JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evidence for Formation of Heterooligosaccharides by *Lactobacillus sanfranciscensis* during Growth in Wheat Sourdough

MARKUS TIEKING, WOLFGANG KÜHNL, AND MICHAEL G. GÄNZLE*

Lehrstuhl Technische Mikrobiologie, TU München, D-85350 Freising, Germany

Lactobacillus sanfranciscensis is a key organism of the lactic microflora in traditional and industrial sourdough fermentations. In this paper we provide evidence for the formation of heterooligosaccharides (HeOS) by *L. sanfranciscensis* during growth in sourdough. To identify the HeOS based on HPAEC−PAD analysis, HeOS standards were synthesized by enzymatic reactions with *L. sanfranciscensis* levansucrase in a chemically defined system in the presence of raffinose, maltotriose, maltose, xylose, or arabinose as acceptor carbohydrates. The oligosaccharides known to originate from the corresponding acceptor reactions, 1^F-β-fructosylraffinose, 1^F-β-fructofuranosylmaltotriose, erlose (1^F-β-fructofuranosylmaltose), xylsucrose, 1^F-β-fructosylxylsucrose, and arabsucrose, were identified by HPAEC−PAD. Evidence for the formation of further tri-, tetra-, and pentasaccharides was provided. Wheat doughs with sucrose were fermented with *L. sanfranciscesis* TMW 1.392 or the isogenic, levansucrase-negative strain TMW 1.392Δlev, and the analysis of dough extracts or invertase-treated dough extracts provided evidence for the formation of arabsucrose and erlose in sourdough in addition to 1-kestose and nystose.

KEYWORDS: ***L. sanfranciscensis*; sourdough fermentation; heterooligosaccharides; levansucrase**

INTRODUCTION

Food applications of inulin-type fructooligosaccharides (FOS) are based mainly on their prebiotic properties (*1*). Furthermore, FOS have a low sweetness compared to sucrose, are essentially calorie-free, and are noncariogenic (*2*). They are not degraded by pancreatic enzymes and reach the lower intestine, where they selectively stimulate the growth of bifidobacteria (*3*, *4*). To date, FOS are produced enzymatically from sucrose using fructosyltransferase enzymes (*4*) or derived from partial hydrolysis of inulin, and are used in food applications as an ingredient. Recently, the formation of 1-kestose by *Lactobacillus reuteri* and *Lactobacillus sanfranciscensis* was reported (*5*, *6*), and the use of these strains may enable the generation of FOS in situ during food fermentations using the low-cost ingredient sucrose as carbon source (*7*).

FOS formation by lactobacilli from sucrose is mediated by levansucrase or inulosucrase activities; both enzymes generally also use raffinose as substrate (*5*, *7*, *8*). Levansucrase enzymes belong to the glycosyltransferase family of enzymes. They use the energy of the osidic bond of sucrose to catalyze the transfer of the fructosyl moiety of sucrose to H_2O, an acceptor carbohydrate, or a growing levan polymer to yield glucose and fructose, oligosaccharides, and levan, respectively (*2*, *9*, *10*):

$$\text{sucrose} + H_2O \rightleftharpoons \text{glucose} + \text{fructose}$$

$$\text{sucrose} + \text{acceptor carbohydrate (C)} \rightleftharpoons \text{oligosaccharide (CF)} + \text{glucose}$$

$$\text{sucrose} + \text{fructan } (GF_n) \rightleftharpoons \text{fructan } (GF_{n+1}) + \text{glucose}$$

Mono-, di-, and trisaccharides vary in their efficiency as fructosyl acceptors to the levansucrase activity. Sucrose acts as fructosyl donor as well as fructosyl acceptor to yield 1-kestose (1-kestotriose) with subsequent transfer reactions that yield the corresponding higher oligomers nystose (1,1-kestotetraose, GF_3) and 1,1,1-kestopentaose (GF_4). Furthermore, maltotriose, raffinose, maltose, arabinose, and xylose are efficient fructosyl acceptors and oligosaccharides resulting from fructosyl transfer catalyzed by levansucrase enzymes of *Aerobacter levanicum* and *Bacillus subtilis* were characterized previously (*9*, *11*−*13*). Generally, levansucrases are specific for anomers of an aldose and the fructosyl transfer to an acceptor is effected with net retention of the fructose configuration (*12*). Because the term FOS is commonly used for inulin-type oligomers only, oligosaccharides resulting from the acceptor reaction of levansucrases are termed heterooligosaccharides (HeOS) (*13*).

The sourdough isolate *L. sanfranciscensis* TMW 1.392 exhibits levansucrase activity and produces 1-kestose and a high-molecular-weight levan from sucrose (*6*, *7*, *14*, *15*). We have previously shown that levan and 1-kestose are produced during growth of *L. sanfranciscensis* in wheat sourdoughs (*7*, *16*).

* Corresponding author. Present address: University of Alberta, Dept. of Agricultural, Food and Nutritional Science, 4−10 Agriculture/Forestry Centre, Edmonton, AB, Canada T6G 2P5. Telephone: + 1 780 492 0774. Fax: + 1 780 492 4265. E-mail: michael.gaenzle@ualberta.ca.

10.1021/jf048307v CCC: $30.25
Published on Web 02/26/2005

When sucrose was present as substrate, up to 5 g kg^{-1} levan and 20 g kg^{-1} 1-kestose were formed. Levan levels produced by *L. sanfranciscensis* significantly improve rheological parameters of wheat doughs and bread volume (*17*). Approximately 0.1 g kg^{-1} 1-kestose is naturally present in wheat flour (*18*), less than 1% of the kestose levels that can be generated by fermentation with levansucrase-positive strains of *L. sanfranciscensis*.

To fully characterize the products from the levansucrase activity during growth of *L. sanfranciscensis* TMW 1.392 in sourdough, the objective of this study was to detect those HeOS that may originate from the acceptor reaction. Mono-, di-, and trisaccharides that are present in substantial levels in wheat and rye doughs were used as acceptor carbohydrates. The qualitative analysis of HeOS was achieved on high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC−PAD). HeOS for use as external standards were synthesized in chemically defined enzymatic reactions with purified levansucrase of *L. sanfrancisensis* TMW 1.392 in the presence of sucrose as fructosyl donor and the corresponding carbohydrates as fructosyl acceptors.

MATERIALS AND METHODS

Strains, Plasmids, Media, and Growth Conditions. *L. sanfranciscensis* TMW 1.392 and *L. sanfranciscensis* TMW 1.392Δlev were cultivated anaerobically at 30 °C in modified MRS medium (mMRS) (*19*), containing 10 g L^{-1} maltose and 5 g L^{-1} fructose (mMRS−maltose). The strain *L. sanfranciscensis* TMW 1.392 (isogenic to LTH 2590) harbors a levansucrase gene; *L. sanfranciscensis* TMW 1.392Δlev is an isogenic, levansucrase-negative deletion mutant derivative of *L. sanfranciscensis* TMW 1.392 (*7*). *Escherichia coli* strain JM109 DE3 carrying the plasmid pLEV1 was cultivated aerobically in Luria-Bertani (LB) medium containing 100 μg L^{-1} ampicillin at 37 °C. The plasmid pLEV1 is based on the expression vector pET3a and harbors the structural gene for the *L. sanfranciscensis* levansucrase downstream of the inducible *lacZ* promotor and in frame upstream of a 6 × histidine tag to obtain C-terminally His-tagged proteins (*7*). Expression of the levansucrase by *E. coli* JM109 DE3 was induced by adding 40 μg L^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) to exponentially growing cultures.

Purification of the *L. sanfranciscensis* Levansucrase and Enzymatic Synthesis of Oligosaccharides. The levansucrase of *L. sanfranciscensis* TMW 1.392 was purified to homogeneity after heterologous expression with a His tag (levΔhis) in *E. coli* JM109 as described (*7*). *E. coli* JM109 carrying pLEV1 was grown to an optical density (590 nm) of 0.6, IPTG was added, and cells were further incubated at 30 °C for 12 h. Cells were harvested, washed with 50 mL of binding buffer A (50 mM NaH_2PO_4, 10 mM imidazole, 300 mM NaCl), and broken by ultrasonification (HD-70, Bandelin Electronics, Germany, 5 × 30 s with 50% cycle, 90% power). Cell debris was removed by centrifugation (10 000*g*, 10 min, 4 °C), and the supernatant was applied to a HiTrap chelating nickel column (Amersham Biosciences, Freiburg, Germany) with a flow of 1.5 mL min^{-1}. The column was washed with 5.0 mL of 20% buffer B (50 mM NaH_2PO_4, 300 mM imidazole, 300 mM NaCl), and the His-tagged levansucrase was eluted with a linear gradient from 20% buffer B to 100% buffer B. Fractions containing levansucrase were dialyzed against 25 mM sodium acetate buffer with pH 5.4. It was verified by SDS−polyacrylamide gel electrophoresis using an miniprotean electrophoresis cell (Bio-Rad Laboratories, USA) with the Laemmly buffer system according to the instructions of the supplier that the levansucrase was not contaminated by other proteins (**Figure 1**), and the electrophoretic mobility of the enzyme was in good agreement with previous results (*7*). The protein concentration of the stock solution with the purified levansucrase was determined with the Bradford method, using the Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) and bovine serum albumin as external standard.

Enzymatic reactions were carried out at 37 °C in 10 mM sodium acetate buffer, pH 5.4, with an enzyme concentration of 0.5 μg mL^{-1}, containing 1 mmol L^{-1} $CaCl_2$, 0.4 mol L^{-1} sucrose or 0.4 mol L^{-1} raffinose, and 0.4 mol L^{-1} acceptor carbohydrate where indicated. The reaction mixture was incubated for 20 h and stored frozen for further analysis.

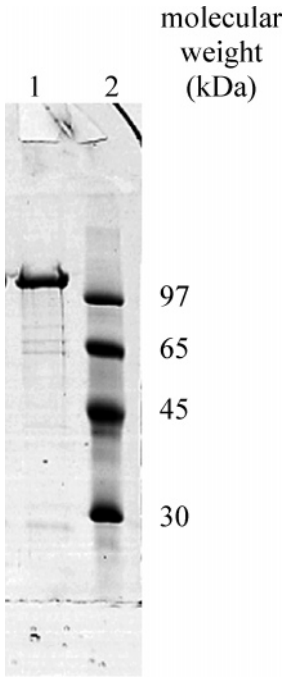

Figure 1. SDS–PAGE analysis of *L. sanfranciscensis* TMW 1.392 levansucrase (levΔhis, lane 1) after heterologous expression in *E. coli* and affinity chromatrography as described (*7*). The molecular weight marker was applied on lane 2, and the electrophoretic mobility of levΔhis was in good agreement with previous results (*7*).

Partial Purification and Characterization of Oligosaccharides from Enzymatic Reactions. Enzymatic reactions were carried out in 1 mL volume with levansucrase using using raffinose, or sucrose and maltose, maltotriose, xylose, or arabinose as acceptor carbohydrates. After overnight incubation, the reaction mixture was separated on a Superdex Peptide gel permeation chromatography (GPC) column (Amersham Pharmacia Biotech, Freiburg, Germany) eluted with deionized water at a flow of 0.4 mL min^{-1}. Carbohydrates were detected with an refractive index (RI) detector (Gynkothek, Germering, Germany). To estimate the degree of polymerization (DP) of the oligosaccharides, the column was calibrated using glucose, sucrose, raffinose, and stachyose as molecular weight standards. Peaks corresponding to trisaccharides, tetrasaccharides, or higher oligosaccharides were collected, pooled, and dried under vacuum. When maltotriose or raffinose was used as acceptor carbohydrate, peaks corresponding to tetra- and pentasaccharides were collected. The dried oligosaccharides were redissolved in 0.5 mL of deionized water and subjected to a second chromatographic separation on the same column. Peaks correponding to oligosaccharides with a DP of 3 or 4 and 4 or 5 were pooled, dried, and redissolved in 0.2 mL of deionized water. These fractions were analyzed for the oligosaccharide composition by HPAEC−PAD as described to verify that no contaminating mono-, di-, or trisaccharides were present. Furthermore, oligosaccharides in these fractions were hydrolyzed by incubation for 1 h with 5% perchloric acid at 90 °C. The hydrolysates were neutralized by addition of 4 M KOH. Monosaccharides in the hydrolysates were quantified by HPLC using the CHPB column (Merck, Darmstadt, Germany) eluted with deionized water at a flow of 0.4 mL min^{-1} and RI detection as described (*16*).

Preparation of Doughs, Determination of pH, and Cell Counts in Sourdough. Wheat doughs were fermented with *L. sanfranciscensis* TMW 1.392 or the isogenic, levansucrase-negative deletion mutant, *L. sanfranciscensis* TMW 1.392Δlev. For inoculation of doughs, about 10^9 cells from an overnight culture in mMRS−maltose were washed with sterile water and resuspended in 10 mL of tap water. Doughs were prepared with 100 g of wheat flour type 550, 90 g of sterile tap water,

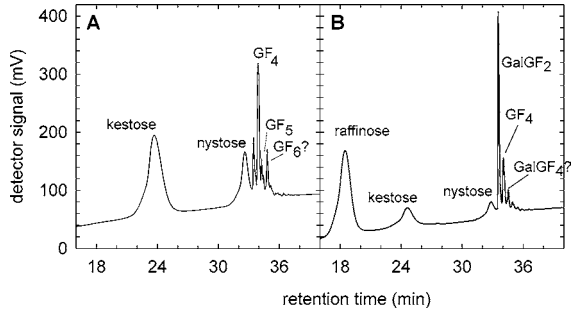

Figure 2. HPAEC–PAD separation of oligosaccharides resulting from the levansucrase reaction with 0.4 mol L^{-1} sucrose (**A**) or raffinose (**B**). Peaks were assigned based on external standards or literature data on products of levansucrase activity as described in the text. Uncertain assignments are marked with question marks. Glucose, fructose, sucrose, and melibiose eluted prior to the retention time cutoff at 16 min (not shown).

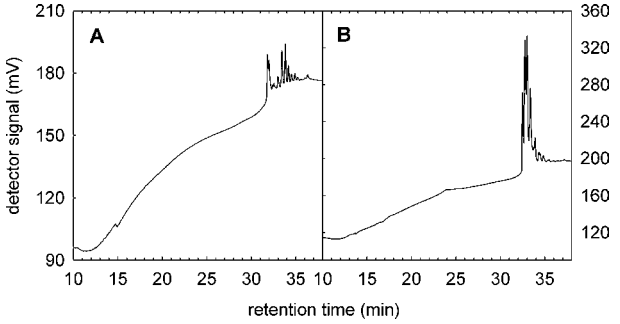

Figure 3. HPAEC–PAD separation of oligosaccharides resulting from the levansucrase reaction in the presence of raffinose (**A**) or sucrose and arabinose (**B**), followed by two consecutive GPC runs to remove mono-, di-, and trisaccharides. Likewise, oligosaccharides from the maltotriose, maltose, and xylose acceptor reactions were prepared (data not shown).

10 mL of cell suspension of strain TMW 1.392 or TMW 1.392Δlev, and 20 g of sucrose. The determination of pH values and cell counts was carried out as described (*20*), and by the observation of the colony morphology it was verified that the strains used to inoculate the doughs dominated the fermentation throughout the incubation time of 24 h. For oligosaccharide analysis, dough samples were weighed, diluted with demineralized water, and centrifuged at 17000*g* for 30 min to remove solids. These supernatants were diluted 1:100 and used for oligosaccharide analysis as described below.

Chromatographic Separation of Oligosaccharides for Qualitative Analysis. The separation of oligosaccharides was achieved by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC−IPAD) using an AminoPac PA10 column and an ED40 electrochemical detector as described (*21*). A ternary gradient was employed using water with a resistance of 18 MΩ or more (solvent A), 1 M sodium acetate (solvent B), and 0.25 M NaOH (solvent C). To improve the separation of oligosaccharides, the gradient was modified as follows: 0 min, 0% B and 12% C; 2 min, 10% B and 12% C; 11 min, 20% B and 12% C; 18 min, 0% B and 30% C; 36 min, 36% B and 24% C; 45 min, 40% B and 24% C. The column was washed with 50% B and 50% C for 20 min after each sample. Maltose, maltotriose, raffinose, melibiose, and mixtures of inulin-type FOS (Actilight, degree of polymerization (DP) 2−8, Gewürzmüller, Stuttgart, Germany) or maltooligosaccharides (DP4-10, Sigma, Deisenhofen, Germany) were used as external standards. Invertase treatment of enzymatic reactions and aqueous dough extracts were carried out by incubation with yeast invertase (Sigma, Deisenhofen, Germany) at a concentration of 100 μg L^{-1} for 3 h at 37 °C.

Statistical Analysis and Experimental Error. All enzymatic reactions with levansucrase, and sourdough fermentations with *L. sanfranciscensis* strains, were carried out in duplicate, triplicate, or quadruplicate independent experiments with consistent results. The retention times of the individual peaks in HPAEC−PAD analysis were reproducible with an error of ±0.5 min, and peak areas were generally reproducible with an experimental error of less than 15%.

RESULTS

Enzymatic Synthesis of FOS and HeOS. To determine the retention times of HeOS on the HPAEC−PAD system, HeOS were synthesized in enzymatic reactions with purified levansucrase using sucrose as fructosyl donor and the corresponding carbohydrates as fructosyl acceptors. Although the elution order of hetergenous sugar oligomers on HPAEC−PAD cannot be predicted, oligomers of a homologous series differing only in their DP generally elute in the order of their molecular weight. Our analytical setup allowed the separation of maltooligosaccharides and inulin-type FOS up to a DP of 8 (*22*) (**Figure 2** and data not shown); however, sufficient separation of HeOS with a DP of 4 or more was not achieved in those cases where oligomers of two or more homologous series were present. Therefore, peaks were assigned to HeOS based on the following considerations: (i) comparison of the retention times to FOS external standards; (ii) assignment of additional peaks in reactions with fructosyl acceptors to HeOS known to result from the (repetitive) acceptor reaction of levansucrase with maltotriose, maltose, xylose, and arabinose, which allowed identification of oligomers with a DP of 3 or less; (iii) preparation of tetra-, penta-, and hexasaccharides in two consecutive GPC runs, followed by HPAEC−PAD analysis of the oligosaccharides, and the determination of the monomer composition of oligosaccharides.

FOS and HeOS from Sucrose or Raffinose as Fructosyl Donor and Acceptor. Levansucrase activity with sucrose as the only substrate is known to yield inulin-type FOS with a DP of up to 5 (*9*). The reaction of the *L. sanfranciscensis* levansucrase with 0.4 M sucrose as fructosyl donor and acceptor yielded 1-kestose (GF_2), nystose (GF_3), and FOS with a DP of 5 or greater (GF_4 and GF_5), which were identified using FOS as external standards (**Figure 2A**).

In reactions of levansucrase with raffinose (GalGF) as fructosyl donor and acceptor, melibiose (GalG) and 1^F-β-fructosylraffinose ($GalGF_2$) are formed (*9*). In the reaction of the *L. sanfranciscensis* levansucrase with 0.4 M raffinose as fructosyl donor, peaks corresponding to tetra-, penta-, and hexasaccharides were present in addition to melibiose, raffinose, kestose, and nystose (**Figure 2B**). The major oligosaccharide peak at 34 min was assigned to $GalGF_2$ (*9*). HeOS from this reaction were prepared by two consecutive runs on a GPC column. Peaks corresponding to tetra- and pentasaccharides were collected, dried under vacuum, and resubjected to analysis by HPAEC−PAD. This oligosaccharide preparation was essentially free of oligomers with a DP of 3 or less, nystose was not present, and $GalGF_2$ was depleted (**Figure 3A**). The molar monomer ratio in this oligosaccharide preparation upon hydrolysis was Gal:Glu:Fru 1:2:5, which demonstrates that raffinose acts as fructosyl acceptor to yield hitherto undescribed oligosaccharides with a DP of 4−5.

HeOS from Maltose and Maltotriose as Fructosyl Acceptors. The levansucrase reaction with sucrose as fructosyl donor and maltotriose or maltose as acceptor yields 1^F-β-fructofuranosylmaltotriose (G_3F) or erlose (1^F-β-fructofuranosylmaltose), respectively, in addition to the FOS series (*12*). In the reaction of the *L. sanfranciscensis* TMW 1.392 levansucrase with 0.4 M sucrose (fructosyl donor) and 0.4 M maltotriose or maltose (fructosyl acceptors), 1-kestose and possibly nystose, which coelutes with maltose, were present (**Figure 4A,B**). The major HeOS in reactions with maltose eluting at 35 min was attributable to erlose. Oligomers with a DP or 4 or greater that

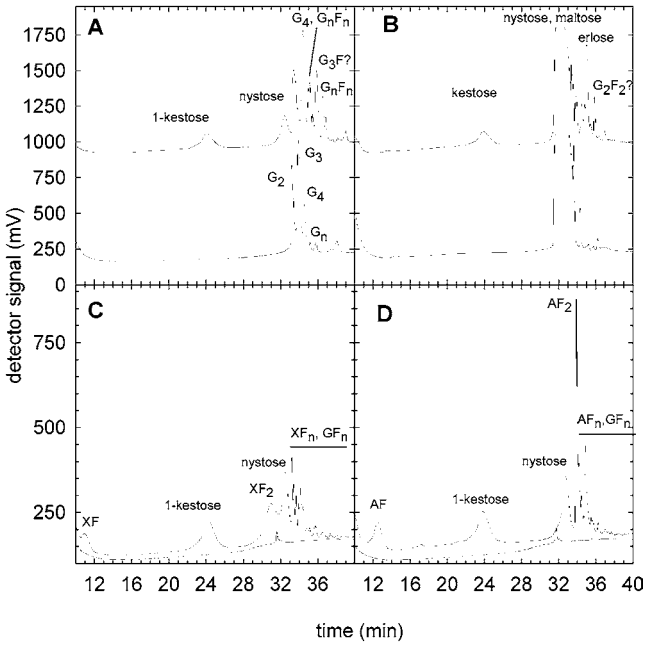

Figure 4. HPAEC–PAD separation of oligosaccharides resulting from the levansucrase reaction with 0.4 mol L^{-1} sucrose as fructosyl donor and 0.4 mol L^{-1} maltotriose (**A**), maltose (**B**), xylose (**C**), or arabinose (**D**) as fructosyl acceptors (upper traces). The same reaction products were separated after hydrolysis of FOS and HeOS by yeast invertase (lower traces). Peaks were assigned based on external standards or literature data on products of levansucrase activity as described in the text. Uncertain assignments are marked with question marks. Glucose, fructose, sucrose, xylose, and arabinose eluted prior to the retention time cutoff at 16 min used to depict oligosaccharide peaks (not shown).

were hydrolyzed by invertase were additionally present (**Figure 4B**). The unambiguous assignment of HeOS from the maltotriose acceptor reaction was not possible because three homologous series of oligosaccharides may have been present: maltooligosaccharides, FOS resulting from the acceptor reaction with sucrose, and additionally the oligosaccharides G_3F_x resulting from the acceptor reaction with maltotriose (**Figure 4A**). This complex mixture of oligomers was not sufficiently separated with our analytical setup. However, all oligomers from the maltotriose acceptor reaction eluting after 35 min were hydrolyzed by invertase treatment (**Figure 4A**) and cannot be assigned to FOS (compare to **Figure 2A**), indicating the presence of hitherto undescribed G_3F_x HeOS. Accordingly, in the monomers from pentasaccharides prepared by GPC (data not shown), a glucose-to-fructose ratio of 2.5:1 was determined. Because the maltotriose preparation used in our experiments contained trace amounts of maltose and maltotetraose (**Figure 4A**, lower trace and data not shown), additional oligosaccharides from maltose and maltotetraose may be present in the reaction products.

HeOS from Xylose or Arabinose as Fructosyl Acceptors. From the levansucrase reaction in the presence of xylose, the products xylsucrose (xylosyl-β-fructofuranoside, XF), 1^F-β-fructosylxylsucrose (XF_2), and di- and trifructosylxylsucrose are obtained, while arabsucrose (arabinosyl-β-fructofuranoside, AF) is produced from arabinose (*9*, *12*, *13*, *23*). In reactions of the *L. sanfranciscensis* levansucrase with xylose and arabinose, xylsucrose and arabsucrose were present in substantial amounts (**Figure 4C,D**), and the major peaks at 31 and 34 min are attributable to fructosylxylsucrose (XF_2) and fructosylarabsucrose (AF_2), respectively. The presence of HeOS resulting from the acceptor reaction with arabinose was substantiated by preparation of oligosaccharides with a DP of 3 or more via GPC. The resulting oligosaccharide preparation was essentially free of mono- and disaccharides as well as kestose and nystose (**Figure 3B**). A comparable oligosaccharide preparation was obtained from acceptor reactions with xylose (data not shown). The ratio of glucose:pentose:fructose in tri, tetra-, and pentasaccharides from reactions with xylose and arabinose were 3:1:5.5 and 2:1:4, respectively, demonstrating the presence of oligosaccharides with a DP of 3 or greater in enzymatic reactions with xylose and arabinose as fructosyl acceptors.

Oligosaccharides Formed during Sourdough Fermentations with *L. sanfranciscensis*. To determine whether HeOS are formed in wheat dough during growth of *L. sanfranciscensis* TMW 1.392, fermentations in wheat dough were carried out in the presence of 10% sucrose. Doughs fermented with *L. sanfranciscensis* TMW 1.392 were compared to doughs fermented with an isogenic levansucrase-negative derivative of this strain, *L. sanfranciscensis* TMW 1.392Δlev. Both strains exhibited growth and acidification kinetics that were virtually identical (data not shown), as was observed previously (*7*). The aqueous extracts of wheat doughs were compared to aqueous extracts treated with yeast invertase to eliminate fructooligosaccharides originating either from the levansucrase reaction or from flour. The chromatograms of dough extracts and invertase-treated dough extracts are shown in **Figure 5**. Major peaks that were present in the doughs fermented with *L. sanfranciscensis* TMW 1.392, but not in doughs fermented with *L. sanfranciscensis* TMW 1.392Δlev, eluted at 12.5, 23.5, 34.0, 34.75, and 35.25 min (**Figure 5A**). Chromatograms of extracts from doughs fermented with TMW 1.392 and TMW 1.392Δlev were virtually identical after treatment with invertase, indicating that all these peaks are indeed attributable to FOS or HeOS (**Figure 5B**). The peaks corresponding to 1-kestose and AF can be assigned on the basis of external standards and the retention times of the respective compounds derived from the enzymatic reactions (**Figure 4D**). The peak eluting at 35.35 min may correpond to erlose, AF_n, or G_3F originating from the acceptor reaction with maltose, arabinose, or maltotriose (**Figure 4A–C**). Because maltose is by far the most abundant carbohydrate in sourdough, the formation of erlose is more likely than that of other HeOS. Low amounts of 1-kestose originating from flour were also present in doughs fermented with *L. sanfranciscensis* TMW 1.392Δlev; nystose coelutes with maltose and therefore cannot be identified unambiguously. FOS or HeOS eluting between 33.5 and 34.75 min could not be assigned to individual compounds because several FOS and HeOS with DP > 3 eluting in that range were generated in enzymatic reactions. Invertase treatments of extracts from doughs fermented with strain TMW 1.392Δlev or TMW 1.392 in the presence of sucrose generated an oligosaccharide eluting at 12 min. This peak was absent in TMW 1.392Δlev fermented doughs but was also oberved in dough extracts from chemically acidified doughs when sucrose was present (data not shown).

DISCUSSION

In this paper we provide evidence for the formation of heterooligosaccharides (HeOS) by *L. sanfranciscensis* TMW 1.392 during growth in sourdough. To identify the various HeOS based on HPAEC–PAD analysis, HeOS were synthesized in a chemically defined system by enzymatic reactions with *L. sanfranciscensis* TMW 1.392 levansucrase in the presence of the corresponding acceptor carbohydrates. Preliminary evidence was provided that hitherto undescribed tri-, tetra-, and pentasaccharides are formed by levansucrase in the acceptor reaction with maltotriose, maltose, arabinose, or xylose.

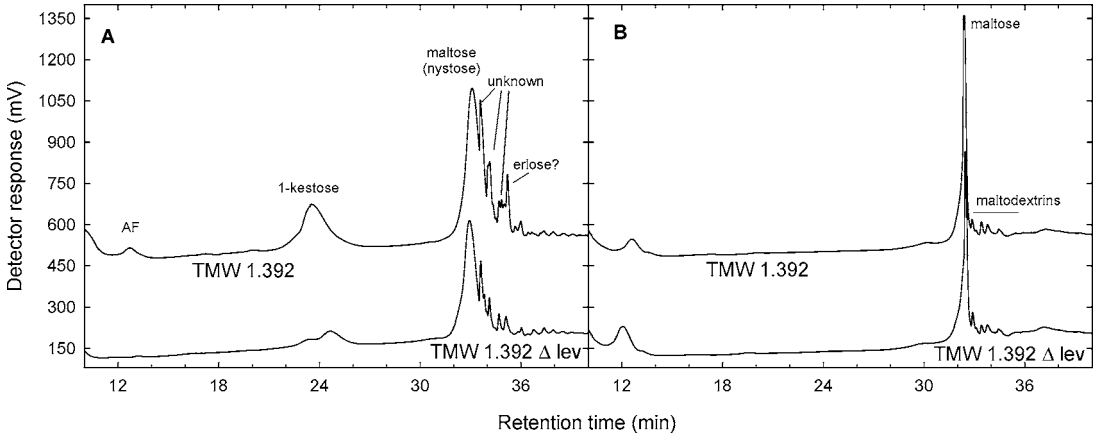

Figure 5. HPAEC–PAD separation of oligosaccharides extracted from wheat doughs. (**A**) Analysis of sourdough aqueous extracts fermented with *L. sanfranciscensis* TMW 1.392 (upper trace) or *L. sanfranciscensis* TMW 1.392Δlev (lower trace). (**B**) Analysis of invertase treated sourdough aqueous extracts fermented with *L. sanfranciscensis* TMW 1.392 (upper trace) or *L. sanfranciscensis* TMW 1.392Δlev (lower trace). Peaks were assigned based on the comparison of the retention times with external standards, and standards derived from enzymatic reactions (**Figure 3**). Uncertain assignments are marked with question marks. Mannitol, glucose, fructose, sucrose, and melibiose eluted prior to the retention time cutoff at 10 min (not shown).

Because external standards for the various HeOS are unavailable, HeOS previously reported as products from transfructosylation were identified by comparison of the products obtained from the levansucrase reaction in the presence and absence of fructosyl acceptors. The HeOS 1^F-β-fructosylraffinose, 1^F-β-fructofuranosylmaltotriose, erlose, xylsucrose, 1^F-β-fructosylxylsucrose, and arabsucrose were characterized from the levansucrase reactions with the corresponding acceptor carbohydrates (*9*, *11*−*13*, *23*). Identical HeOS were found using levansucrase enzymes from the phylogenetically diverse bacteria *Rahnella aquatilis* and *Bacillus subtilis*. The strain designated *Aerobacter levanicum* by Hestrin et al. (*9*) was later reclassified as *Erwinia herbicola* and is now available as *Rahnella aquatilis* ATCC 15552 (*24*). Bacterial levansucrases share a substantial degree of homology in their primary sequences, and the catalytic residues and substrate binding domains are well conserved (*5*, *7*, *25*, *26*). The catalytic properties of the *L. sanfranciscensis* TMW 1.392 levansucrase with respect to substrate specificity, polymer formation, and oligomer formation are in good agreement with kinetic data from other bacterial levansucrases (*7*), and levansucrase products other than HeOS were readily identified by HPAEC−PAD. Therefore, additional products could be identified as HeOS. The major HeOS from the acceptor reactions with *L. sanfranciscensis* levansucrase are expected to be identical to those described previously using levansucrases from other bacterial species. To demonstrate the presence of hitherto undescribed, heterologous tetra- and pentasaccharides, compounds with the appropriate molecular weights were separated from mono-, di-, and trisaccharides by two consecutive GPC runs. The determination of the monomer composition of these oligomers demonstrated for xylose and arabinose as acceptor molecules that HeOS with a DP of 3 or more were present in the HeOS mixture prepared by GPC. In the case of raffinose and maltotriose, HeOS with a DP of >4 were formed. The characterization of these oligomers, however, requires improved analytical methods to achieve the separation of the complex mixture of oligosaccharides generated by the levansucrase enzyme.

The ability of *L. sanfrancsicensis* to form HeOS in wheat sourdough was determined by comparing the oligosaccharide patterns in dough fermented with the isogenic, levansucrase-positive and levansucrase-negative strains TMW 1.392 and TMW 1.392Δlev, respectively. *L. sanfranciscensis* TMW 1.392Δlev is unable to metabolize sucrose because levansucrase is the only enzyme acting on sucrose in the parent strain (*7*). The development of viable cell counts, lactate levels, and pH values during fermentation with these two strains was virtually identical (*7* and data not shown). Therefore, any effect of flour enzymes on the formation or removal of endogenous oligosaccharides was identical in the two doughs, and differences in the levels of oligosaccharides result from levansucrase activity. Indeed, the oligosaccharide levels in these two doughs were identical after removal of FOS and HeOS with yeast invertase. The unidentified carbohydrate generated by treatment of dough extracts with yeast invertase may result from the hydrolysis of FOS originating from flour, or from the transferase activity of invertase (*11*, *27*).

Based on the comparison of oligosaccharides present in extracts from doughs fermented with *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev, at least five FOS or HeOS could be identified that result from levansucrase activity. The formation of 1-kestose was previously reported (*7*), and evidence for the formation of arabsucrose and erlose was provided in this work. Further HeOS in dough remain to be characteirzed. Maltose is the major carbohydrate in either wheat or rye sourdoughs, and is therefore readily available as fructosyl acceptor. Substantial levels of arabinose, xylose, and maltodextrins are additionally present (*28*, *29*). Their levels are enhanced by the use of pentosanases and amylases in bread applications (*30*). Leavening of bread doughs may be achieved through the metabolic activity of heterofermentative lactic acid bacteria in the absence of yeasts (*31*); however, in industrial practice, baker's yeast is the leavening agent most commonly used. Because FOS and HeOS produced in dough by the *L. sanfranciscensis* levansucrase are hydrolyzed by yeast invertase, these compounds are not likely to be present in bread.

In conclusion, the oligosaccharides known to originate from the acceptor reaction of levansucrase with raffinose, maltotriose, maltose, xylose, and arabinose, i.e., 1^F-β-fructosylraffinose, 1^F-β-fructofuranosylmaltotriose, erlose (1^F-β-fructofuranosylmaltose), xylsucrose, 1^F-β-fructosylxylsucrose, and arabsucrose, were identified in enzymatic reactions with the levansucrase of *L. sanfranciscensis* TMW 1.392, sucrose, and the corresponding acceptor carbohydrates. In addition to the FOS 1-kestose and nystose, arabsucrose and erlose were detected in wheat sourdoughs fermented with *L. sanfranciscensis* TMW 1.392. Hitherto undescribed tri-, tetra-, and pentasaccharides that remain to be characterized on the structural level were furthermore

detected in the various acceptor reactions. The formation of HeOS by *L. sanfranciscensis* needs to be taken into account when the sucrose metabolism is studied to optimize the levan synthesis through the levansucrase activity during growth in sourdough. Moreover, improved knowledge on the formation of FOS and HeOS during growth of levansucrase-positive lactobacilli may enable the directed generation of cereal-based, functional ingredients for food and feed use.

LITERATURE CITED

(1) Rhee, S.-K.; Song, K.-B.; Kim, C.-H.; Park, B.-S.; Jang, E.-K.; Jang, K.-H. Levan. In *Biopolymers*; Steinbüchel, A., Baets, S. D., Vandamme, E. H., Eds.; Wiley VCH: Weinheim, Germany, 2002; pp 351−378.

(2) Yun, J. W. Fructooligosaccharides—occurrence, preparation, and application. *Enzyme Microb. Technol.* **1996**, *19*, 107−117.

(3) Simmering, R.; Blaut, M. Pro- and prebiotics—the tasty guardian angels? *Appl. Microbiol. Biotechnol.* **2001**, *55*, 19−28.

(4) Hidaka, H.; Hirayama, M.; Yamada, K. Fructooligosaccharides. Enzymatic preparation and biofunctions. *J. Carbohydr. Chem.* **1991**, *10*, 509−522.

(5) van Hijum, S. A.; van Geel-Schutten, G. H.; Rahaoui, H.; van der Maarel, M. J.; Dijkhuizen, L. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Appl. Environ. Microbiol.* **2002**, *68*, 4390−4398.

(6) Korakli, M.; Pavlovic, M.; Ganzle, M. G.; Vogel, R. F. Exopolysaccharide and kestose production by *Lactobacillus sanfranciscensis* LTH2590. *Appl. Environ. Microbiol.* **2003**, *69*, 2073−2079.

(7) Tieking, M.; Ehrmann, M. A.; Vogel, R. F.; Ganzle, M. G. Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Appl. Microbiol. Biotechnol.* **2005**, in press.

(8) Tieking, M.; Korakli, M.; Ehrmann, M. A.; Ganzle, M. G.; Vogel, R. F. In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *Appl. Environ. Microbiol.* **2003**, *69*, 945−952.

(9) Hestrin, S.; Feingold, D. S.; Avigad, G. The mechanism of polysaccharide production from sucrose. 3. Donor-acceptor specificity of levansucrase from *Aerobacter levanicum*. *Biochem. J.* **1956**, *64*, 340−351.

(10) Monsan, P.; Bozonnet, S.; Albenne, C.; Joucla, G.; Willemot, R.-M.; Remaud-Siméon, M. Homopolysaccharides from lactic acid bacteria. *Int. Dairy J.* **2001**, *11*, 675−685.

(11) Feingold, D. S.; Avigad, G.; Hestrin, S. The mechanism of polysaccharide production from sucrose. 4. Isolation and probable structures of oligosaccharides formed from sucrose by a levansucrase system. *Biochem. J.* **1956**, *64*, 351−361.

(12) Hestrin, S.; Avigad, G. The mechanism of polysaccharide production from sucrose. 5. Transfer of fructose to C-1 of aldose by levansucrase. *Biochem. J.* **1958**, *69*, 388−398.

(13) Tanaka, T.; Yamamoto, S.; Oi, S.; Yamamoto, T. Structures of heterooligosaccharides synthesized by levansucrase. *J. Biochem. (Tokyo)* **1981**, *90*, 521−526.

(14) Korakli, M.; Schwarz, E.; Wolf, G.; Hammes, W. P. Production of mannitol by *Lactobacillus sanfranciscensis*. *Adv. Food Sci.* **2000**, *22*, 1−4.

(15) Sebastien, V.; Brandt, M.; Cavadini, C.; Hammes, W.; Neeser, J.-R.; Waldbüsser, S. Lactobacillus strain producing levan and its use in human or pet food products. International Patent WO 02/50311 A3, 2002.

(16) Korakli, M.; Rossmann, A.; Ganzle, M. G.; Vogel, R. F. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *Lactobacillus sanfranciscensis*. *J. Agric. Food Chem.* **2001**, *49*, 5194−5200.

(17) Brandt, M. J.; Münscher, I.; Hammes, W. P. Einfluss von Laktobazillen aus Sauerteigen auf Weizenteigeigenschaften. *Getreide, Mehl Brot* **2003**, *57*, 15−17.

(18) Campbell, J. M.; Bauer, L. L.; Fahey, G. C.; Hogarth, A. J. C. L.; Wolf, B. W.; Hunter, D. E. Selected fructooligosaccharide (1-kestose, nystose, and 1^F-β-fructofuranosylnystose) composition of foods and feeds. *J. Agric. Food Chem.* **1997**, *45*, 3076−3082.

(19) Stolz, P.; Böcker, G.; Hammes, W. P. Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco*. *Z. Lebensm.-Unters.-Forsch.* **1995**, *201*, 91−96.

(20) Thiele, C.; Gänzle, M. G.; Vogel, R. F. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. *Cereal Chem.* **2002**, *79*, 45−51.

(21) Thiele, C.; Gänzle, M.; Vogel, R. F. Sample preparation of amino acid determination by integrated pulsed amperometric detection in foods. *Anal. Biochem.* **2002**, *310*, 171−178.

(22) Tieking, M.; Kaditzky, S.; Valcheva, R.; Korakli, M.; Vogel, R. F.; Gänzle, M. G. Extracellular homopolysaccharides and oligosaccharides from intestinal lactobacilli. *J. Appl. Microbiol.* **2005**, in press.

(23) Othsuka, K.; Hino, S.; Fukushima, T.; Ozawa, O.; Kanematsu, T.; Uchiuda, T. Characterization of levansucrase from *Rhahnella aquatilis* JCM-1683. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1373−1377.

(24) Cote, G. L.; Ahlgren, J. A. Metabolism in microorganisms part I. Levan and levansucrase. In *Science and technology of fructans*; Suzuki, M., Chatterton, N. J., Eds.; CRC Press: Boca Raton, FL, 1993; pp 141−168.

(25) van Hijum, S. A.; Szalowska, E.; van der Maarel, M. J.; Dijkhuizen, L. Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. *Microbiology* **2004**, *150*, 621−630.

(26) Meng, G.; Fütterer, K. Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. *Nat. Struct. Biol.* **2003**, *10*, 935−941.

(27) Farine, S.; Versluis, C.; Bonnici, P. J.; Heck, A.; Peschet, J. L.; Puigserver, A.; Biagini, A. Separation and identification of enzymatic sucrose hydrolysis products by high-performance anion-exchange chromatography with pulsed amperometric detection. *J. Chromatogr., A* **2001**, *920*, 299−308.

(28) Boskov-Hansen, H.; Andreasen, M. F.; Nielsen, M. M.; Larsen, L. M.; Bach Knudsen, K. E.; Meyer, A. S.; Christensen, L. P.; Hansen, A. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread making. *Eur. Food Res. Technol.* **2002**, *214*, 33−42.

(29) Rouzaud, O.; Martinez-Anaya, M. A. Effect of processing conditions on oligosaccharide profile of wheat sourdoughs. *Z. Lebensm.-Unters.-Forsch.* **1993**, *197*, 434−439.

(30) di Cagno, R.; de Angelis, M.; Corsetti, A.; Lavermicocca, P.; Arnault, P.; Tossut, P.; Gallo, C.; Gobbetti, M. Interactions between sourdough lactic acid bacteria and exogenous enzymes: effects on the microbial kinetics of acidification and dough textural properties. *Food Microbiol.* **2003**, *20*, 67−75.

(31) Gänzle, M.; Häusle, S.; Hammes, W. P. Wechselwirkungen zwischen Laktobazillen und Hefen des Sauerteiges. *Getreide, Mehl Brot* **1997**, *51*, 209−215.

Received for review October 12, 2004. Revised manuscript received January 17, 2005. Accepted January 21, 2005. The work was supported by funds of the German Bundesministerium für Wirtschaft und Technologie (BMWi) via the Arbeitsgemeinschaft industrieller Forschungsvereinigungen "Otto von Guericke" e.V. (AiF), Grant 14037N/2.

JF048307V