Substrate Recognition by Two Members of Glycoside Hydrolase Family 32 Involved in Fructo-oligosaccharide Metabolism in *Lactobacillus acidophilus* NCFM

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(Received Decsmber 6, 2011)

Summary

Two fructo-oligosaccharide active enzymes, BfrA and ScrB, of glycoside hydrolase family 32 are found in *Lactobacillus acidophilus* NCFM and belong to phylogenetic clusters of bacterial β-fructosidase and sucrose 6-phosphate hydrolase, respectively, involved in the intracellular metabolism of kesto-oligosaccharides [β-D-fructofuranosyl-(2,1)]$_n$-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose (n, 1-3) and sucrose. Recombinant ScrB, produced in Escherichia coli, showed 20-1500-fold higher catalytic efficiency (k_{cat}/K_m) toward sucrose than raffinose [α-D-galactopyranosyl-(1,6)-α-D-glucopyranosyl-(1,2)-β-D-fructofuranose] and kesto-oligosaccharides. At high concentration of sucrose (0.4-1.0 M) ScrB catalysed formation of 1-kestose [β-D-fructofuranosyl-(2,1)-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose] in 2.3-15% yield by transglycosylation. Recombinant BfrA in contrast efficiently hydrolysed kesto-oligosaccharides, in particular 1-kestose with 70-300-fold higher k_{cat}/K_m than for sucrose and raffinose and the k_{cat}/K_m values decreased slightly with increasing degree of the polymerization of the kesto-oligosaccharides. Neither BfrA nor ScrB degraded the β-(2,1)- and β-(2,6)-linked fructans, inulin and levan. The outcome of the present detailed specificity analysis of the β-fructosidase and sucrose 6-phosphate hydrolase towards fructo-oligosaccharides combined with modelling of BfrA and ScrB, using the structure of *Thermotoga maritima* β-fructosidase as template and superimposition of β-fructose from a complex with *Aspergillus awamori* exo-inulinase, sucrose and 1-kestose from complexes with *Cichorium intybus* fructan β-(2,1)-fructosidase/1-exohydrolase, suggested that loop 1 in the fifth blade of the characteristic GH32 β-propeller catalytic domain controls the specificity toward 1-kestose. Furthermore, a ScrB His71 is strictly conserved in the first blade of sucrose 6-phosphate hydrolase and is suggested to recognise the phosphate group of the α-glucose 6-phosphate moiety in the substrate sucrose 6-phosphate at subsite +1.

Bull.Facul.Agric.Niigata Univ., 64(2):115-126, 2012

Key words : glycoside hydrolase family 32, β-fructosidase, sucrose 6-phosphate hydrolase, fructo-oligosaccharide, *Lactobacillus acidophilus* NCFM

Lactobacillus acidophilus is a well-known inhabitant of the human gastrointestinal tract and a member of the "acidophilus complex" of six related lactobacilli, *L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri, and L. johnsonii* (Johnson *et al*., 1980 ; Fujisawa *et al*., 1992), which constitute less than 1% of the total gut microbiota (Sghir *et al*., 2000). These bacteria are recognized as probiotics, *i.e.* "live microorganisms that administered in adequate amounts confer health benefits on the host" (Sanders *et al*., 2001 ; Sui *et al*., 2002 ; Reid *et al*., 2003) and have beneficial effects in treatment of gastrointestinal disorders (Sandine *et al*., 1972). Since the isolation of *L. acidophilus* in 1900 (Moro, 1900), numerous investigations of the physiological and biochemical properties resulted in a variety of suggestions for the mechanism of the probiotic effect (Sanders, 1999). Recently the complete genome sequence of *L. acidophilus* NCFM (Gilliland *et al*., 1975) has improved the insight into the relationship between the genetics and probiotic effects (Altermann *et al*., 2005). *L. acidophilus* NCFM is able to use prebiotics, *i.e.* non-digestible food ingredients (Voragen, 1998 ; Barrangou *et al*., 2005) such as plant cell-wall derived carbohydrates (Hartemink *et al*., 1995 ; van Laere *et al*., 2000) resistant to degradation and absorption in the upper intestinal tract. Prebiotics modulate the intestinal microbiota (Sui *et al*., 2002) by selectively stimulating growth of beneficial commensals, which consumes the non-digestible carbohydrates (Benno *et al*., 1987 ; Gibson *et al*., 1995).

Fructo-oligosaccharides (FOS), *e.g.* kesto-oligosaccharides [β-D-fructofuranosyl-(2,1)]$_n$-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose (n= 1-3) derived from inulin, a fructan polymer, are considered as prebiotics (Gibson *et al*., 1995; Orrhage *et al*., 2000 ; Rycroft *et al*., 2001) and not utilised by humans but degraded by bacteria in the gastrointestinal tract (Hartemink *et al*., 1995 ; Hartemink *et al*., 1997 ; van Laere *et al*., 2000 ; Kaplan and Hutkins, 2000). In *L. acidophilus* NCFM the metabolic pathways of FOS including sucrose [β-D-fructofuranosyl-(2,1)-α-D-glucopyranose] were mapped (Fig. 1) based on cDNA microarray and real-time

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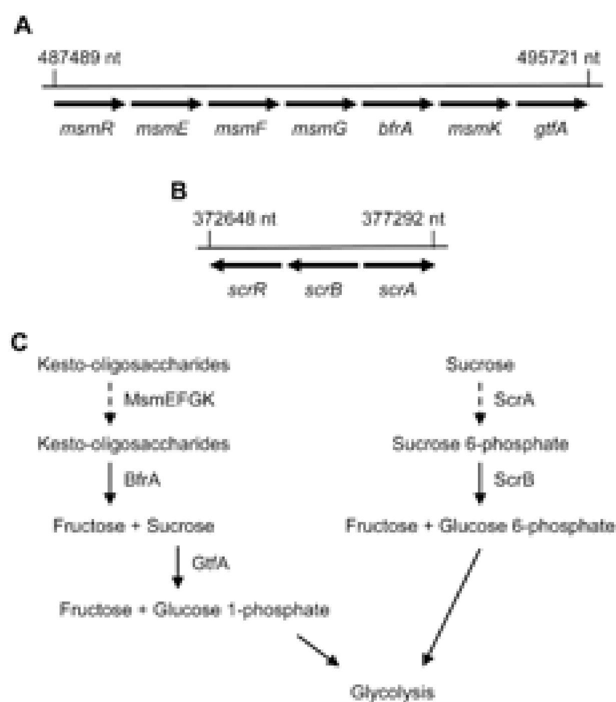

Fig 1. FOS utilization in *Lactobacillus acidophilus* NCFM. Gene loci of gene clusters involved in transport and metabolism of kesto-oligosaccharides (A) and sucrose (B): msmR, transcriptional regulator of LacI family; *msmE*, ABC transporter sugar-binding protein; *msmF* and *msmG*, ABC transporter permeases; *bfrA*, β-fructosidase; *msmK*, ABC transporter ATP-binding protein; *gtfA*, sucrose phosphorylase; *scrR,* sucrose operon repressor of LacI family; *scrB,* sucrose 6-phosphate hydrolase; *scrA*, sucrose PTS transporter. The numbers (nt) indicate the locations of the first bases of *msmR* (A) and scrR (B) and last bases of *gtfA* (A) and *scrA* (B) on the *L. acidophilus* NCFM chromosome (*Altermann et al*., 2005). (C) predicted pathways of transport (dotted arrows) and metabolism (arrows) of FOS in *L. acidophilus* NCFM.

qRT-PCR data (Barrangou *et al*., 2005 ; Barrangou *et al*., 2006). Kesto-oligosaccharides are taken up *via* an ATP-binding cassette (ABC) transporter that includes a sugar-binding protein (MsmEFGK) and subsequently degraded by β-fructosidase (BfrA; EC 3.2.1.26) and sucrose phosphorylase (GtfA; EC 2.4.1.7) (Barrangou *et al*., 2005), whereas sucrose uptake occurs via a phosphoenolpyruvate: sugar phosphotransferase transporter (ScrA) and the sucrose 6-phosphate is hydrolysed intracellularly by sucrose 6-phosphate hydrolase (ScrB; EC 3.2.1.26) (Barrangou *et al*., 2006). The transcription of the kesto-oligosaccharides and sucrose operons is controlled by the regulators MsmR and ScrA, respectively, which belong to the LacI repressor family (Barrangou *et al*., 2005 ; Barrangou *et al*., 2006).

Although the metabolism of FOS in *L. acidophilus* NCFM has been outlined (Barrangou *et al*., 2005 ; Barrangou *et al*., 2006), the BfrA and ScrB involved in the intracellular degradation remain to be characterized in detail. Both are exo-acting glycosidases releasing fructose from substrates with net retention of the anomeric configuration (Reddy and Maley, 1990a ; Reddy and Maley, 1996). They occur widely in bacteria, fungi, and plants (Pons *et al*., 1998) and sequence analysis assigned β-fructosidase (Liebl *et al*., 1998 ; Ehrmann *et al*., 2003 ; Huang *et al*., 2003) and sucrose 6-phosphate hydrolase (Li and Ferenci, 1996 ; Reid *et al*., 1999 ; Bogs and Geider, 2000) to glycoside hydrolase family 32 (GH32; http://www.cazy.org/) (Cantarel *et al*., 2008) together with five fructoside hydrolases; exo-inulinase (EC 3.2.1.80); fructan β-(2,1)-fructosidase/1-exohydrolase (EC 3.2.1.153)]; levanase (EC 3.2.1.65); β-2,6-fructan 6-levanbiohydrolase (EC 3.2.1.64); and fructan β-(2,6)-fructosidase/6-exohydrolase (EC 3.2.1.154). Five fructosyltransferases that catalyse biosynthesis of FOS and fructans by transglycosylation also belong to GH32; sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99); fructan:fructan 1-fructosyltransferase (EC 2.4.1.100); sucrose:fructan 6-fructosyltransferase (EC 2.4.1.10); fructan:fructan 6G-fructosyltransferase (EC 2.4.1.243); and cycloinulo-oligosaccharide fructanotransferase (EC 2.4.1.-). Crystal structures have been solved of different GH32 fructoside hydrolases (Nagem *et al*., 2004 ; Alberto *et al*., 2006 ; Verhaest *et al*., 2007 ; Lammens *et al*., 2008). They possess a five-blade β-propeller fold similar to levansucrase of GH68 (EC 2.4.1.10) (Meng and Fütterer, 2003 ; Martínez-Fleites *et al*., 2005) and belonging to clan GH-J (Henrissat and Davies, 1997). Although different FOS active enzymes in GH32, including β-fructosidase and sucrose 6-phosphate hydrolase, have been described, structural elements involved in specificity, *e.g.* recognition of kesto-oligosaccharides of different degree of polymerization (DP) and of phosphate in sucrose 6-phosphate are not yet identified.

The present study focuses on *L. acidophilus* NCFM GH32 BfrA and ScrB involved in kesto-oligosaccharide and sucrose metabolism and characterises the properties of these enzymes produced in *Escherichia coli*. This allows identification of structural features concerned with substrate specificity in GH32 towards FOS of varying DP using modelled BfrA and ScrB three-dimensional structures based on the *Thermotoga maritima* β-fructosidase structure (Alberto *et al*., 2006) and superimposing β-fructose from a complex with *Aspergillus awamori* exo-inulinase (Nagem *et al*., 2004) or sucrose and 1-kestose [β-D-fructofuranosyl-(2,1)-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose] from complexes with *Cichorium intybus* fructan β-(2,1)-fructosidase/1-exohydrolase (Verhaest *et al*., 2007).

MATERIALS AND METHODS

Materials

Glucose, fructose, raffinose, sucrose, inulin from chicory, and levan from *Zymomonas mobilis* were from Sigma (St. Louis, MO, USA). 1-Kestose, 1,1-kestotetraose, and 1,1,1-kestopentaose were from Megazyme (Bray, Ireland). Other reagents were of analytical grade.

Sequence analysis

Similarity searches were performed using the BLAST version 2.2.18 at the DNA Data Bank Japan website (http://blast.ddbj.nig.ac.jp/top-j.html). The blastp program was used against UniProt/Swiss-Prot+UniProt/TrEMBL database. BLOSUM62 was used as comparison matrix. Phylogenetic analysis using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) comprised full-length amino acid sequences of functionally characterized GH32 enzymes (http://www.cazy.org/GH32.html) (Cantarel *et al.*, 2008); the phylogentic tree was generated with Treeview version 1.6.6 using the bootstrap test based on 1000 resamplings (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Prediction of protein localization and signal peptide was done by PSRT-B version 2.0 (http://www.psort.org) (Gardy *et al.*, 2005) and SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson *et al.*, 2007), respectively.

Cloning, mutagenesis, and construction of expression plasmids

The *bfrA* (GenBank, gi: 58336834) and *scrB* (GenBank, gi: 58336737) were cloned by PCR from genomic DNA of *L. acidophilus* NCFM (ATCC SD5221; Danisco USA Inc, Madison, WI, USA) grown in de Mann, Rogosa and Sharpe medium (Oxoid Ltd., Basingstoke, UK) under anaerobic conditions at 37℃ until late exponential phase. Cells were harvested by centrifugation, washed twice with phosphate-buffered saline, resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0), and highly pure DNA was isolated as described (Apajalahti *et al.*, 1998). Expand High Fidelity PCR System (Roche, Basel, Switzerland) was used as DNA polymerase with oligonucleotide pairs constructed based on the genomic sequence (Altermann *et al.*, 2005): 5′-AAAGAGCTCAAGGCAAATACTTACTTATATAAAAAA-3′ and 5′-AAAGAGCTCGAATGGACAAGAGAAAAACGTTATTTAC-3′ (SacI site is underlined) as 5′ forward primers for *bfrA* and *scrB*, respectively, and 5′-AAAAAGCTTTTACAAATCCACTTTTTTAAGTTCCATTC-3′ and 5′-AAAAAGCTTTTAAAGAATTGTTTTCATATTCCAAAG-3′ (HindIII site is underlined) as corresponding 3′ reverse primers. PCR products were purified (QIAquick Gel Extraction Kit; Qiagen, Germantown, MD), digested by SacI and HindIII (New England Biolabs, Ontario, Canada), and cloned into pCold I (Takara, Kyoto, Japan) resulting in expression plasmids *bfrA*/pCold I and *scrB*/pCold I propagated in E. *coli* DH5a (Novagen, Madison, WI), purified (QIAprep Spin Miniprep Kit; Qiagen), and verified by sequencing (MWG Biotech, Ebsersberg, Germany).

Production of recombinant BfrA and ScrB

E. *coli* BL21(DE3) (Novagen) harbouring *bfrA*/pColdI and *scrB*/pColdI were grown at 12℃ in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/mL ampicillin (6 × 1 L in 2 L shake flasks) and expression was induced by 0.1 mM isopropyl-1-thio-β-galactopyranoside at 12℃ for 24 h. Cells were harvested (9000g, 10 min, 4℃), resuspended in 60 mL BugBuster Protein Extraction Reagents (Novagen) containing 2 μL Benzonate Nuclease (Novagen), incubated 30 min at room temperature, and centrifuged (19000g, 15 min, 4℃). The supernatant was filtered (0.45 μm; GE Infrastructure Water & Process Technologies Life Science Microseparations, Trevose, PA, USA) and applied to HisTrap HP (5 mL; GE Healthcare UK Ltd., Buckinghamshire, England) equilibrated with 20 mM HEPES pH 7.5, 0.5 M NaCl, 10 mM imidazole and washed with 20 mM HEPES pH 7.5, 0.5 M NaCl, 22 mM imidazole. Enzyme-containing fractions eluted by a linear 22-400 mM imidazole gradient in the same buffer were pooled, concentrated (Amicon Ultra Ultracel-10k; Millipore Corporation, Billerica, MA), and applied to HiLoad™ 75 Superdex™ 26/60 column (GE Healthcare, Uppsala, Sweden) equilibrated with 10 mM MES pH 6.8, 0.15 M NaCl. Enzyme-containing fractions were pooled, dialyzed against 10 mM HEPES pH 7.0, and concentrated as above. All purification steps were performed at 4℃. Protein concentration was determined spectrophotometrically at 280 nm using $E^{0.1\%}$ = 2.14 (BfrA) and 1.83 (ScrB) determined by aid of amino acid analysis. The molecular mass was estimated by SDS-PAGE stained with Coomassie Brilliant Blue and by gel filtration (HiLoad™ 200 Superdex™ 16/60 column; flow rate 0.5 mL/min; ÄKTAexplorer; GE Healthcare) in 10 mM MES pH 6.8, 0.15 M NaCl using the Gel Filtration Calibration kit HMW (GE Healthcare)

Standard enzyme assay

BfrA (101-126 nM) and ScrB (0.55-3.3 nM) hydrolysed 120 mM sucrose in 40 mM sodium acetate pH 5.3 and 40 mM sodium phosphate pH 6.3, respectively, for 10 min at 40℃ (60 μL). The reaction was stopped by 2 M Tris-HCl (120 μL) and released glucose was quantified (D-Glucose Assay kit; Megazyme).

Characterization of enzymatic properties

pH optimum of 126 nM BfrA and 0.55 nM ScrB was determined towards 120 mM sucrose in 40 mM Britton-Robinson buffer (Britton and Robinson, 1931) (60 μL; pH 2.4-10.9; 40 mM acetic acid, 40 mM phosphoric acid, and 40 mM boric acid, pH-adjusted by NaOH) at 40℃ for 10 min, stopping the reaction and quantifying liberated glucose as above. Temperature optimum of activity toward 120 mM sucrose at 20-90℃ was determined in 40 mM sodium acetate pH 5.3 for 101 nM BfrA or in 40 mM sodium phosphate pH 6.3 for 3.3 nM ScrB. The pH and temperature stabilities were deduced from residual activity for 1.3 μM BfrA or 5.5 μM ScrB in 90 mM Britton-Robinson buffer (pH 2.2-10.8) incubated at 4℃ for 24 h and for 101 nM BfrA or 17 nM ScrB in 10 mM HEPES pH 7.0 incubated at 20-90℃ for 15 min. Each experiment was made in triplicate.

Hydrolytic activity towards 120 mM sucrose, raffinose, 1-kestose, 1,1-kestotetraose, and 1,1,1-kestopentaose, and 0.2% inulin and levan was measured in 40 mM sodium acetate pH 5.3 for 4.7-470 nM BfrA or in 40 mM sodium phosphate pH 6.3

for 1.6-804 nM ScrB at 40℃ (60 μL). The reaction was stopped after 10 min by 2 M Tris-HCl (120 μL) and released fructose was quantified (D-Glucose and D-Fructose Assay kit; Megazyme). One unit of activity was defined as the amount of enzyme that liberates 1 μmol fructose per min under the above conditions.

Initial rates of hydrolysis of sucrose, raffinose, 1-kestose, 1,1-kestotetraose, and 1,1,1-kestopentaose were measured at 8 different substrate concentrations (10-240 mM sucrose; 10-360 mM raffinose; 1.2-120 mM 1-kestose; 1.0-48 mM 1,1-kestotetraose; 1.2-48 mM 1,1,1-kestopentaose) in 40 mM sodium acetate pH 5.3 for BfrA (1 mL; 24 nM for sucrose, 47 nM for raffinose, 4.7 nM for 1-kestose, 12 nM for 1,1-kestotetraose, 16 nM for 1,1,1-kestopentaose) or in 40 mM sodium phosphate pH 6.3 for ScrB (1 mL; 1.6 nM for sucrose, 20 nM for raffinose, 40 nM for 1-kestose, 402 nM for 1,1-kestotetraose, 402 nM for 1,1,1-kestopentaose) at 40℃. Aliquots (60 μL) were removed at 0, 5, 10, 20, and 30 min, mixed with 2 M Tris-HCl (120 μL), and released fructose was quantified as above. Kinetic parameters (K_m and k_{cat}) were obtained from the initial velocities by fitting the Michaelis-Menten equation (Curve Expert software). Each experiment was made in triplicate.

Fig 2. Phylogenetic tree constructed from deduced full-length amino acid sequences of functionally characterized GH32 enzymes using ClustalW. Values at nodes represent percentage of bootstrap confidence level on 1000 resamplings. Bacterial β-fructosidases: *Ba*BfrA, *Bifidobacterium animalis* subsp. lactis DSM 10140^T (GenBank, gi: 46277678); *Ec*CscA, *Escherichia coli* B-62 (gi: 3462879); *La*BfrA, *Lactobacillus acidophilus* NCFM (gi: 58254151); *Tm*BfrA, *Thermotoga maritima* MSB8 (gi: 4981979); *Zm*SacA, *Zymomonas mobilis* ZM1 (gi: 155616); bacterial sucrose 6-phosphate hydrolases: *Cb*ScrB, *Clostridium beijerinckii* NCIMB 8052 (gi: 4091872); *Ea*ScrB, *Erwinia amylovora* CFBP1430 (gi: 11071663); *Gs*SurA, *Geobacillus stearothermophilus* NUB36 (gi: 1737498); *Kp*ScrB, *Klebsiella pneumoniae* 1033-5P14 (gi: 43934); *La*ScrB, *Lactobacillus acidophilus* NCFM (gi: 33149354); *La*ScrB, *Lactococcus lactis* NIZO R5 (gi: 149490); *Sx*ScrB, *Staphylococcus xylosus* C2a (gi: 288269); bacterial exo-inulinases: *An*LevJ, *Actinomyces naeslundii* T14V (gi: 515690); *Bf*FruA, *Bacteroides fragilis* BF-1 (gi: 143972); *Bs*SacC, *Bacillus subtilis* subsp. *subtilis* strain 168 (gi: 2635149); *Gd*LsdB, *Gluconacetobacter diazotrophicus* SRT4 CBS 550.94 (gi: 6540587); bacterial cycloinulo-oligosaccharide fructanotransferases: *Bc*Cft, *Bacillus circulans* MCI-2554 (gi: 2780736); *Pm*Cft, *Paenibacillus macerans* CFC1 (gi: 12056574); bacterial levanase: *Bs*LevB, *Bacillus* sp. L7 (gi: 2546884); bacterial β-2,6-fructan 6-levanbiohydrolases: *Bs*LevB, *Bacillus subtilis* subsp. *subtilis* strain 168 (gi: 1945681); *Ml*LevM, *Microbacterium laevaniformans* ATCC 15953 (gi: 14289197); fungal β-fructosidase: *Sc*Suc2, *Saccharomyces cerevisiae* S288C (gi: 3834); fungal exo-inulinases: *Aa*Inu1, *Aspergillus awamori* var. 2250 (gi: 14787237); *As*InuE, *Aspergillus niger* strain 12 (gi: 38194174), *Km*Inu1, *Kluyveromyces* marxianus var. *marxianus* ATCC 12424 (gi: 2910); *Pg*Inu1, *Pichia guilliermondii* OUC1 (gi: 190333341); plant β-fructosidases: *At*INV1, *Arabidopsis thaliana* L. Heynh. Ecotype Columbia (gi: 15027839); *Dc*INVDC4, *Daucus carota* L. cv Nantaise (gi: 407078); *Ib*Fruct1, *Ipomoea batatas* L. cv Taiong 57 (gi: 4102864); plant fructan β-(2,1)-fructosidases/1-exohydrolases: *Ci*1-FEHIIa, *Cichorium intybus* var. foliosum cv Flash (gi: 13940209); Ta1-FEHw3, *Triticum aestivum* L. cv Pajero (gi: 41581373); plant fructan β-(2,6)-fructosidases/6-exohydrolases: *At*INV3, *A. thaliana* L. Heynh. Ecotype Columbia (gi: 16974575); *Bv*6-FEH, *Beta vulgaris* L. cv Opus (gi: 38488412); plant sucrose:sucrose 1-fructosyltransferases: *At*1-SST, *Agave tequilana* Weber var. azul (gi: 108735978); *Ac*1-SST, *Allium cepa* L. BMCCB (gi: 3559801); plant sucrose:fructan 6-fructosyltransferase *Hv*6-SFT, *Hordeum vulgare* L. cv Express (gi: 1552333); plant fructan:fructan 6G-fructosyltransferases/fructan:fructan 1-fructosyltransferases: *Ac*6G-FFT, *Allium cepa* L. (gi: 1769832); *Ao*FT1, *Asparagus officinalis* L. cv Zuiyuu (gi: 59796645), *Lp*6G-FFT, *Lolium perenne* L. cv Bravo (gi: 20153218).

Transglycosylation by ScrB

ScrB (804 nM) catalysed transglycosylation of 400-1000 mM sucrose in 40 mM sodium phosphate pH 6.3 (1 mL) at 40℃ as monitored for aliquots (50 μL) removed at 0, 10, 30, 60, 120, and 180 min and added 0.5 M NaOH (500 μL) to stop the reaction. Transglycosylation product and remaining sucrose were quantified from peak areas by high-performance ion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD; ICS-3000 Ion Chromatography System; Dionex, CA, USA) eluted by a linear 0-75 mM sodium acetate gradient in 100 mM NaOH (35 min; flow rate, 0.35 mL/min) using 1-kestose and sucrose as standards. Released fructose and glucose were quantified (as above). Transglycosylation yields were calculated based on sucrose concentrations.

Formation of transglycosylation products for structural analysis

Transglycosylation by 804 nM ScrB of 1 M sucrose in 40 mM sodium phosphate pH 6.3 (1 mL) at 40℃ was stopped after 3 h by heating (15 min, 90℃). The product was desalted (Amberlite MB20; Sigma) and purified by high performance liquid chromatography (HPLC; UltiMate 300; Dionex) equipped with a TSKgel Amide-80 column (4.6 × 250 mm; Tosoh Bioscience, Tokyo, Japan) and refractive index detector (Shodex RI-101; Showa Denko K.K., Kanagawa, Japan) by acetonitrile/water (85:15, v/v) at 70℃ (flow rate, 1.0 mL/min). Fractions containing product were collected (Foxy Jr. Fraction collector; Teledyne Isco, Lincoln, Nebraska), freeze-dried (CoolSafe 55; ScanVac, Lynge, Denmark), and analysed by TLC (TLC Silica gel 60 F_{254}; Merck, Darmstadt, Germany) developed by acetonitrile/water (85:15, v/v), sprayed by α-naphthol/sulphuric acid/methanol (0.03:15:85, w/v/v), and tarred at 120℃.

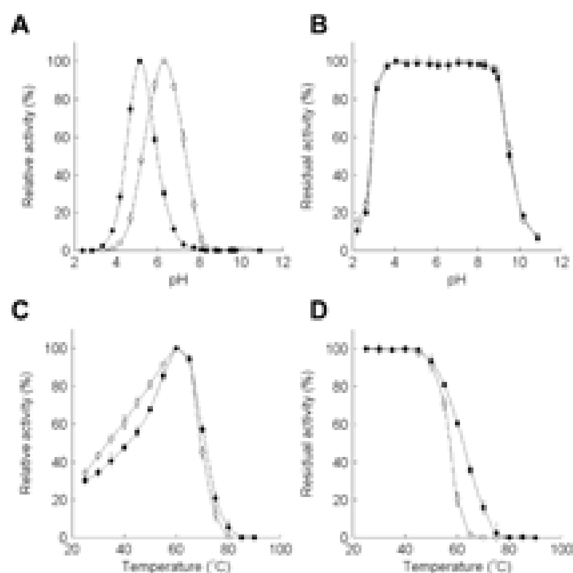

Fig 3. Effect of pH and temperature on activity and stability of BfrA (●) and ScrB (○). (A) pH-dependence for hydrolysis of 120 mM sucrose by 126 nM BfrA and 0.55 nM ScrB in 40 mM Britton-Robinson buffer pH 2.4-10.9. (B) pH-stability of 1.3 μM BfrA and 5.5 μM ScrB in 90 mM Britton-Robinson buffer pH 2.2-10.8. (C) temperature-activity dependence for 101 nM BfrA and 3.3 nM ScrB at 20-90℃ with 10 min reaction. (D) stability of 101 nM BfrA and 17 nM ScrB in the temperature range 20-90℃ for 15 min. Each experiment was made in triplicate. Standard deviations are shown as error bars.

Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS was performed using an LTQ XL Ion Trap MS (Thermo Scientific, San Jose CA, USA) (Westphal *et al*., 2010). Samples were introduced through a Thermo Accela UHPLC system equipped with a Hypercarb column (100 × 2.1 mm, 3 μm; Thermo) eluted with a gradient of acetonitrile and 0.2% (w/v) TFA in millipore water (0.4 mL/min; 70℃) (Westphal *et al*., 2010). In this research 25 μM lithium acetate was added to each eluent resulting in lithium adducts during MS analysis without affecting the separation conditions. MS-detection was performed in positive mode using spray voltage of 4.6 kV, capillary temperature of 260℃ and the mass spectrometer was auto-tuned on glucohexaose (*m/z* 997).

Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectra were recorded (Bruker DRX 600 spectrometer) in 5 mm NMR tubes at 300 K. Relative amounts were obtained by integration of one-dimensional proton spectra. A series of two dimensional homo- and heteronuclear correlated spectra were obtained in Bruker standard COSY, NOESY, TOCSY, HSQC and HMBC experiments. The following parameters were used: acquisition time 0.4 s, NOESY mixing time 0.8 s, 0.12 s TOCSY spinlock, and data points 4096*512 with zero filling in F1 dimension.

Homology modelling of BfrA and ScrB

Structures of BfrA and ScrB were modelled by SWISS MODEL (http://swissmodel.expasy.org/) (Kiefer *et al*., 2009) using the crystal structure of *T. maritima* β-fructosidase (PDB ID: 1W2T) (Alberto *et al*., 2006) as template. β-Fructose from a complex with *Aspergillus awamori* exo-inulinase (1Y9G) (Nagem *et al*., 2004), sucrose and 1-kestose from complexes with *Cichorium intybus* fructan β-(2,1)-fructosidase/1-exohydrolase (2ADD and 2AEZ, respectively) (Verhaest *et al*., 2007) were superimposed onto the modelled BfrA and ScrB structures using PyMOL (http://www.pymol.org/).

RESULTS AND DISCUSSION

Prediction of enzymatic function of BfrA and ScrB

The deduced amino acid sequences encoded by *bfrA* and *scrB* (GenBank, gi: 58254151 and 33149354, respectively; Fig. 1A,B) showed 9-45% sequence identity with functionally characterised GH32 fructoside hydrolases and fructosyltransferases (Cantarel *et al*., 2008). Phylogenetic

Table 1. Kinetic parameters for hydrolysis of sucrose, raffinose, and kesto-oligosaccharides by BfrA and ScrB.

Substrate	BfrA			ScrB		
	K_m (mM)	k_{cat} (s^{-1})	$k_{cat} \cdot K_m^{-1}$ ($s^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	$k_{cat} \cdot K_m^{-1}$ ($s^{-1} \cdot mM^{-1}$)
Sucrose	57 ± 5.9	95 ± 2.6	1.6	31 ± 1.6	1105 ± 27	35
Raffinose	155 ± 5.9	62 ± 0.58	0.40	–	–	1.6
1-Kestose	6.4 ± 0.44	726 ± 9.7	113	–	–	1.6
1,1-Kestotetraose	4.3 ± 0.01	368 ± 9.2	85	–	–	0.16
1,1,1-Kestopentaose	5.2 ± 0.37	391 ± 18	75	–	–	0.023

Kinetic parameters were calculated from initial velocities of release of fructose at different substrate concentrations (see 2.6.).

analysis clearly showed BfrA and ScrB in clusters of GH32 bacterial β-fructosidase and sucrose 6-phosphate hydrolase, respectively (Fig. 2). Neither BfrA nor ScrB possessed a signal peptide as predicted by PSRT-B (Gardy *et al*., 2005) and SignalP (Emanuelsson *et al*., 2007). The bioinformatic analysis thus supports the function of BfrA and ScrB as β-fructosidase and sucrose 6-phosphate hydrolase, respectively, involved in the intracellular catabolism of kesto-oligosaccharides and sucrose 6-phosphate transported into *L. acidophilus* NCFM (Fig. 1C).

Production and purification of recombinant BfrA and ScrB

Recombinant BfrA and ScrB were produced in *E. coli* BL21(DE3) and purified from 6 L cultures in yields of 2.2 and 73 mg, respectively. The molecular masses were estimated by SDS-PAGE to 52 and 57 kDa, respectively, consistent with theoretical values of 51603 and 57496 Da. Gel filtration showed BfrA and ScrB to be monomers similarly to other GH32 fructoside hydrolases (Thompson *et al*., 1991 ; Thompson *et al*., 1992 ; Liebl *et al*., 1998 ; Huang *et al*., 1998 ; Arand *et al*., 2002 ; van den Ende *et al*., 2003) and fructosyltransferases (Shiomi *et al*., 1981 ; Sprenger *et al*., 1995 ; Fujishima *et al*., 2005), although dimeric (Schmid *et al*., 1982 ; Reddy *et al*., 1990b ; Rouwenhorst *et al*., 1990 ; Li and Ferenci, 1996) and tetrameric (Rouwenhorst *et al*., 1990) GH32 enzymes have also been reported.

Enzymatic properties of BfrA and ScrB

BfrA and ScrB catalysed hydrolysis of sucrose with pH optimum 5.3 and 6.3, respectively (Fig. 3A) and were stable (>95% residual activity) at pH 3.6-8.9 and 4℃ for 24 h (Fig. 3B). Both BfrA and ScrB showed maximum activity at 55℃ (Fig. 3C) and retained >95% activity during 15 min incubation up to 40℃ at the pH optimum (Fig. 3D).

ScrB hydrolysed 120 mM sucrose with 10-fold higher specific activity (554 U/mg) than BfrA (56.3 U/mg) at the pH optimum and 40℃ . BfrA and ScrB catalysed cleavage of the β -fructoside linkage in the sucrose moiety of raffinose [α-D-galactopyranosyl-(1,6)-α-D-glucopyranosyl-(1,2)-β-D-fructofuranose] as found for other GH32 β-fructosidases (Liebl *et al*., 1998 ; Ehrmann *et al*., 2003 ; Huang *et al*., 2003) and sucrose 6-phosphate hydrolases (Schmid *et al*., 1982 ; Reid *et al*., 1999). Both enzymes hydrolysed a series of kesto-oligosaccharides [β-D-fructofuranosyl-(2,1)]n-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose (n, 1-3)], but did not hydrolyse inulin [β-(2,1)-linked] or levan [β-(2,6)-linked], two polymer substrates for exo-inulinase (Arand *et al*., 2002 ; Moriyama *et al*., 2003), fructan β-(2,1)-fructosidase/1-exohydrolase (van den Ende *et al*., 2001 ; van Riet *et al*., 2008), levanase (Miasnikov, 1997), β-2,6-fructan 6-levanbiohydrolase (Song *et al*., 2002 ; Daguer *et al*., 2004), and fructan β-(2,6)-fructosidase/6-exohydrolase (van den Ende *et al*., 2003 ; de Coninck *et al*., 2005).

Specificity of BfrA and ScrB towards FOS

The present detailed kinetic analysis for GH32 β-fructosidase and sucrose 6-phosphate hydrolase acting on kesto-oligosaccharides of different DP is the first thorough analysis for FOS-active enzyme from a probiotic inhabitant of the human gastrointestinal tract. The catalytic efficiency (k_{cat}/K_m) for BfrA and ScrB hydrolysis of kesto-oligosaccharides decreased with increasing DP (Table 1). BfrA thus gave a value of 113 s-1・mM-1 for 1-kestose, being 71- and 283-fold higher than for sucrose and raffinose, respectively. This emphasised the importance for BfrA of the recognition of fructose and glucose residues at subsites +1 and +2, respectively (see 3.5.).

Whereas this decreasing k_{cat}/K_m values for kesto-oligosaccharides with increasing DP and the poor binding and affinity for sucrose and raffinose was in accordance with three functionally important subsites (-1, +1, and +2) at the active site of BfrA, ScrB largely prefers sucrose rather than the kesto-oligosaccharides, despite a high K_m value for sucrose as found also for several other GH32 sucrose 6-phosphate hydrolases (Schmid *et al*., 1982 ; Rouwenhorst *et al*., 1990 ; Thompson *et al*., 1991 ; Thompson *et al*., 1992 ; Li and Ferenci, 1996 ; Bogs and Geider, 2000). Noticeably, however, up to 1000-fold lower K_m values were reported for sucrose 6-phosphate hydrolases from *E. coli* (Schmid *et al*., 1982), *Fusobacterium mortiferum* (Thompson *et al*., 1992), *Klebsiella pneumonia* (Thompson *et al*., 2001), and *Lactococcus lactis* (Thompson *et al*., 1991) using sucrose 6-phosphate rather than sucrose as substrate. This indicates that the

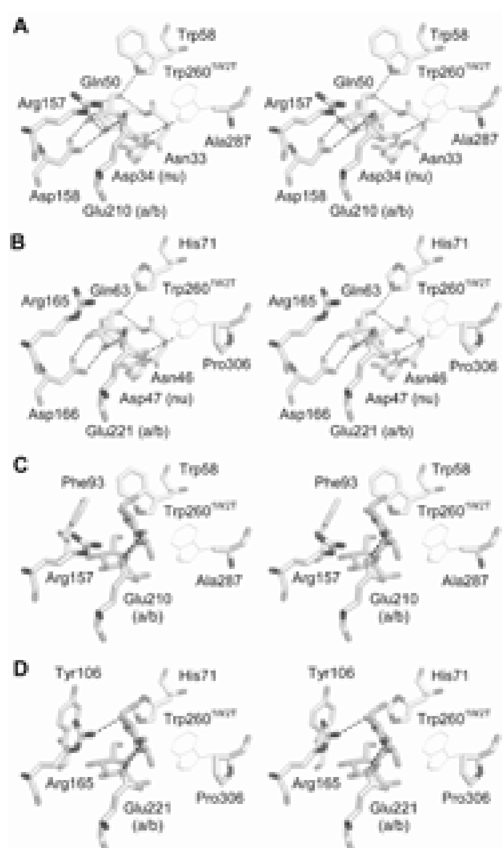

Fig 4. Stereo views of β-fructose and sucrose interactions at subsite -1 (A, B) and +1 (C, D) in active sites of BfrA and ScrB. (A, B) β-fructose from the complex of *A. awamori* exo-inulinase (PDB ID: 1Y9G) superimposed on the BfrA (A) and ScrB (B) structures modelled using *T. maritima* β-fructosidase (PDB ID: 1W2T) as template. Asp34 and Glu210 for BfrA and Asp47 and Glu221 for ScrB are predicted to be nucleophile (nu) and acid/base catalyst (a/b), respectively, as indicated by sequence alignment with S. cerevisiae β-fructosidase. (C, D) sucrose from the complex of *C. intybus* fructan β-(2,1)-fructosidase/1-exohydrolase (PDB ID: 2ADD) superimposed on the modelled BfrA (C) and ScrB (D) structures. Hydrogen bonds (< 3.0 Å) are shown as dotted lines.

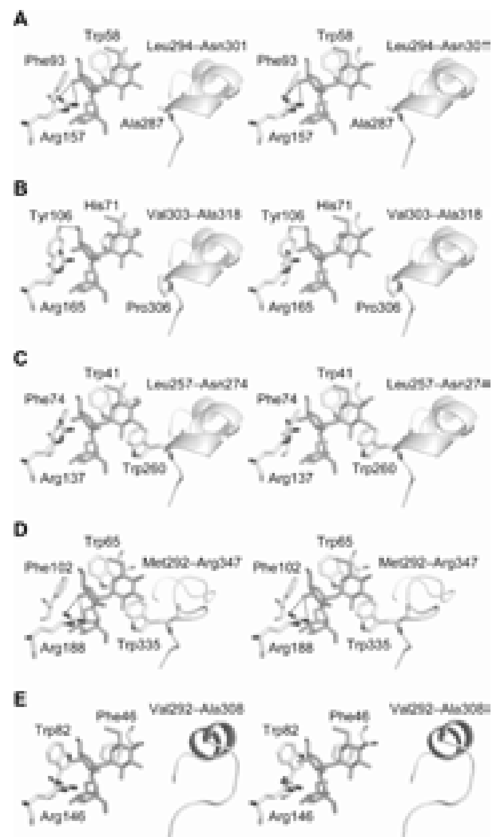

Fig 5. Structural elements of GH32 hydrolases involved in specificity toward 1-kestose. 1-Kestose from the complex of *C. intybus* fructan β-(2,1)-fructosidase/1-exohydrolase (E; PDB ID: 2AEZ) superimposed on modelled structures of BfrA (A) and ScrB (B) and on the crystal structures of *T. maritima* β-fructosidase (C; PDB ID: 1W2T) and *A. awamori* exo-inulinase (D; PDB ID: 1Y9G). Loop 1 in the fifth blade of the catalytic β-propeller-fold domain of each of the enzymes is shown as ribbon representation (to the right).

phosphate group of the α-glucose 6-phosphate moiety in sucrose 6-phosphate is an important determinant of substrate recognition by GH32 sucrose 6-phosphate hydrolases (see 3.5.). The k_{cat}/K_m decreased 22-1500-fold for raffinose and kesto-oligosaccharides compared to sucrose, due to the high K_m estimated to be > 500 mM. The preference of sucrose rather than raffinose was also reported for *E. coli* sucrose 6-phosphate hydrolase (Schmid *et al.*, 1982).

Substrate recognition by BfrA and ScrB

Structural elements involved in the distinctly different specificity of BfrA and ScrB (Table 1) and for several other FOS-active enzymes of GH32 (Cantarel *et al.*, 2008) remain to be identified. To disclose such specificity determinants three-dimensional structures of BfrA and ScrB were modelled with the *T. maritima* GH32 β-fructosidase (Alberto *et al.*, 2006) as template, having 30 and 22% sequence identity (50 and 40% similarity) to BfrA and ScrB, respectively. To better illustrate substrate recognition at subsite -1, β-fructose from a complex with *A. awamori* GH32 exo-inulinase (Nagem *et al.*, 2004) was superimposed onto the modelled BfrA (Fig. 4A) and ScrB (Fig. 4B) structures. In both enzymes several direct hydrogen bonds seem to be formed to β-fructose at subsite -1. This would probably involve Asn33, Asp34, Gln50, and Asp158 of BfrA and Asn46, Asp47, Gln63, and Asp166 of ScrB, which are strictly conserved in GH32. Moreover, of the other well-conserved residues, BfrA Arg157 and ScrB Arg165, only BfrA Arg157 Nε was within appropriate hydrogen bonding distance (2.8 Å) to the 3-OH of β-fructose in these models,

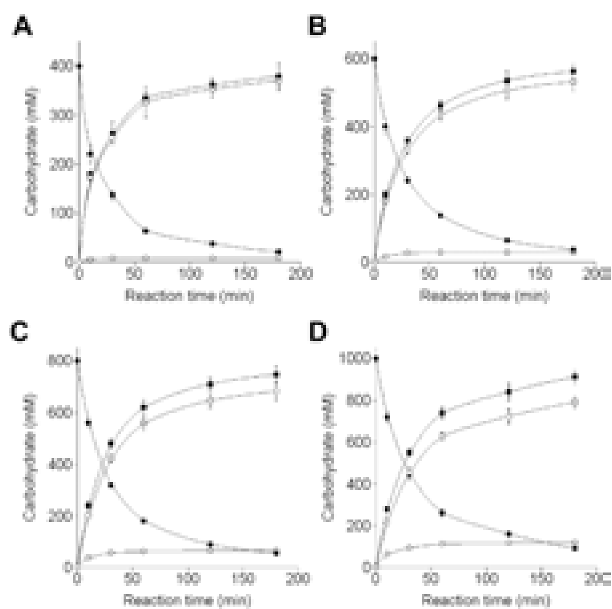

Fig 6. Progress of transglycosylation catalysed by ScrB with sucrose as substrate. ScrB (804 nM) and different concentration of sucrose: 0.4 M (A), 0.6 M (B), 0.8 M (C), 1.0 M (D) in 40 mM sodium phosphate pH 6.3 reacted at 40℃ . (●) sucrose, (○) 1-kestose, (□) fructose, (■) glucose.

and also in the structures of β-fructose complexes with *A. awamori* exo-inulinase (Nagem *et al*., 2004) and *C. intybus fructan* β-(2,1)-fructosidase/1-exohydrolase (Verhaest *et al*., 2007). In the present ScrB structural model, however, the longer distance of 3.6 Å between Arg165 Nε and 3-OH of β-fructose makes hydrogen bond formation doubtful. Noticeably, neither BfrA nor ScrB seems to have a hydrogen bond interaction with 1-OH of β-fructose, which was observed for Nε1 of Trp260 in the *T. maritima* β -fructosidase, at the same position as the differing residues, Ala287 in BfrA and Pro306 in ScrB. However also Trp335 Nε1 in *A. awamori* GH32 exo-inulinase forms this hydrogen bond with 1-OH of the β-fructose (Nagem *et al*., 2004), whereas Trp residues were predicted to suppress binding of α-glucosyl moiety from 1-kestose at subsite +2 (see below).

Focusing on the different preference for kesto-oligosaccharides and sucrose by BfrA and ScrB, respectively (Table 1), sucrose and 1-kestose in structures of complexes with *C. intybus* fructan β-(2,1)-fructosidase/1-exohydrolase (Verhaest *et al*., 2007) were superimposed onto the modelled BfrA (Fig. 4C,5A) and ScrB (Fig. 4D,5B) structures. In the ScrB model hydrogen bonds are suggested to be formed with 3-OH and 4-OH of the α-glucosyl moiety in sucrose by Glu221 Oε2 and Arg165 NH2, respectively (Fig. 4D). Only Glu210 Oε2 in BfrA appears to hydrogen bond with the 3-OH group (Fig. 4C), which supports the observed higher catalytic efficiency toward sucrose of ScrB compared to BfrA (Table 1). BfrA Arg157 thus seemed unable to hydrogen bond with 4-OH of α-glucose in sucrose (3.9 Å), but may form hydrogen bonds with 4-OH of the β-fructosyl moiety in 1-kestose at subsite +1 (Fig. 5A). In case of ScrB, steric clashing between the Arg165 and 4-OH of the β-fructosyl moiety at subsite +1 may prevent binding of 1-kestose (Fig. 5B). These oligosaccharide enzyme interactions are in agreement with the higher and lower k_{cat}/K_m values of BfrA and ScrB, respectively, for 1-kestose compared to sucrose (Table 1). Furthermore, in loop 1 (Leu294-Asn301; Fig. 5A) and (Val303-Ala318; Fig. 5B), situated between β1 and β2 in the fifth blade of the catalytic five-bladed β-propeller domain, Ala287 of BfrA and Pro306 of ScrB make space for binding of the α-glucosyl moiety in 1-kestose at subsite +2, whereas the corresponding Trp260 in *T. maritima* β-fructosidase and Trp335 in *A. awamori* exo-inulinase which hydrolyse inulin (Liebl *et al*., 1998 ; Arand *et al*., 2002), spatially block for binding of the α-glucosyl moiety in 1-kestose (Fig. 5C,D). Noticeably, *C. intybus* fructan β-(2,1)-fructosidase/1-exohydrolase that hydrolyses 1-kestose as well as inulin (de Roover *et al*., 1999), has spatially no counterpart of Trp260 in *T. maritima* β-fructosidase and its loop 1 (Val292-Ala308) seems able to provide space for binding of 1-kestose (Fig. 5E).

The Trp58 of BfrA is well-conserved in GH32 and predicted to be involved in hydrophobic stacking at subsite +2 onto the α-glucosyl moiety in 1-kestose (Fig. 5A). However, this position has a His in sucrose 6-phosphate hydrolases and a Phe in fructan β-(2,1)-fructosidase/1-exohydrolase. While this Phe is also predicted to participate in a stacking interaction (Fig. 5E) similar to the BfrA Trp58, ScrB His71 Nδ1 conserved in sucrose 6-phosphate hydrolase (Fig. 5B) is at a distance of about 5 Å from the 6-OH of the α-glucosyl moiety in sucrose at subsite +1 (Fig. 4D). These observations made on the structural models suggest that the conserved His in GH32 sucrose 6-phosphate hydrolases has a key role in the recognition of the phosphate group of the α-glucose 6-phosphate moiety in sucrose 6-phosphate.

Transglycosylation catalysed by ScrB

The poor hydrolytic activity of ScrB for kesto-oligosaccharides (Table 1) together with the modelled structure (Fig. 5B) motivated attempts of accumulation of these oligosaccharides from sucrose by transglycosylation. ScrB was thus found to catalyse formation of an isomer of β-fructosyl-sucrose in 15% yield from 1.0 M sucrose after 180 min reaction (Fig. 6), which gave *m/z* of 511 by ESI-MS (for $C_{18}H_{32}O_{16} + Li^+$). The product yield increased from 2.3, 5.1, 8.0 to 15% produced from 0.4, 0.6, 0.8, and 1.0 M sucrose. ^{1}H- and ^{13}C-NMR spectra indicated formation of a single product identified to β-D-fructofuranosyl-(2,1)-β-D-fructofuranosyl-(2,1)-α-D-glucopyranose (1-kestose; Table 2). ScrB thus catalysed transglycosylation with strict β-(2,1)-regioselectivity similarly to *Triticum aestivum* β -fructosidase (van den Ende *et al*., 2009), whereas *Xanthophyllomyces dendrorhous* β-fructosidase was reported to have dual β-(2,6)- and β-(2,1)-regioselectivity (Linde *et al*., 2009). The 1-kestose produced by ScrB accumulated without degradation, when sucrose remained in the reaction mixture, due to the very

Table 2. ^{1}H and ^{13}C NMR data assignment for 1-kestose produced from sucrose as substrate by transglycosylation catalysed by ScrB.

	Chemical shifts (δ, p.p.m.)						
	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
β-Fru*f*-(2→1)-	3.66 61.0	 104.3	4.15 77.2	4.04 75.0	3.82 81.7	3.78 62.9	3.74
β-Fru*f*-(2→1)-	3.73 61.5	 103.9	4.23 77.2	4.00 74.4	3.82 81.7	3.76 62.8	3.76
α-Glc*p*	5.39 93.1	3.49 71.7	3.71 73.2	3.43 69.8	3.80 73.0	3.76 60.7	3.76

low hydrolytic activity of ScrB for 1-kestose (Table 1). 1-Kestose is not utilised by humans, but degraded by probiotic bacteria including *L. acidophilus* NCFM (Hartemink *et al*., 1995 ; van Laere *et al*., 2000 ; Orrhage *et al*., 2000 ; Rycroft *et al*., 2001) and considered to be able to exert a prebiotic effect by selectively stimulating growth of probiotic bacteria (Gibson *et al*., 1995 ; Kaplan and Hutkins, 2000 ; Hartemink *et al*., 1997).

ACKNOWLEGEMENT

This work was supported by the Danish Strategic Research Council's Committee on Food and Health (FφSu, to the project "Biological Production of Dietary Fibres and Prebiotics" no. 2101-06-0067 and to the project "Gene discovery and molecular interactions in prebiotics/probiotics systems. Focus on carbohydrate prebiotics" no. 2102-07-0105), the Center for Advanced Food Studies (LMC), and the Carlsberg Foundation.

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乳酸菌由来糖質加水分解酵素ファミリー32タンパク質の基質認識機構の解明

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（平成23年12月6日受付）

要　約

乳酸菌 *Lactobacillus acidophilus* NCFM でのフルクトオリゴ糖代謝に関与する２つの糖質加水分解酵素（BfrA, *β*- フルクトシダーゼ; ScrB, スクロース6- リン酸ハイドロラーゼ）の基質認識機構を明らかにすることを目的とし、各種組み換え酵素を用いてフルクトオリゴ糖に対する加水分解反応の速度論的解析を行った。ScrB はフルクトオリゴ糖よりスクロースに対して高い加水分解活性を示し、BfrA は短鎖なフルクトオリゴ糖に対して特異性を示すことを明らかにした。加えて、両酵素の基質特異性に関与する領域（BfrA ループ１）およびアミノ酸残基（ScrB His71）を、ホモロジーモデリング法により推定した。

新大農研報, 64(2):115-126, 2012

キーワード：グルコシドハイドロラーゼファミリー32, *β*- フルクトシダーゼ , スクロース6- リン酸ハイドロラーゼ , *Lactobacillus acidophilus* NCFM

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