Levansucrase from *Halomonas smyrnensis* AAD6^T: first halophilic GH-J clan enzyme recombinantly expressed, purified, and characterized

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Abstract

Fructans, homopolymers of fructose produced by fructosyltransferases (FTs), are emerging as intriguing components in halophiles since they are thought to be associated with osmotic stress tolerance and overall fitness of microorganisms and plants under high-salinity conditions. Here, we report on the full characterization of the first halophilic FT, a levansucrase from *Halomonas smyrnensis* AAD6^T (*Hs*Lsc; EC 2.4.1.10). The encoding gene (*lsc)*

was cloned into a vector with a 6xHis Tag at its C-terminus, then expressed in *Escherichia coli*. The purified recombinant enzyme (47.3 kDa) produces levan and a wide variety of fructooligosaccharides from sucrose, but only in the presence of high salt concentrations (> 1.5 M NaCl). *Hs*Lsc showed Hill kinetics and pH and temperature optima of 5.9 and 37 °C, respectively. Interestingly, *Hs*Lsc was still very active at salt concentrations close to saturation (4.5 M NaCl) and was selectively inhibited by divalent cations. The enzyme showed high potential in producing novel saccharides derived from raffinose as both fructosyl donor and acceptor and cellobiose, lactose, galactose, and L-arabinose as fructosyl acceptors. With its unique biochemical characteristics, *Hs*Lsc is an important enzyme for future research and potential industrial applications in a world faced with drought and diminishing freshwater supplies.

Keywords

Levansucrase
Fructosyltransferase
Halophilic enzyme
Halomonas smyrnensis
Levan
Fructan

Electronic supplementary material

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Introduction

Levansucrases (EC 2.4.1.10) are enzymes that catalyze the formation of levan polymers and fructooligosaccharides (FOSs) by cleaving sucrose and transferring the resulting fructose moiety to acceptor saccharides. Together with inulosucrases (EC 2.4.1.9), they belong to the family 68 of glycoside hydrolases (GH68), which all contain a five-bladed β-propeller structure harboring the catalytic triad at the bottom of the active site cavity (Lammens et al. 2009). Levan and levan-type FOSs contain a terminal glucose residue while the rest of the molecule is comprised of fructose moieties with β-2,6 bonds and occasional branches at β-2,1 positions. Due to its wide acceptor specificity, levansucrase can produce a variety of sucrose derivatives, but it can also fructosylate trisaccharides (such as lactosucrose, erlose, raffinose) and phenolic compounds

(Li et al. 2015b), which makes it a highly interesting enzyme for both research and industrial applications.

Levan polysaccharide has been known and well-studied for over a century, and there is a vast number of reports on its microbial and enzymatic production (for a recent review, Toksoy Öner et al. 2016). Levansucrases from various bacteria such as *Bacillus megaterium* (Strube et al. 2011), *Bacillus subtilis* (Meng and Fütterer 2003), *Erwinia amylovora* (Wuerges et al. 2015), *Gluconacetobacter diazotrophicus* (Martínez-Fleites et al. 2005), *Pseudomonas syringae* (Visnapuu et al. 2015), and *Zymomonas mobilis* (Santos-Moriano et al. 2015) have been reported and characterized in depth; however, all are mesophiles, meaning they all originate from environments with mild conditions in temperature, salinity, pH, etc. It was not until 2009 when the halophilic bacterium *Halomonas smyrnensis* AAD6^T (=DSM 21644 =JCM 15723) was isolated from a saltern in Turkey and reported as the first levan-producing halophile (Poli et al. 2009), and since then, *Halomonas* levan and its chemical derivatives have been the subject of various high-value applications ranging from laser deposited surfaces (Axente et al. 2014) to drug delivery systems (Osman et al. 2017; Sezer et al. 2017) and tissue engineering (Avsar et al. 2018; Gomes et al. 2018). The whole genome sequence of *H. smyrnensis* AAD6^T was determined (Diken et al. 2015) and used to identify critical network elements related to levan production to improve the metabolic capacity of the cells via genome-scale mathematical models (Ates et al. 2013; Aydin et al. 2018). Besides many mesophilic levan producers, *Halomonas* cultures carry significant industrial potential owing to their advantages such as high yields and eliminating the need for aseptic production under high salinity (Chen and Jiang 2018; Kazak Sarilmiser et al. 2015). In fact, recently, a cost-effective *Halomonas* levan production process was developed using the advantage of halophilicity under non-sterile conditions and, with this halophilic microbial process, *H. smyrnensis* AAD6^T exhibited the highest reported sucrose conversion efficiency ever for levan production in submerged cultures (Erkorkmaz et al. 2018).

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Not only halophiles but also halophilic enzymes (or, in short, halozymes) are of great industrial and scientific interest since they are active at very high salt concentrations and display unique physicochemical characteristics, which may lead to the discovery of novel enzymatic applications (DasSarma and DasSarma 2012; Yin et al. 2015). Considering that halozymes may be linked to survival and overall fitness (Versluys et al. 2018) under water-restricted environments as that no information is available on halophilic fructosyltransferases (FTs), studies

were initiated to investigate the kinetic and biochemical characteristics of levansucrase enzyme of *H. smyrnensis* AAD6^T (*Hs*Lsc). In this article, recombinant production and purification of levansucrase from *H. smyrnensis* AAD6^T and its detailed biochemical characterization are discussed elaborately, with special emphasis put on its unique properties. Since this enzyme is the first extensively characterized levansucrase functioning under extreme salt concentrations, this work sets a cornerstone in halozyme research.

Materials and methods

Plasmids, bacterial strains, media, and cloning details

Complete genome sequence of *H. smyrnensis* AAD6^T was deposited to GenBank (accession number AJKS02000000), containing a levansucrase-encoding gene (*lsc*; NCBI accession number: KC480580.1) (Diken et al. 2015). The *lsc* gene was amplified by polymerase chain reaction (PCR) on 1 µg of genomic DNA in a reaction mixture volume of 50 µl. This mixture contained (final concentrations): *Pfu* pol amplification buffer 1X (Promega), dNTP (0.2 mM), both oligonucleotide primers (0.5 mM), and *Pfu* polymerase enzyme (0.05 U/µl) (Thermo Scientific). The amplification conditions were 2 min denaturation at 95 °C in an Automatic Temperature Programmer (MinicyclerTM, MJ Research Inc., USA), followed by 40 cycles with 1 min at 95 °C, 1 min at 55 °C, and 1.5 min extension at 72 °C, with a final extension of 5 min at 72 °C.

Forward and reverse primer sequences were 5′-TCGCCCTTCATCATGAGTATCCAAGATAAG-3′ and 5′-TCGCGCCTGTCGACTTTGGTTACCACATTG-3′ (restriction sites for *Bsp*HI and *Sal*I are underlined). The PCR band of 1250 bp from the *lsc* gene was inserted in the *Nco*I-*Xho*I sites of the expression vector pET28a (Novagen) under the control of the T7 RNA polymerase promoter. The resulting construct (plasmid pALS238) was introduced into competent *Escherichia coli* DH5α cells, and the transformed colonies were selected on Luria-Bertani (LB) agar supplemented with kanamycin (75 µg/ml). The identity of plasmid pALS238 recovered from kanamycin-resistant colonies was confirmed by DNA sequencing. Plasmid pALS238 was then introduced into competent *E. coli* BL21(DE3) cells, and the transformed colonies were selected on LB agar supplemented with 2% (*w*/*v*) glucose and kanamycin (75 µg/ml).

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Recombinant expression and purification of *Hs*Lsc

E. coli BL21(DE3) containing the plasmid pALS238 was grown in 10 ml of LB medium at 37 °C until OD_{600} reached ~ 0.4. This culture (2 ml) was used to

inoculate 200 ml of LB medium in 1-l Erlenmeyer flasks, and incubated for around 3 h at 37 °C in a rotatory shaker with agitation at 180 rpm. When OD_{600} reached ~ 0.7, expression of *lsc* was induced via the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Cells were incubated at 26 °C and 180 rpm overnight. A bacterial culture without IPTG was used as negative control.

For the purification of recombinant *Hs*Lsc, cells were pelleted via centrifugation at 10,000×*g* and 4 °C for 20 min, and then resuspended in 20 mM phosphate buffer with 300 mM NaCl (pH 6.6). The suspension was kept on ice and cells were disrupted by ultrasonication (30% of nominal power, 30 s on, 30 s off, 30 cycles). Cell debris was centrifuged at 10,000×*g* and 4 °C for 20 min, and the resulting supernatant was subjected to immobilized metal affinity chromatography (IMAC; Ni-NTA Superflow, QIAGEN) for the purification of *Hs*Lsc fused to the hexa-histidine tag at the C-terminus.

Equilibration, wash, and elution buffers used for IMAC contained 10, 20, and 300 mM imidazole, respectively, in 20 mM phosphate buffer with 300 mM NaCl. pH values of all buffers were set to 7.4. IMAC slurry was packed in a glass column and equilibrated with 10 column volumes (CV) of equilibration buffer. Supernatant containing *Hs*Lsc was mixed with the equilibration buffer (1:1) prior to loading to the column. A peristaltic pump was used to provide a constant flow rate (around 175 cm^3/h). At the second step, the column was washed with 10 CV of wash buffer to elute proteins with non-specific binding. For the last step, 10 CV of elution buffer was added to the column. Fractions of 10 ml were collected and analyzed for their total protein concentrations and enzymatic activities.

Protein and enzyme activity assays

The Bradford method was used with Bio-Rad Protein Assay Dye Reagent Concentrate for the determination of total protein amount (Bradford 1976). Bovine serum albumin was used as the standard. Protein concentrations of collected IMAC fractions were determined by measuring their absorbance at 280 nm and calculating the corresponding protein concentration via the molar extinction coefficient for recombinant *Hs*Lsc (A_{280} value of 1 g/l of *Hs*Lsc in water is equal to 1.56, according to ExPASy ProtParam, Gasteiger et al. 2005). To determine the enzyme activity, fractions were incubated in 50 mM phosphate buffer (pH 6.0) with 0.15 M sucrose and 2.5 M NaCl at 37 °C for 10 min, with a final total protein concentration of 20 µg/ml. Activity of the enzyme was terminated by boiling reaction mixtures for 5 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol reducing

sugars in one minute. Concentration of the released reducing sugars was determined via DNS method (Miller 1959).

SDS-PAGE analysis

To determine the purity and molecular weight of *Hs*Lsc, protein fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A Bio-Rad Mini Protean Tetra System was used following a modified version of the protocol developed by Laemmli (1970). Acrylamide concentrations of stacking and separating gels were 5 and 12%, respectively. Protein bands were visualized with Coomassie Brilliant Blue R-250. PeqGOLD Protein Marker I (VWR™, UK) was used as the protein ladder.

Effects of temperature, pH, and various compounds on enzyme activity

To determine the optimum temperature values for enzyme activity and levan formation, enzymatic reactions were carried out at different temperatures (4, 15, 30, 37, 50, and 55 °C). Levan formation was determined via measuring the absorbance at 405 nm. To evaluate the effect of pH, enzymatic reactions were carried out at 14 different pH values (pH 3.6–5.4, sodium acetate buffer; pH 6.0–7.5, potassium phosphate buffer; pH 8.0–8.5, Tris buffer; pH 9.0–10.1, glycine buffer; all buffer concentrations were 50 mM). Effects of various divalent cations (Fe^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Ba^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}), detergents (SDS, Triton X-100), and EDTA were also evaluated in the presence of 1 and 5 mM of those compounds, in a 50 mM sodium acetate buffer system. All reactions contained 0.15 M sucrose and 2.5 M NaCl.

Determination of enzyme kinetics

Kinetics of *Hs*Lsc was determined as total enzyme activity at a sucrose concentration range of 0–525 mM and constant NaCl concentration of 2.0 M. Released total reducing sugar concentrations were determined via DNS method, and the kinetic model was obtained with GraphPad Prism 5 software (GraphPad Software, Inc.).

Substrate and product specificities of *Hs*Lsc

To get a better understanding of the enzymatic product profile under different conditions as well as transfructosylation to different acceptors, various reactions were carried out and resulting products were analyzed with Dionex ICS 5000 + HPAEC-IPAD (High-Performance Anion Exchange Chromatography with Integrated Pulsed Amperometric Detection; Thermo Scientific). Samples were diluted 100× in 20 μM rhamnose, which was used as an internal standard. Using

a full loop injection, samples were run for 32 min. Starting from 100% solution A (90 mM NaOH), a gradient was used for solution B (90 mM NaOH + 0.5 M NaOAc), going from 0 to 35% in 26 min, followed by a cleaning step using 100% solution B. The flow rate was 0.25 ml/min, and a Dionex CarboPac PA100 column (2 × 50 mm) was used. Amperometric detection was carried out with a Ag/AgCl reference electrode and an Au electrode using a carbohydrate quadruple waveform. The following reference samples were used for identification and quantification of specific carbohydrates: a standard containing 10 µM D-glucose, D-fructose, and sucrose (Sigma-Aldrich®); a kestose standard containing 10 µM 1-kestotriose (TCI Europe), 6-kestotriose (kind gift from Dr. Masaru Iizuka, Kobe Shoin Women's University), and neokestose; sugar extracts from wheat stem and forced chicory root after purification using a mixed bed ion exchange column (Dowex® Ac^- and H^+ resins). Neokestose from *Xanthophyllomyces dendrorhous* and sugar extracts were produced in the Laboratory of Molecular Plant Biology, KU Leuven.

For the analyses of product profiles under different conditions, reactions were carried out at various sucrose (0.1, 0.5, and 1.5 M) and NaCl (1.5, 2.0, and 2.5 M) concentrations, in a total of nine different combinations.

To reveal the potential transfructosylation reactions from 1% (*w*/*v*) sucrose to various saccharides, the following sugars were used as fructosyl acceptors at 2% (*w*/*v*) concentrations unless stated otherwise: D-lactose (1%), D-mannose, D-cellobiose (1%), D-xylose, D-sorbitol, D-arabinose, L-arabinose, L-rhamnose, D-galactose (1%), and D-glucuronic acid, with all reactions containing 2.0 M NaCl. Additionally, providing 0.3 M raffinose as a fructosyl donor and acceptor along with sucrose was also investigated.

To determine the effect of D-glucose on the enzyme activity, reactions containing 0.15 M sucrose and 2.0 M NaCl were supplied with 5.6, 10, 20, or 50 mM D-glucose and then analyzed with HPAEC-IPAD.

Intrinsic levanase activity of *Hs*Lsc

Intrinsic levanase activity of *Hs*Lsc against the following fructans (0.5%, *w*/*v*) at 37 °C for 3 weeks was investigated: *Halomonas* levan (produced by *H. smyrnensis* cultures), *Z. mobilis* levan (Fluka™), *B. subtilis* levan (kindly provided by Dr. Joan Combie, Montana Polysaccharides Inc., USA) and inulin from chicory (Orafti® HPX). Also, a standard enzymatic reaction mixture was incubated under the same conditions to investigate the degradation of levan produced by *Hs*Lsc itself. The course of fructan degradation was followed by measuring the amount of reducing sugars released via the DNS method.

Salt dependency of the enzyme

Replacement of NaCl in the reaction medium with salts like NaBr, KCl, KI, $NaNO_3$, and $Na_2O_5S_2$ was carried out to investigate salt dependency of *Hs*Lsc. All salts were provided at 2.5 M Na^+ or K^+. Also, enzyme activity at nine different NaCl concentrations ranging from 0 M to 4.5 M was evaluated in the presence of 0.15 M sucrose.

Homology modeling of *Hs*Lsc

For homology modeling studies, SWISS-MODEL (http://swissmodel.expasy.org) (Biasini et al. 2014) was used. *Erwinia amylovora* levansucrase (PDB ID: 4D47) was chosen as the most suitable template for modeling *Hs*Lsc.

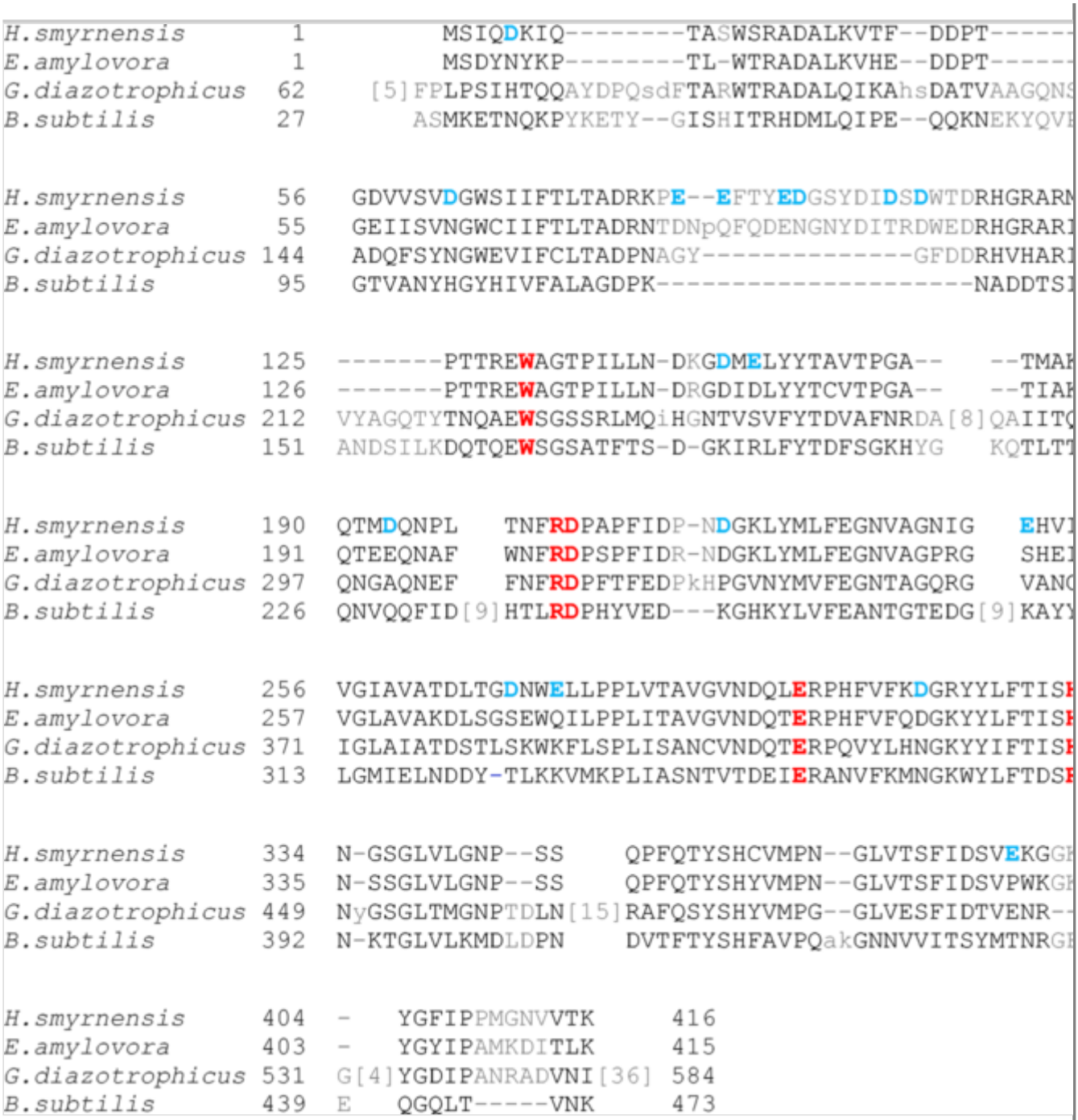
Results

Recombinant expression and purification of *Hs*Lsc

In this work, the levansucrase-encoding gene (*lsc*) from the first-reported halophilic levan producer *H. smyrnensis* AAD6^T was expressed in *E. coli* using the T7 RNA polymerase/promoter system. The recombinant enzyme fused to a C-terminal 6xHisTag comprises 424 amino acids with a theoretical molecular weight of 47.3 kDa and pI of 4.6. As expected for an extracellular halozyme, the enzyme is highly negatively charged/hydrophilic: the total number of negatively and positively charged residues are 64 and 30, respectively. Similar to some other levansucrases from Gram-negative bacteria, *Hs*Lsc lacks a predictable N-terminal signal peptide according to SignalP 4.1 server (Petersen et al. 2011). In Fig. 1, a multiple sequence alignment of native *Hs*Lsc with three other levansucrases with known crystal structures (*Erwinia amylovora*, PDB ID: 4D47; *Gluconacetobacter diazotrophicus*, PDB ID: 1W18; *Bacillus subtilis*, PDB ID: 3BYN) is presented. Sequence identity values of *Hs*Lsc with these proteins were 68.5, 43.6, and 27.0%, respectively.

Fig. 1

Multiple sequence alignment of *Hs*Lsc against three levansucrases with known 3D structures (*Erwinia amylovora*, PDB ID: 4D47; *Gluconacetobacter diazotrophicus*, PDB ID: 1W18; *Bacillus subtilis*, PDB ID: 3BYN). Alignment was carried out with NCBI COBALT (Papadopoulos and Agarwala 2007). Red letters indicate amino acid residues involved in overall catalysis/substrate specificity. Blue letters indicate acidic residues that are not commonly observed in the other three sequences



Purification of recombinant *Hs*Lsc has been carried out by passing the crude cell extract through a metal affinity column, and as shown in Table 1, the enzyme has been purified almost fivefold with high specific activity values, which was increased from 0.83 U/mg in crude cell extract to 4.12 U/mg in the fraction collected from IMAC. Cell sonicate of the control (uninduced *E. coli* BL21(DE3) cells) showed only minimal specific activity (0.001 U/mg), which may be attributed to the “leaky” characteristic of the T7 promoter system.

Table 1

Protein purification table for recombinant *Hs*Lsc

	Total protein (mg)	Volume (ml)	Total activity (U)	Specific activity (U/mg protein)	Purity (fold)

	(mg)		(U)		
Crude cell extract	27.55	25	1157 ± 9.7	42 ± 0.6	1
IMAC (peak fraction)	1.64	10	338 ± 5.1	206 ± 3.2	4.9
Uninduced cell extract (control)	11.93	10	0.01	0.001	–

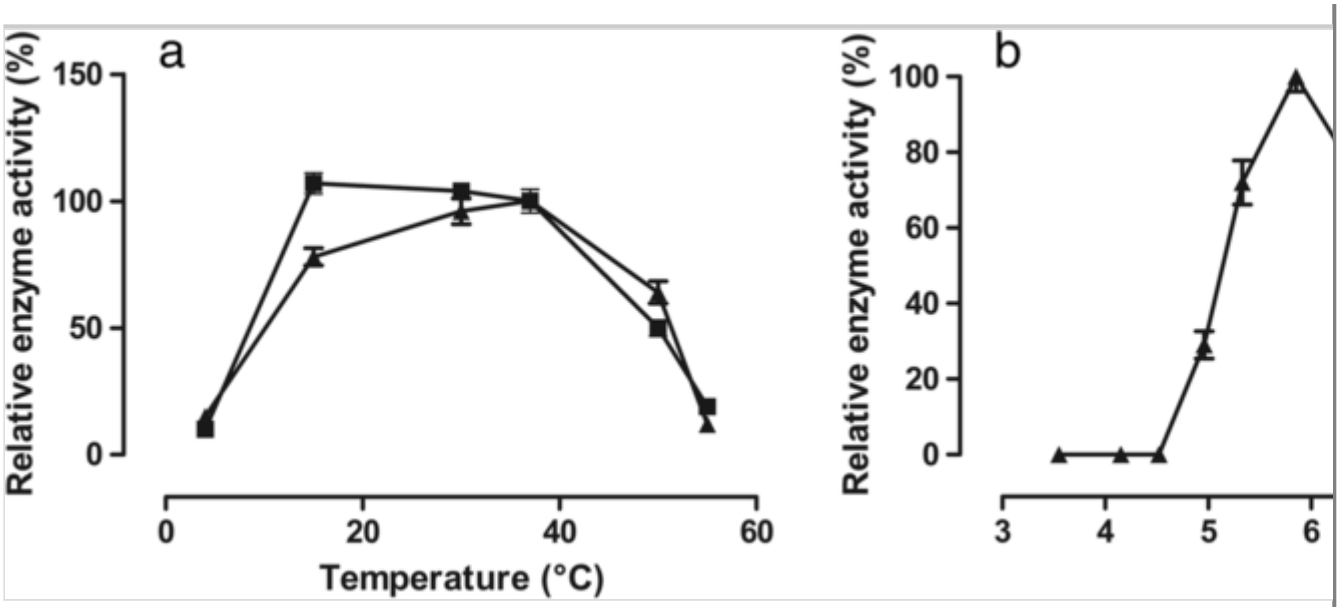
SDS-PAGE analysis revealed that proteins from purified fractions with activity appeared on the gel at around 45 kDa (Fig. S1), which is expected since the molecular weight of *Hs*Lsc is 47.3 kDa. Two bands appeared on lanes with active fractions (fractions 3, 4, and 5), with the main band around 45 kDa probably belonging to *Hs*Lsc.

Effects of temperature, pH, and various substances on enzyme activity

Total activity (*U*) and levan biosynthesis ability (absorbance at 405 nm) of the purified enzyme were assessed at various temperatures, ranging from 4 to 55 °C. As seen in Fig. 2a, *Hs*Lsc shows maximal activity at 37 °C, while temperatures below 4 °C and above 55 °C severely inhibit it. Levan formation was more prominent at 15 °C compared to that at 37°C, suggesting that increasing temperature values render sucrose or water molecules more attractive fructosyl acceptors compared to growing levan chains, resulting in a reaction shift to FOS formation and/or sucrose hydrolysis rather than polymerization into high-DP levans. Exceptionally, when a standard enzymatic reaction mixture was left at − 20 °C, it was still in liquid form thanks to high amounts of NaCl, and *Hs*Lsc was still able to produce levan at such low temperature, though very slowly (in about 30 days, data not shown). Optimal pH for *Hs*Lsc activity was determined as 5.9, while at pH values below 5.0 and above 7.0, the activity dropped drastically (Fig. 2b).

Fig. 2

a Effect of temperature on total activity (triangles) and polymerase activity (squares) of *Hs*Lsc. **b** Effect of pH on total activity of *Hs*Lsc. Error bars represent standard error. 100% of activity corresponds to 192 ± 3.7 U/mg



Various metal ions, EDTA, and two detergents at 1 and 5 mM concentrations were added to *Hs*Lsc reaction mixtures (Table 2). Interestingly, all tested divalent cations inhibited the enzyme activity to various extents. The enzyme was extremely sensitive to the presence of 1 mM Fe^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}, all of which completely inhibited the enzyme activity, while Ba^{2+}, Mg^{2+}, and Mn^{2+} were tolerated better. Intriguingly, 1 mM EDTA enhanced the enzyme activity by 31%. This suggests that *Hs*Lsc is sensitive towards the presence of divalent cations, and EDTA may be enhancing its activity by chelating trace amounts of metal ions which may be originating from the commercial chemicals used throughout the experiments. As for the detergents tested, Triton X-100 was tolerated better than SDS.

Table 2

Relative *Hs*Lsc activity values at 1 and 5 mM concentrations of various substances. All reactions contained 0.15 M sucrose and 2.5 M NaCl. One hundred percent of activity corresponds to 210 ± 2.9 U/mg

Compound	Activity at 1 mM (%)	Activity at 5 mM (%)
None	100	100
Triton X-100	79	71
SDS	67	2
EDTA	131	94
Ba^{2+}	58	0
Ca^{2+}	1	6
Co^{2+}	0	0
Cu^{2+}	0	0

Compound	Activity at 1 mM (%)	Activity at 5 mM (%)
Fe^{2+}	0	0
Mg^{2+}	66	61
Mn^{2+}	25	6
Ni^{2+}	0	0

Enzyme kinetics

According to the model obtained with GraphPad software, *Hs*Lsc fits well to Hill kinetics under the conditions tested ($R^2 = 0.9969$; Fig. S2). The Hill coefficient $n = 2.271$ suggests a positive cooperation of multiple subsites in substrate binding; however, this hypothesis requires further experimental validation. V_{max} and $K_{0.5}$ values were determined as 296 ± 4.0 U/mg protein and 104.79 ± 4.17 mM sucrose, respectively.

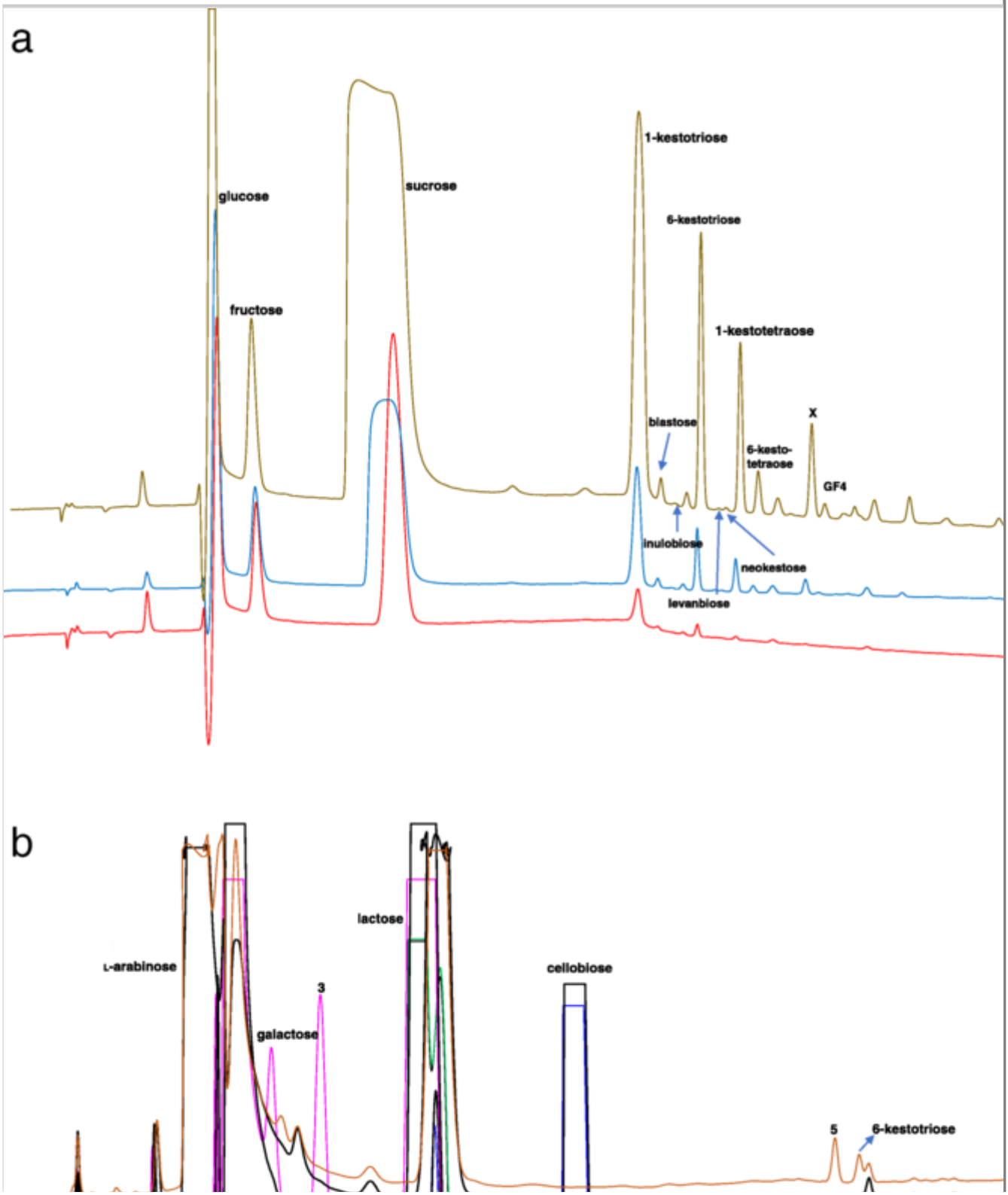
Substrate and product specificities of *Hs*Lsc

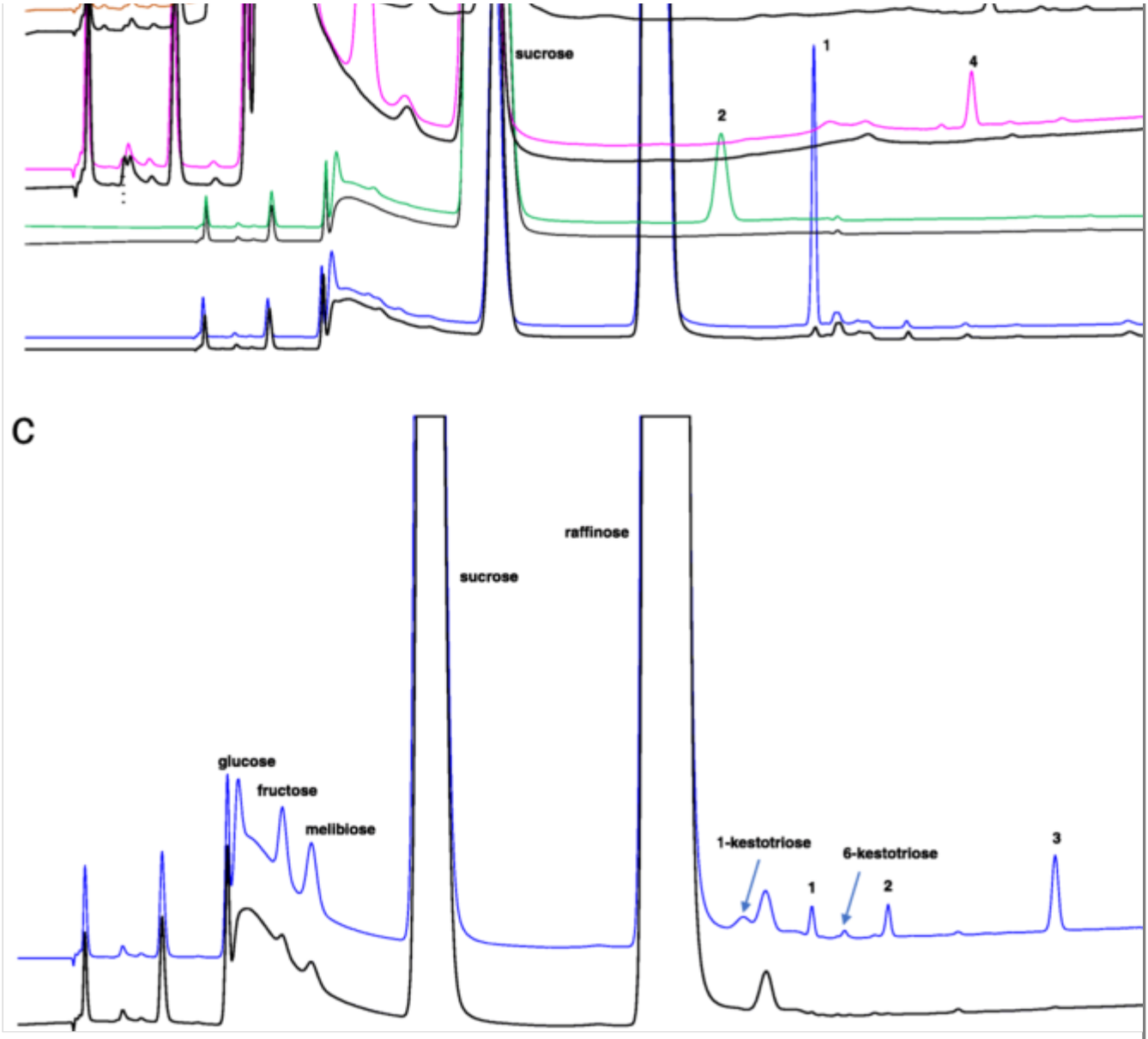
As shown in HPAEC-IPAD chromatograms (Fig. 3a), FOSs formation became more prominent with increasing initial sucrose concentration. At 0.1 M sucrose, 1-kestotriose, 6-kestotriose and 1-kestotetraose and few other unidentified FOSs peaks were detected. At 0.5 M sucrose, additional peaks for blastose, inulobiose, 6-kestotetraose, a GF_3 (most probably Glc (β-2,1) Fru (β-2,1) Fru (β-2,6)Fru), and a GF_4 were detected. At 1.5 M sucrose, in addition to all the FOSs above, levanbiose and neokestose were also detected. Transfructosylation experiments between sucrose and cellobiose or lactose revealed that both act as fructosyl acceptors, with cellobiose being the more efficient one. Clear peaks representing putative fructosylated cellobiose and fructosylated lactose products were observed (Fig. 3b). As for the monosaccharides tested, mannose, xylose, sorbitol, D-arabinose, and rhamnose showed little or no acceptor activity (not shown), while L-arabinose was a better acceptor and galactose was the best acceptor among monosaccharides tested. Both putative Fru (β-2,1) Gal and Fru (β-2,6) Gal were formed, with putative Fru (β-2,1) Gal being the prominent one. When raffinose was supplied along with sucrose, it was observed that *Hs*Lsc can use it both as a fructosyl donor (melibiose formation) and acceptor, forming significant amounts of three different unidentified oligosaccharides (Fig. 3c).

Fig. 3

HPAEC-IPAD chromatograms of different *Hs*Lsc reactions. **a** Reactions under constant NaCl (2.5 M) and increasing sucrose concentrations; from bottom to top, 0.1, 0.5, and 1.5 M sucrose. X, putative Fru (β-2,1) Fru (β-2,1) Glc (β-2,6) Fru. **b**

Reactions with sucrose (1%) as the fructosyl donor and cellobiose (1%), lactose (1%), galactose (1%), and L-arabinose (2%) as fructosyl acceptors. Numbered peaks are 1, putative fructosylated cellobiose; 2, putative fructosylated lactose; 3, putative Fru (β-2,1) Gal; 4, putative Fru (β-2,6) Gal; 5, putative fructosylated L-arabinose. Each bottom chromatogram represents (in black) the corresponding t0 sample. All reactions contained 2.0 M NaCl. **c** Reactions supplied with raffinose (0.3 M) along with sucrose (0.15 M). Bottom chromatogram represents the t0 sample. Numbered peaks indicate raffinose-derived type oligosaccharides. All reactions contained 2.0 M NaCl

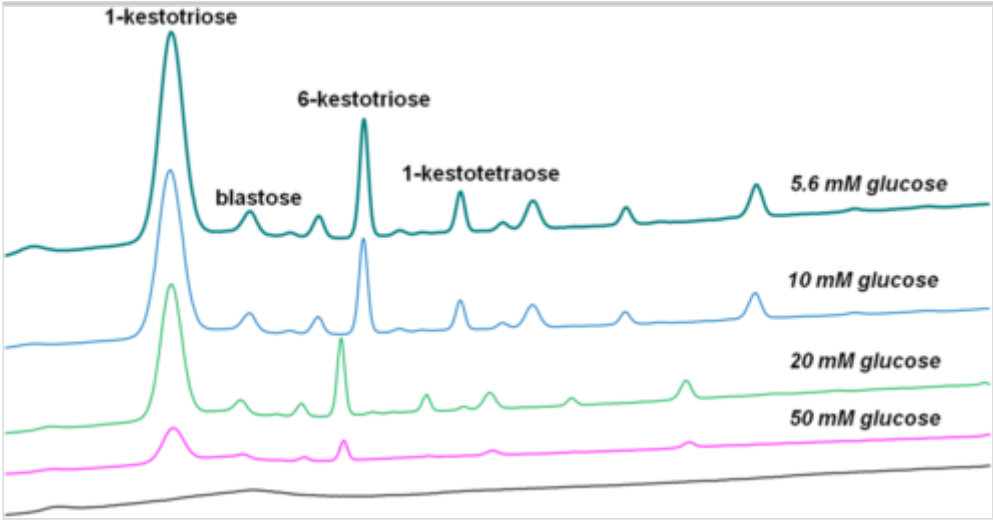




As derived from the HPAEC-IPAD profiles (Fig. 4), concentrations of the end products started to decrease in the presence of 20 mM glucose, and the enzyme activity was remarkably inhibited with 50 mM glucose. However, increasing glucose concentrations did not result in a marked increase in futile transfer of sucrose-derived fructosyl units to glucose, resulting in blastose (Fru (β-2,6)Glc) formation.

Fig. 4

HPAEC-IPAD chromatograms showing the fructosylation products of the *Hs*Lsc reaction with constant sucrose (150 mM) and different glucose concentrations (from bottom to top: t0 sample, 50, 20, 10, and 5.6 mM glucose). All reactions contained 2.0 M NaCl



Intrinsic levanase activity of *Hs*Lsc

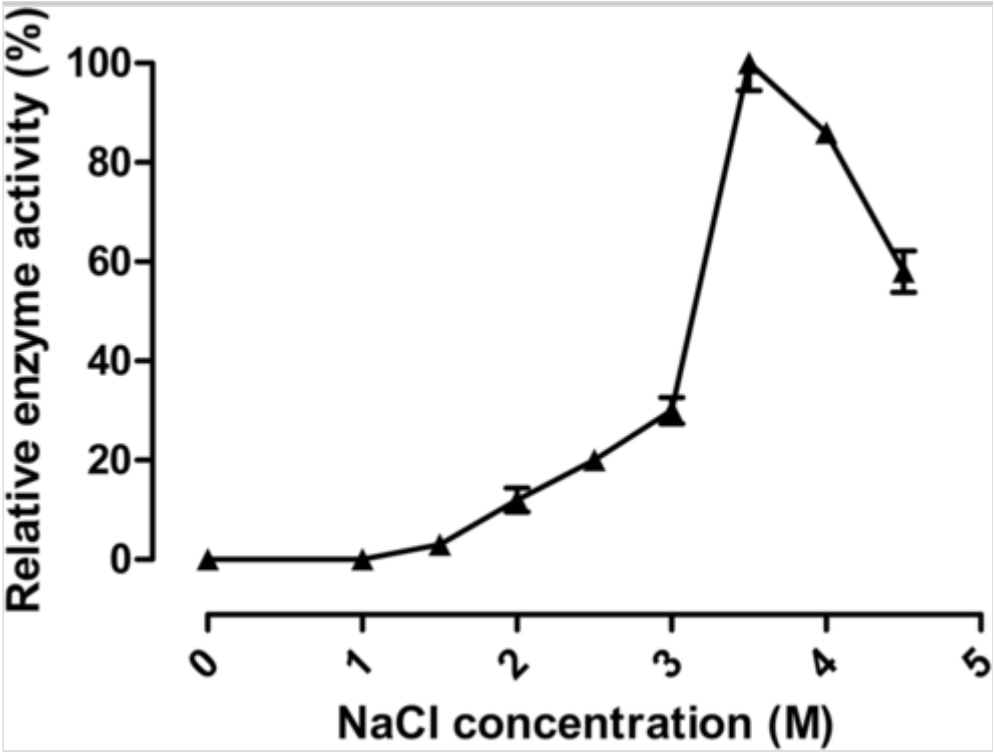
Intriguingly, *Hs*Lsc could not hydrolyze any of the fructans (levan from *H. smyrnensis*, *Z. mobilis*, and *B. subtilis*, and inulin from chicory) provided as the sole substrate at 37 °C. However, studying *Hs*Lsc/sucrose reaction mixture dynamics on a very long term showed two phases: (1) polymerization of sucrose into levan and (2) depolymerization of levan into hexoses.

Salt dependency of *Hs*Lsc activity

Among all salts tested, NaCl was the most favorable one in terms of enzyme activity, followed by KCl (86% of the activity observed with NaCl). There was no activity in reactions with KI, NaBr, $NaNO_3$, or $Na_2S_5O_2$. Enzymatic reactions were also carried out under increasing NaCl concentrations from 0 to 4.5 M. *Hs*Lsc shows maximum activity in the presence of 3.5 M NaCl (five times the activity observed with 2.5 M NaCl) and requires at least 1.5 M NaCl to be active (Fig. 5). Exceptionally, *Hs*Lsc was still soluble and remarkably active (58% of maximal activity) in the presence of 4.5 M NaCl and 0.15 M sucrose. Additionally, activity loss at low salt concentrations was reversible, since purification of *Hs*Lsc was carried out at a NaCl concentration of only 0.3 M.

Fig. 5

Enzyme activity (relative to the maximum) at increasing NaCl concentrations. All reactions contained 0.15 M sucrose. Error bars represent standard error. One hundred percent of activity (at 3.5 M NaCl) corresponds to 951 ± 9.4 U/mg

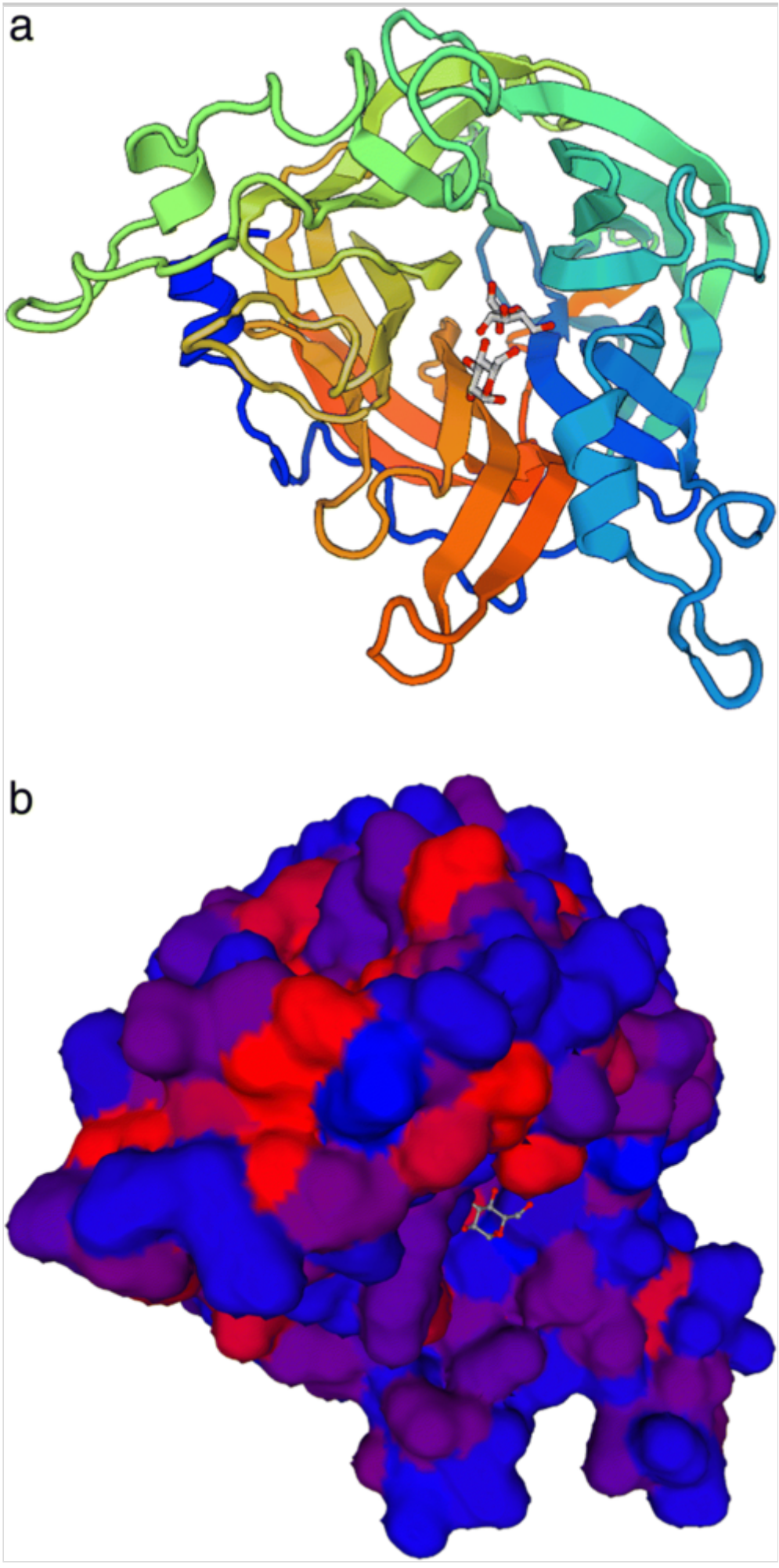


Homology modeling of *Hs*Lsc

According to the SWISS-MODEL, *Hs*Lsc and *E. amylovora* levansucrase (PDB ID: 4D47) show 68.5% sequence identity and 95% coverage (between amino acid residues 12-415 in *Hs*Lsc). A high-quality homology model was obtained with Global Model Quality Estimate (GMQE) and QMEAN scores of 0.83 and 0.33, respectively. *Hs*Lsc folds into a five-bladed β-propeller (Fig. 6a), which is typical for all GH68 family enzymes. The deep central cavity formed by this five-bladed β-propeller harbors ligands: fructose (in contact with W46, D47, L70, H96, W130, R201, D202, E219, E286, and Y351) and glucose (in contact with R95, H96, E286, H304, Y351, D366, and ligand: fructose) in the model. Figure 6b shows the surface of the model being significantly rich with hydrophilic residues, which is a common trait of extracellular halozymes, allowing them to remain soluble in hypersaline environments. Additionally, like many other halozymes, *Hs*Lsc shows a similar structure to its mesophilic counterparts despite its increased number of acidic and hydrophilic residues.

Fig. 6

Structure of *Hs*Lsc according to homology modeling using *E. amylovora* levansucrase as template. **a** Cartoon model of *Hs*Lsc with rainbow color scheme. **b** Surface model of *Hs*Lsc. Red, strongly hydrophobic residues; blue, strongly hydrophilic residues; purple, mildly hydrophobic/hydrophilic residues


a
b

Discussion

Although there have been many reports in the literature on recombinant levansucrase expression, purification, and characterization (Ishida et al. 2016; Jang et al. 2001; Kim et al. 1998; Li et al. 2017; Liu et al. 2017; Lu et al. 2014), levansucrases from extremophilic microorganisms have been largely overlooked. In this work, levansucrase enzyme from *H. smyrnensis* AAD6^T (*Hs*Lsc) was recombinantly expressed, purified, and characterized. Specific activity of purified *Hs*Lsc (206 ± 3.2 μmol reducing sugar/min mg) was in accordance with other levansucrases reported in the literature (El-Refai et al. 2009; Gao et al. 2017; Liu et al. 2017; Morales-Arrieta et al. 2006; Rairakhwada et al. 2010). Moreover, it should be noted that the specific activity of the enzyme was almost fivefold of this value at 3.5 M NaCl (Fig. 5). As a halophilic GH-J clan enzyme, *Hs*Lsc exhibits some unprecedented characteristics as discussed below.

AQ5

According to the alignment result (Fig. 1), residues that act as the nucleophile (D47), the transition-state stabilizer (D202), and the general acid/base catalyst (E286) are strictly conserved in *Hs*Lsc like in all other levansucrases. Another strictly conserved residue, W130 in *Hs*Lsc and W163 in *B. subtilis* levansucrase, is known to take part in substrate-binding processes (Lammens et al. 2009). The arginine residue adjacent to the transition-state stabilizer (R201 in *Hs*Lsc) has been reported to form a salt bridge with the acid/base catalyst in *G. diazotrophicus* levansucrase (Martínez-Fleites et al. 2005). Interestingly, the strictly conserved 'D (E/Q)(T/I/V)ER' motif that contains the acid/base catalyst (E286 in *Hs*Lsc) appears as 'DQ**L**ER' in *Hs*Lsc. Similar to other levansucrases from Gram-negative bacteria, a histidine (H304) is found at the position of R360 homolog of *B. subtilis* levansucrase, which is known to be a key residue that takes part in the polymerization process (Chambert and Petit-Glatron 1991; Homann et al. 2007; Goldman et al. 2008). As a result of *Hs*Lsc's halophilic nature, the sequence is significantly richer with acidic residues (D and E, shown in blue color in Fig. 1) in regions predicted to be far from the active site and making up the protein surface according to the model constructed (Fig. 6).

AQ6

For *Hs*Lsc, optimum temperatures for levan formation and total activity were determined at 15 and 37 °C, respectively (Fig. 2a). Santos-Moriano et al. (2015) reported that levansucrase from *Z. mobilis* showed maximum levan production activity at 4 °C, while total enzyme activity peaked at 40 °C. Similar results were reported by Visnapuu et al. (2015) for levansucrase (Lsc3) from *Pseudomonas syringae* pv. *tomato*. When the enzyme was incubated with sucrose at 20 °C, conversion rate of sucrose to levan was 80%, which was 67%

higher than the conversion rate at 60 °C. For *Brenneria goodwinii* levansucrase, optimum temperatures for transfructosylation and sucrose hydrolysis were 35 and 45 °C, respectively (Liu et al. 2017). In the case of *B. subtilis* 168 levansucrase, both transfructosylation/hydrolysis rate and the molecular weight of levan increased at lower temperatures (4 °C instead of 37 °C; Porras-Domínguez et al. 2015). However, both *Bacillus* sp. TH4-2 and *Bacillus licheniformis* RN-01 levansucrases produced higher molecular weight levan at 50 °C as compared to 30 °C, demonstrating that transfructosylation was more favorable as compared to sucrose hydrolysis at elevated temperatures (Ammar et al. 2002; Nakapong et al. 2013). The ability to form high molecular weight levan at elevated temperatures may be attributed to the thermotolerant nature of these two strains.

*Hs*Lsc showed highest activity at pH 5.9 (Fig. 2b). For most levansucrase enzymes, reported optimal pH values are slightly acidic (around 6.0; Hernández et al. 1995; Choi et al. 2004; Zhang et al. 2014; Santos-Moriano et al. 2015; Gao et al. 2017). It is also known that during the course of levan production, *H. smyrnensis* AAD6^T cultures rapidly reduce the pH of the fermentation medium from 7.0 to around 6.0 (Erkorkmaz et al. 2018), creating a more suitable environment for levansucrase action.

Several metal ions and detergents are known to have significant impacts on various levansucrases (Gao et al. 2017; Ishida et al. 2016; Shaheen et al. 2017). The strong sensitivity of *Hs*Lsc to divalent cations (Table 2) seems to be a unique property among levansucrases. Several levansucrase and inulosucrase enzymes from Gram-positive bacteria are known to carry amino acid residues that take part in Ca^{2+} binding. For instance, levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121 were reported to be more durable against inactivation at elevated temperatures in the presence of Ca^{2+} ions (1 mM) (Ozimek et al. 2005). Liu et al. (2017) reported that 1 mM Ca^{2+}, Mn^{2+}, or Ni^{2+} enhanced *Brenneria goodwinii* levansucrase activity, while Cu^{2+}, Fe^{2+}, Zn^{2+}, or Mg^{2+} only decreased it by less than 25%. Activity of levansucrase from *Z. mobilis* KIBGE-IB14 was increased with 1 mM Ba^{2+}, Ca^{2+}, Cu^{2+}, or Mg^{2+}, and hardly inhibited by Co^{2+}, Ni^{2+}, or Zn^{2+} (Shaheen et al. 2017). Moreover, 5 mM Ca^{2+} did not affect levansucrases Lsc2, Lsc3 from *Pseudomonas syringae* pv. *tomato* (Visnapuu 2012) and Lsc from *Pseudomonas syringae* pv. *phaseolicola* (Hettwer et al. 1995). However, while 5 mM Cu^{2+} severely inhibited Lsc^a activity (18% relative activity), it hardly impeded Lsc2 or Lsc3 (92 and 99% relative activities, respectively). These results suggest that the effects of divalent cations on levansucrases can be highly variable even between different strains of the same species.

Levansucrases are known to exhibit different product profiles at different initial sucrose concentrations. Also, being a halophilic enzyme, activity of *Hs*Lsc is expected to be crucially dependent on the NaCl concentration in the reaction. Experiments with *Hs*Lsc at various combinations of initial sucrose and NaCl concentrations demonstrated that FOS production was more prominent as initial sucrose concentration was increased (up to 1.5 M; Fig. 3a). Similar to other levansucrases of Gram-negative bacteria, *Hs*Lsc synthesized both 1-kestotriose (inulin-type FOS) and 6-kestotriose (levan-type FOS) (Caputi et al. 2013; Santos-Moriano et al. 2015). Although NaCl concentration did not have any effect on the product profile, it increased the overall activity significantly and, thus, the product concentrations too. For most other levansucrases, water-restricted environments promote fructan synthesis and polymerization (Toksoy Öner et al. 2016). These results show that *Hs*Lsc can be utilized to produce a wide range of FOSs at initially high sucrose concentrations.

In levansucrase reactions, addition of various mono- or disaccharides as fructosyl acceptors along with the sucrose as donor substrate leads to the formation of various fructosylated saccharides (Li et al. 2015b; Seibel et al. 2006; Visnapuu et al. 2011). *Hs*Lsc showed good acceptor specificity towards cellobiose, lactose, galactose, and L-arabinose (Fig. 3). Fructosylated forms of cellobiose and lactose have the potential to be used as low-calorie sugar substitutes and prebiotics in the food industry (Biton et al. 1995; Silvério et al. 2015). Transfer of fructose to galactose resulted in the production of both putative Fru (β-2,6) Gal and Fru (β-2,1) Gal (Fig. 3b). Baciu et al. (2005) studied the production of fructosylated galactose via a FT (EC 2.4.1.162) from *B. subtilis* NCIMB 11871, and successfully produced and purified Fru (β-2,1) Gal. The authors stated that the reaction exhibited an equilibrium between sucrose and Fru (β-2,1) Gal. Since this disaccharide is structurally almost identical to sucrose (Fru (β-2,1) Glc), and the retention times of those two substances are very close in ion exchange chromatography, suggesting peak number 1 in Fig. 3b most probably represents Fru (β-2,1) Gal, while the putative Fru (β-2,6) Gal probably elutes much later (peak number 5). The fact that inulin-type FOSs always precede their levan-type counterparts in HPAEC-IPAD chromatograms also supports this hypothesis. Formation of two types of linkages mimics what occurs when the fructosyl moiety is transferred from sucrose to glucose (synthesis of sucrose and blastose; Homann et al. 2007; Méndez-Lorenzo et al. 2015). An interesting phenomenon occurred in the presence of glucuronic acid, where sucrose hydrolysis was significantly prominent instead of transfructosylation (Fig. S3). It is possible that the negatively charged glucuronic acid forms electrostatic interactions with the H304 residue of *Hs*Lsc (homologue of H419 in *G. diazotrophicus* levansucrase and R360 in *B. subtilis* levansucrase),

thus blocking the polymerization ability of the enzyme. In *B. subtilis* levansucrase, when the above-mentioned residue was mutated to Lys, Ser, or Leu, the enzyme lost its ability to form levan (Chambert and Petit-Glatron 1991). In *Z. mobilis* levansucrase, homologue H296 is essential for the formation of levansucrase microfibrils, which are related to levan polymerization (Goldman et al. 2008). Visnapuu et al. (2011) determined H321 and T302 as crucial residues for polymerization activity of *P. syringae* pv. *tomato* DC3000 Lsc3. When H321 was replaced by Arg, Lys, Ser, or Leu, the enzyme's polymerization ability was greatly reduced.

*Hs*Lsc was able to use raffinose, when supplied along with sucrose, both as a fructosyl donor and acceptor (Fig. 3c). Most levansucrases characterized to date are able to use raffinose in a similar manner, however still preferring sucrose as the main fructosyl donor (Li et al. 2015a; Park et al. 2003; Visnapuu et al. 2015; Xu et al. 2017). Levansucrase from *Z. mobilis* 113S, on the other hand, showed higher reaction velocities when raffinose was used as a fructosyl donor instead of sucrose (Andersone et al. 2004).

In levansucrase reactions, the presence of glucose along with sucrose mostly results in the formation of sucrose and glucose again (futile transfer of fructose) with some blastose (Fru (β-2,6) Glc) formation (Homann et al. 2007; Méndez-Lorenzo et al. 2015). It seems that increasing glucose concentration in the reaction (up to 50 mM) does not increase blastose yield in this case (Fig. 4), suggesting that as its concentration increases, glucose starts to bind better at the donor site of *Hs*Lsc than its acceptor site, inhibiting enzyme activity, thus overall resulting in less product (either levan or hexose) formation. During fructan production, the accumulation of the glucose released from sucrose in the culture or reaction media is a serious problem which results in reduced fructan yields. Kazak Sarilmiser et al. (2015) reported that in *H. smyrnensis* AAD6^T cultures supplemented with 50 g/l of initial sucrose, glucose concentration in the medium has shown an almost linear increase and exceeded 20 g/l, after 150 h. Optimization of medium composition reduced the accumulated glucose concentrations to 9–10 g/l with improved levan titers but not eliminating it completely (Erkorkmaz et al. 2018). Removal of accumulating glucose by co-culturing with yeast (Avigad 1957; Szwengiel et al. 2007) or glucose oxidase enzyme (Yun et al. 1994) has proven to be effective in some microbial and enzymatic fructan production systems. However, implementing such strategies to hypersaline media presents a challenging aspect and requires novel approaches.

It is known that most levansucrases exhibit an intrinsic exo-levanase activity when sucrose concentration in the environment is depleted. Méndez-Lorenzo et al. (2015) observed that levansucrase from *B. subtilis* 168 hydrolyzes levan

through a first-order exo-type kinetic, and also acts as a transferase in the presence of glucose and fructose as fructosyl acceptors. Ua-Arak et al. (2017) also reported the possibility of a similar case in *Gluconobacter albidus* TMW 2.1191 cultures. *Hs*Lsc could not hydrolyze any of the fructans provided exogenously, including *Halomonas* levan. Free *Hs*Lsc may not be able to bind exogenous long-chain fructans as fructosyl donor. However, incubation of an enzymatic reaction over a long period of time first resulted in levan formation, followed by its complete hydrolysis. This may be due to the fact that levansucrases generally remain bound to the elongating levan chain during the polymerization reaction (Ozimek et al. 2006), enabling the enzyme to start hydrolyzing the attached polymer when sucrose is no longer available in the reaction medium. The formation of fructose and FOS through the hydrolysis of the synthesized levan by levansucrase may be crucial to provide the microbial community with an energy source during starvation. Additionally, the small sugars formed may be involved in signaling events (Versluys et al. 2018).
AQ7

Extracellular halozymes owe their high solubility to greater number of acidic residues they harbor compared to mesophilic enzymes (Graziano and Merlino 2014; Oren 2013). Elevi Bardavid and Oren (2012) studied isoelectric point (p*I*) profiles of the proteomes of two moderately halophilic bacterial species, namely *Halomonas elongata* and *Chromohalobacter salexigens*, which are taxonomically close species to *H. smyrnensis* AAD6, and found out that proteins with p*I* values around 4.4–5.1 make up the largest group of their proteomes. It is thought that increased number of negatively charged residues on halozymes' surfaces enables the retention of the hydration shell even under extremely saline conditions, thus preventing protein aggregation and denaturation. *Hs*Lsc harbors 64 negatively charged and 30 positively charged amino acid residues in its sequence, with a grand average of hydropathicity (GRAVY) value of − 0.403, according to ExPASy ProtParam. Evidently, these traits enable *Hs*Lsc to be able to remain active and soluble under extremely high salt concentrations. The NaCl dependency (at least 1.5 M, with an optimum of 3.5 M, Fig. 5) of *Hs*Lsc activity has not been reported for any other GH-J clan enzyme characterized to date.

As discussed in Kırtel et al. (2018), presence of fructosyltransferases in saline or hypersaline environments presents an intriguing research aspect. Microorganisms thriving in such water-restricted habitats have evolved unique ways to cope with high salinity, such as excluding Na^+ ions from the cytoplasm while accumulating K^+ ions as much as possible (the salting-out strategy) and/or accumulating intracellular compatible solutes (i.e., ectoine, glycine betaine, trehalose). In hypersaline environments, Archaea make up the largest biomass as a rule (Oren 2015), and the presence of putative GH-J clan enzymes only in the

halophilic class of Haloarchaea and not in other archaeal classes suggests a potential role of fructans in salt tolerance. However, presence of fructosyl donors such as sucrose or raffinose in these environments is essential for these enzymes to be active. Although, to our knowledge, there is no experimental data on GH-J clan enzymes in saline or hypersaline environments actively using fructosyl donors, the occurrence of several algal (*Dunaliella tertiolecta*, MacRae and Lunn 2012) and bacterial (two *Methylobacter* strains, Khmelenina et al. 1997; and several cyanobacterial species, Loukas et al. 2018) sucrose producers in these habitats has been reported.

With characteristics such as the ability to form a wide range of FOSs, high acceptor specificity on various saccharides, retaining its activity at sub-zero temperatures, and exceptionally high specific activity values at NaCl concentrations close to saturation levels, *Hs*Lsc presents a novel halozyme with huge potential for use in future research and industrial applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Electronic supplementary material

ESM 1

(PDF 492 kb)

References

AQ8

AQ9

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