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Microbial sucrose isomerases: Producing organisms, genes and enzymes

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ABSTRACT

Sucrose isomerase (SI) activity is used industrially for the conversion of sucrose into isomers, particularly isomaltulose or trehalulose, which have properties advantageous over sucrose for some food uses. All of the known microbial SIs are TIM barrel proteins that convert sucrose without need for any cofactors, with varying kinetics and product specificities. The current analysis was undertaken to bridge key gaps between the information in patents and scientific publications about the microbes and enzymes useful for sucrose isomer production.

This analysis shows that microbial SIs can be considered in 5 structural classes with corresponding functional distinctions that broadly align with the taxonomic differences between producing organisms. The most widely used bacterial strain for industrial production of isomaltulose, widely referred to as "*Protaminobacter rubrum*" CBS 574.77, is identified as *Serratia plymuthica*. The strain producing the most structurally divergent SI, with a high product specificity for trehalulose, widely referred to as "*Pseudomonas mesoacidophila*" MX-45, is identified as *Rhizobium* sp.

Each tested SI-producer is shown to have a single SI gene and enzyme, so the properties reported previously for the isolated proteins can reasonably be associated with the products of the genes subsequently cloned from the same isolates and SI classes. Some natural isolates with potent SI activity do not catabolize the isomer under usual production conditions. The results indicate that their industrial potential may be further enhanced by selection for variants that do not catabolize the sucrose substrate.

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1. Introduction

Sucrose (α-D-glucopyranosyl-1,2-D-fructofuranose) is the world's most abundant disaccharide. It is produced by essentially all plants, where it has a vital role as the transport carbohydrate and sometimes also as a storage carbohydrate. Enjoyment of its sweet taste has been innate in humans for tens of thousands of years, possibly arising much earlier as an indicator of safety and nutritional value in foods [1]. Today it is harvested and purified by humans on a scale around 5×10^8 tonnes per annum, predominately for use in foods and as a feedstock for fermentative production of fuel ethanol. Chemically, sucrose is the unique non-reducing (α-1,2) disaccharide from glycosidic linkage of glucose and fructose. Several structural isomers are commonly present at low levels in honey and in environments where sucrose is available, including various sucrose-containing foods. These natural sucrose isomers involve glycosidic bonds between the anomeric carbon of glucose and non-anomeric carbons of the fructose moiety, resulting in five reducing disaccharides with individual chemical properties that for some applications may be advantageous over sucrose [2].

The isomers leucrose (α-1,5), maltulose (α-1,4) and turanose (α-1,3) are formed as occasional side-products of polyglucan-synthesising or starch-hydrolysing reactions [3–5]. Some microbes convert sucrose with remarkable yields into the structural isomers isomaltulose (palatinose™, α-1,6) and trehalulose (α-1,1), possibly to sequester the sugar in a form that confers an advantage against competing species [6–9]. For humans, the sucrose isomers are nutritional sugars that combine the sweet taste profile of sucrose with potential benefits for consumers including acariogenicity and slower digestion into monosaccharides. The isomers have greater acid stability than sucrose, and they differ in properties including solubility that are important for use in foods and as substrates for conversion to other biomaterials [10–15].

Use of sucrose isomers is currently limited by the expense of microbial or enzymatic conversion from more abundant, plant-derived sucrose [16]. Sucrose isomerase (SI) enzymes can convert dissolved sucrose to isomers without any need for cofactors, and the conversion can be remarkably complete because of the multi-step mechanism and the lower free energy of the isomers [9,17–20]. The known SI enzymes from various microbes are all in the same structural family (TIM-barrel family 13 glycoside hydrolases) but they vary in kinetic efficiency and in product ratios, ranging from fairly specific isomaltulose (IM) or trehalulose (TH) synthases to

Abbreviations: IM, isomaltulose; SI, sucrose isomerase; TH, trehalulose.

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mixed-isomer producers and hydrolases producing mostly hexoses from sucrose under certain conditions. The enzymes with highest reported product specificity are from *Pantoea dispersa* UQ68J (91% IM and 3% TH yield from sucrose at 30–35 °C; [8]) and "*Pseudomonas mesoacidophila*" strain MX-45 (91% TH and 8% IM yield from sucrose at 20 °C; [21]). Most industrial production is catalysed by SI in fixed cells of "*Protaminobacter rubrum*" strain CBS574.77 [2], one of the earliest organisms investigated for this capability [22,23].

Initially, scientific insight into SIs came from studies of isolated microbes and their (partially) purified enzymes [21,22,24–35]. Subsequently, the genes encoding several SIs were cloned and sequenced [7,9,36–41]. Because of potential industrial utility, substantial characterization of SIs has been disclosed in the patent literature. This has not always been followed by validation in the peer-reviewed scientific literature and there are many gaps in integration across these literatures. For example, despite recent detailed studies of several cloned SI gene products to X-ray crystal structure level [19,42–44]: (i) the identity of the "archetypical" SI-producing organisms is obscure as the currently applied names have no valid taxonomic standing and (ii) it has not been formally established whether the previously characterized (but not sequenced) SI proteins correspond with the products of the subsequently cloned SI genes from the same species. These are important questions in regulatory (safety and patent) contexts and for the scientific community to build appropriately on earlier biochemical characterization of enzymes that were purified from the native SI-producing isolates.

The aim of this article is to integrate and extend information from the patent and journal literature about diversity of SI genes and enzymes from microbes. In order to do this, we tested the environment for useful SI activity beyond the existing classes of SI enzymes; we used rRNA sequence analysis to establish the unresolved identity of key SI-producing organisms; and we used peptide sequencing and gene inactivation to establish the relationship between the enzymes in the foundation literature and the genes in subsequent studies. Finally, we explored the potential for strain improvement from the best natural isolates.

2. Materials and methods

2.1. Reference bacterial strains

"*P. rubrum*" strain CBS 574.77 [36] was obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. "*P. mesoacidophila*" strain MX-45 [21] was obtained from the International Patent Organism Depository, Tsukuba, Japan. *P. dispersa* UQ68J is as described [45].

2.2. Environmental screening, culture media and SI assays

Screening for additional microbes that can produce sucrose isomers was done using the media and assays described by Wu and Birch [45] with variations including enrichment for TH metabolism. Samples from diverse environments including plant tissues, soils and residues in sugar mills (typically about 0.2 g) were suspended into 2 mL of sterile water or buffer (0.05% v/v Tween 20 in 20 mM $NaPO_4$ pH 7.0). For enrichments, some soils were pre-incubated after seeding with perlite beads soaked in 4% (w/v) TH (produced by reaction to completion using MX-45 cells on sucrose substrate). Alternatively, samples were added to minimal medium (MM) supplemented with 0.5% (w/v) yeast extract and 50 μg/mL cycloheximide plus either 50 mM IM, 50 mM leucrose, 4% (w/v) TH or 40% (w/v) sucrose. Samples were incubated with shaking at 28 °C until cultures became cloudy (typically 2 days), then streaked to single colonies on plates of LB medium amended with 10% sucrose (SLB). Morphologically different colonies were grided onto fresh plates and grown in 1 mL SLB cultures to provide cells for SI assays. The cells from fresh overnight cultures were harvested by centrifugation, suspended in 400 μL citrate–phosphate buffer pH 6 containing 10% (w/v) sucrose, and incubated with shaking at 28 °C for 8 h. The cells were then removed by centrifugation and the sugar composition of the supernatant was analysed by HPAEC-PAD.

2.3. DNA sequencing and analysis and PCR primers

To eliminate ambiguity from incomplete or conflicting published sequences, the full SI genes were sequenced from genomic DNA of strains CBS 574.77 and MX-45. For selected isolates from environmental screening, a central portion of the SI gene was amplified using degenerate primers:

SI_F2_Sal: 5′-GATACSGTRGCKACYTWNTC-3′ or
SI_ F3_Sal: 5′-GGCGTDTCHRGHHTRCGNTTTGATAC-3′ and
SI_R2-Sal: 5′-CGCCATTGYGGNSDRTCRTC-3′.

The amplified products were directly sequenced using the Australian Genome Research Facility, to identify groups with near-identical sequences, of which one representative was advanced for complete double stranded sequencing after cloning into a pGEM-T vector. Sequences were aligned using Clustal W (http://align.genome.jp/). Pairwise comparisons were made using Needle (www.ebi.ac.uk/Tools/emboss/align).

16S rDNA was amplified from genomic DNA using primers:
16SF: 5′-AGAGTTTGATCCTGGCTCAG-3′ and
16SR: 5′-GGTTACCTTGTTACGACTT-3′.

PCR specificity was checked by running a small aliquot of PCR reaction on a gel. The sequences were subject to comparative analysis at the Ribosomal Database Project site (http://rdp.cme.msu.edu/); to identify strains within the phylogenetically consistent bacterial taxonomy proposed by Garrity et al. [46]

2.4. Enzyme sequencing

Native SI enzymes were isolated from strains CBS 574.77 and MX-45 as detailed below. Automated Edman degradation was used for N-terminal amino acid sequence determination (Applied Biosystems). Tandem mass spectrometric (MS) analysis of endoproteinase-cleaved, purified SI enzyme was used to obtain further sequence. Sixty micrograms (approximately 1 nmol) of SI was dissolved in 60 μL of 25 mM NH_4HCO_3 pH 7.8, for cleavage by endoproteinase Glu–C (Protease V8 sequencing grade, Roche); or in 100 mM NH_4HCO_3 pH 8.5 for cleavage by trypsin (Proteomics sequencing grade, Sigma). One microlitre of a 1 μg/μL protease stock was added to the protein (1:60 w/w) and incubated at either 37 °C (trypsin) or 25 °C (Glu–C) for 16 h. The digests were analyzed by HPLC–MALDI-MS/MS and HPLC–ESI-MS/MS at the Molecular and Cellular Proteomics Mass Spectrometry Facility, UQ Institute for Molecular Biosciences, using ProteinPilot™ 2.0.1 software (Applied Biosystems). Leucine and isoleucine alternatives were generated manually for non-redundant fragments with >90% sequence confidence and similarity with sequences in the GenBank 'nr' and 'pat' databases were identified by conducting BLAST searches using the BLASTP algorithm.

2.5. Site-specific disruption of the SI genes in selected bacteria

Insertional mutagenesis was achieved by homologous recombination of a suicide vector into the bacterial genome at a site within the SI gene of strains CBS 574.77, MX-45, *Klebsiella planticola* UQ14S, and *Erwinia rhapontici* WAC2928, using methods described previously [47,48]. For example in the case of CBS 574.77, primers 5′-TTCGGATCCCAACCCTTGCTTAACG-3′ and
5′-ACGAATTCCGTAACCATTATCCGTGTTCGG-3′ were used to amplify a 246 bp fragment of the SI gene, which was cloned (using the underscored restriction sites) into pJP5603 to create suicide construct pJPPRSI1, then transferred via *Escherichia coli* S17-1 (λ*pir*) into CBS 574.77. Transconjugants selected on kanamycin-supplemented M9 medium were screened for SI activity.

Insertional disruption was verified by PCR using primers outside of the targeted insertion site, for example using primers 5′-TCAAGGATTGAAAACTGCACTAGCG-3′ and 5′-TTGGGTTGACCTTGAACCAAGG-3′ for CBS 574.77. Southern blot analysis was used to determine copy number of integrations. For example, genomic DNA from CBS 574.77 wild type and SI^- mutants was digested using *Eco*RI, *Bam*HI and *Kpn*I, which do not cut within the probed region of the SI gene (and which do not cut more than once in the entire SI gene). Digested DNA was gel electrophoresed, transferred to Hybond N^+ membrane and probed with ^{32}P-labelled pJP5603.

2.6. Confirmation of the SI activity of the target gene in CBS 574.77

The gene targeted for insertional disruption in CBS 574.77 was cloned and expressed in *E. coli*. The full-length gene including the periplasmic signal sequence was cloned into the expression vector pET17b (Novagen) and transferred into *E. coli* BL21 (DE3). Recombinant *E. coli* were sub-cultured from an overnight culture, grown for 3 h, then induced using IPTG and grown for a further 1.5 h. CBS 574.77 was cultured the same way except IPTG induction was omitted. Cells were harvested by centrifugation and mixed with 25% sucrose in citrate–phosphate buffer (pH 6.0), at a cell density that achieved near-complete removal of the sucrose during 2 h incubation with gentle shaking at 30 °C. The cells were then removed by centrifugation and the sugar composition of the supernatant was analysed by HPAEC-PAD.

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