Proteomic analysis and characterization of a novel glucansucrase from a newly isolated strain of Leuconostoc mesenteroides AA1

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Citation

Abstract
Purpose Glucansucrase is an industrially important enzyme that is produced by different species of bacteria like Leuconostoc, Streptococcus and Acetobacter. Among them Leuconostoc mesenteroides is used for the production of glucansucrase that catalyzes the synthesis of a commercially important long chain glucose polymer known as glucan on industrial scale. Due to its increasing demand in various biotechnological industries, isolation of new strains of Leuconostoc mesenteroides for the production of glucansucrase and glucan is of valuable importance.

Methods New strain of Leuconostoc mesenteroides AA1 was isolated and various biochemical conditions and aspects regarding extracellular glucansucrase activity have been optimized. Glucansucrase was purified to homogeneity using conventional chromatographic techniques. Amino acid analysis and N-terminal sequence of the purified enzyme was also performed.

Results Glucansucrase [EC 2.4.1.5] was purified to homogeneity with an overall purification factor of 162.5 times, with a specific activity of 2692 DSU/mg. The native protein showed a molecular mass of 177,000 Da by SDS PAGE. The enzyme exhibited optimum catalytic activity at 35°C and pH 5.0 with 0.1 M citrate phosphate buffer. The kinetic constants K_m and V_max were estimated as 69.88 mM and 61.75 DSU/ml/hr, respectively. The amino acid composition of the enzyme showed that the enzyme is rich in both the basic and polar/hydrophilic amino acid and is less rich in acidic amino acids. The first six amino acid residues at the N-terminal end are D-S-T-N-T-V.

Conclusions This novel extracellular glucansucrase is significant for industrial perspective and is therefore suggested that this strain can be used for the production of glucansucrase and high molecular weight glucan on a large scale.

Abbreviations: DSU, Dextranucrase Unit; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HPLC, high performance liquid chromatography

TrEMBL UniProtKB Accession #: P85080

E.C. 2.4.1.5 (glucosyltransferase)

INTRODUCTION
Glucansucrase is a glucosyltransferase (E.C. 2.4.1.5) that catalyzes the transfer of glucosyl residues from sucrose to glucan polymer and liberates fructose. Glucan is a high-molecular-mass polymer (10^7 to 10^8 Da) and composed of a linear chain of glucosyl residues all linked through ß (1→6) glucosidic bond and several ß (1→2), ß (1→3), or ß (1→4) branched linkages [12]. The frequency and nature of the branch points mainly depend on the origin of the glucansucrase (i.e., the producing microorganism) [14]. Glucansucrase can catalyze the synthesis of several types of linkages that leads to the formation of a branched polymer [16]. The enzyme responsible for the production of glucan (dextran) from sucrose is a glucansucrase, which belong to the family 70 of the glucosidases and transglycosidases in the CAZy classification [15].

Glucansucrase have broad applications in the biotechnology industries. They have made a remarkable impact in the world of biotechnology because of their applications in the food, cosmetic, agricultural, photography and fermentation industries. The most promising application of glucansucrase and glucan is their use as protective colloid in blood plasma volume expander, flocculation, stabilization, lyophilization
and cosmetic ingredient formulation [8]. Other applications include its use as gel permeation matrices in research and various industries for the separation purposes of various products.

Several species of genera *Leuconostoc*, *Lactobacillus* and *Streptococcus* have been found to synthesize extracellular glucansucrase. Its expression is constitutive in *Streptococcus* strains [10], while it is inducible in *Leuconostoc* strains [11]. These bacterial strains have been shown to produce glucan of various structures, and this is attributed to the excretion of different kind of dextranucrases by the microorganisms [12-14].

To satisfy the industrial need of glucansucrase, it is imperative to explore new microbial strains. In this study a new strain of *Leuconostoc mesenteroides* AA1 was isolated and its extracellular glucansucrase was purified. A purification procedure for glucansucrase, characterization and its catalytic properties are also described. Glucan from this strain was also produced and characterized.

**MATERIALS AND METHODS**

**REAGENTS**

DEAE Sephadex A50, Sepharose CL-6B, Sephacryl 300 HR and all other reagents of analytical grade were purchased from Sigma (Sigma Chemicals Co., St. Louis, MO, USA). Molecular weight markers were purchased from Promega (Promega Corporation, USA). Media used in this study were purchased from Oxoid Ltd., Hampshire, UK.

**ISOLATION AND SCREENING OF STRAIN**

Bacterial culture was isolated from *Brassica oleracea* var *Capitata* L. (Cabbage) purchased from local market using enrichment media technique. Initially the culture was cultivated on MRS medium (BioM Laboratories, USA). The isolates were screened for glucan producing strains by inoculating in medium containing: (g l\(^{-1}\)) Sucrose, 50.0; Tryptone, 10.0; Yeast extract, 1.0; K\(_2\)HPO\(_4\), 2.5; the pH was adjusted at 7.0 and autoclaved at 121ºC for 15 minutes. After autoclaving 0.005 % sodium azide was added aseptically. Bacterial strain was selected showing highly viscous slimy growth on sucrose agar plate [15].

**STRAIN IDENTIFICATION**

*Leuconostoc mesenteroides* AA1 was selected for optimization and characterization due to high enzyme activity and glucan producing characteristic. The bacterial strain AA1 was isolated and identified by the physiological and biochemical tests according to Bergey’s Manual of Systematic Bacteriology [16], which confirmed the strain AA1 belong to genus *Leuconostoc mesenteroides*. The culture was maintained on sucrose broth medium at 4ºC.

**CULTURE MEDIA AND GROWTH CONDITIONS**

For fermentation purpose, the organism was grown at 25ºC in a medium containing (g l\(^{-1}\)): Sucrose, 25.0; Bactopeptone, 5.0; yeast extract, 5.0; K\(_2\)HPO\(_4\), 15.0; MnSO\(_4\).H\(_2\)O, 0.01; NaCl, 0.01; MgSO\(_4\).7H\(_2\)O, 0.01; CaCl\(_2\).2H\(_2\)O, 0.1. The pH of the medium was adjusted to 7.5 before sterilization at 121ºC for 15 minutes.

**ENZYME ASSAY & PROTEIN QUANTIFICATION**

Glucansucrase activity was determined by measuring the reducing sugar by Nelson Somogyi method as described earlier [17]. Units of glucansucrase activity are represented in DSU [18]. “One unit of enzyme activity was defined as the enzyme quantity that converts 1.0 milligram of sucrose into fructose and glucan in 1.0 hour using 0.1M citrate phosphate buffer of pH 5.00 at 35ºC”. Total protein content of the samples was estimated using the standard method described with bovine serum albumin as a standard [19].

**CULTIVATION AND CRUDE ENZYME PREPARATION**

Sterile sucrose broth medium was inoculated by a growing culture of L. mesenteroides AA1 and incubated at 25ºC for 8 hours. The culture was then centrifuged at 35,000 x g for 15 minutes at 4ºC and the cell free supernatant containing the extracellular crude enzyme was stored at -20ºC.

**ISOLATION AND PURIFICATION OF GLUCANSUCRASE**

**PEG 4000 PRECIPITATIONS**

The cell free supernatant was first subjected to partial purification by treating it with PEG 4000. A solution of 30 % (w/v) PEG 4000 was added to the crude enzyme to give 25 % saturation at 4ºC and stirred for 10 minutes. The suspension was left for 24 hour and centrifuged at 35,000 x g for 15 min at 4ºC. The precipitate obtained after centrifugation was re-suspended in 0.1 M citrate phosphate buffer (pH 5.0) and dialyzed overnight against the same buffer.

**DEAE SEPHADEX A50 ION EXCHANGE**
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CHROMATOGRAPHY

LKB gel filtration system was used for this purpose. The sample was applied through automatic sample applicator on XK16/70 glass column packed with DEAE Sephadex A50 column. The fractions were collected through automatic fraction collector Ultro Rac II (Model LKB 2070).

The dialyzed sample was applied to a DEAE Sephadex A50 column (1.6 x 40), pre-equilibrated with 0.1M citrate phosphate buffer (pH 5.0). After washing the column with 500 ml of equilibration buffer, bound proteins were eluted with the same buffer containing 0-4M guanidine-HCl. The flow rate was adjusted to 20ml/hr and each fraction of 0.5 ml was collected. The collected fractions were assayed for enzyme activity. The fractions showing glucansucrase activity were pooled and concentrated using freeze-drying method.

SEPHAROSE CL6B GEL FILTRATION CHROMATOGRAPHY

The concentrated fraction was then further applied on a Sepharose CL6B gel filtration column (1.6 x 49) earlier equilibrated with 0.1M citrate phosphate buffer (pH 5.0). The proteins were eluted at a flow rate of 20 ml/hr with the same buffer and fractions (1.0 ml each) were collected. Active fractions showing enzyme activity were pooled, dialyzed against the same buffer and then freeze dried. This freeze-dried gel filtration fraction was stored at −20°C and was used as the purified enzyme preparation for the characterization studies.

ASSESSMENT OF HOMOGENEITY

The homogeneity of the purified enzyme was analyzed by HPLC using C18 column (Teknokroma HPLC Column, Kromasil, 5 μm 25 x 0.46). Protein was eluted isocratically using 0.1 M citrate phosphate buffer (pH 5.0) at a flow rate of 0.5ml/minute. The protein profile was monitored at 280nm using a UV detector (Perkin Elmer, USA). Purified dextransucrase from Leuconostoc mesenteroides (Sigma Chemicals Co., St. Louis, MO, USA), 10 Units/mg protein, Cat.# D9909) was used as a standard.

MOLECULAR MASS DETERMINATION BY SDS PAGE AND IN-SITU ELECTROPHORESIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of the enzyme was resolved on 7.5 % polyacrylamide gel according to the standard protocol [20]. The proteins were stained with Coomassie brilliant blue R-250. The molecular mass of the purified glucansucrase was estimated from its position relative to those with the broad range protein molecular weight markers (Promega, Promega Corporation, USA). In-situ electrophoresis was also carried out to distinguish protein bands capable of synthesizing polymer from sucrose. For in-situ activity analysis, the gel after electrophoresis was washed three times with 20 mM sodium acetate buffer (pH 5.4) containing 0.05 g/L CaCl2 and 0.1 % (v/v) Triton X-100 to eliminate SDS. The gel was incubated in the same buffer containing sucrose (100 g/L) at 30°C for 24 hours. The active bands were detected by the appearance of dextran as a white polymer inside the gel [21].

CATALYTIC PROPERTIES OF GLUCANSUCRASE

KINETIC DETERMINATION

Initial rate of sucrose utilization was determined at various substrate concentrations. The kinetic constants Km and Vmax were estimated by the method of Lineweaver and Burk plot using sucrose as substrate and plotting the values of 1/v as a function of 1/S [22].

EFFECT OF PH & TEMPERATURE

The activity of purified glucansucrase was measured in a pH range of 3.0 to 7.0 at 35°C for 15 minutes.

The glucansucrase assay mixture was incubated with substrate at different temperatures ranging from 15°C to 45°C for a period of 15 minutes followed by the standard assay procedure.

AMINO ACID ANALYSIS

Acid hydrolysis of the purified enzyme was carried out using 6N HCl. The sample was sealed in a Pyrex test tube under vacuum. The test tube was kept at 110°C for 24 hours. After 24 hours the sample was washed thrice with deionized water, concentrated and filtered through 0.45 μm membrane filter. Derivatization of the sample carried out using o-phthalaldehyde (OPA). The amino acid analysis of the purified enzyme was carried out using an amino acid analyzer amino acid standard solution was purchased from Sigma (Sigma Chemicals Co., St. Louis, MO, USA). Amino acids are in 0.1 N HCl at the indicated concentration of ± 4 %. Amino acid standard solution was stored at 4°C.
ANALYSIS OF N-TERMINAL PROTEIN SEQUENCE

Purified enzyme was resolved by SDS-PAGE and was electronically blotted onto the polyvinylidene difluoride membrane (PVDF), using a semi-dry blotting device \[^{[23]}\]. Blots were stained with Coomassie brilliant blue R-250, protein band of interest was excised and amino terminal amino acid sequence was determined using an automated protein sequencer (ABI Procise 491 Protein Sequencer, USA).

ACCESSION NUMBER

The EMBL accession of the sequence reported in this paper is UniProtKB P85080.

RESULTS AND DISCUSSION

In the present study isolation, purification and characterization of extracellular glucansucrase and glucan from a newly isolated strain of Leuconostoc mesenteroides AA1 is reported.

PURIFICATION OF GLUCANSUCRASE

Leuconostoc mesenteroides AA1 secreted extracellular glucansucrase into the culture medium, when sucrose was used as an inducer. After fermentation the cells were removed by centrifugation and the cell free supernatant contained about 53.0 DSU/ml of glucansucrase activity with a specific activity of 16.56 DSU/mg (Table-1).

ASSESSMENT OF HOMOGENEITY

The apparent purity of the enzyme was further demonstrated by HPLC for purity check analysis. HPLC profile obtained from the standard shows a single peak with a retention time of 4.24 minutes (Figure 3A). When glucansucrase purified sample was injected, the profile also showed a single peak with retention time of 4.24 minutes (Figure 3B) confirming that glucansucrase has been purified to homogeneity.
Proteomic analysis and characterization of a novel glucansucrase from a newly isolated strain of *Leuconostoc mesenteroides* AA1

**Figure 4**

Figure 3: HPLC profile of glucansucrase from AA1: A, Purified dextranucrase from (Sigma) was used as standard; B, Purified glucansucrase fraction after fractionation through Sepharose CL-6B

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**CATALYTIC PROPERTIES OF GLUCANSUCRASE**

**EFFECT OF SUBSTRATE CONCENTRATION ON GLUCANSUCRASE ACTIVITY**

Sucrose was used as a substrate for the determination of substrate saturation kinetics. A wide range of sucrose concentration was used to measure the initial rate of reaction. $K_m$ and $V_{max}$ values at 35°C were calculated as 69.88 mM and 61.75 DSU/ml/hr, respectively. Substrate saturation kinetic showed that after reaching to the maxima, the substrate inhibitory effect started due to higher substrate concentration and no further increase in glucansucrase activity was detected. Dextranucrase from *L. mesenteroides* NRRL 1299 was specified as enzyme I and II, with $K_m$ values as 10.7 mM and 250 mM sucrose, respectively [13]. Similarly in case of *Streptococcus mutans* FA1, when sucrose was used as a substrate a $K_m$ value of 55mM was reported [26]. $K_m$ value of 12 to 16 mM of a purified dextranucrase from *L. mesenteroides* NRRL B512F has also been reported [27].

**EFFECT OF TEMPERATURE & PH ON GLUCANSUCRASE ACTIVITY**

Glucansucrase activity was determined at various temperatures ranging from 15°C to 45°C. The purified enzyme displays an optimal activity at 35°C (Figure 4A). Glucansucrase from *L. mesenteroides* strain B-742, B-742CA and B-742 (parent strain) had temperature optima at 35°C while for *L. mesenteroides* B-742CB glucansucrase activity, prepared on both glucose and sucrose medium, had a temperature optima at 45°C [28]. In another reported temperature optima of 30°C for *L. mesenteroides* B-512F have also been recorded [13] while two forms of extracellular dextranucrase from *L. mesenteroides* NRRL B-1299 named as enzyme I and N had exhibited optimum activity at 40°C and 35°C, respectively [29].

The pH optimum, determined at optimal temperature (35°C) was found at pH 5.0 with a rapid decline in the activity as pH moved to either extreme (Figure 4B). The results showed that the activity of the glucansucrase depends, inter alia, on pH. Two forms of extracellular dextranucrase from *L. mesenteroides* NRRL B-1299, I and N had an optimum pH value of 6.0 and 5.5, respectively [29]. It was also reported by group of scientist that an important loss of activity occurred, when the pH fell to values that were lower than pH 5.0 [30]. It has been reported that dextranucrase produced by *Leuconostoc mesenteroides* NRRL B512F is active only in a pH range between 4.8 and 6.2 [27].

**Figure 5**

Figure 4A: Effect of pH on extracellular glucansucrase activity

**Figure 6**

Figure 4B: Effect of temperature on extracellular glucansucrase activity
Proteomic analysis and characterization of a novel glucansucrase from a newly isolated strain of Leuconostoc mesenteroides AA1

MOLECULAR MASS DETERMINATION

SDS PAGE profile showed three protein bands that were detected in the partially purified sample (Figure 5, Lane B). While, after purification a single protein band of extracellular glucansucrase (Figure 5, Lane C) was detected confirming that the glucansucrase has been purified to homogeneity with an approximate molecular mass of 177,000 Da which is similar to the molecular mass of glucansucrase isolated from Leuconostoc mesenteroides NRRL B512F and mutant strain of Leuconostoc mesenteroides NRRL B512 FMC [13], but it significantly differ from the molecular mass of the same protein isolated from other Leuconostoc mesenteroides and Streptococcus strains [32,33,34]. Zymography of the purified enzyme was also performed and homogeneity of the purified enzyme was confirmed (Figure 5, Lane D). The molecular mass of the monomeric enzyme from L. mesenteroides B512 (f) had a molecular mass of 64,000 Da [34]. Whereas, multiple active lower molecular weight dextran sucrase forms from L. mesenteroides B512F have also been reported and these forms arise from proteolytic processing of a 177,000 Da precursor into 155,000 and 129,000 Da [13]. Purified dextran sucrase from L. mesenteroides B512 FMC had a molecular size of 184,000 Da [33].

Figure 5: SDS-PAGE profile of glucansucrase: Lane A, High molecular weight standards; Lane B, Partially purified glucansucrase; Lane C, Purified glucansucrase (Coomassie blue staining); Lane D, Assay for soluble glucan-synthesis corresponding to lane

AMINO ACID ANALYSIS

Amino acid analysis of purified glucansucrase was performed after acid hydrolysis. Standard amino acid solution (Sigma) was used for the detection purpose with reference to retention time. Amino acid analysis profile of standard solution is shown in Figure 6A, where as amino acid analysis profile of glucansucrase is presented in Figure 6B. Compositional analysis data provides a unique distribution profile of all amino acids in the protein (data not shown). Amino acid analysis of the purified extracellular glucansucrase shows that the enzyme is rich in both the basic (His, Lys, & Arg) and polar/hydrophilic amino acid and is less rich in acidic amino acids (Asp & Glu).
Proteomic analysis and characterization of a novel glucansucrase from a newly isolated strain of _Leuconostoc mesenteroides AA1_

**Figure 8**
Figure 6: Amino acid analysis profile: A, Amino acid standard (Sigma); B, Purified glucansucrase

**N- TERMINAL SEQUENCE**
The first six amino acid residues obtained from the sequencing of the N-terminus of the purified glucansucrase are: Asp-Ser-Thr-Asn-Thr-Val (D-S-T-N-T-V) (UniProtKB P85080). Further more the protein is not N-terminally blocked. The N-terminal amino acid sequence of glucansucrase from _Leuconostoc mesenteroides AA1_ is aligned in comparison with other glucosyltransferases (Table-2).

**Figure 9**
Table 2: N-Terminal Protein sequencing of glucansucrase from AA1

<table>
<thead>
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<th>Organism</th>
<th>Reference</th>
<th>Sequence</th>
<th></th>
</tr>
</thead>
<tbody>
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<td><em>Leuconostoc mesenteroides AA1</em></td>
<td>Current study</td>
<td>DSTNTV</td>
<td></td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides Lee4</em></td>
<td>Neubauer et al. 2003</td>
<td>DSSVPD</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus sobrinus Mf28</em></td>
<td>Ferretti et al. 1987</td>
<td>DTETVS</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus reuteri Strain 121</em></td>
<td>Kraji et al. 2002</td>
<td>DQVVOQ</td>
<td></td>
</tr>
</tbody>
</table>

A comparison of the N-terminus sequence of the glucansucrase AA1 showed no strong homology with the previously characterized glucansucrase from _Leuconostoc mesenteroides_ [35], _Streptococcus sobrinus_ [36] and _Lactobacillus reuteri_ [36] hence suggesting it is a unique novel enzyme sequence. However, the first amino acids, of the N-terminal region of all the four glucansucrases were strongly similar i.e. starting with aspartic acid. The glucosyltransferase (GTF) enzymes of oral Streptococci and the dextranucrase (DSRs) along with alternansucrase (ASRs) from _Leuconostoc mesenteroides_ strains have been studied in most detail. So far, one dextranucrase expressed by _Leuconostoc mesenteroides_ NRRL B-512F and two dextranucrases of _Leuconostoc mesenteroides_ NRRL B-1299 have been cloned and characterized in more detail [37,38]. These enzymes are all phylogenetically closely related and are large proteins with an average molecular mass of 160 kDa. Although 16 different glucansucrase has been sequenced, no 3D structure is yet available and much remains to be known about the mechanism and structure/function relationship of these important enzymes.

**CONCLUSIONS**
A new strain taxonomically identified as _Leuconostoc mesenteroides AA1_ was isolated that produces a novel extracellular glucansucrase. This enzyme catalyzes the synthesis of a commercially important long chain glucose polymer known as glucan. The biochemical characteristics of the enzyme were studied and extracellular glucansucrase activity was optimized. Glucansucrase was purified using affinity chromatography with DEAE Sephadex A-50 followed by gel permeation chromatography using Sepharose CL6B to homogeneity with 162 fold purification. The molecular mass of the enzyme was determined as 177,000 Daltons. Amino acid analysis of the purified glucansucrase showed that the enzyme is rich in both basic and polar amino acids and less rich in acidic amino acids. The N-terminal characterized glucansucrase from _Leuconostoc mesenteroides AA1_ showed no strong homology with the other N-terminal glucansucrase sequences and hence it is concluded that this enzyme has a unique sequence. Hence, this novel glucansucrase is significant for industrial perspective and is therefore suggested that this strain can be used for the elaboration of glucansucrase and glucan on a large scale.

**ACKNOWLEDGEMENT**
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