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Structure-function relationships of bacterial and enzymatically produced reuterans and dextran in sourdough bread baking application

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ABSTRACT

Exopolysaccharides from lactic acid bacteria may improve texture and shelf life of bread. The effect of exopolysaccharides on bread quality, however, depends on properties of the EPS and the EPS producing strain. This study investigated structure-function relationships of EPS in baking application. The dextransucrase DsrM and the reuteransucrase GtfA were cloned from *Weissella cibaria* 10M and *Lactobacillus reuteri* TMW1.656, respectively, and heterologously expressed in *Escherichia coli*. Site-directed mutagenesis of GtfA was generates reuterans with different glycosidic bonds. NMR spectrum indicated reuteranPI, reuteranNS and reuteranPINS produced by GtfA-V1024P:V1027I, GtfA-S1135N:A1137S and GtfA-V1024P:V1027I:S1135N:A1137S, respectively, had a higher proportion of α-(1 → 4) linkages when compared to reuteran. ReuteranNS has the lowest molecular weight as measured by asymmetric flow-field-flow fractionation. The reuteransucrase negative mutant *L. reuteri* TMW1.656Δ*gtfA* was generated as EPS-negative derivative of *L. reuteri* TMW1.656. Cell counts, pH, and organic acid levels of sourdough fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA* were comparable. Reuteran produced by *L. reuteri* TMW1.656 during growth in sourdough and reuteran produced *ex situ* by GtfA-ΔN had comparable effects on bread volume and crumb hardness. Enzymatically produced dextran improved volume and texture of wheat bread, and of bread containing 20% rye flour. ReuteranNS but not reuteranPI or reuteran was as efficient as dextran in enhancing wheat bread volume and texture. Overall, reuteran linkage type and molecular weight are determinants of EPS effects on bread quality. This study established a valuable method to elucidate structure-function relationships of glucans in baking applications.

1. Introduction

Sourdough improves texture, flavour, shelf life, and nutritional properties of baked and steamed bread (Gänzle et al., 2007; Gänzle, 2014; Liu et al., 2016). These beneficial effects are related to the metabolism of lactic acid bacteria (LAB) during sourdough fermentation. Exopolysaccharides (EPS) produced by LAB increase bread volume, decrease bread firmness, and function as prebiotics (Galle et al., 2010; Kaditzky et al., 2008; Katina et al., 2009; Rühmkorf et al., 2012; Tieking and Gänzle, 2005). Because of their beneficial effects on bread quality, EPS from LAB may replace or reduce the use of hydrocolloids as bread improvers (Galle and Arendt, 2014).

Most EPS produced in sourdough fermentation are high molecular weight polymers composed of glucose (glucan) or fructose (fructan). Among these EPS, α-(1 → 6) linked dextrans have been regarded as the most promising bread improvers. Dextran from *Weissella cibaria* 10M decreased firmness and improved freshness of sorghum bread (Schwab et al., 2008); however, levan produced by *Lactobacillus reuteri* in the same bread formula showed no effect on bread quality. Reuteran, an α-(1 → 4) and α-(1 → 6) linked glucan produced by *L. reuteri*, and dextran from *W. cibaria* MG1 also exhibited differential effect on wheat bread quality (Galle et al., 2012a). It remains unclear, however, whether differences relate to functional differences of the EPS, or to other differences between the EPS producing strains. For example, *L. reuteri* but not *W. cibaria* produce high levels of acetate in presence of sucrose (Galle et al., 2010; Schwab et al., 2008). Thus, different acidification levels may confound the beneficial impact of *in situ* produced EPS. To assess the effect of EPS that differ in linkage type and molecular weight on sourdough bread quality, it is necessary to eliminate the differential impact of EPS producing strains on bread quality as confounding factor.

Reuteran and dextran are synthesized by the glycoside hydrolase family 70 (GH70) enzymes reuteransucrase and dextransucrase, respectively. Glucansucrases catalyze the alternative reactions sucrose hydrolysis, EPS synthesis, and oligosaccharide synthesis when suitable acceptor sugars are present (Korakli and Vogel, 2006, van Hijum et al., 2006). Glucansucrases harbor of four distinct domains, a signal peptide, an N variable region, a catalytic domain and a C-terminal domain

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(Monchois et al., 1999). Sequence alignments, site directed mutagenesis and three-dimensional structure analysis identified the catalytic sites of glucansucrases, namely the catalytic nucleophile, the acid/base catalyst, and the transition-state stabilizer (van Hijum et al., 2006). Linkage specificity is determined by several catalytic residues. The amino acids V1024, V1027 in nucleophile region, and S1135, N1136, A1137 in the transition-state stabilizer region (GtfA of *L. reuteri* TMW1.656 numbering) determined the linkage specificity of glucansucrases (Kralj et al., 2005; van Leeuwen et al., 2008). Knowledge on the amino acids that determine glucan structure thus allows the generation of mutant glucansucrases producing glucans differing in linkage type and/or molecular weight.

This study aimed to investigate structure-function relationships of reuteran produced from *L. reuteri* TMW1.656 and dextran produced from *W. cibaria* 10M in baking application. The primary structure of reuteransucrase was modified to produce reuterans with different linkage types and molar masses, and the effect of these EPS on bread quality was determined. Moreover, the effect of *in situ* produced reuteran on bread quality was compared to the effect of reuteran used as additive.

2. Material and methods

2.1. Bacteria strains, plasmids, media and growth condition

L. reuteri TMW1.656 was cultivated anaerobically at 37 °C in modified DeMan-Rogosa-Sharpe (mMRS) medium (Stolz et al., 1995) containing either 10 g/L maltose, 5 g/L glucose and 5 g/L fructose, or 100 g/L sucrose. *W. cibaria* 10M was cultivated anaerobically at 30 °C in the same medium. *Escherichia coli* strains TOP10 (Invitrogen, Toronto, ON, Canada), *E. coli* XL1 Blue (Agilent Technologies, Santa Clara, CA, USA) containing plasmids pUC18, pUC19 (Thermo scientific, Burlington, ON, Canada) were cultivated aerobically at 37 °C in LB (BD, Mississauga, ON, Canada) medium with 50 mg/L ampicillin. *E. coli* strain BL21 Star (DE3) (Invitrogen) with plasmid pET28a$^+$ (Novagen, Etobicoke, ON, Canada) was cultivated aerobically at 37 °C in LB broth with 50 mg/L kanamycin (Invitrogen) for the purpose of expression *dsrM*, *gtfA* and derived mutant genes.

2.2. General molecular techniques

Bacterial DNA was isolated with DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada). Bacterial plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen). DNA was amplified by PCR using PfuUltra High-Fidelity DNA polymerase (Agilent Technologies). DNA fragments were purified from agarose gel using MinElute Gel Extraction Kit (Qiagen). Cloning, *E. coli* transformation, DNA manipulations and agarose gel electrophoreses were performed as described (Green and Sambrook, 2012). Restriction endonuclease (Thermo Scientific, Ottawa, Canada) digestion and DNA ligation with T4 ligase (Thermo Scientific) were performed following procedures provided by the suppliers. Chromosomal and plasmid DNA were sequenced by service of Macrogen (Macrogen, Rockville, MD, USA). Nucleotide and amino acid sequences analysis was performed using DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA).

2.3. Glucansucrase gene cloning and plasmid construction

The reuteransucrase *gtfA* in *L. reuteri* TMW1.656 was identified in the genome of that strain by nucleotide blast with *gtfA* from *L. reuteri* TMW1.106 (EF189716) as query sequence. GtfA from *L. reuteri* TMW1.656 is identical to GtfA from *L. reuteri* TMW1.106. For construction of the expression plasmid, *gtfA* without the *N*-terminal variable region was cloned into two fragments with a site silent mutation to inactive NcoI restriction site and ligated into pUC19 plasmid. pUC19-GtfA-ΔN-2571 and GtfA-ΔN-616 were ligated after the digestion at BamHI and PaeI sites to allow the generation of pUC19-GtfA-ΔN. pE-GtfA-ΔN was constructed by ligating GtfA-ΔN with pET28a$^+$ plasmid at NcoI and NotI sites after digestion (Table 1). To simplify further mutagenesis, pUC18-gtfA1500 was constructed by ligating pUC18 plasmid and 1500 bp catalytic domain of *gtfA* at PstI and KpnI sites (Table 1).

The dextransucrase gene *dsrM* from *W. cibaria* 10M was amplified by using primer pair (DSRf 5′-TTACCAAGTGAACAACGTGCAA-3′ and DSRr 5′ TTAAAWCGTCACCAACGTACC-3′); these primers were designed based on the alignment the dextransucrase genes from *W. cibaria* LBAE-K39 (GU237484), *W. cibaria* CMU (EU885339), *W. cibaria* CH2 (CP012873), *W. cibaria* TN610 (HE8118409) and *L. fermentum* strain Kg3 (AY697433). The amplified fragment was sequenced (KU363982). The signal peptide truncated *dsrM* was cloned into pUC18 plasmid (Table 1). DsrM-ΔSP was ligated with pET28a$^+$ plasmid at BamHI and NotI sites.

2.4. Amino acid sequence alignment and site-directed mutagenesis of GtfA

The amino acid sequence of GtfA was aligned together with 8 glucansucrases and 3 GtfA mutants with known catalytic preferences by using DNAMAN software (Lynnon Biosoft) (Table S1 of the online supplementary material). Catalytic residues with putative glucosidic bond preference were identified. QuikChange II Site Directed Mutagenesis Kit (Agilent Technologies) was used to construct site-directed mutants in pUC18-gtfA1500 (Table 1). Successful mutants resulted in the generation or elimination of restriction sites, followed by sequence confirmation. The desired mutant pUC18-gtfA1500 was ligated into the corresponding site of pUC19-GtfA-ΔN after the digestion by PstI and FspI restriction endonucleases. Whole sequence of the mutated GtfA-ΔN was ligated into pET28a$^+$ plasmid to generate GtfA-ΔN-V1024P:V1027I, GtfA-ΔN-S1135N:A1137S and GtfA-ΔN-V1024P:V1027I:S1135N:A1137S.

2.5. Heterologous expression and purification of glucansucrases

DsrM-ΔSG, GtfA-ΔN and its mutant derivatives were expressed and purified as described previously (Kralj et al., 2011). The purity of glucansucrases was determined by SDS-PAGE (Bio-Rad, Mississauga, ON, Canada).

2.6. Recombinant enzyme characterization

Protein concentration was determined by using protein assay reagent (Bio-Rad) with bovine serum albumin as standard. Enzyme activities of reuteransucrase GtfA-ΔN and mutant enzymes were evaluated essentially as described (Kralj et al., 2004). In brief, enzyme activities were determined in 25 mM sodium acetate buffer (pH = 4.7) containing 1 mM $CaCl_2$, 100 mM sucrose, and 1 μM enzyme. Samples were taken in 5 min intervals and the reaction was stopped by heating to 95 °C for 10 min. The concentration of glucose and fructose was determined enzymatically (Glucose and Fructose Assay Kit, Sigma-Aldrich, Oakville, ON, Canada). The amount of free glucose represents hydrolysis activity and the amount of fructose represents the total enzyme activity. Transferase activity was calculated as difference between total and hydrolysis activity. One unit of enzyme activity was defined as the release of 1 μmol of monosaccharides per min.

2.7. Analysis of acceptor reaction products

Oligosaccharides were synthesized from acceptor reaction of 1 μM of GtfA-ΔN or mutant derivatives incubated with 500 mM sucrose and 500 mM maltose in reaction buffer for 24 h. Samples were analyzed by HPAEC-PAD with a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada) (Galle et al., 2010). Fructose, glucose, sucrose, maltose, maltotriose and panose were identified by using external standards (Sigma Aldrich).