COMPARISON OF CATALYTIC PROPERTIES OF LEVANSUCRASES LSC2 AND LSC3 OF PSEUDOMONAS SYRINGAE PV. TOMATO

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Introduction

Pseudomonas syringae is a pathogenic bacterium with numerous plant-specific pathovars. The P. syringae pathovars possess two or three levansucrase genes suggesting that levansucrase proteins may act as virulence factors. Still, since now a role of the levansucrase in plant pathogenesis has clearly been shown only for the levansucrase of Erwinia amylovora, the bacterium which causes fire blight in plants from Rosaceae family1,2. Levansucrases (EC 2.4.1.10; sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferases) are bacterial extracellular enzymes that act on sucrose as their major substrate producing from it β-2,6-linked fructans: short-chain fructooligosaccharides (FOS) and polymeric levan3. It has been suggested that polymeric levan synthesized on site may protect a pathogen from plant defence mechanisms. Recent data on E. amylovora indicate that levan polymer binds copper, a chemical that is widely used to control plant pathogenic microorganisms, and induces a persistor state to the bacterium. Thereby, levan certainly contributes to the survival of this pathogen in sucrose-rich plant environment4. It has been shown that polymerization products of levansucrases may also have applications in medicine and food technology. These substrates have exhibited beneficial effects for humans: short-chain FOS as prebiotics5 and levan as immune system stimulator6.

The genome of P. syringae pv. tomato, a pathogen of tomato and Arabidopsis thaliana, contains three levansucrase genes: lsc1, lsc2 and lsc3 (also designated as lsc-1, lsc-2 and lsc-3). We have shown that these genes encode functional levansucrases (Lsc1, Lsc2 and Lsc3) if expressed in Escherichia coli from a heterologous promoter7. The lsc1 and lsc2 genes are chromosomal, whereas the lsc3 resides on the plasmid pDC3000A which is rich in virulence genes8. All three levansucrases of P. syringae pv. tomato have highly similar protein sequence, except for a 16-amino acid (aa) N-terminal extension which is present only in Lsc1 and Lsc3 (see Fig. 1). Due to that, Lsc1 and Lsc3 are 431 aa long, whereas the Lsc2 consists of 415 amino acids9 (see Fig. 1). According to Srivastava et al.9, the 431 aa levanseucrase proteins may have emerged through insertion of a phage-derived DNA ahead of the gene encoding a 415 aa levanseucrase, creating a functional promoter to a silent gene and simultaneously adding 16 N-terminal amino acids to the protein.

We have thoroughly characterized the biochemical properties of the Lsc3 protein of P. syringae pv. tomato and disclosed many catalytically important positions of it10-13. In this study, we characterize the Lsc2 protein in detail. Properties of heterologously synthesized N-terminally His-tagged Lsc2 and Lsc3 proteins are also compared.

Materials and methods

Bacterial strains and plasmids

E. coli strain DH5α (supE44 ΔlacU169 (φ80 lacZAM15) recA1 endA1 hsdR17 thi-1 gyrA96 relA1) (Invitrogen, CA, USA) was used for gene cloning. E. coli strain BL21 (DE3) (hsdS gal (φλde857 ind1 Sam7 nin5 lacUV5-T7 gene 1) was used as a host in overexpression of levansucrases. pUR3-TEV was applied as cloning vector providing N-terminal His6-tag to the expressed protein10. pUR3-lsc3TEV was constructed by us earlier14. The lsc2 gene was cloned into pUR3-TEV using a PCR-based ligation-free method described in 14. Primers for the lsc2 were designed to insert the lsc2 vector into the plasmid vector Lsc2LSEL-TEV_Fw (5’TGAAGCTTGAATTCACGAGCCGATGACAACTACTACGACCTACTG 3’) and Lsc2TEV_Rev (5’AAAGCTTGGATCCTTTFTGCTATCGCTATTATACCTACTATTAGCGATGC 3’). The nucleotides pairing with the expression vector are in italics, the nucleotides pairing with the lsc2 gene are underlined and the start codon of the lsc2 is shown in bold. In these cloning procedures, Pfu DNA polymerase (Thermo Scientific, MA, USA) was used with pHPMalprom-Lsc2 as template for the lsc2 gene. Details of the cloning method are described by us earlier10.

Cultivation of bacteria, protein expression and purification

Plasmid-carrying bacterial cells were grown on LB medium supplemented with 0.15 mg/ml ampicillin. Overexpression and purification of His6-tagged levansucrases was conducted as shown previously11.

Assay of total and transfructosylating activities, determination of kinetic parameters of Lsc2 and Lsc3

Levanseurace reactions were conducted in McIlvaine’s buffer of pH 6.0 as in 11. To calculate total levansucrase activity, initial velocity of sucrose cleavage was measured according to glucose release from sucrose at 37 °C. To determine the Km reaction was conducted at varied concentrations (10 to 1200 mM) of sucrose. Enzyme Kinetics Module 1.1 of the Sigma Plot 2001 (SYSTAT, CA, USA) was used to calculate the kinetic parameters of the reaction. Catalytic constant of sucrose cleavage (kcat; 1/s) was calculated using maximal reaction velocity (Vmax) and molecular weight (Mw) of a purified protein. Theoretical Mw values of levansucrases and extinction coefficients for protein concentration measurements according to absorbance at 280 nm were computed at ExPASy Proteomics Server as in 12.

Polymerizing properties of levansucrases were evaluated by 1) transfructosylating activity (TA) which indicates the percentage of fructose residues from reacted sucrose used for transfructosylation and 2) pattern and amount of FOS produced per mg of protein. For those experiments 2.7 total activity units per 1 ml of reaction mixture of levansucrase proteins (His6-Lsc3 – 7 µg/ml; His6-Lsc2 – 8 µg/ml) were reacted with 1200 mM sucrose at 37 °C for 20 h. The enzyme was then inactivated by heating the samples for 5 min at 96 °C. Amounts of glucose, fructose, sucrose and FOS in the samples were determined by high performance liquid chromatography (HPLC). The obtained data were used to calculate TA as described in 11 and to characterize the spectrum and amount of produced FOS.
Assay of raffinose/sucrose and stachyose/sucrose cleavage ratio of levansucrases

Initial velocities of reducing sugar release from sucrose, raffinose and stachyose (100 mM) by levansucrases were measured in reaction with dinitrosalicylic acid reagent. Initial velocities of reducing sugar production with raffinose and sucrose were divided to calculate the raffinose to sucrose or stachyose to sucrose cleavage ratio of the enzyme that was presented in percentages.

Quantification of sugars by liquid chromatography

Glucose, fructose, sucrose and FOS were quantified from the reaction mixtures as in using HPLC. Chromatography was performed on Acquity UPLC system (Waters, MA, USA) coupled with evaporative light scattering (ELS) detector. Products in the samples were separated using an Alltech Prevail Carbohydrate ES column (Grace, IL, USA). The mobile phase consisted of LC grade water and acetonitrile. Appropriate sugar solutions were used as standards.

Results and discussion

Comparison of amino acid sequences of Lsc2 and Lsc3

Figure 1 illustrates the main difference between the sequences of Lsc2 and Lsc3 and Lsc1 proteins. Lsc1 and Lsc2 have 16 additional amino acids that constitute an N-terminal extension to the proteins. Otherwise, the three of them are highly identical. Lsc1 and Lsc3 proteins have a 96% identity value and they both have 95% identity with the Lsc2 protein. Lsc2 and Lsc3 have only 22 positions out of 415 that are different between the sequences in the shared region. Dissimilarities are mostly detected at positions that are not conserved between the levansucrases (data not shown).

Fig. 1. The N-terminal regions of *P. syringae* pv. tomato levansucrases. Protein sequences with their SwissProt numbers indicated were aligned using the MUSCLE program and the N-terminal part of the alignment is presented. Lsc1 and Lsc2 are encoded chromosomally (C) and lsc3 locates on plasmid (P).

Then we compared the sequences of *P. syringae* pv. tomato levansucrases Lsc1 (431 aa), Lsc2 (415 aa) and Lsc3 (431 aa) with those of levansucrases from *Erwinia amylovora* (415 aa), *Rahnella aquatilis* (415 aa) and *P. chlororaphis* subsp. *aurantiaca* (424 aa) using the MUSCLE program for the alignment. The highest identity (77-78%) with Lsc2 and Lsc3 proteins revealed for levansucrases of *R. aquatilis* and *E. amylovora*. Identity of Lsc2 and Lsc3 with the levansucrase LscA of *P. chlororaphis* subsp. *aurantiaca* was close to 74%.

Comparison of biochemical properties of Lsc2 and Lsc3

Lsc2 and Lsc3 were heterologously expressed in *E. coli* as N-terminally His<sub>6</sub>-tagged proteins, purified and biochemically characterized. The substrate specificity data are presented in Table 1. Both proteins cleave fructose residues and produce reducing sugars from sucrose (α-Glc-1,2-β-Fru; GF), raffinose (α-Gal-1,6-α-Glc-1,2-β-Fru; GalGF) and stachyose (α-Gal-1,6-α-Gal-1,6-α-Glc-1,2-β-Fru; Gal<sub>3</sub>GF). Notably, all these three substrates share a sucrose moiety in their molecules.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sucrose</th>
<th>Raffinose</th>
<th>Stachyose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (mM)</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (1/s)</td>
<td>Km (mM)</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-Lsc2</td>
<td>17.1 ± 1.4</td>
<td>328.3</td>
<td>49.0 ± 2.2</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-Lsc3</td>
<td>19.9 ± 2.0*</td>
<td>481.7*</td>
<td>44.8 ± 3.4</td>
</tr>
</tbody>
</table>

* data from 15

Lsc2 and Lsc3 proteins certainly prefer sucrose over raffinose and stachyose as can be concluded from the data in Table 1. Notably, the substrate specificity of the Lsc1 protein of *P. syringae* pv. tomato is similar to that of Lsc2 and Lsc3. We have earlier compared catalytic properties of the Lsc3 protein of *P. syringae* pv. tomato and the LscA of *P. chlororaphis* subsp. *aurantiaca*. Our studies indicated that the LscA protein also prefers sucrose over raffinose: the Km of this enzyme for sucrose cleavage reaction is ~24 mM and for raffinose ~88 mM. Also, Lsc3 and LscA could both use nonconventional fructosyl acceptors producing heterooligosaccharides.

Herein it should be noted that not all levansucrases prefer sucrose to other substrates. For example, relative activity of the *Zymomonas mobilis* levansucrase with raffinose is 17% higher than with sucrose or stachyose.

Catalytic turnover (k<sub>cat</sub>) of the Lsc3 protein at sucrose cleavage is somewhat higher compared to the Lsc2 (Table 1). Still, the k<sub>cat</sub> values of both these proteins exceed respective parameters of most levansucrases. The k<sub>cat</sub> values for the levansucrases from *Gluconacetobacter diazotrophicus*<sup>19</sup>, *Zymomonas mobilis*<sup>18</sup> and *Bacillus subtilis* (cited in 20) are respectively 65, 28.3 and up to 60 1/s. We have shown that the k<sub>cat</sub> of the LscA from *P. chlororaphis* subsp. *aurantiaca* (purchased from Sigma-Aldrich, Germany) is only 7.2 1/s. The highest k<sub>cat</sub> of sucrose cleavage (2272 1/s) has been reported...
for the levansucrase of *Bacillus megaterium*\(^{20}\). Recently, a paper on properties of the *E. amylovora* levansucrase was published\(^{21}\). This protein is interesting for us, because it has high sequence identity (77-78%) with levansucrases of *P. syringae* pv. *tomato*. According to \(^{22}\), the *k*\(_m\) of *E. amylovora* levansucrase for sucrose is 33.6 mM and the *k*\(_{cat}\) (502.7 1/s) is highly similar to that of the Lsc3 protein (see Table 1). The *E. amylovora* enzyme was also capable of raffinose and stachyose usage\(^{21}\).

Transfructosylation properties of Lsc2 and Lsc3 were compared with sucrose as the substrate. Reactions were conducted at three concentrations (300, 600 and 1200 mM) of sucrose for 20 h and concentrations of glucose, fructose and FOS were measured using HPLC. From these data, TA was calculated as in \(^{11}\) and FOS yield was determined as in \(^{12}\). Table 2 shows that TA depends on sucrose concentration being highest at 1200 mM sucrose. Also, DP of the produced FOS increases concomitantly with sucrose concentration in the reaction medium. So, the FOS yield by 20 h of reaction was rather low (~1 g/mg of protein) at 300 mM sucrose and was elevated up to ~13 g/mg protein in case of the Lsc2 protein when reaction was conducted with 1200 mM sucrose.

**Table 2.** Transfructosylating activities, spectra and the amounts of produced FOS in reaction of Lsc2 and Lsc3 with sucrose.

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Transfructosylating activity (g/mg protein)</th>
<th>DP range</th>
<th>FOS yield (g/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose (mM)</td>
<td>Sucrose (mM)</td>
<td>Sucrose (mM)</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>600</td>
<td>1200</td>
</tr>
<tr>
<td>His(_s)-Lsc2</td>
<td>26.9±1.2</td>
<td>44.1±0.5</td>
<td>71.3±1.5</td>
</tr>
<tr>
<td>His(_s)-Lsc3</td>
<td>25.3±0.5</td>
<td>43.2±0.4</td>
<td>71.4±1.8</td>
</tr>
</tbody>
</table>

*Enzymes were added to reaction mixture at 2.7 U/ml and incubation time was 20 h.

The data in Table 2 clearly shows that polymerizing properties of Lsc2 and Lsc3 proteins are highly similar. From this aspect they both differ from the levansucrase of *E. amylovora* that exhibits enhanced polymerization ability at low sucrose concentration. For example, already at 200 mM sucrose concentration, its transfructosylating activity was slightly over 80%. At 1200 mM, it was around 96%\(^{21}\).

**Conclusions**

We show here that the Lsc2 and Lsc3 proteins of *P. syringae* pv. *tomato* have highly similar catalytic properties. Both proteins favour sucrose over raffinose and stachyose as substrates. Notably, the *k*\(_{cat}\) values of Lsc2 and Lsc3 proteins were shown superior to most other levansucrases and they also produced FOS with a high yield.

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**REFERENCES**