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Glycosylation of caffeic acid and structural analogues catalyzed by novel glucansucrases from *Leuconostoc* and *Weissella* species

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ABSTRACT

Twelve *Leuconostoc* and seven *Weissella* strains with extracellular glucansucrase activity were obtained from an analysis of 41 lactic acid bacteria. Culture supernatants of all glucansucrase positive strains catalyzed the glycosylation of caffeic acid with sucrose as donor substrate. Eighteen enzymes produced one major peak, which was identified as caffeic acid-4′-*O*-α-D-monoglucoside by LC-MS and NMR spectroscopy. Only *W. beninensis* DSM 22752 formed significant amounts of the corresponding 3´-O-α-D-monoglucoside. The *Weissella* strain and five *Leuconostoc* strains with high glycosylation activity were selected for further studies. All glucansucrases catalyzed the glycosylation of the catechol protocatechuic acid, a side-chain truncated analogue of caffeic acid. The *Leuconostoc* enzymes displayed a preference for the 4′-*O*-α-D isomer, while the DSM 22752 glucansucrase also produced the protocatechuic acid-3′-*O*-α-D-monoglucoside. Lower activities with non-catecholic caffeic acid derivatives and no activity with mono-methylated caffeic acid were observed with all glucansucrases. Time-course analyses confirmed that glucansucrase from *L. citreum* DSM 5577 was the most efficient biocatalyst for catechol glucosylation with yields of up to 74% caffeic acid glucosides after 24 h. The enzyme displayed a high regio-preference for the 4′-*O*-α-D-isomer and formed less than 10% oligoglucosides. Gel electrophoretic analysis and activity staining of the PEG-enriched enzyme showed a single protein band with a molecular mass of 171 kDa. The DSM 5577 glucansucrase was tolerant against the co-solvents dimethyl sulfoxide and ethanol. Kinetic analysis revealed a K_M of 27.6 mM for caffeic acid and 31 mM for sucrose with k_{cat} values of 131 s^{-1} and 438 s^{-1}.

1. Introduction

Natural polyphenols are widely distributed secondary plant metabolites with attributed health effects and applications in dietary supplements, cosmetics and pharmaceuticals (Nadim et al., 2014; Quideau et al., 2011; Sauer and Plauth, 2017). Covalent attachment of a glycosyl-residue protects polyphenols from oxidation, increases water solubility and can lead to enhanced bioavailability (Desmet et al., 2012; Křen, 2008; Nadim et al., 2014; Thuan and Sohng, 2013; Xu et al., 2016). Chemical glycosylation of polyphenols, requiring extensive protective group chemistry, needs greener alternatives (Desmet et al., 2012; Roode et al., 2003). Glucansucrases of glycoside hydrolase family 70 (Lombard et al., 2014), naturally catalyzing extracellular polysaccharide formation in lactic acid bacteria, are promising glycosylation catalysts. They are secreted to the outer medium (Meng et al., 2016; van Hijum et al., 2006), from which they can be isolated and purified (Majumder et al., 2007). Glucansucrases use sucrose as cost efficient donor substrate in a one-step transglycosylation and possess sufficient process stability in the presence of solvents (Andre et al., 2010; Girard and Legoy, 1999; Meng et al., 2016).

Glucansucrase catalyzed glycosylation of non-physiological polyphenols was originally shown with the flavanol (+)-catechin by Nakahara et al. (1995). Other successfully glycosylated catechols include epigallocatechin gallate (Kim et al., 2016), L-DOPA (Yoon et al., 2010) and caffeic acid (Auriol et al., 2012). The glucoside of caffeic acid is marketed as a cosmetic bioactive with antioxidant properties under the brand name Inoveol® CAFA (Induchem). Also a few non-catecholic phenols like resveratrol (Shim et al., 2003), hydroquinone (Seo et al., 2009) and salicyl alcohol (Seo et al., 2005) were glycosylated by glucansucrases, though with comparably lower activity.

The two aerotolerant genera *Leuconostoc* and *Weissella* were shown to be rich sources of glucansucrases (Bounaix et al., 2009), however; studies concerning polyphenol glycosylation are currently limited to enzymes from a few *Streptococcus* species, *Leuconostoc mesenteroides* and *Lactobacillus reuteri*. Glucansucrases from the species *Weissella* have not been analyzed for polyphenol acceptor specificity, so far. This study

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presents the isolation and characterization of novel glucansucrases from *Leuconostoc* and *Weissella* and the evaluation of their glycosylation potential with caffeic acid and structurally related polyphenol acceptors.

2. Materials and methods

2.1. Bacterial strains and chemicals

Forty-one *Leuconostoc* and *Weissella* strains obtained from DSMZ, USDA, Agroscope, Sacco S.r.l., Ernst Böcker GmbH and Moguntia Food Group AG were used in this study (Table 1). All strains were maintained at −80 °C as 20% (v/v) glycerol stocks obtained from single colonies and propagated in De Man, Rogosa and Sharpe (MRS) medium containing 2% (w/v) glucose at 30 °C. Caffeic acid, 4-coumaric acid and umbellic acid were purchased from Sigma Aldrich. Protocatechuic acid was from Abcr Gmbh, ferulic acid from Acros organics and resveratrol was obtained from Carl Roth.

2.2. Strain cultivation and selection

Bacterial cultures from glycerol stocks were grown for 15 h in 2 mL pre-culture containing MRS media with 2% (w/v) glucose at 30 °C, 288 rpm in 48 well round plates covered with an air permeable sealing tape in an 80% humidified atmosphere using a BioLector® micro bioreactor system. For cultivation of *Weissella* strains, the medium was supplemented with 0.1% (w/v) cysteine hydrochloride. Cells from the pre-cultures were centrifuged (4 °C, 15 min at 2800 × *g*), washed once with sodium acetate buffer, pH 5.4, and suspended in 2 mL main culture media (MRS) supplemented with 2% (w/v) sucrose to a final $OD_{(600\ nm)}$ of 0.25. Main cultures were incubated in the Biolector® system at 25 °C and 288 rpm for 48 h. Samples of 250 µL were taken periodically, the cells were removed by centrifugation and the supernatants were used for the determination of glucansucrase activity and caffeic acid glycosylation.

Table 1
List of strains and analysis of glucansucrase activities in supernatants.

Microorganism	Designation, supplier and glucansucrase activity
L. citreum	[1]DSM 20188 (++), [1]DSM 5577 (++), [6]50018 (−)
L. fallax	[1]DSM 20189 (◯)
L. gellidum subsp. *gellidum*	[1]DSM 5578 (−)
L. kimchi	[2]B-65330 (−), [2]B-65337 (++)
L. lactis	[4]LN19 (−), [4]LN24 (−), [3]FAM 22733 (−)
L. mesenteroides	[4]LN34 (−), [4]LN27 (+), [4]LN32 (−), [4]LN07 (−), [3]FAM 22488 (+)
L. mesenteroides subsp. *cremoris*	[1]DSM 20346 (−)
L. mesenteroides subsp. *dextranicum*	[1]DSM 46216 (◯), [1]DSM 20484 (−), [1]DSM 20187 (−)
L. mesenteroides subsp. *suionicum*	[1]DSM 20241 (++)
L. pseudomesenteroides	[1]DSM 20193 (++), [4]LN23 (◯), [2]B-65331 (++), [2]B-65333 (−), [2]B-65334 (◯)
W. beninensis	[1]DSM 22752 (◯)
W. cibaria	[1]DSM 15878 (◯), [1]DSM 14295 (−)
W. confusa	[1]DSM 20196 (−), [5]BSM 1107 (◯), [5]BSM 1193 (+), [5]BSM 1390 (+), [4]WS01 (+), [4]WS02 (−), [3]FAM 22479 (−)
W. fabaria	[1]DSM 21416 (◯)
W. ghanensis	[1]DSM 19935 (−)
W. kandleri	[1]DSM 20593 (−)
W. paramesenteroides	[3]FAM 18796 (−)
W. thailandensis	[3]FAM18792 (−)
W. viridescens	[2]B-1951 (−)

Supernatant activities: = < 0.1 U mL^{-1}, ◯ = > 0.1 U mL^{-1}, + = > 0.3 U mL^{-1}, ++ = > 0.6 U mL^{-1}; Strains were obtained from [1] DSMZ, [2] ARS culture collection (USDA), [3] Agroscope, [4] Sacco S.r.L, [5] Ernst Böcker GmbH and [6] Moguntia Food Group AG.

2.3. Isolation and concentration of glucansucrases

Pre-cultures of selected strains were grown in a volume of 15 mL and main cultures in a volume of 200 mL in shake flasks at 100 rpm essentially according to the Biolector® micro-scale method described in 2.2. After harvesting of the main cultures glucansucrases were isolated according to the method of Paul et al. (1986). An aqueous (50% w/v) polyethylene glycol 4000 solution was added under continuous stirring to the supernatants to reach a final concentration of 25% (w/v). The mixtures were centrifuged at 2800 × *g*, 4 °C for 15 min and the precipitates containing glucan and glucansucrase were suspended in 20 mM sodium acetate, pH 5.4. The PEG precipitation was repeated twice and the final precipitates were dissolved in 20 mM sodium acetate, 0.45 mM $CaCl_2$, pH 5.4 containing protease inhibitor cocktail (EDTA-free, Roche) and stored at 4 °C.

2.4. Biocatalytical glycosylation and glycoside purification

Glycosylation mixtures contained 200 mM sucrose, 10 or 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 glucansucrase-containing supernatants or concentrated glucansucrases (to a final activity of 0.33 U mL^{-1} or 1.0 U mL^{-1}) and incubated statically for up to 24 h 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 3300 × *g* for 20 min at 4 °C according to the method of Overwin et al. (2015).

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 equipped with a Kromasil 100 C18 column (20 × 250 mm, 5 µm) and a PDA detector. The eluents were water (eluent A) and acetonitrile (eluent B) each supplemented with 0.2% (v/v) formic acid. The flow-rate was 8 mL min^{-1}. Caffeic acid and umbellic acid glycosides were purified in a water/acetonitrile gradient and protocatechuic acid glycosides were purified isocratically with 90% eluent A. Glycoside containing fractions were pooled and lyophilized.

2.5. Determination of 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 according to Miller (1959). Glucansucrase preparations were diluted to an appropriate concentration and incubated in 20 mM sodium acetate buffer (pH 5.4) containing 292 mM sucrose and 0.45 mM $CaCl_2$ for up to 120 min at 30 °C (Dols et al., 1997). Samples were drawn at t = 0, 15, 30, 60 and 120 min and stopped by addition of an equal amount of DNS reagent. Samples were incubated at 100 °C for exactly 5 min and cooled down prior to dilution with 2 vol of H_2O. Glucansucrase activities were determined at least in triplicate. Calibration of the plate reader with the DNS-solution was done with fructose and the linear correlation of absorbance to fructose concentration was determined. One unit of glucansucrase activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of reducing monosaccharide from sucrose per min at 30 °C.

2.6. Biochemical methods and kinetic analysis

Protein concentration was determined as described previously by Bradford (1976). Analysis of purified glucansucrase was analyzed by SDS PAGE (7% w/v acrylamide) by the method of Laemmli (1970) and stained with Coomassie Brilliant Blue G-250. *In situ* periodic acid schiff activity staining of glucan on SDS gel was done according to the method of Miller and Robyt (1986). Protein mass was calculated by comparison

with prestained protein ladder (Thermo Scientific) using the software Image Lab (Biorad). Kinetic parameters were analyzed for sucrose and caffeic acid by determining initial reaction rates at different substrate concentration. Physiological glucansucrase activity in dependence of sucrose concentration was analyzed by the release of fructose according to chapter 2.7 and glucosylation of caffeic acid was done according to the method of chapter 2.4 with quantification by LC-UV according to chapter 2.8. Kinetic constants were obtained by non-linear regression using the software Graphpad Prism 6.

2.7. Carbohydrate analysis

Sucrose, glucose and fructose were analyzed spectrophotometrically with an enzymatic kit from R-Biopharm GmbH at 340 nm according to the manufacturers protocol scaled down to microplate format as described in Vermeir et al. (2007). Glucose-6-phosphate was quantified by the NAD^+-dependent oxidation to 6-phospho-gluconate catalyzed by glucose-6-phosphate dehydrogenase. In consecutive reaction steps sucrose was hydrolyzed to glucose and fructose with β-fructosidase, the monosaccharides were phosphorylated with hexokinase and fructose-6-phosphate was isomerized to glucose-6-phosphate with phosphoglucose isomerase.

2.8. LC-UV and LC-MS analysis of polyphenol glycosides

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 × 250 mm, 5 µm) using water (eluent A) and acetonitrile (eluent B) each supplemented with 0.1% (v/v) formic acid as eluents. Glycoside samples were separated at a constant flow rate of 1 mL min^{-1} and 30 °C in a gradient to 100% eluent B with a linear increase of 2.1% min^{-1}. Detection of phenols and corresponding glycosides was done at 280 nm except for protocatechuic acid, which was analyzed at 254 nm. The substrates were stable under conditions of glucansucrase transformation. According to Meulenbeld et al. (1999) the molar absorption coefficients of the aromatic ring systems were used for quantification of substrates and corresponding glycosides which was verified by calibration of the LC-UV with caffeic acid and caffeic acid-4′-*O*-α-D-glucoside. 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 a Hitachi LaChrom II C18 reversed phase column (4.6 × 250 mm, 5 µm). The mass spectrometer was run in positive and negative scan mode in the m/z range from 150 to 1000 at 10,000 u sec^{-1} and an event time of 0.1 s. In parallel, the mass spectrometer was run in SIM mode for selective detection of m/z values for reactants and products. Time program and eluents were the same as in LC-UV analysis with the Accela system.

2.9. Structure elucidation with NMR spectroscopy

Purified glycosides were suspended in deuterated methanol or deuterated DMSO. NMR spectra were recorded with a 400 MHz Bruker Ascend™ 400. Data reported were referenced to residual protons in methanol at 3.31 ppm or DMSO at 2.50 ppm (Gottlieb et al., 1997). Besides ^{1}H- und ^{13}C NMR analysis, 2D-NMR experiments were performed for structural analysis. Homonuclear bond correlation was elucidated with COSY experiments, heteronuclear bond correlation with HSQC and HMBC experiments and through space correlation with NOESY experiments.

3. Results & discussion

3.1. Identification of Leuconostoc and Weissella culture supernatants for caffeic acid glycosylation

Forty-one *Leuconostoc* and *Weissella* strains were analyzed for glucansucrase secretion in a micro-cultivation screening. Both genera secreted glucansucrases with 12 out of 25 *Leuconostoc* and 7 out of 16 *Weissella* strains exceeding activities of 0.10 U mL^{-1} (Table 1). Culture supernatants of the glucansucrase positive strains were tested for caffeic acid glycosylation. After 24 h of incubation in the presence of 40 mM caffeic acid, 200 mM sucrose and 15% co-solvent DMSO, samples were analyzed by LC-UV for caffeic acid glucoside quantification and LC-MS for assignment of glycoside species. Two distinct monoglucoside peaks with mass assignments of m/z = 341 as well as multiple glucosylated caffeic acid species were detected (Fig. 1A). Formation of oligoglucosides correlated to glycosylation yield and significant amounts were only detected at monoglucoside concentrations exceeding 30% (Fig. 1B).

The caffeic acid monoglucosides were purified by preparative HPLC for NMR based structure elucidation (Table 2). The isomers exhibited a

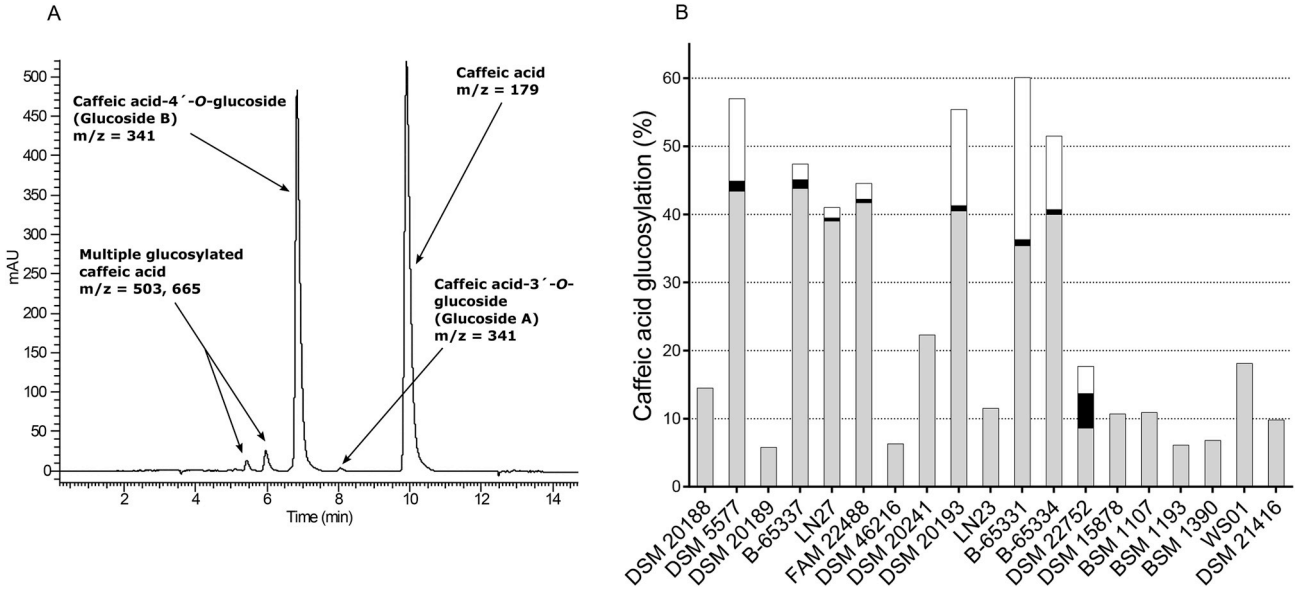
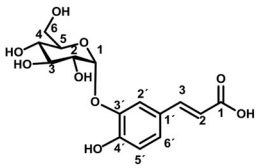
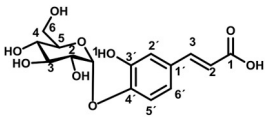


Fig. 1. A) LC-UV example chromatogram of reaction products from caffeic acid conversion by DSM 5577 glucansucrase with peak assignment according to LC-MS and NMR analysis; **B)** Caffeic acid glucoside distribution of all glucansucrase positive strains after 24 h incubation of c with 40 mM caffeic acid, gray bar = 4′-*O*-α-D-monoglucoside, black bar = 3′-*O*-α-D-monoglucoside, white bar = oligoglucosides.

Table 2
^{1}H NMR und ^{13}C NMR data of caffeic acid glucosides A and B in comparison to their aglycon.

	Caffeic acid		Glucoside A		Glucoside B	
						
	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)
2	115.47	6.22 (1H, d, *J* = 15.9 Hz)	116.44	6.31 (1H, d, *J* = 15.9 Hz)	118.16	6.32 (1H, d, *J* = 15.9 Hz)
3	147.04	7.53 (1H, d, *J* = 15.9 Hz)	146.50	7.58 (1H, d, *J* = 15.9 Hz)	145.86	7.54 (1H, d, *J* = 15.9 Hz)
1′	127.75		128.00		131.25	
2′	115.04	7.04 (1H, d, *J* = 2.1 Hz)	118.51	**7.55 (1H, d, J = 2.0 Hz)**	115.98	7.10 (1H, d, *J* = 2.2 Hz)
3′	146.77		146.76		148.91	
4′	149.44		151.38		148.73	
5′	116.46	6.78 (1H, d, *J* = 8.2 Hz)	117.54	6.87 (1H, d, *J* = 8.3 Hz)	118.49	**7.29 (1H, d, J = 8.4 Hz)**
6′	122.85	6.93 (1H, dd, *J* = 8.2, 2.1 Hz)	125.85	7.19 (1H, dd, *J* = 8.4, 2.1 Hz)	122.00	7.03 (1H, dd, *J* = 8.4, 2.2 Hz)
Glucose						
1			101.48	5.37 (1H d, *J* = 3.7 Hz)	100.82	5.42 (1H, d, *J* = 3.7 Hz)
2			73.39	3.61 (1H, dd, *J* = 9.8, 3.7 Hz)	73.38	3.60 (1H, dd, *J* = 9.7, 3.7 Hz)
3			74.79	3.88 (1H, t, *J* = 9.4 Hz)	74.79	3.88 (1H, t, *J* = 9.3 Hz)
4			71.42	3.42 (1H, t, *J* = 9.3 Hz)	71.30	3.43 (1H, t, *J* = 9.4 Hz)
5			74.67	3.83–3.69 (1H, m)	74.68	3.82–3.65 (1H, m)
6			62.40	3.83–3.69 (2H, m)	62.30	3.82–3.65 (2H, m)
NOESY			glucose H1 couples with caffeic acid H2′		glucose H1 couples with caffeic acid H5′	

small coupling constant of 3.7 Hz for the anomeric glucose proton implying an α-configuration. Downshifts of the 2′-proton of glucoside A pointed towards a glucose linkage at position 3′ and downshift of the 5′-proton of glucoside B revealed glucose binding at the 4′-position. In agreement, coupling of the anomeric glucose proton with the 2′-proton of caffeic acid was observed in NOESY experiments with glucoside A and coupling of the anomeric H1 with the 5′-proton for glucoside B. Thus the isomers were confirmed as caffeic acid-3′-*O*-α-D-glucoside and the corresponding caffeic acid-4′-*O*-α-D-glucoside. NMR data were in good agreement with the results of Nishimura et al. (1995), who synthesized both α-D-glucosides with α-amylase.

Astonishingly all glucansucrase positive strains catalyzed the glycosylation of caffeic acid demonstrating the widespread ability for the promiscuous acceptor reaction (Fig. 1B), which is shown here for the first time with glucansucrases from the genus *Weissella*. Caffeic acid-4′-*O*-α-D-glucoside was the major product, which is in good agreement with previous results pointing to a glucansucrase preference for the catecholic hydroxyl group in para-position (Meulenbeld and Hartmans, 2000). In this context it was shown that the 4′-*O*-α-D-glucoside has a remarkably 35-fold better water solubility than the corresponding caffeic acid-3′-*O*-α-D-glucoside (Nishimura et al., 1995). Only culture supernatants of *W. beninensis* DSM 22752 produced significant amounts of the corresponding 3′-*O*-α-D-glucoside in our screening. So far only one *L. mesenteroides* dextransucrase was reported to possess a preference for the caffeic acid-3′-*O*-α-D-glucoside (Nam et al., 2017).

3.2. Glycosylation of caffeic acid analogues with selected glucansucrases

Five *Leuconostoc* strains with high caffeic acid glycosylation activity (DSM 5577, B-65337, DSM 20193, B-65331 and B-65334) were selected exemplarily for further studies. In addition, *W. beninensis* DSM 22752 was chosen due to its divergent regioselectivity. With an aqueous two-phase separation glucansucrase activities were successfully concentrated by factors of 6.4 for *L. pseudomesenteroides* B-65334 to over 37 for *W. beninensis* DSM 22752 with isolated yields of at least 40% for all glucansucrases. Substrate specificities of the PEG fractionated glucansucrases were comparatively analyzed with structural analogues of caffeic acid (Table 3A) comprising side-chain truncated protocatechuic

Table 3
A) Structural comparison of caffeic acid derivates; **B)** Analysis of polyphenol glucosylation, molar yields are given in % glucosides formed (sum of all monoglucoside and oligoglucoside species) within 9 h at an enzyme dosage of 0.33 U mL^{-1} for caffeic acid and 1.0 U mL^{-1} for all other substrates at 10 mM substrate concentration (nd = no glucoside detected).

A)	C1′	C2′	C3′	C4′	C5′	C6′
Caffeic acid	-C=C-COOH	-H	-OH	-OH	-H	-H
Protocatechuic acid	**-COOH**	-H	-OH	-OH	-H	-H
Ferulic acid	-C=C-COOH	-H	**-O-CH3**	-OH	-H	-H
Umbellic acid	-C=C-COOH	**-OH**	**-H**	-OH	-H	-H
p-Coumaric acid	-C=C-COOH	-H	**-H**	-OH	-H	-H
Resveratrol	**-C=C-Phe-(p)-OH**	-H	-OH	**-H**	**-OH**	-H

B)	DSM 5577	B-65337	DSM 20193	B-65331	B-65334	DSM 22752
Caffeic acid *(ratio 4′-O-: 3′-O-)*	66% (*99:1*)	20% (*97:3*)	28% (*99:1*)	29% (*99:1*)	42% (*99:1*)	32% (*50:50*)
Protocatechuic acid *(ratio 4′-O-: 3′-O-)*	54% (*92:8*)	10% (*93:7*)	9% (*99:1*)	16% (*93:7*)	12% (*99:1*)	34% (*23:77*)
Ferulic acid	nd	nd	nd	nd	nd	nd
Umbellic acid	1%	< 1%	nd	nd	nd	5%
4-Coumaric acid	1%	nd	nd	nd	nd	1%
Resveratrol	< 1%	2%	< 1%	1%	< 1%	< 1%

acid, 3′-*O*-methylated ferulic acid and non-catecholic derivatives.

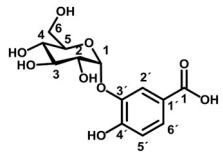
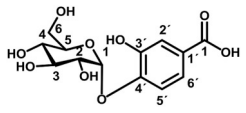
Beside caffeic acid being the best glucansucrase acceptor in this screening, high substrate conversions were obtained with protocatechuic acid. All glucansucrases accepted protocatechuic acid and two monoglucosides as well as oligoglucosides were detected. Glucansucrase of L. *citreum* DSM 5577 glucansucrase was the most efficient biocatalyst for caffeic acid and protocatechuic acid glucosylation with yields of 66% and 54% after 9 h, illustrating its high affinity towards aromatic catechols.

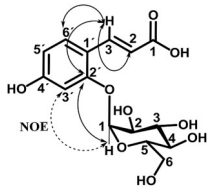
The protocatechuic monoglucosides were purified and structures were analyzed by NMR (Table 4A). Downshift and NOE patterns were in agreement with data of the caffeic acid glucosides. The glucose proton 1 (5.28 ppm) showed a coupling to proton 2′ (7.72 ppm) in glucoside C and a coupling to proton 5′ (7.34 ppm) in glucoside D confirming glucoside C as protocatechuic acid-3′-*O*-α-D-glucoside and glucoside D as protocatechuic acid-4′-*O*-α-D-glucoside. Both glucosides have not been prepared before with glucansucrases. Again, all *Leuconostoc* glucansucrases displayed a regio-preference for the 4′-hydroxy position as shown for caffeic acid. The regioselectivity, however, was lower with protocatechuic acid than with caffeic acid, which may be attributed to higher freedom of orientation in the active site due to the smaller side chain in protocatechuic acid. The *W. beninensis* DSM 22752 glucansucrase showed a different regio-preference and produced the 3′-*O*-monoglucoside in a 77:23 excess.

In contrast to caffeic and protocatechuic acid, polyphenol glycosylation activity was completely suppressed upon methylation of the catecholic 3′-hydroxy position in ferulic acid (Table 3B). No activity was observed with any glucansucrase though the preferred 4′-hydroxy position is accessible. Steric hindrance seems to be responsible and in accordance glycosylation of both catecholic hydroxyl groups has not been observed with glucansucrases.

Non-catecholic caffeic acid derivatives umbellic acid, *p*-coumaric acid and resveratrol were partially accepted and transformed with significantly lower yields (Table 3B). Glucansucrase of *W. beninensis* DSM 22752 was the only biocatalyst converting umbellic acid to its corresponding glucoside in significant amounts. The monoglucoside was isolated for structure elucidation (Table 4B). The coupling constant of 3.6 Hz verified an α-configuration of the glucose. The downfield shift of 0.34 ppm in deuterated DMSO for proton 3′ and coupling of the anomeric proton of glucose with proton 3′ could be assigned to glucosylation either at position 2′ or 4’. Thus an unambiguous assignment of the similar carbons C2′ and C4′ was needed for structure elucidation. In HMBC experiments a 3-bond coupling of umbellic acid proton 3 with carbon C2′ was observed, but no interference with carbon C4′ in a 5-bond distance could be detected. Additionally coupling of the anomeric glucose proton to C2’ was observed in NOE experiments. Therefore the structure of glucoside E was assigned to umbellic acid-2′-*O*-α-D-glucoside, a result confirming the unique regioselectivity of DSM 22752

Table 4
^{1}H and ^{13}C NMR data of **A)** protocatechuic acid glucosides and **B)** umbellic acid glucoside in comparison to their aglycons.

A)	Protocatechuic acid		Glucoside C		Glucoside D	
						
	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)
1	167.4	–	167.1	–	169.8	–
1‘	122.0	–	122.2	–	126.8	–
2‘	117.0	7.33 (d, *J* = 2.1 Hz, 1H)	118.4	**7.72 (d, J = 2.0 Hz, 1H)**	118.1	7.49 (d, *J* = 2.0 Hz, 1H)
3‘	145.3	–	144.7	–	148.1	–
4‘	150.4	–	151.8	–	150.2	–
5‘	115.6	6.78 (d, *J* = 8.2 Hz, 1H)	115.6	6.88 (d, *J* = 8.3 Hz, 1H)	117.2	**7.34 (d, J = 8.3 Hz, 1H)**
6‘	122.4	7.28 (dd, *J* = 8.2, 2.1 Hz, 1H)	125.3	7.52 (dd, *J* = 8.3, 2.0 Hz, 1H)	123.0	7.51 (dd, *J* = 8.2, 2.0 Hz, 1H)
Glucose			Signals corresponding to caffeic acid glucoside		Signals corresponding to caffeic acid glucoside	
NOESY			Glucose H1 couples with protocatechuic acid H2′		Glucose H1 couples with protocatechuic acid H5′	

B)	Umbellic acid		Glucoside E	
				
	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)	$^{\delta}$C (ppm)	$^{\delta}$H (ppm)
1	168.6	11.91 (s, 1H)	168.2	12.00 (s, 1H) (br)
2	114.1	6.28 (d, *J* = 16.0 Hz, 1H)	116.4	6.34 (d, *J* = 16.0 Hz, 1H)
3	140.0	7.71 (d, *J* = 16.0 Hz, 1H)	138.9	7.82 (d, *J* = 16.0 Hz, 1H)
1‘	112.7	–	114.7	–
2‘	158.3	9.82 (s, 1H)	157.1	–
3‘	102.5	6.34 (d, *J* = 2.3 Hz, 1H)	102.4	**6.68 (d, J = 2.3 Hz, 1H)**
4‘	160.7	10.07 (s, 1H)	160.7	10.05 (s, 1H)
5‘	107.8	6.26 (dd, *J* = 8.5, 2.3 Hz, 1H)	109.9	6.45 (dd, *J* = 8.5, 2.3 Hz, 1H)
6‘	130.2	7.37 (d, *J* = 8.5 Hz, 1H)	129.3	7.51 (d, *J* = 8.6 Hz, 1H)
Glucose			Signals corresponding to caffeic acid glucoside	
NOESY			Glucose H1 couples with umbellic acid H3′	

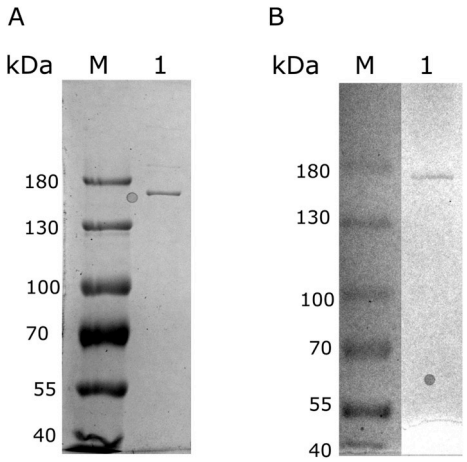


Fig. 2. SDS-PAGE analysis of *L. citreum* DSM 5577 glucansucrase (2 µg) after **A)** Coomassie staining and **B)** activity staining with periodic acid Schiff reagent for visualization of *in situ*-synthesized glucan.

glucansucrase.

All glucansucrases catalyzed the glycosylation of resveratrol to some extent (Table 3B). Glucosides of resveratrol were proposed as useful prodrugs with increased bioavailability in this context (Falomir et al., 2016). Conversion of resveratrol could be increased from 2% to 8% with *L. kimchi* B-65337 glucansucrase by increasing the resveratrol concentration to 40 mM. Thus glucansucrase of B-65337 is an interesting target for further optimization.

3.3. Biochemical characterization of L. citreum DSM 5577 glucansucrase

Glucansucrase of L. *citreum* DSM 5577, displaying highest turnover with the catechols caffeic acid and protocatechuic acid, was characterized biochemically in more detail. After PEG fractionation of the supernatants a protein concentration of 35.4 ± 4.6 µg mL^{-1} was obtained. SDS-PAGE revealed that the enzyme preparation was essentially free of contaminating proteins (Fig. 2A). A defined protein band with a molecular mass of 171 kDa was verified by activity staining (Fig. 2B) matching data for glucansucrase enzymes with 160 kDa on average (van Hijum et al., 2006).

Caffeic acid glucosylation by DSM 5577 glucansucrase was analyzed in a time-course experiment at 10 mM acceptor concentration supplying 200 mM co-substrate sucrose. Transformation with 0.33 U mL^{-1} of enzyme was monitored over a period of 24 h (Fig. 3A). Release of glucose was not observed; therefore sucrose hydrolysis can be neglected. The clearing of sucrose was faster than the formation of caffeic acid glucosides caused by simultaneous glucan formation. Regioselectivity towards the caffeic acid-4′-*O*-α-D-glucoside remained high over the course of the experiment. The total amount of glucosides increased until 24 h yielding 74% total conversion whereas the 4′-*O*-monoglucoside reached its maximum after 9 h. The formation of oligoglucosides caused a slow decrease of monoglucoside content afterwards.

The K_M-value for caffeic acid was determined with a surplus of

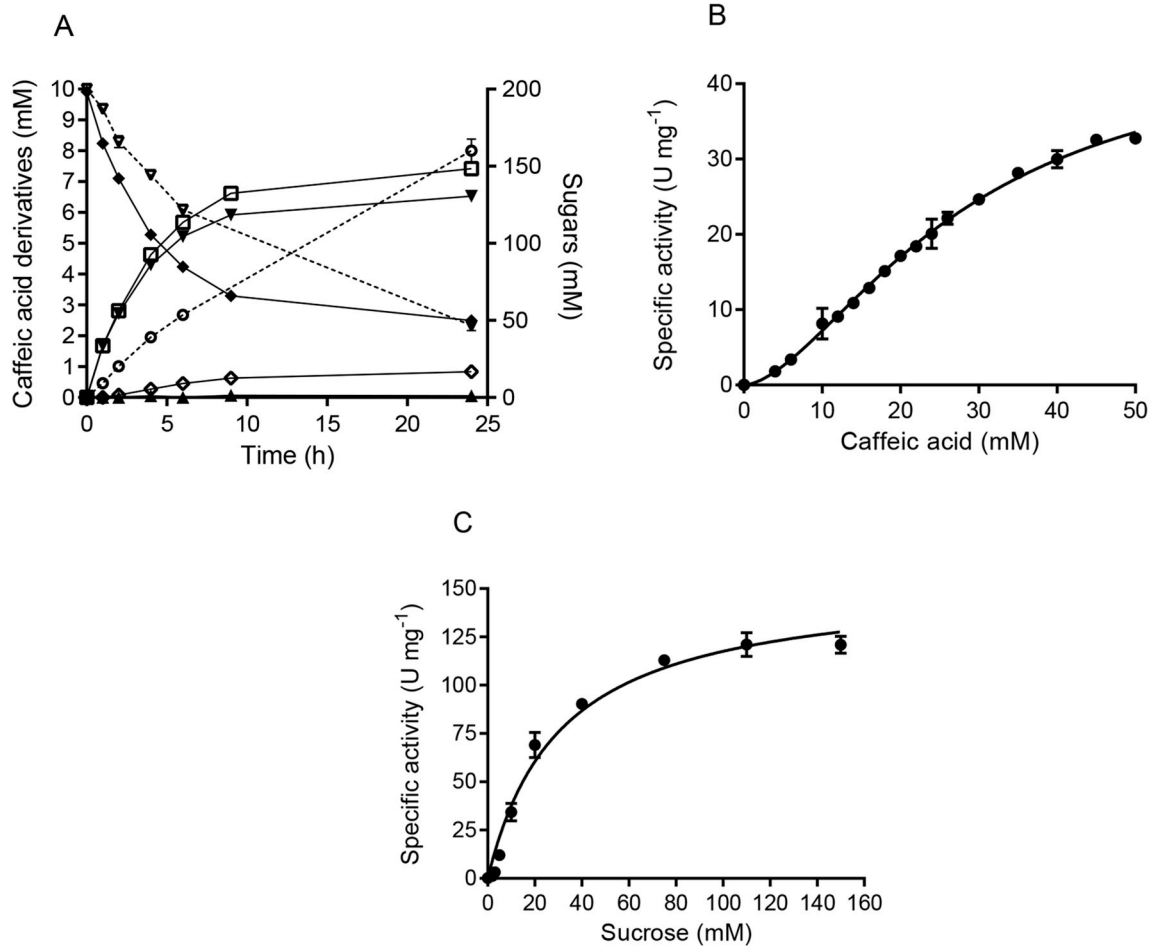


Fig. 3. A) Time-course analysis of caffeic acid glucosylation with glucansucrase from *L. citreum* DSM 5577 with ◆ = caffeic acid, ▲ = caffeic acid-3′-*O*-α-D-glucoside, ▼ = caffeic acid-4′-*O*-α-D-glucoside, ◇ = sum of multiple glucosylated caffeic acid; □ = caffeic acid glucosides in total, ◯ = fructose and ▽ = sucrose; **B + C)** Determination of kinetic constants of *L. citreum* DSM 5577 glucansucrase for acceptor substrate caffeic acid and donor substrate sucrose.

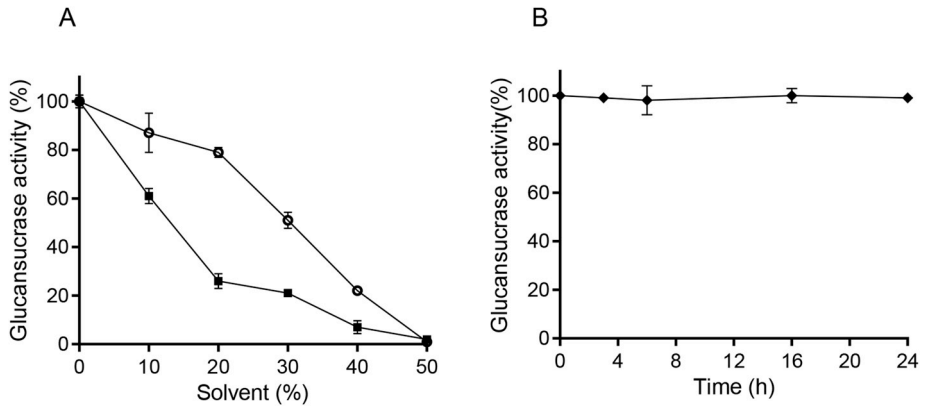


Fig. 4. A) Activity profile of *L. citreum* DSM 5577 glucansucrase in DMSO (circles) and ethanol (black squares), **B)** Stability of L. *citreum* DSM 5577 glucansucrase in 15% (v/v) DMSO at 30 °C.

200 mM sucrose allowing for a first-order approximation. An apparent K_M of 27.6 mM and a v_{max} of 46.1 U mg^{-1} were obtained (Fig. 3B). The sigmoidal kinetic curve indicates a complex interaction of the enzyme with the artificial acceptor substrate caffeic acid and sucrose, which in parallel serves as the substrate for glucan formation. Sucrose conversion into glucan by DSM 5577 glucansucrase followed a Michaelis-Menten-type kinetic behavior (Fig. 3C) and the deduced K_M- and v_{max}-values were in the same order of magnitude as determined for *L. mesenteroides* B-512 glucansucrase before (Arguello-Morales et al., 2005). Based on the molecular weight of 171 kDa, turnover frequencies of 131 s^{-1} for caffeic acid and 438 s^{-1} for sucrose were calculated. Hence, glucansucrase of *L. citreum* DSM 5577 almost reached ⅓ of the turnover frequency of the natural donor sucrose with the non-physiological acceptor caffeic acid.

The glycosylation of hydrophobic natural products needs cosolvents as substrate solubilizer. Therefore the activity and stability of *L. citreum* DSM 5577 glucansucrase was analyzed in different polar solvents. Low residual activities of < 5% were observed in 15% ethyl acetate and tetrahydrofuran. Acetonitril served as a slightly better solvent with 24% residual activity at a concentration of 15%. Sufficient residual activities in DMSO and ethanol were obtained up to concentrations of 20% in aqueous buffer with a comparative lower activity in ethanol (Fig. 4A). The glucansucrase fully retained its activity in 15% DMSO during storage at 30 °C for 24 h (Fig. 4B).

4. Conclusions

Novel glucansucrases from *Leuconostoc* and *Weissella* were isolated and evaluated for their ability to glycosylate the antioxidant caffeic acid and structural analogues. Surprisingly, all glucansucrases accepted the non-physiological acceptor substrate, which was shown with *Weissella* enzymes for the first time. Most enzymes displayed a high regio-preference for the caffeic acid-4′-*O*-α-D-glucoside. *L. citreum* DSM 5577 glucansucrase exhibited the highest glucoside yields with catechols. The high total turnover of > 70% with a v_{max} of 46.1 U mg^{-1} for caffeic acid and sufficient solvent tolerance make this enzyme a candidate for technical applications. Additionally, interesting glucansucrase activities were identified whose characterization might be of interest for further studies: *W. beninensis* DSM 22752 possessed a divergent regioselectivity with catechols and synthesized umbellic acid-2′-*O*-α-D-glucoside and *L. kimchi* B-65337 glucansucrase glycosylated the non-catecholic bioactive resveratrol.

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