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Levanase from *Bacillus subtilis* hydrolyses β-2,6 fructosyl bonds in bacterial levans and in grass fructans

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ABSTRACT

A Levanase, LevB, from *Bacillus subtilis* 168, was expressed as a His_6-tagged protein in *Escherichia coli*. The enzyme was purified and characterised for its activity and substrate specificity. LevB has a pH optimum of 6.0-6.5 and a maximum observed specific activity of 3 U mg^{-1} using levan from *Erwinia herbicola* as substrate. Hydrolysis products were analysed by HPAEC, TLC, and NMR using chicory root inulin, mixed linkage fructans purified from ryegrass (*Lolium perenne)* and levan from *E. herbicola* as substrates. This revealed that LevB is an endolevanase that selectively cleaves the (β-2,6) fructosyl bonds and does not hydrolyse inulin. Ryegrass fructans and bacterial levan was hydrolysed partially releasing oligosaccharides, but together with exoinulinase, LevB hydrolysed both ryegrass fructans and bacterial levan to near completion. We suggest that LevB can be used as a tool to achieve more structural information on complex fructans and to achieve complete degradation and quantification of mixed linkage fructans.

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1. Introduction

Fructans are non-structural polymers of fructose units which are accumulated by microorganisms and in the vegetative tissues of many higher plants, including temperate forage grasses and cereals. Fructans are typically composed of a single glucose unit and one or more chains of fructose units which are linked by either (β-2,1) or (β-2,6) fructosyl bonds. The precise structure depends on the type of organism from which the fructans are derived. Bacteria, such as *Bacillus subtilis*, typically contain levans (β-2,6 polyfructose), whereas plants may contain inulin (β-2,1 polyfructose), levan or mixed linkage fructans [1]. The linkage to the glucose unit varies, and the degree of polymerization range from 3 (one glucose and two fructose units) to hundreds. This gives a large structural variation and the precise structure depends on both the botanical origin and environmental conditions.

In plants, fructans fulfil several important roles as long- and short term energy storage. Their biosynthesis will affect the source-sink balance and can serve as carbon reserve for survival e.g. during a winter season. In many plants, the accumulation of fructans in shoots or meristematic tissues is related to cold stress responses [2], and fructans have been suggested to provide a degree of cold resistance by operating as either osmolytes or by working directly as membrane stabilising agents [3]. In addition, fructans have been suggested to provide protection against oxidative stress [4]. The precise function of fructans may depend on their structure, biosynthesis and mobilization. To achieve a better understanding on fructan structure and function more precise and reliable methods are required.

For commercial production of fructans, the most important crop is chicory (*Cichorium intybus* L.), which accumulates high levels of inulin in the storage roots [1]. The large amount and relatively simple structure of this polymer allows for easy and specific detection as fructose released by acid hydrolysis or by enzymatic degradation (Megazyme Fructan Assay Procedure, Megazyme, Bray, Ireland). Analysis of fructans with a more complex structure constitutes a greater challenge. In plants of the grass family, Poaceae, fructans are found in many of the species that grow in temperate climate regions [5]. This includes some of our important fodder grasses as well as cereal crops, such as wheat, barley, oat and rye. The fructans found in the grass family, also called graminans, are typically of mixed

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linkage type and have a complex and variable structure. Some grass species primarily have (β-2,6) chains with branches of (β-2,1) whereas others primarily have (β-2,1) chains with branches of (β-2,6) [1] and the degree of polymerization varies from mainly short chain oligosaccharides to polysaccharides with degree of polymerisation (DP) estimated to 200–300 [5]. In *Lolium perenne*, the grass which we have analysed in this study, the fructans are of intermediate DP with longer chains in the range of DP 50-100 [5,6] as well as shorter oligosaccharides (down to DP3) [6–8], it has also been shown that the core structure of the *L. perenne* fructans may be of kestose, isokestose as well as neokestose type and the profiles of fructans depend on both tissue origin and physiological status of the plant [8]. Consequently the quantification of individual fructans and analysis of their structures in *Lolium* and other grasses is a challenge. Availability of levanases with specific substrate specificities represents a valuable tool in these analyses.

In food production, fructans may represent extra energy intake and add sweetness and texture. They also play a central role in the field of preventive nutrition due to their prebiotic action [9,10]. The prebiotic effects are almost exclusively documented for inulin type fructans, and while inulin type fructans are well characterized with respect to their functional properties, much still remains to be learned about the mixed linkage fructans [11]. Any prebiotic effects of mixed linkage fructans still need to be characterized, and such documentation will have to rely on precise quantification and structural characterization of the fructan in question. Despite the relatively low content of fructans in cereal derived food items, the typical human intake of fructan, e.g. in a wheat based diet, can be dominated by the graminans [11]. This is due to the large intake of grain derived food items compared to other fructan containing foods. Thus, because of both the agronomic and the nutritive value, there is a considerable interest in fructans, and a need for both structural characterization and convenient quantitative analysis. New methods for analysis have been devised, for example based on mild acid hydrolysis [12] and enzymatic analysis [13] and LC–MS [14]. In this context, an enzyme with levanase activity would offer a basis for a convenient assay and could provide additional information on structural details of graminans.

To facilitate the degradation of levans and mixed linkage fructans we decided to express and characterise a bacterial levanase. Preferably the enzyme should be an endolevanase that could hydrolyse (β-2,6) fructosyl bonds within a more complex structure. Based on this, we chose the enzyme LevB from *B. subtilis*. This enzyme has previously been identified as a levanase [15,16] since cell fractions enriched in the expressed enzyme were shown to hydrolyse a high molecular weight levan produced *in vitro*. In those studies the enzyme was demonstrated predominantly to produce levanbiose and to a lesser extent fructose and levantriose [15]. However, the enzyme has not yet been characterised in any detail with respect to e.g. specific activity, substrate specificity or pH optimum. In this study we report the expression, purification and characterization of a His_6-tagged version of LevB. We demonstrate its ability to assist the degradation of mixed linkage grass fructans derived from *L. perenne* where it specifically hydrolyses the (β-2,6) bonds, and together with an exoinulinase LevB hydrolyses *Lolium* fructan to fructose units.

2. Materials and methods

2.1. Construction of vector for expression of His_6 tagged LevB

Primers were designed to amplify the sequence encoding a levanase, LevB, from *B. subtilis* based on the available database entry (GenBank: AL009126.3). The sequence was amplified using DNA isolated from *B. subtilis* strain 168 (A1) as template and two primers, forward primer: 5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACTATATAAAAGCA-3′ and reverse primer: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTCATGAATCGAATC-3′. The underlined sequences are complementary to the bacterial DNA sequences and the remaining sequences add the *att*B1 and *att*B2 recombination sites to the PCR product to prepare the sequence for cloning using the Gateway system (Life Technologies). A proofreading polymerase (Phusion High-Fidelity DNA Polymerase, FINNZYMES) was used for amplification, and the expected amplified 1612 bp product was verified by electrophoresis (1% agarose) and extracted from the gel with QIAquick Gel Extraction Kit (QIAGEN). Purified PCR product (7 μl) was recombined with 150 ng pDONR201 (Life Technologies), incubating at 25 °C for 20 h with 2 μl BP Clonase II (Invitrogen). The reaction was stopped the following day by adding 1 μl Proteinase K solution (Invitrogen) and incubating 10 min at 37 °C. The mixture (1 μl) was transformed by electroporation (200 Ω, 2.5 kV, 25 μFD) into electrocompetent *Escherichia coli* strain DH5α (50 μl). Positive colonies were selected on LB agar plates containing 50 μg ml^{-1} kanamycin. Positive colonies were selected, subcultivated and liquid cultures were grown (LB + 50 μg ml^{-1} kanamycin, 24 h, 37 °C, 220 rpm agitation) for plasmid purification. The entry clones was purified using QIAprep Spin Miniprep Kit (QIAGEN). The correct sequence of the entry clone, covering the entire open reading frame and recombination sites, was verified by sequence analysis (MWG-Biotech AG).

The entry clone was used for LR-recombination with the destination vector pDEST17 (Life Technologies) to yield an expression vector encoding a fusion protein His_6-tagged at the NH_2-terminus behind a T7 promoter. LR Recombination was including entry vector, destination vector and LR Clonase II enzyme mix was incubated at 25 °C (20 h), treated with 1 μl of the Proteinase K (10 min, 37 °C) and transformed into *E. coli* strain DH5α. Colonies containing recombined vectors were selected on LB agar plates containing 100 μg ml^{-1} ampicillin. Final expression vector was purified from liquid cultures of subcloned colonies using QIAprep Spin Miniprep Kit (QIAGEN) and the vector was transformed into *E. coli* strain BL21-A1 by electroporation as described above. The correct sequence of the final expression vector was verified by sequencing, and encodes a protein with 538 amino acid residues comprising 4 upstream amino acids, a His-tag (6 aa) a short linker (12 aa), and the entire LevB-sequence (516 aa), with a predicted total mass of 61.7 kDa.

2.2. Expression and purification of LevB

LB medium (1L) containing ampicillin (100 μg ml^{-1}) was inoculated with the E. coli carrying recombinant plasmid and grown at 37 °C (220 rpm) to an OD600 of 1.1. Expression was induced by addition of arabinose to a final concentration of 0.1% and proceeded for 18 hours at 18 °C at constant agitation of 150 rpm. The cells were harvested by centrifugation and resuspended in lysis buffer (25 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl, 10 mM imidazole, 5% glycerol, 0.1% TritonX100, and 1 mM DTT). The cell extract was obtained by sonication (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT, USA). After centrifugation (20,000 × *g*, 20 min) the supernatant was passed through a 5 ml HisTrap FF column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was washed with 20 ml buffer containing 25 mM Tris–HCl (pH 7.5) 100 mM NaCl, 50 mM imidazole, 5% glycerol, and 1 mM DTT. Elution was done with 25 mM Tris–HCl buffer (pH 7.5) containing 5% glycerol, 200 mM imidazole and 1 mM DTT collecting 0.9 ml fractions. Fractions containing LevB, monitored by SDS-PAGE, were pooled and passed through a 6 ml Resource Q column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the column was washed with (30 ml) 25 mM Tris–HCl buffer (pH

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