PURIFICATION AND CHARACTERIZATION OF GLUCOSYLTRANSFERASES FROM NEW STRAINS LEUCONOSTOC MESENTEROIDES

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ABSTRACT
The Leuconostoc species require sucrose in the culture medium as an inducer for the elaboration of glucosyltransferase (GTF) and glucans. Among all the reported purification methods, polyethylene glycol (PEG) is an effective and single-step purification method for GTF from Leuconostoc mesenteroides.

The extracellular GTFs from Leuconostoc mesenteroides L.m.28 and mutant Leuconostoc mesenteroides L.m.M2860 were purified by phase partitioning using polyethylene glycol (PEG) of different molecular weights. The cell-free extracts were subjected to fractionation by PEG-400, 1500, 4000 and 6000. In a single-step precipitation, at a final concentration of 25% (w/v) PEG-1500, the enzyme from parent strain L.m.28 gave the maximum specific activity – 12.17 U/mg protein. The purified enzyme by all using PEGs showed a single band corresponding to 180 kDa molecular size when run on SDS-PAGE for in situ activity detection by Periodic Acid Schiff’s staining. The 20% (w/v) PEG-6000 gave GTF from mutant strain L.m.M2860 with maximum specific activity of 10.93 U/mg protein and a single band of protein corresponding to 180 kDa. In a single-step precipitation, at a final concentration of 20% (w/v), 25% (w/v) and 30% (w/v) PEG-1500, the purified enzyme from L.m.M2860 showed a few molecular forms on SDS-PAGE corresponding to 180 kDa, 140 kDa, 120 kDa and 86 kDa. In addition, the purified GTF from L.m.M2860 showed higher thermal stability at 45°C for 30 minutes.

Keywords: glucan, glucosyltransferase, Leuconostoc mesenteroides, polyethylene glycol

Introduction
Lactic acid bacteria Leuconostoc mesenteroides and Streptococcus mutans produce enzymes synthesizing glucans. The glucosyltransferases (GTFs) of Streptococcus mutans are produced constitutively, but those from Leuconostoc mesenteroides are produced by sucrose induction, except for its recently isolated constitutive mutants (6,10).

Several researches have reported that glucosyltransferase exists in either single or multiple forms having molecular weights in the range of 64-245 kDa (7,17). The enzyme remains in an aggregated form in the presence of dextran resulting in a high molecular weight. Various methods like ultra-filtration, salt and PEG precipitation, chromatography and a combination of these methods have been standardized and successfully used for purification of glucosyltransferase from different strains Leuconostoc mesenteroides (13,16). High molecular weight proteins have been purified by precipitation using the nonionic hydrophilic polymer polyethylene glycol (PEG) (4). Goyal and Katiyar, (1993) have been reported an effective method of purification of dextranucrase from L. mesenteroides NRRL B-512F by fractionation with PEG of different molecular weights (4).

Dextranase treatment removes dextran from the enzyme, which simplifies the chromatographic processes, while dextran is required for efficient phase partitioning by PEG. Some GTFs are rendered resistant to dextranase hydrolysis by producing branched glucans. Most of these difficulties are overcome by the use of constitutive mutants that can produce glucosyltransferases in the absence of sucrose.

The aim of the present work is to optimize the purification of extracellular GTFs from Leuconostoc mesenteroides L.m.28 and mutant Leuconostoc mesenteroides L.m.M2860 by phase partitioning using polyethylene glycol of different molecular weights and to study their temperature resistance.
Materials and methods
Bacterial strains and culture media. Strain *Leuconostoc mesenteroides* Lm 28 was obtained from the bacterial culture collection of the Department of General and Industrial Microbiology, Sofia University and “SIBIO-93” Ltd – Plovdiv. *Leuconostoc mesenteroides* Lm M2860 is a mutant strain, receiving after chemical mutagenesis (5). The strains were cultured 6-8 h in GTF culture medium at 27°C on a rotary shaker (200rpm) (5).

**Glucosyltransferase assay.**
One unit of glucosyltransferase is defined as the amount of enzyme that catalyzes the production of 1 μmol of fructose per min. at 30°C in 20 mM sodium acetate buffer, pH 5,4, with 100 g of sucrose per liter, 0,05 g of CaCl₂ per liter, and 1 g of NaN₃ per liter. It was ascertained that the reducing sugar measured by DNS assay was due to glucosyltransferase and not to levansucrase, inveratase, or sucrose phosphorylase activity as described by Dols, et al., 1998 (2,3). Glucose concentration was measured enzymatically with glucose oxidase using a Beckman Glucose Analyzer 2.

Purification of glucosyltransferase.
The culture media was first centrifuged for 20 min at 7000 x g and 4°C. The supernatant was filtered with a Sartorius membrane (0,2 μm cutoff) to remove any traces of cells in the supernatant. The filtered supernatants on glucose cultures concentrated 10-fold by using Centricon tubes (10 000 Da cutoff; Amicon).

GTF was separated from the supernatants and concentrate solutions by using aqueous two-phase partition between dextran (native or exogenous) and polyethyleneglycol (PEG) (12). After addition of PEG-400, 1500, 4000 and 6000 to get the final concentrations of 10, 15, 25 and 30 (% w/v), the dextran – rich phase containing GTF was separated by centrifugation at 7000 x g for 20 min at 4°C, collected in the pellet, and diluted in 20 mM sodium acetate buffer, pH 5.4.

**Thermal stability** of GTFs was studied 45°C for 30 minutes.

**Protein Determination.** Proteins were assayed by method of Bradford, 1990 (1).

Electrophoresis analysis.
SDS-PAGE (70 x 80 mm slab gels, 7 % acrilamide gels ) was conducted by the method of Laemmli, 1970 (8). Protein was stained with Coomassie Brilliant Blue R 250(Sigma Chemical Co.). Glucosyltransferase activities were detected by incubating the gels in 10% sucrose overnight, followed by staining for polysaccharide by a periodic acid-Schiff procedure (9). Myosin, α-macroglobulin, β-galactosidase, transferin were used as molecular mass protein standards.

Results and Discussion
In the present study we screened PEGs of different molecular weights to purify extracellular GTFs from *L. mesenteroides* Lm28 and mutant strain *L. mesenteroides* LmM2860. The GTFs were also analyzed for purity and confirmed by staining the glucan produced by them.

The extracellular GTFs were subjected to fractionation in a single step precipitation by PEG 400, 1500, 4000 and 6000. Table 1 shows the maximum specific activity of GTF from Lm28 with corresponding concentrations of PEG 400, 1500, 4000 and 6000. Fractionation with PEG 1500 gave GTF with higher specific activity than PEGs with low and high molecular weights.

<table>
<thead>
<tr>
<th>PEG (%)</th>
<th>GTF (U/ml)</th>
<th>Total units</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude</td>
<td>5.80</td>
<td>580.0</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td>20% PEG</td>
<td>25.00</td>
<td>250.0</td>
<td>43.10</td>
<td>2.28</td>
</tr>
<tr>
<td>25% PEG</td>
<td>36.50</td>
<td>365.0</td>
<td>62.90</td>
<td>3.00</td>
</tr>
<tr>
<td>10% PEG</td>
<td>11.94</td>
<td>119.4</td>
<td>20.53</td>
<td>1.23</td>
</tr>
<tr>
<td>10% PEG</td>
<td>24.10</td>
<td>241.0</td>
<td>41.55</td>
<td>2.51</td>
</tr>
</tbody>
</table>

In a single step precipitation at a final concentration of 25% (w/v) PEG 1500 the enzyme from parent strain Lm28 showed the maximum specific activity – 12.17 U/mg protein. A concentration of 30%(w/v) PEG 400 showed GTF with 10.96 U/mg protein and a concentrations of 10%(w/v) PEG 4000 and PEG 6000 gave the enzyme with 9.70 and 9.60 U/mg, respectively. A lower percent of PEG was required for fractionating the GTF with its increasing molecular weight.

A maximal specific activity of the GTF from mutant LmM2860 was determined by 30% PEG 400, 30% PEG 1500, 15% PEG 4000 and 20% PEG 6000, in comparison with other using PEG concentrations (Table 2).
Fractionation of GTF from *Leuconostoc mesenteroides* LmM2860 after fermentation on media with 4% sucrose.

<table>
<thead>
<tr>
<th>PEG (%)</th>
<th>GTF (U/ml)</th>
<th>Total units</th>
<th>Overall yield (%)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude</td>
<td>2.30</td>
<td>115.00</td>
<td>-</td>
<td>0.32</td>
<td>7.19</td>
</tr>
<tr>
<td>30% PEG 400</td>
<td>17.20</td>
<td>17.20</td>
<td>14.96</td>
<td>3.70</td>
<td>4.65</td>
</tr>
<tr>
<td>30% PEG 1500</td>
<td>10.83</td>
<td>10.83</td>
<td>9.42</td>
<td>2.12</td>
<td>9.82</td>
</tr>
<tr>
<td>15% PEG 4000</td>
<td>24.67</td>
<td>24.67</td>
<td>21.45</td>
<td>8.00</td>
<td>3.08</td>
</tr>
<tr>
<td>20% PEG 6000</td>
<td>34.44</td>
<td>34.44</td>
<td>29.95</td>
<td>3.15</td>
<td>10.93</td>
</tr>
</tbody>
</table>

In a single step precipitation using 20% PEG 6000, the maximal specific activity was measured – 10.93 U/mg protein, as compared to those obtained with appropriate concentrations for other using PEGs. This suggested that the most appropriate concentration for fractionation and purification of GTF from LmM2860 is 20% PEG 6000. The cell free extract after fermentation on media with 4% glucose was subjected to fractionation by the same PEGs in the same concentrations after preliminarily addition of exogenous Dextran T70.

Table 3 shows the maximum specific activities of GTF from LmM2860 with corresponding concentrations of PEGs. An increase in the molecular weights of PEG 4000 and 6000 led to an increase in the recovered activity. In a single step of precipitation at a final concentration of 15% PEG 4000 and 6000 the enzyme from mutant LmM2860 indicated the maximum specific activity – 6.12 U/mg protein.

The GTF fractions from Lm28 and LmM2860 were also analyzed by SDS-PAGE for in situ activity detection by Periodic Acid Shiff’s staining. The purified enzyme from Lm28 by all using PEGs showed a single band corresponding to 180 kDa molecular size (Fig.1C). GTF preparation from LmM2860 obtained after fermentation on media with glucose and fractionation by all using PEGs and concentrations was homogeneous as shown by the single band of 180 kDa on SDS-PAGE (Fig.1A). The same band was found by in situ detection of GTF fractions obtained from LmM2860 after fermentation on media with sucrose and precipitation by PEG 400, 4000 and 6000 in all using concentrations also by PEG 1500 only at a final concentrations 10% (w/v) and 15% (w/v) (Fig.1A,B). This confirmed that the purified fractions contained only dextransucrase. In a single step precipitation at a final concentration of 20% (w/v), 25% (w/v) and 30%(w/v) PEG 1500 the purified enzyme from L.m.M2860 showed a few molecular forms on SDS-PAGE corresponding to 180 kDa, 140 kDa, 120 kDa and 86 kDa (Fig.1 B). The molecular form with 180 kDa molecular size predominated in the crude as well as purified enzyme preparations.

The stabilities of purified enzymes from Lm28 and LmM2860 at 45°C for 30 minutes were tested. To confirm GTF activity SDS-PAGE analysis were performed and the obtained bands were specifically stained with Shiff’s reagent (Fig.1D).

With the exception of mutant, the band disappeared after the temperature treatment, which resulted from inactivation of GTF from Lm28. The resistance to thermal inactivation of GTF from LmM2860 is of great importance to optimization of reaction conditions on the synthesis of oligosaccharides with specific properties.

The present results are similar to the results of other authors. Purama and Goyal, 2007 have been reported an effective purification of dextranase from *L. mesenteroides* NRRLB-640 by PEGs with different molecular weights (14). A final concentration of 10% PEG1500 have been resulted in maximum specific activity of 23 U/mg in a single step. The purified enzyme have been existed in multiple molecular forms (14). Otts and Day, 1988 have been purified glucosyltransferase from *L. mesenteroides* ATTC 10830 (11). They have been obtained purified enzyme with specific activity of 30 U/mg protein using 10% dextran T-500 and 20% PEG3350 (11).
Fig. 1. SDS-PAGE of GTFs from \textit{L. mesenteroides} LmM2860 fractionated at various concentrations of PEG400 (A), PEG1500 (B) and from \textit{L. mesenteroides} Lm28 fractionated at various concentrations of PEG 1500 (C), after temperature treatment at 45°C for 30 minutes (D).

(A) 1 - *30\% PEG400; 2 - *25\% PEG400; 3 - *20\% PEG400; 4 - *15\% PEG400; 5 - *10\% PEG400; 6 - **25\% PEG400; 7 - **30\% PEG400; (B) 1 - *30\% PEG1500; 2 - *15\% PEG1500; 3 - *10\% PEG1500; 4 - **25\% PEG1500; 5 - **20\% PEG1500; 6 - **15\% PEG1500; 7 - **10\% PEG1500; 8 - **20\% PEG1500; 9 - **25\% PEG1500; (C) 1- 20\% PEG1500; 2- Supernatant fraction 20\% PEG1500; 3- Supernatant fraction 25\% PEG1500; 4- Supernatant fraction 15\% PEG1500; 5- 25\% PEG1500; 6- 15\% PEG1500; 7- 10\% PEG1500; 8- 20\% PEG1500; 9- 30\% PEG1500; (D) 1, 2, 3 – GTF from Lm28; 4, 5, 6 – GTF from LmM2860;

\text{R - Protein standards( myosin-220 kDa, macroglobulin-170 kDa, β-galactosidase-116 kDa, transferin-76 kDa, glutamatdehydrogenase-53 kDa);}

* - after fermentation on media with 4\% sucrose; ** - after fermentation on media with 4\% glucose.

Remaud-Simeon et al., 1991 have been obtained purified GTF from \textit{L. mesenteroides} B-742 with an activity of 83 U/mg protein by PEG1500 (15).

We optimized the purification of extracellular GTFs from \textit{Leuconostoc mesenteroides} Lm28 and mutant \textit{Leuconostoc mesenteroides} LmM2860 by precipitation with PEGs with different molecular weights. In a single step precipitation, at a final concentration of 25\% PEG-1500, the enzyme from parent strain L.m.28 showed the maximum specific activity – 12.17 U/mg protein. A maximal specific activity of GTF - 10.93 U/mg protein was determined after precipitation of the enzyme from mutant strain LmM2860, cultivated in media with sucrose, using 20\% PEG-6000. A final concentration of 15\% PEG4000 and 6000 indicated the best purification of GTF from LmM2860 after fermentation in media with glucose. The results in this study give a good base for doing additional researches in order to optimize other methods for purification of GTFs especially from mutant L.m.M2860 also in order to use the purified enzymes with higher thermal stability for the production of specific oligosaccharides.

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