**
Caffeic acid	92	66	28	52	32
(4-O- : 3-O-)	99 : 1	99 : 1	99 : 1	40 : 60	50 : 50
NDGA	81	20	79	36	< 5
Umbellic acid	nd	1	nd	11	5
Coumaric acid	nd	1	nd	4	1

Conclusions

Alternan- and mutansucrase are efficient glucosylation biocatalysts. Mutansucrase is especially suited for the high yield transformation of catecholic substrates, while alternansucrase allows for the glucosylation of a broader spectrum of phenolic compounds. Reaction engineering was not yet done and according to our optimization studies done with *Leuconostoc* glucansucrases [17,18] further increase of glucosylation yields should be possible with both enzymes.

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The authors have declared no conflict of interest.

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