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Enzymatic analysis of levan produced by lactic acid bacteria in fermented doughs

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ABSTRACT

Levans and inulins are fructans with mainly β-(2→6) and β-(2→1) linkages, respectively. Levans are produced by many lactic acid bacteria, e.g. during sourdough fermentation. Levans have shown prebiotic properties and may also function as *in situ*-produced hydrocolloids. So far, levan contents have been measured by acid hydrolysis, which cannot distinguish levans from e.g. inulins. In order to develop a specific analysis for levan in food matrices, a *Paenibacillus amylolyticus* endolevanase was combined with exoinulinase for levan hydrolysis. A separate endoinulinase treatment was used to detect the possible presence of inulin. Interfering sugars were removed by a pre-wash with aqueous ethanol. Levan content was estimated from fructose and glucose released in the hydrolysis, with a correction made for the residual fructose and glucose-containing sugars. The method was validated using wheat model doughs spiked with commercial *Erwinia* levan, and tested by analyzing levan content in *Leuconostoc mesenteroides* DSM 20343-fermented fava bean doughs.

1. Introduction

Fructans are polysaccharides produced from sucrose by some plants and microorganisms (Öner, Hernández, & Combie, 2016). The two most common structural forms of fructans are levans and inulins, with mainly β-(2→6) and β-(2→1)-linked fructofuranosyl residues, respectively. Both carry a terminal sucrose moiety, and can be β-(2→1) branched in the case of levans and β-(2→6) branched in the case of inulins. There are other types of fructans in plants, namely graminans with mixed β-(2→6) and β-(2→1)-linkages, and neo-inulins and neo-levans, both containing an internal glucose moiety instead of a terminal one (Van den Ende, 2013). In bacteria, levans and inulins are respectively synthesized by levansucrases and inulosucrases. The gene encoding levansucrase is widely distributed in both gram-positive and gram-negative bacteria, whereas the gene encoding inulosucrase has only been found in a limited number of gram-positive bacteria (Öner et al., 2016). Bacterial fructans have much higher molecular weights (at least 10^3 fructosyl units) than plant fructans (normally less than 50 fructosyl units) (Vijn & Smeekens, 1999).

Fructans can be produced by various lactic acid bacteria (LAB) belonging to genera *Leuconostoc, Lactobacillus*, *Streptococcus, Weissella*, etc., with most of them harboring solely levansucrase and only a few possessing inulosucrase (Anwar et al., 2010; Anwar, Kralj, van der Maarel, & Dijkhuizen, 2008; Malang, Maina, Schwab, Tenkanen, & Lacroix, 2015; Olivares-Illana, Wacher-Rodarte, Le Borgne, & López-Munguía, 2002; van Hijum, van Geel-Schutten, Rahaoui, van der Maarel, & Dijkhuizen, 2002). In the rare case where a bacterium, *Lactobacillus reuteri* 121, possessed both levansucrase and inulosucrase, analysis of cultures detected only a single fructan (levan) and fructooligosaccharides, i.e. 1-kestose (1-kestotriose) and nystose (1,1-kestotetraose) (van Hijum et al., 2002). These fructooligosaccharides and even 1,1,1-kestopentaose can be produced by levansucrases, in addition to levan (Kang et al., 2005; Tieking, Ehrmann, Vogel, & Gänzle, 2005).

LAB that produce homopolymeric exopolysaccharides (EPS), namely fructans and glucans (e.g. dextrans), are increasingly used as fermentation starters. Their EPS can enhance the texture of e.g. vegetable, cereal, and legume-based foods and beverages (Juvonen et al., 2015; Peyer, Zannini, & Arendt, 2016; Xu et al., 2017), offering a potential solution for producing foods free of hydrocolloid additives. The application of EPS-producing LAB is especially promising in sourdough bread making (Di Cagno et al., 2006; Galle, Schwab, Arendt, & Gänzle, 2010; Katina et al., 2009). For example, levans from LAB can improve

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sourdough bread volume and shelf life (Tieking et al., 2005). Levans have also shown prebiotic potential (Dal Bello, Walter, & Hammes, 2001; Korakli, Gänzle, & Vogel, 2002) and have found applications in the personal care and medical sectors (Öner et al., 2016). To date, however, there remains no method for their specific analysis in complex samples such as food matrices. Levans produced by LAB in fermented foods have been analyzed by the quantification of fructose (and glucose) after acid hydrolysis (Juvonen et al., 2015; Schwab, Mastrangelo, Corsetti, & Gänzle, 2008; Tieking, Korakli, Ehrmann, Ganzle, & Vogel, 2003). Since acid hydrolysis is nonspecific towards all fructan oligo- and polysaccharides and other fructose-containing sugars such as sucrose and raffinose family oligosaccharides, i.e. raffinose, stachyose, and verbascose (Verspreet et al., 2012), it is thus of interest to assess enzymatic hydrolysis as a potentially more specific method for *in situ* levan analysis.

There are three types of enzymes that catalyze fructan hydrolysis: exo-β-fructosidases, endoinulinases, and endolevanases. Exo-β-fructosidases, including exoinulinases (EC 3.2.1.80), can nonspecifically hydrolyze both β-(2→1) and β-(2→6)-linkages from the non-reducing end of fructooligosaccharides and fructans (Muñoz-Gutiérrez, Rodríguez-Alegría, & Munguía, 2009). Exoinulinases also hydrolyze sucrose and raffinose family oligosaccharides (Verspreet et al., 2012). Endoinulinases (EC 3.2.1.7) and endolevanases (EC 3.2.1.65) hydrolyze the internal β-(2→1)-linkage in inulin and the internal β-(2→6)-linkage in levan, respectively. Enzymatic analysis methods have long been established for plant inulins, based on the joint action of endo- and exoinulinases, as in AOAC Methods 997.08 and 999.03 (McCleary & Blakeney, 1999; Quemener, Thibault, & Coussement, 1994; Steegmans, Iliaens, & Hoebregs, 2004). Recently, commercial endo- and exoinulinases have been supplemented with an endolevanase (levanase henceforth) in an improved Megazyme assay kit (K-FRUC) for the analysis of plant fructans (Megazyme, 2018). The combined action of levanase and exoinulinase has been tested on pure *Erwinia herbicola* levan, and near complete hydrolysis (with no hydrolysis yield reported) was observed (Jensen et al., 2016).

So far, levans in LAB-fermented foods have mostly been analyzed by acid hydrolysis method, which involved lengthy polysaccharide isolation and background correction against a levan-free control sample (Schwab et al., 2008; Tieking et al., 2003). Moreover, no study has addressed the possible interference of inulin, which can be coproduced by starter bacteria. Therefore, this work aimed to explore hydrolysis specificity of different enzymes towards fructans so as to establish a novel *in situ* enzymatic analysis specific for levan, without the need for a separate blank control. A combination of levanase and exoinulinase was first selected using pure bacterial levans. The interference from inulin, if coexisting, could be detected by single use of endoinulinase at a high dosage. The feasibility of this method for the *in situ* analysis of levan in food matrices was evaluated in spiked wheat and fava bean doughs, as sourdough is one potential source of levan in foods. To remove interference from matrix-derived sugars, aqueous ethanol washing was adopted, followed by correction for residual sugars. Finally, the method was applied to the analysis of levan in fava bean dough fermented by *Leuconostoc mesenteroides* DSM 20343, which produces both dextrans and levan (Olvera, Centeno-Leija, & López-Munguía, 2007; Xu et al., 2017). This test successfully demonstrated the usability of this enzymatic assay for levan in complex dough matrices.

2. Materials and methods

2.1. Commercial inulinases, fructan substrates, and raw materials

Commercial exoinulinase from *Aspergillus niger* (E-EXOIAN) and endoinulinase from *A. niger* (E-ENDOIAN) were both purchased from Megazyme (Bray, Ireland). Levan from *Erwinia herbicola* (Lot No. 101M4038 V) and inulin from chicory (Lot No. 079F7105), used as enzyme substrates, were purchased from Sigma-Aldrich (St. Louis, MO).

Wheat flour was purchased from Raisio Group (Raisio, Finland) with ash content 0.52% (on a dry weight basis), protein content 13.6% and falling number 250. Fava bean (*Vicia faba* major) flour was purchased from CerealVeneta (San Martino di Lupari, Italy) with the composition on a dry weight basis as follows: protein (35.7%), carbohydrates (49.3%), lipids (1.6%), moisture (9.5%) and ash (3.9%).

2.2. Levanase preparation

The GH32 *Paenibacillus amylolyticus* levanase described in WO 00/17331-A1 (Moller, Johansen, Schafer, Ostergaard, & Hoeck, 2000) was heterologously expressed and purified. Briefly, the levanase gene was cloned into an expression vector for *Bacillus subtilis*. The enzyme (GenBank: AAO30665.1) was purified using hydrophobic interaction chromatography followed by anion-exchange chromatography to electrophoretic homogeneity. The protein concentration was estimated from the absorbance at 280 nm and the molar extinction coefficient (1.62 /mg) calculated using the values from Gill and Von Hippel (1989). In detail, the cultivation broth was diluted 1:1 with Milli-Q water and added ammonium sulphate to 1.6 M. The sample was applied to a 4-liter Phenyl Sepharose FF column with a column diameter of 14 cm and a flow of 100 ml/min (GE Healthcare Life Sciences, Piscataway, NJ) equilibrated with 20 mM KH_2PO_4/NaOH, pH 7.0, containing 1.6 M ammonium sulphate and eluted with the same buffer without ammonium sulphate over 5 column volumes. The pooled material was buffer exchanged on a G25 Sephadex column (GE Healthcare Life Sciences) equilibrated with 20 mM HEPES/NaOH, pH 8.0. The last purification step was applying this buffer-exchanged enzyme sample to a 4-liter Q Sepharose FF column with a column diameter of 14 cm and a flow of 100 ml/min (GE Healthcare Life Sciences) equilibrated in 20 mM HEPES/NaOH, pH 8.0, and eluted with a 3-column volume gradient to the same buffer with 0.2 M NaCl.

The activity of this levanase preparation was assayed by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945), where one unit is the amount of enzyme releasing 1 μmol of reducing sugars in 1 min. The reaction was conducted in 20 mM sodium citrate buffer, pH 6.0, containing 1% w/v *Erwinia herbicola* levan and 2 μg/ml levanase, with incubation at 35 °C for 15 min. D-fructose was used as a standard. Inulinase activity was assayed similarly, using 1% w/v chicory inulin as the substrate instead of levan.

The production of mono- and oligosaccharides by levanase was determined by incubating 400 U/g levan of levanase with 2 mg/ml of levan (final concentration) in sodium citrate buffer, pH 6.0, at 30 °C. Products were profiled after 5, 10, 24, and 48 h reactions by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) as described in Section 2.7.

2.3. Fructan hydrolysis conditions

Enzymatic hydrolysis conditions were selected using commercial *Erwinia* levan and chicory inulin as substrates. Considering that optimal pH values are around 4.0 for exoinulinase and 5.0 for endoinulinase (according to the manufacturer), and around 6.0 for levanase (Moller et al., 2000), pH 5.0 was selected for their combined use in this work. All hydrolysis was conducted in 50 mM sodium citrate buffer, pH 5.0, at 30 °C for either 5 or 24 h. The final fructan concentration was approximately 3 mg/ml. Enzymes were tested at varying dosages alone or in combination to obtain the maximum hydrolysis of fructans (Table 1). The dosages tested included 40 and 400 U/g fructan of levanase, 1000 and 100,000 U/g fructan of commercial exoinulinase, and 40 and 400 U/g fructan of commercial endoinulinase. Fructose, glucose, and residual oligosaccharides in the digests were analyzed by HPAEC-PAD to determine the degree of hydrolysis. Fructan yield was calculated by multiplying the released fructose and glucose content by 0.9 to account for anhydro monosaccharide units in the polymer. Sucrose hydrolysis was also assayed for exoinulinase (1000 U/g fructan), endoinulinase

Table 1
Hydrolysis yields of *Erwinia* levan and chicory inulin under different conditions.

Hydrolysis method[a]	Hydrolysis yield[b] (% w/w, dry matter)	
	Levan	Inulin
Acid hydrolysis	81.21 ± 1.40 [a]	86.45 ± 1.70 [a]
Exoinulinase	19.10 ± 0.69 [b]	+
Endoinulinase (40)	nd	23.34 ± 1.36 [b]
Endoinulinase (400)	nd	85.16 ± 1.48 [a]
Exoinulinase/Endoinulinase (40)	+	84.67 ± 2.13 [a]
Exoinulinase/Levanase	80.73 ± 1.98 [a]	+
Exoinulinase/Levanase/ Endoinulinase (40)	78.78 ± 1.54 [a]	+

nd, fructose generation not detected.
+, fructan recovery not determined but partial hydrolysis observed.
[a] The dosages used were 1000 U/g fructan for exoinulinase, 40 or 400 U/g fructan for endoinulinase, and 400 U/g fructan for levanase. Enzymatic hydrolysis was conducted in sodium citrate buffer, pH 5.0, at 30 °C for 24 h.
[b] Hydrolysis yield is the sum of glucose and fructose released in fructan hydrolysis (in terms of dry fructan weight) multiplied by 0.9 to account for anhydro monosaccharides. Values in the same column with different letters (a–b) are significantly different ($p < 0.05$).

(40 U/g fructan), and levanase (400 U/g fructan), respectively. The hydrolysis was carried out at 30 °C for 24 h.

Mild acid hydrolysis of fructans, as described by Juvonen et al. (2015), was conducted for comparison to the enzymatic hydrolysis results. Commercial fructan was dissolved in 0.5 M trifluoroacetic acid (TFA) to a concentration of approximately 1.5 g/l. The solution (400 µl) was incubated at 50 °C for 2 h before 100 µl of 1.0 M sodium carbonate was added to stop the reaction. Fructose and glucose in the hydrolysate were analyzed by HPAEC-PAD (Section 2.7). Pure fructose treated under the same conditions was used as the standard for quantification.

2.4. Hydrolysis of Leuconostoc mesenteroides levan

Leuconostoc mesenteroides DSM 20343 (also known as ATCC 8293 and NRRL B-1118) was grown on MRS agar medium (Oxoid, Basingstoke, UK) supplemented with 5% w/v raffinose at 30 °C for five days in anaerobic conditions for levan production. The cell mass was harvested from the plates and suspended in 10 mM phosphate buffered saline for isolation of water-soluble EPS as previously described (Juvonen et al., 2015). The suspension was shaken for 10 min and subsequently centrifuged for 20 min at 10,000 *g* to separate cells. The supernatant was collected by decanting and centrifuged again under the same conditions. Levan was recovered from the supernatant by adding two volumes of ethanol and centrifugation. The precipitate was washed twice with Milli-Q water before being lyophilized. The *Leuconostoc* levan extract was treated with enzymes, i.e. 400 U/g fructan of levanase together with 1000 U/g fructan of exoinulinase, or with TFA in the same manner as the commercial levan described above. The hydrolysis yield from enzyme treatment was compared with that from acid treatment. The hydrolysate was analyzed by HPAEC-PAD for residual oligosaccharides (Section 2.7).

2.5. Analysis of levan in spiked model doughs

To evaluate the recovery of levan from a complex dough matrix, model doughs were prepared by mixing 10 g wheat flour or fava bean flour (Section 2.1), 0.13 g *Erwinia* levan, and 15 g water. The levan content was 0.52% w/w of wet matter (1.28% of dry matter). In addition, wheat dough was prepared with levan spiked in as 1.00% of wet matter (2.47% of dry matter). For the assay, the spiked doughs and corresponding blank doughs were freeze-dried, homogenized, and then washed twice with aqueous ethanol (80% v/v) to remove interfering free sugars and short oligosaccharides originating from the flour. About 100 mg of freeze-dried dough was placed in 10 ml centrifuge tubes and suspended evenly in 6 ml 80% aqueous ethanol by thorough mixing. The mixture was kept in boiling water for 10 min and then centrifuged at 10,000 *g* for 10 min. After discarding the supernatant, the pellet was re-suspended in 5 ml 80% aqueous ethanol and centrifuged again, then re-suspended in 4.5 ml 50 mM sodium citrate buffer, pH 5.0, before the addition of enzymes (final volume 5 ml). Spiked and blank doughs for each flour were treated with no enzyme, exoinulinase/levanase pair (400 U/g fructan of levanase together with 1000 U/g fructan of exoinulinase), or 400 U/g fructan of endoinulinase alone, or the three enzymes together. After 24 h of hydrolysis, the samples were centrifuged and the supernatants incubated in a boiling water bath for 10 min to inactivate the enzymes. Sugars in the resultant samples were analyzed by HPAEC-PAD (Section 2.7).

2.6. Analysis of levan in fermented fava bean doughs

Fava bean doughs were prepared and fermented as in our previous study (Xu et al., 2017). Briefly, the flour milled from untreated fava bean seeds was mixed with distilled water or sucrose water solution. Dough composition was as follows: 40 g flour and 60 ml water for unsupplemented dough, and 30 g flour, 10 g sucrose, and 60 ml water for sucrose-enriched dough. These doughs were inoculated with *Ln. mesenteroides* DSM 20343 cell suspensions at an initial cell density of 6–7 log cfu/g and were fermented at 30 °C for 24 h. Fermented doughs were freeze-dried and homogenized, after which levan content determined following the procedure described above. Enzymes dosages were determined based on the assumption that one half of the 25% (w/w dry matter) added sucrose would be used by dextransucrases and one half of the remainder would be hydrolyzed by levansucrase. Under that assumption, approximately 3.1% levan (31 g/ kg freeze-dried dough) could be produced by the transferase activity of levansucrase; therefore, the dosages of exoinulinase, levanase, and endoinulinase were 31, 12.4, and 12.4 U/g of freeze-dried dough, respectively. Residual sugars in aqueous ethanol-washed doughs before and after enzymatic hydrolysis were analyzed by HPAEC-PAD. Levan content was determined from the yield of fructose and glucose after hydrolysis, minus the matrix-derived background. Levan recovery loss was accounted for by applying a correction factor determined using fava bean model dough.

2.7. Chromatographic analysis of mono- and oligosaccharides

Sugars were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD), equipped with a CarboPac PA-1 column (250 × 4 mm, i.d., Dionex, CA), a Waters 2465 pulsed amperometric detector (Waters Corporation, Milford, MA), and a Waters 2707 autosampler (Shi et al., 2016). The gradient elution (1 ml/min) started from 2 mM NaOH (3 min), then to 60 mM NaOH (32 min), and finally to 200 mM NaOH (8 min), followed by regeneration and re-equilibration. Residual oligosaccharides in levan hydrolysates were monitored by another HPAEC-PAD, equipped with a CarboPac PA-100 column (250 × 4 mm, i.d., Dionex), a Decade detector (Antec Leyden, The Netherlands), and a Waters 717 autosampler (Shi et al., 2016). The gradient elution (1 ml/min) started from 100 mM NaOH (15 min), then to 120 mM NaAc in 100 mM NaOH (20 min), followed by regeneration and re-equilibration.

All the samples were properly diluted and filtered with a 10 kDa of Amicon Ultra-0.5 centrifugal filter (Millipore, Billerica, MA) and the injection volume was 10 µl in all the measurements. Glucose, fructose, sucrose (Merck, Darmstadt, Germany), raffinose, stachyose, 1-kestose (Sigma-Aldrich), and verbascose (Megazyme) were used as standards, and 2-deoxy-D-galactose (Sigma-Aldrich) was used as the internal standard for quantification.

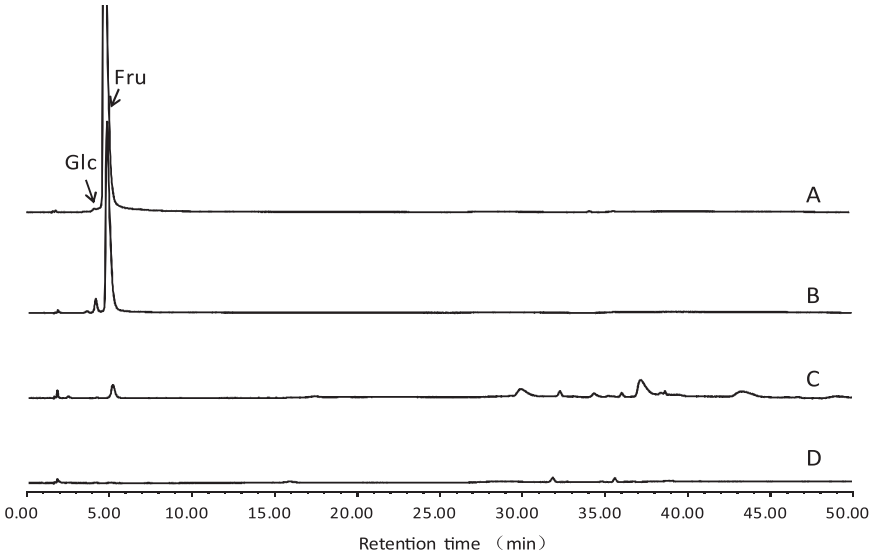

Fig. 1. HPAEC-PAD profiles of mono- and oligosaccharide products after treating *Erwinia* levan with (A) levanase and exoinulinase, (B) endoinulinase and exoinulinase, (C) levanase, and (D) no enzyme for 24 h. Levanase, exoinulinase, and endoinulinase were used at dosages of 400, 1000, and 400 U/g fructan, respectively. Labeled peaks are: Glc, glucose; and Fru, fructose. The peak was identified using authentic standard. The samples were of the same dilution and signal intensity scale.

2.8. Statistical analysis

All analyses in this study were done in triplicate. The effect of treatment was determined by one-way analysis of variance (ANOVA) using Origin 8.6 (OriginLab Inc., Northampton, MA), and considered significant when $p < 0.05$. Means of treatments were compared using Tukey test ($p < 0.05$).

3. Results and discussion

3.1. Levanase preparation

The levanase preparation exhibited an activity of 1116 U/ml towards levan and no activity towards inulin as determined by the reducing value method. Given a protein concentration of approximately 13.6 mg/ml, its specific activity was 82 U/mg. The levanase was incubated with *Erwinia* levan at pH 6.0, and the mono- and oligosaccharides in the digests were detected over time by HPAEC-PAD. The oligosaccharide profile after 24 h (Fig. 1C) was similar to that after 5, 10, and 48 h (Fig. 1S), with fructose content increasing slightly as the reaction proceeded. The low fructose content in the digests indicated the endo-activity of the levanase used. Most resulting levan fragments were probably too large to be analyzed by HPAEC-PAD. Release of free fructose from *Erwinia* levan was also observed for an endo-acting *Bacillus subtilis* levanase, which was hypothesized to be due to minor exo-activity or cleavage from the reducing end (Jensen et al., 2016).

3.2. Selection of enzymatic hydrolysis conditions for bacterial levans

Commercial levan was treated with exoinulinase and endoinulinase singly and jointly, as well as paired levanase/exoinulinase or supplemented with endoinulinase for the highest degree of hydrolysis. For comparison, commercial inulin was treated in parallel. Neither exoinulinase and levanase alone nor the combination of exoinulinase and endoinulinase at their highest dosages sufficiently hydrolyzed levan after 24 h reaction (Table 1 and Fig. 1). Levans from two *Ln. mesenteroides* strains (including the strain DSM 20343 used in the present study) have previously been found resistant to hydrolysis catalyzed by a commercial exoinulinase/endoinulinase combination, i.e. Fructozyme L from Novozymes (Muñoz-Gutiérrez et al., 2009). As expected, when exoinulinase and levanase were used in concert, levan yield increased to 80.73% of dry matter, not significantly different from that obtained by acid hydrolysis (81.21%), demonstrating the effectiveness of levanase in assisting levan degradation. The degree of hydrolysis could not be further improved with high dosage of exoinulinase (100,000 U/g fructan of exoinulinase with 40 U/g fructan of levanase, with a similar enzyme ratio as used by Jensen et al., 2016) (Table 1S), or with the addition of endoinulinase (Table 1). Near complete degradation (without quantifying the hydrolysis degree) of pure *Erwinia* levan after 24 h incubation has also been reported for the pairing of *Bacillus subtilis* levanase (approximately 1 U/g levan) with commercial *A. niger* exoinulinase (3175 U/g levan) (Jensen et al., 2016).

Based on these results, the exoinulinase/levanase pair (1000 U/g fructan of exoinulinase in combination with 400 U/g fructan of levanase) was selected for levan analysis. As these enzymes were selected using *Erwinia* levan containing 5% β-(2→1) branches (Blake, Clarke, Jansson, & McNeil, 1982), and levans from LAB have been reported to contain branch degrees ranging from 4%–30% (Lewis, Cyr, & Smith, 1967; Sims et al., 2011; van Hijum, Bonting, van der Maarel, & Dijkhuizen, 2001), the effect of the enzyme treatment on more branched levans was evaluated with *Ln. mesenteroides* DSM 20343 levan extract. The 1D ^{1}H NMR spectrum of this levan was similar to that of *Ln. citreum* BD1707 levan (Han, Xu, Gao, Liu, & Wu, 2015; Juvonen et al., 2015). Both showed an additional minor signal as compared with the spectrum of *Erwinia* levan, probably attributable to β-(2→1)-linked fructofuranosyl residues (Juvonen et al., 2015). The *Leuconostoc* levans were thus presumed to contain a higher degree of branching. The yield from hydrolysis of *Ln. mesenteroides* levan with the enzyme pair amounted to 99.53% of that obtained by TFA hydrolysis. The HPAEC-PAD profile of the digest showed no resistant oligosaccharides remaining after hydrolysis (data not shown). Thus, the enzymatic hydrolysis method was considered effective for analyzing low to moderately branched levans from LAB.

3.3. Applicability of the proposed enzymatic analysis of levan

As the exoinulinase used in this analysis indiscriminately hydrolyzes both levan and inulin types of fructans and oligosaccharides, as well as sucrose and raffinose family oligosaccharides (Verspreet et al., 2012), the possible interfering substances in a sample need to be identified. Interfering sugars, including both common oligosaccharides originating from the food matrix and those produced by LAB, can be largely removed through washing. In contrast, inulin with its longer chain length cannot be readily separated from levan, and thus if present will hamper the applicability of the levan analysis. Therefore, an analytic step to detect interfering inulin needs to be carried out beforehand. Unlike exoinulinase, endoinulinase is regarded as selective towards inulin-type substrate and was thus evaluated as a candidate enzyme for inulin

detection. As presented in Table 1, we confirmed that bacterial levan treated with 400 U/g fructan of endoinulinase did not release detectable fructose, whereas chicory inulin degraded as completely as in acid hydrolysis or exoinulinase/endoinulinase hydrolysis. Similar near-complete hydrolysis of chicory inulin was reported when incubated with 50 U/g inulin of commercial *A. niger* endoinulinase for about 17 h (McCleary & Blakeney, 1999). Low activity of endoinulinase towards levan was also reported for two *A. niger* endoinulinases, which were active towards dahlia inulin in a 30-min assay but did not release detectable reducing sugars from a commercial *Serratia levanicum* levan after 3 h (Nakamura, Nagatomo, Hamada, Nishino, & Ohta, 1994). In contrast, another *A. niger* endoinulinase at 120 U/g levan was observed to liberate fructose from *Bacillus* levans, but this was suggested to result from the presence of contaminating exoinulinase (Tian, Karboune, & Hill, 2014). Thus, the difference in endoinulinase activity towards levan and inulin could be used to discriminate the two types of fructans, i.e. fructose would be generated in a sample containing inulin, but not in one containing only levan.

Notably, commercial endoinulinase alone at high dosage resulted in a maximal hydrolysis yield of chicory inulin, a typical linear inulin of plant origin, indicating the prospect of endoinulinase quantifying such inulin. Bacterial inulins, on the other hand, have high molecular weights and may be slightly branched (Anwar et al., 2008; Muñoz-Gutiérrez et al., 2009; van Hijum et al., 2002), and thus more resistant to endoinulinase hydrolysis. Given the rare, but possible cases of co-production of inulin and levan by LAB starters (Anwar et al., 2010, 2008; Malang et al., 2015; Olivares-Illana et al., 2002; van Hijum et al., 2002), analysis by endoinulinase hydrolysis was not considered quantitative for inulin. Thus, endoinulinase treatment was proposed for detecting inulin interference, i.e. levan analysis employing exoinulinase/levanase is workable when no fructose is formed after treatment with a high dosage of endoinulinase.

The susceptibility of sucrose to endoinulinase hydrolysis was also determined, with 8% of sucrose hydrolyzed by endoinulinase (40 U/g sucrose) after 24 h incubation. Under similar conditions, levanase (400 U/g sucrose) showed no activity towards sucrose, while exoinulinase (1000 U/g sucrose) hydrolyzed all sucrose. Fructose could also be released from other sucrose-containing oligosaccharides, such as kestose, by the action of endoinulinase. It is thus necessary to remove them in order to confirm the presence of inulin, which in this study was done through a washing step.

3.4. Analysis of levan in model wheat and fava bean doughs

Wheat and fava bean doughs are complex food matrices containing interfering carbohydrates, and are effective models for establishing analytical procedures for *in situ* levan analysis and examining matrix effects on levan recovery. As the exoinulinase used in this analysis is also active towards e.g. sucrose and raffinose family oligosaccharides, an aqueous ethanol washing step was introduced prior to enzymatic hydrolysis to remove sugars from the food matrices. An ethanol concentration of 80% was selected for washing (Xu et al., 2017), as lower ethanol concentration resulted in low levan recovery, indicating loss due to the solubility of levan in more aqueous solutions. Wheat and fava bean model doughs spiked or unspiked with *Erwinia* levan were washed twice and then subjected to enzyme treatment (Table 2). The HPAEC-PAD profiles of mono- and oligosaccharides in model doughs before and after exoinulinase/levanase treatment are shown in Fig. 2. Treating wheat and fava bean doughs with a high dosage of endoinulinase led to the release of a trivial amount of glucose (Table 2), which might result from side activity of the endoinulinase preparation on other substrates, possibly starch and maltooligosaccharides. No detectable fructose was formed after endoinulinase treatment, indicating the absence of any appreciable amount of interfering fructan in the washed matrix.

In washed wheat doughs, no obvious residual sugars were detected by HPAEC-PAD (Fig. 2A). After exoinulinase/levanase treatment, the total background yield of fructose (0.03%) and glucose (0.01%) from blank dough was 0.04% of dry matter, which was subtracted from the total hydrolysis yield of the spiked dough to determine recovery (Table 2). This background yield was negligible and probably derived from residual wheat fructans. An earlier study tested different extraction parameters for wheat fructans and determined that extraction with 80% ethanol at 21 °C for 60 min was insufficient to extract fructans with a higher degree of polymerization (Haskå, Nyman, & Andersson, 2008). The use of 80% ethanol in the present study was a trade-off between retention of levan and washing away of most wheat fructans.

In order to determine whether supplementation with endoinulinase enhances levan recovery, hydrolysis yields were compared between wheat samples treated with exoinulinase/levanase alone and those given the three-enzyme treatment. In both blank and spiked wheat samples, a higher glucose yield (0.06% dry matter) was obtained after the three-enzyme treatment. This additional yield likely also resulted from side activity of the endoinulinase preparation used. Fructose yields were not significantly different (Table 2), indicating the efficiency of the exoinulinase/levanase pair alone. Levan recovery from wheat dough spiked with levan at 1.28% dry matter (corresponding to 0.52% wet matter) was determined to be 67.49%.

Wheat dough was also spiked with a higher amount of levan, 2.47% dry matter (i.e. 1.00% wet matter) and a recovery of 65.12% was obtained from the same exoinulinase/levanase treatment. The recovery was thus reproducible for different spiked amounts. This relatively low recovery was probably due to enzyme-resistant complexes formed with starch or proteins during freeze-drying, or to loss of polysaccharides during sample handling (Katina et al., 2009). Therefore, when estimating levan content in wheat dough, the yield of enzymatic hydrolysis should be multiplied by a correction factor of approximately 1.5 (1/0.6749).

Fava bean was selected to represent legume matrices that contain high amounts of interfering raffinose family oligosaccharides. HPAEC-PAD analysis of mono- and oligosaccharides in fava bean dough has shown that verbascose, sucrose, and stachyose are the major components (Xu et al., 2017). After washing fava bean model doughs twice with aqueous ethanol (80%), traces of all three sugars were still present (Table 2 and Fig. 2B1). Residual stachyose and verbascose were not detected after the two-enzyme treatment (Table 2), which is in agreement with the β-fructosidase activity of exoinulinase on raffinose family oligosaccharides, giving rise to manninotriose and manninotetraose, respectively (Verspreet et al., 2012). Thus, the two additional peaks appearing after hydrolysis probably correspond to manninotriose and manninotetraose (Fig. 2B2). Exoinulinase/levanase treatment of fava bean blank dough gave a hydrolysis yield of 0.27% dry matter, which represented the background yield derived from endogenous sugars in the matrix and was subtracted from the hydrolysis yield of 1.03% obtained for exoinulinase/levanase-treated spiked dough. Levan recovery was thus 58.85%. When estimating levan content in fava bean dough, the net yield of glucose and fructose after exoinulinase/levanase hydrolysis should be multiplied by a correction factor of 1.7 (1/0.5885).

For the fava bean matrix, which contains large amounts of endogenous sugars, washing twice with 80% aqueous ethanol could not remove all sucrose, stachyose, and verbascose. The residual sugars were degraded completely by exoinulinase/levanase, causing background glucose or fructose to be present during the levan analysis. The total background that could be released from residual sucrose, stachyose, and verbascose was calculated to be 0.07 ± 0.01% glucose and 0.20 ± 0.01% fructose, not significantly different from the actual yield of 0.08 ± 0.01% glucose and 0.22 ± 0.01% fructose from exoinulinase/levanase-treated blank dough (Table 2). Assay background could thus be deduced from the amount of degraded interfering sugars, without the need for a separate blank control. A protocol for levan analysis of LAB-fermented doughs is presented in Fig. 3. Free glucose and fructose and the sugars that release them under enzyme treatment, mainly sucrose and raffinose family oligosaccharides, can be readily

Table 2
Sugar contents in and levan recovery from model doughs spiked with 1.28% dry matter *Erwinia* levan after aqueous ethanol washing and enzyme treatment.

Sample	Treatment[a]	Sugars[b] (% w/w, dry matter)						Levan recovery[d] (%)
		Glc	Fru	Suc	Sta	Ver	Hydrolysis yield[c]	
Wheat dough								
Blank	No enzyme	nd	nd	nd	nd	nd	-	-
	Endoinulinase	0.04 ± 0.00	nd	nd	nd	nd	-	-
	Exoinulinase/levanase	0.01 ± 0.00	0.03 ± 0.00	nd	nd	nd	0.04 ± 0.01	-
	Three enzymes	0.06 ± 0.00	0.02 ± 0.00	nd	nd	nd	-	-
Spiked	No enzyme	nd	nd	nd	nd	nd	-	-
	Endoinulinase	0.04 ± 0.00	nd	nd	nd	nd	-	-
	Exoinulinase/levanase	0.01 ± 0.00	1.01 ± 0.07	nd	nd	nd	0.91 ± 0.07	67.49 ± 5.11
	Three enzymes	0.06 ± 0.00	1.03 ± 0.08	nd	nd	nd	-	-
Fava bean dough								
Blank	No enzyme	nd	nd	0.13 ± 0.01	0.17 ± 0.01	0.37 ± 0.02	-	-
	Endoinulinase	0.06 ± 0.00	nd	0.12 ± 0.01	0.18 ± 0.01	0.36 ± 0.03	-	-
	Exoinulinase/levanase	0.08 ± 0.01	0.22 ± 0.01	nd	nd	nd	0.27 ± 0.01	-
Spiked	No enzyme	nd	nd	0.13 ± 0.01	0.17 ± 0.01	0.38 ± 0.02	-	-
	Endoinulinase	0.06 ± 0.00	nd	0.13 ± 0.01	0.17 ± 0.01	0.37 ± 0.02	-	-
	Exoinulinase/levanase	0.06 ± 0.01	1.09 ± 0.02	nd	nd	nd	1.03 ± 0.02	58.85 ± 1.50

-, not applicable for levan recovery determination.
nd, not detected.
[a] The dosages used were 400, 1000, and 400 U/g fructan for endoinulinase, exoinulinase, and levanase, respectively. Enzymatic hydrolysis was conducted in sodium citrate buffer, pH 5.0, at 30 °C for 24 h.
[b] In order to present sugar contents with up to two decimal digits, they are expressed on a dry weight basis. The contents on a wet weight basis are equal to these values multiplied by 0.4. Glc, glucose; Fru, fructose; Suc, sucrose; Sta, stachyose; and Ver, verbascose.
[c] Hydrolysis yield is the sum of the glucose and fructose released by exoinulinase/levanase treatment (in terms of dry dough weight) multiplied by 0.9 to account for anhydro monosaccharides.
[d] Levan recovery was calculated based on the difference of glucose and fructose yield between exoinulinase/levanase-treated blank and spiked doughs.

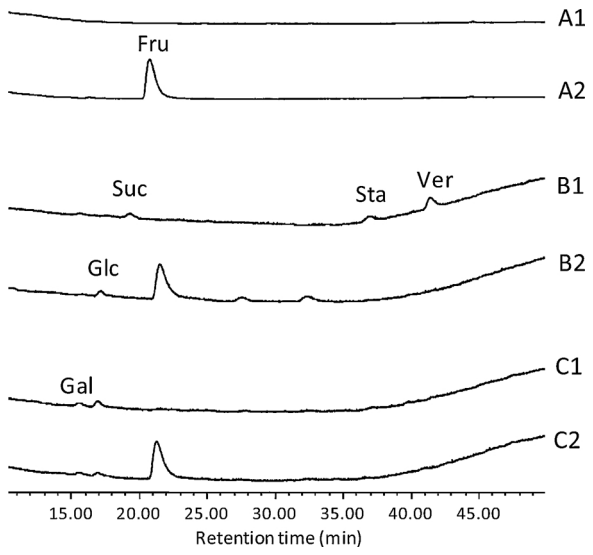

Fig. 2. HPAEC-PAD profiles of sugars in (A) wheat dough spiked with levan, (B) fava bean dough spiked with levan, and (C) sucrose-enriched fava bean dough fermented by levan-producing *Ln. mesenteroides* DSM 20343, all after washing with aqueous ethanol (80%) and treating with (1) no enzyme and (2) the exoinulinase/levanase enzyme pair. Labeled peaks are: Fru, fructose; Suc, sucrose; Sta, stachyose; Ver, verbascose; Glc, glucose; and Gal, galactose. The peak was identified using authentic standard. The samples were of the same dilution and signal intensity scale.

analyzed by HPAEC-PAD and used to calculate background glucose and fructose originating from the food matrix. Levan content can then be estimated by subtracting that background from the total hydrolysis yield of an exoinulinase/levanase-treated sample.

In inulin analyses employing a combination of endo- and exoinulinases, interfering sucrose and starch (and maltosaccharides) can be hydrolyzed specifically by sucrase and starch-degrading enzymes, respectively (McCleary & Blakeney, 1999; Quemener et al., 1994). Reducing sugars are then removed by borohydride reduction prior to hydrolysis of inulin. Alternatively, two separate assays can be performed, one for free fructose and glucose and those released from sucrose and starch and the other for the total fructose and glucose after inulinase hydrolysis; inulin content is then calculated from their difference. When analyzing fructan in wheat milling fractions, α-galactosidase has been used to hydrolyze raffinose family oligosaccharides before the degradation of sucrose and starch to avoid overestimating the fructan content (Haskå et al., 2008). In the present study, starch and maltooligosaccharides were not degraded by side activity of the selected levanase/exoinulinase pair, thus did not interfere with levan analysis. Instead, the major sources of interference were residual sucrose and raffinose family oligosaccharides, which can be determined by HPAEC-PAD. The matrix-derived background can then be estimated by mathematical conversion of the degraded sugars, rendering it unnecessary to hydrolyze interfering sugars with specific enzymes. HPAEC-PAD is also applied in e.g. AOAC Method 997.08 for inulin analysis and is advantageous in separating sugar mixtures and for exhibiting a low detection limit (Quemener et al., 1994). If sucrose and raffinose family oligosaccharides cannot be readily measured, e.g. due to a lack of HPAEC-PAD capability, a control sample treated with sucrase and α-galactosidase can be incorporated in the proposed levan analysis for interference correction.

3.5. Analysis of levan in fermented fava bean doughs

The proposed procedure was applied to the *in situ* analysis of levan produced by *Ln. mesenteroides* DSM 20343 in fava bean doughs. In our previous study, unfermented fava bean dough without added sucrose was shown to contain 4.3% verbascose, 3.7% sucrose, 2.3% stachyose, and 0.2% each raffinose, glucose, and galactose of dry matter, respectively (sugar contents in the following text are expressed on a dry weight basis, with the contents on a wet weight basis equal to these values multiplied by 0.4) (Xu et al., 2017). After fermentation, there remained 0.9% galactose, 0.4% verbascose, and 0.3% stachyose, while the endogenous sucrose, glucose, and raffinose were consumed. For

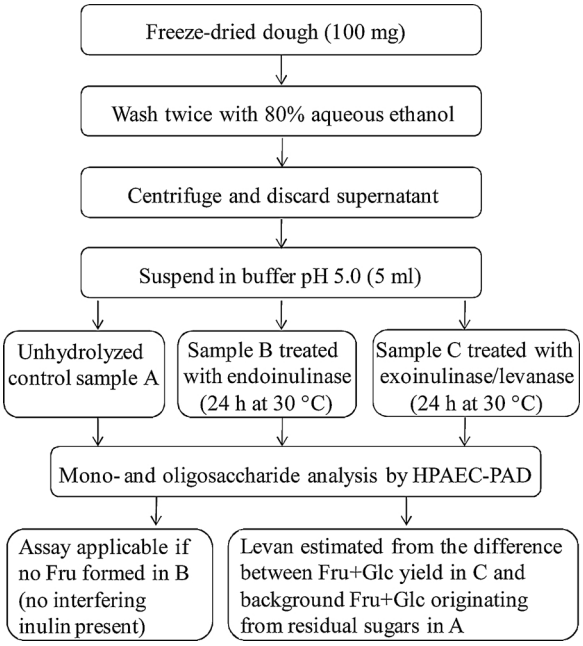

Fig. 3. Schematic representation of the enzymatic assay developed for semi-quantification of levan in LAB-fermented doughs. The dosages of levanase, exoinulinase, and endoinulinase were 400, 1000, and 400 U/g fructan, respectively. Inulin, if present, interferes with the assay and thus endoinulinase, which liberates fructose from inulin but not levan, is used to detect its presence (sample B). Glucose, fructose, sucrose, raffinose, stachyose, and verbascose are quantified by HPAEC-PAD. Assay background derived from dough matrix is subtracted from the total yield of glucose and fructose in sample C for the net yield originating from levan. The background is estimated as the sum of glucose and fructose, both present as free sugars and released from sucrose and raffinose family oligosaccharides. The release of glucose or fructose from sugars is calculated from the difference between sample A and C sugar contents (multiplied by 180/sugar molecular weight). Levan content (%) can be calculated as.

$$\frac{\text{net yield of Glc+Fru mg/ ml} \times\ 0.9\ \times\ 5\,\text{ml} \times \text{correction factor}}{\text{weight of freeze–dried sample (~100) mg}} \times 100$$

where 0.9 and the correction factor account for anhydro units and recovery loss, respectively. For example, 58.85% levan was recovered from fava bean dough, thus the correction factor is 1.7 (1/0.5885).

fava bean dough enriched with sucrose as 25% of dry matter, the ferment contained 2.4% glucose, 1.3% fructose, 1.0% galactose, and 0.5% verbascose. Due to the action of plant-derived α-galactosidase and possibly also bacterial levansucrase, the major oligosaccharides in fava bean matrix, i.e. verbascose and stachyose, were mostly degraded in the ferments, with concomitant release of galactose. The endogenous or added sucrose was also depleted during fermentation. Thus, interfering sucrose, stachyose, and verbascose were absent or only present in small amounts in fermented doughs. In the present study, these sugars were no longer detected after aqueous ethanol washing (Fig. 2C). Neither was any bacterial fructooligosaccharide, such as 1-kestose, detected by HPAEC-PAD (Fig. 2C). Traces of glucose and galactose did remain in the sucrose-enriched dough after washing. This free glucose was subtracted from the total glucose and fructose in exoinulinase/levanase-treated samples. Endoinulinase treatment did not release fructose (Table 3), indicating that the sample was devoid of interfering inulin.

Using the enzymatic analysis developed in this study, the yield of levan produced by *Ln. mesenteroides* DSM 20343 in unsupplemented and sucrose-supplemented fava bean doughs was estimated to be 0.35% and 1.92% dry matter (i.e. 0.14% and 0.77% wet matter), respectively (Table 3). If all sucrose was used for levan production, the theoretical maximum yield would be approximately half the amount of available sucrose, i.e. 1.85% and 14.35% dry matter for unsupplemented and supplemented doughs, respectively. However, in addition to levansucrase, this strain produces more than one type of dextransucrase (Côté & Skory, 2012), which can divert a major proportion of sucrose to the production of dextrans and glucooligosaccharides (Malang et al., 2015). Some sucrose may be used by levansucrase to synthesize fructooligosaccharides other than levan (Galle & Arendt, 2014). In addition to transferase activities, both glycansucrases catalyze the hydrolysis of sucrose; in this strain, sucrose hydrolytic activity constitutes 30% of the enzyme's total levansucrase activity (Olvera et al., 2007).

Previously, lengthy polysaccharide isolation was needed in order to quantify levan in sourdoughs by acid hydrolysis. Moreover, control dough fermented with a levansucrase-negative strain was required to correct for interference from flour polysaccharides (Schwab et al., 2008; Tieking et al., 2003). In contrast, the present enzymatic assay is not affected by flour polysaccharides, allows *in situ* analysis of levan in the matrix, and does not need an acidified control dough. The present method is also advantageous over acid hydrolysis in its ability to distinguish between levan and inulin by exploiting the specificity of endoinulinase. The method could be further improved if exolevanase would be available to be used instead of less specific exoinulinase.

4. Conclusion

EPS-producing LAB have been increasingly used in food fermentation to produce texture-modifying and prebiotic EPS. Compared to nonspecific acid hydrolysis, enzymatic hydrolysis enables *in situ* analysis of EPS without the need for EPS isolation or background correction against a blank control. Enzymatic analysis of dextran (Katina et al., 2009) has proven usable in estimating dextran yield in food ferments. However, corresponding analysis of another common EPS from LAB, i.e. levan, has been lacking. In this study, an *in situ* semi-quantitative

Table 3
Determination of levan content in *Leuconostoc mesenteroides* DSM 20343-fermented fava bean doughs by the method presented in Fig. 3.

Fermented fava bean dough	Treatment[a]	Sugars[a] (% w/w, dry matter)						Levan content[b] (% dry matter)
		Glc	Fru	Suc	Sta	Ver	Hydrolysis yield	
Without sucrose addition	No enzyme	nd	nd	nd	nd	nd	nd	–
	Endoinulinase	nd	nd	nd	nd	nd	–	–
	Exoinulinase/levanase	0.01 ± 0.00	0.22 ± 0.01	nd	nd	nd	0.21 ± 0.01	0.35 ± 0.02
With sucrose (25% dry matter)	No enzyme	0.05 ± 0.01	nd	nd	nd	nd	0.05 ± 0.01	–
	Endoinulinase	0.05 ± 0.00	nd	nd	nd	nd	–	–
	Exoinulinase/levanase	0.05 ± 0.01	1.27 ± 0.09	nd	nd	nd	1.18 ± 0.08	1.92 ± 0.14

[a] Detailed information can be found in Table 2. All values are presented on a dry weight basis. The contents on a wet weight basis are equal to these values multiplied by 0.4.

[b] To determine levan content, the background glucose and fructose derived from sugars in unhydrolyzed samples were subtracted from the yields of exoinulinase/levanase-treated samples. Levan content was calculated by multiplying the net yield of glucose and fructose by 1.7 to correct for recovery loss.

analysis specific for levan was established using an endo-acting levanase and commercial endo- and exoinulinases. An exoinulinase/levanase enzyme pair was selected to hydrolyze levans to a degree comparable to that obtained with acid hydrolysis. Levan content was then estimated by measuring the glucose and fructose released by the hydrolysis. However, interfering sugars such as free glucose and fructose, sucrose, raffinose family oligosaccharides, and short-chain fructooligosacchairdes, if present, cause assay background under the enzyme action. To remove interfering sugars in e.g. sourdough, the freeze-dried dough sample was first washed with 80% aqueous ethanol; levan recovery was reduced when washed with more aqueous solutions. After washing, the assay background derived from matrix in wheat dough was found negligible, but higher in fava bean dough due to the degradation of residual sucrose, stachyose, and verbascose. To correct for interference from sucrose and raffinose family oligosaccharides, their contents before and after exoinulinase/levanase treatment were determined by HPAEC-PAD and used to calculate their contribution to the yield of fructose and glucose, which together with endogenous free fructose and glucose were subtracted from the total hydrolysis yield to estimate levan content. In contrast with the nonspecific acid hydrolysis method commonly used for levan analysis, endoinulinase used alone at high dosage could distinguish between inulin and levan, as the former generated free fructose while the latter did not. A separate endoinulinase treatment was thus incorporated to detect the possible presence of interfering inulin. The proposed method was applied to the *in situ* analysis of levan produced by *Ln. mesenteroides* DSM 20343 in fava bean doughs, using a correction factor accounting for levan recovery from the fava bean model dough. To the best of our knowledge, this is the first report on *in situ* enzymatic analysis of levan in foods.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.12.044.

References

Anwar, M. A., Kralj, S., Pique, A. V., Leemhuis, H., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2010). Inulin and levan synthesis by probiotic *Lactobacillus gasseri* strains: Characterization of three novel fructansucrase enzymes and their fructan products. *Microbiology, 156*(4), 1264–1274.

Anwar, M. A., Kralj, S., van der Maarel, M. J., & Dijkhuizen, L. (2008). The probiotic *Lactobacillus johnsonii* NCC 533 produces high-molecular-mass inulin from sucrose by using an inulosucrase enzyme. *Applied and Environmental Microbiology, 74*(11), 3426–3433.

Blake, J. D., Clarke, M. L., Jansson, P. E., & McNeil, K. E. (1982). Fructan from *Erwinia herbicola*. *Journal of Bacteriology, 151*(3), 1595–1597.

Côté, G. L., & Skory, C. D. (2012). Cloning, expression, and characterization of an insoluble glucan-producing glucansucrase from *Leuconostoc mesenteroides* NRRL B-1118. *Applied Microbiology and Biotechnology, 93*, 2387–2394.

Dal Bello, F., Walter, J. H., & Hammes, W. P. (2001). *In vitro* study of prebiotic properties of levan-type exopolysaccharides from Lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Systematic and Applied Microbiology, 24*(2), 232–237.

Di Cagno, R., De Angelis, M., Limitone, A., Minervini, F., Carnevali, P., Corsetti, A., et al. (2006). Glucan and fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum*. *Journal of Agricultural and Food Chemistry, 54*, 9873–9881.

Galle, S., & Arendt, E. K. (2014). Exopolysaccharides from sourdough lactic acid bacteria. *Critical Reviews in Food Science and Nutrition, 54*, 891–901.

Galle, S., Schwab, C., Arendt, E., & Gänzle, M. (2010). Exopolysaccharide-forming *Weissella* strains as starter cultures for sorghum and wheat sourdoughs. *Journal of Agricultural and Food Chemistry, 58*, 5834–5841.

Gill, S. C., & Von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Analytical Biochemistry, 182*(2), 319–326.

Han, J., Xu, X., Gao, C., Liu, Z., & Wu, Z. (2015). Levan-producing *Leuconostoc citreum* strain BD1707 and its growth in tomato juice supplemented with sucrose. *Applied and Environmental Microbiology, 82*(5), 1383–1390.

Haskå, L., Nyman, M., & Andersson, R. (2008). Distribution and characterisation of fructan in wheat milling fractions. *Journal of Cereal Science, 48*(3), 768–774.

Jensen, S. L., Diemer, M. B., Lundmark, M., Larsen, F. H., Blennow, A., Mogensen, H. K., et al. (2016). Levanase from *Bacillus subtilis* hydrolyses β-2, 6 fructosyl bonds in bacterial levans and in grass fructans. *International Journal of Biological Macromolecules, 85*, 514–521.

Juvonen, R., Honkapää, K., Maina, N. H., Shi, Q., Viljanen, K., Maaheimo, H., et al. (2015). The impact of fermentation with exopolysaccharide producing lactic acid bacteria on rheological, chemical and sensory properties of pureed carrots (*Daucus carota* L.). *International Journal of Food Microbiology, 207*, 109–118.

Kang, H. K., Seo, M. Y., Seo, E. S., Kim, D., Chung, S. Y., Kimura, A., et al. (2005). Cloning and expression of levansucrase from *Leuconostoc mesenteroides* B-512 FMC in *Escherichia coli*. *Biochimica Et Biophysica Acta (BBA)-Gene Structure and Expression, 1727*(1), 5–15.

Katina, K., Maina, N. H., Juvonen, R., Flander, L., Johansson, L., Virkki, L., et al. (2009). *In situ* production and analysis of *Weissella confusa* dextran in wheat sourdough. *Food Microbiology, 26*, 734–743.

Korakli, M., Gänzle, M., & Vogel, R. (2002). Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *Journal of Applied Microbiology, 92*(5), 958–965.

Lewis, B., Cyr, M. S., & Smith, F. (1967). The constitution of the fructan produced by *Leuconostoc mesenteroides* strain C. *Carbohydrate Research, 5*(2), 194–201.

Malang, S. K., Maina, N. H., Schwab, C., Tenkanen, M., & Lacroix, C. (2015). Characterization of exopolysaccharide and ropy capsular polysaccharide formation by *Weissella*. *Food Microbiology, 46*, 418–427.

McCleary, B., & Blakeney, A. (1999). Measurement of inulin and oligofructan. *Cereal Foods World, 44*, 398–406.

Megazyme (2018). *Fructan assay kit.* Retrieved August 23, 2018, fromhttps://secure.megazyme.com/Fructan-Assay-Kit.

Moller, S., Johansen, C., Schafer, T., Ostergaard, P. R., & Hoeck, L. H. (2000). World Patent No. WO200017331-A1. Switzerland: World Intellectual Property Organization.

Muñoz-Gutiérrez, I., Rodríguez-Alegría, M. E., & Munguía, A. L. (2009). Kinetic behaviour and specificity of β-fructosidases in the hydrolysis of plant and microbial fructans. *Process Biochemistry, 44*(8), 891–898.

Nakamura, T., Nagatomo, Y., Hamada, S., Nishino, Y., & Ohta, K. (1994). Occurrence of two forms of extracellular endoinulinase from *Aspergillus niger* mutant 817. *Journal of Fermentation and Bioengineering, 78*(2), 134–139.

Nelson, N. (1944). A photometric adaptation of the somogyi method for the determination of glucose. *The Journal of Biological Chemistry, 153*, 375–380.

Olivares-Illana, V., Wacher-Rodarte, C., Le Borgne, S., & López-Munguía, A. (2002). Characterization of a cell-associated inulosucrase from a novel source: A *Leuconostoc citreum* strain isolated from *Pozol*, a fermented corn beverage of Mayan origin. *Journal of Industrial Microbiology & Biotechnology, 28*(2), 112–117.

Olvera, C., Centeno-Leija, S., & López-Munguía, A. (2007). Structural and functional features of fructansucrases present in *Leuconostoc mesenteroides* ATCC 8293. *Antonie Van Leeuwenhoek, 92*(1), 11–20.

Öner, E. T., Hernández, L., & Combie, J. (2016). Review of levan polysaccharide: From a century of past experiences to future prospects. *Biotechnology Advances, 34*(5), 827–844.

Peyer, L. C., Zannini, E., & Arendt, E. K. (2016). Lactic acid bacteria as sensory biomodulators for fermented cereal-based beverages. *Trends in Food Science & Technology, 54*, 17–25.

Quemener, B., Thibault, J., & Coussement, P. (1994). Determination of inulin and oligofructose in food products, and integration in the AOAC method for measurement of total dietary fibre. *LWT-Food Science and Technology, 27*(2), 125–132.

Schwab, C., Mastrangelo, M., Corsetti, A., & Gänzle, M. (2008). Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. *Cereal Chemistry, 85*, 679–684.

Shi, Q., Juvonen, M., Hou, Y., Kajala, I., Nyyssola, A., Maina, N. H., et al. (2016). Lactose- and cellobiose-derived branched trisaccharides and a sucrose-containing trisaccharide produced by acceptor reactions of *Weissella confusa* dextransucrase. *Food Chemistry, 190*, 226–236.

Sims, I. M., Frese, S. A., Walter, J., Loach, D., Wilson, M., Appleyard, K., et al. (2011). Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23. *The ISME Journal, 5*(7), 1115–1124.

Somogyi, M. (1945). A new reagent for the determination of sugars. *The Journal of Biological Chemistry, 160*, 61–68.

Steegmans, M., Iliaens, S., & Hoebregs, H. (2004). Enzymatic, spectrophotometric determination of glucose, fructose, sucrose, and inulin/oligofructose in foods. *Journal of AOAC International, 87*(5), 1200–1207.

Tian, F., Karboune, S., & Hill, A. (2014). Synthesis of fructooligosaccharides and oligolevans by the combined use of levansucrase and endo-inulinase in one-step bi-enzymatic system. *Innovative Food Science & Emerging Technologies, 22*, 230–238.

Tieking, M., Ehrmann, M. A., Vogel, R. F., & Gänzle, M. G. (2005). Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Applied Microbiology and Biotechnology, 66*(6), 655–663.

Tieking, M., Korakli, M., Ehrmann, M. A., Ganzle, M. G., & Vogel, R. F. (2003). *In situ* production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *Applied and Environmental Microbiology, 69*(2), 945–952.

Van den Ende, W. (2013). Multifunctional fructans and raffinose family oligosaccharides. *Frontiers in Plant Science, 4*, 247.

van Hijum, S. A., Bonting, K., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2001). Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. *FEMS Microbiology Letters, 205*(2), 323–328.

van Hijum, S. A., van Geel-Schutten, G. H., Rahaoui, H., van der Maarel, M. J., & Dijkhuizen, L. (2002). Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Applied and Environmental Microbiology, 68*(9), 4390–4398.

Verspreet, J., Pollet, A., Cuyvers, S., Vergauwen, R., Van den Ende, W., Delcour, J. A., et al. (2012). A simple and accurate method for determining wheat grain fructan content and average degree of polymerization. *Journal of Agricultural and Food Chemistry, 60*(9), 2102–2107.

Vijn, I., & Smeekens, S. (1999). Fructan: More than a reserve carbohydrate. *Plant Physiology, 120*(2), 351–360.

Xu, Y., Coda, R., Shi, Q., Tuomainen, P., Katina, K., & Tenkanen, M. (2017). Exopolysaccharides production during the fermentation of soybean and fava bean flours by *Leuconostoc mesenteroides* DSM 20343. *Journal of Agricultural and Food Chemistry, 65*(13), 2805–2815.