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Search for a dextransucrase minimal motif involved in dextran binding

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Abstract **Fourteen truncated forms of *Leuconostoc mesenteroides* NRRL B512-F dextransucrase, involving N-, C- or N- plus C-terminal domain truncations were tested for their ability to bind dextrans. The shortest fragment (14 kDa molecular weight) that still exhibited a strong interaction with dextran was localized between amino acids N1397 and A1527 of the C-terminal domain (GBD-7) and consists of six YG repeats. With a dissociation constant K_d of 2.8×10^{-9} M, this motif shows a very high affinity for isomaltohexaose and longer dextrans, supporting the proposed role of GBD in polymer formation. The potential application of GBD-7 as an affinity tag onto cheap resins like Sephacryl® S300HR for rapid purification was evaluated and is discussed.**

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1. Introduction

Glucansucrases (EC. 2.4.1.5, family 70 of the glycoside-hydrolases (GH) [1]) are extracellular enzymes mainly produced by *Streptococcus*, *Leuconostoc* and *Lactobacillus* bacteria [2]. From sucrose, they naturally catalyze the synthesis of glucans that vary in terms of their size, the types of osidic linkages and the degree of branching. The dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (DSR-S), a member of the GH family 70 produces dextran, a soluble glucan with more than 95% of the D-glucosyl units α-1,6 linked.

Primary structure analyses revealed that most of glucansucrases possess a C-terminal domain often called the glucan-binding domain (GBD). The GBD is typically about 30–50 kDa in size and consists of several closely related repeated units, which all contain the same YG repeat structural element, characterized by the presence of clusters of aromatic residues and occurrence of glycine from three to five amino acids downstream these clusters [3]. The type, number and organization of these YG repeats vary among the 70 members of the GH-family of enzymes [4].

Over the past decade several studies have revealed the role of the GBD in dextran binding. For example, the strong affinity for dextran has been used to purify glucansucrases by dextran/polyethyleneglycol phase demixion [5], or affinity chromatography onto Sephadex® gels [6–8], which consist of cross-linked dextrans. Kaseda et al. [9] proposed the use of the 30 kDa GBD of *Streptococcus sobrinus* GTF-I glucansucrase as an affinity tag for recombinant protein purification. More recently, Shah et al. [10] revealed the crucial role of the aromatic residues found in the YG repeats, which were suggested to "stack" with the sugar units of dextran or mutan. Finally, Moulis et al. [11] showed that YG repeats are involved in the DSR-S polymerization reaction, in which they are proposed to act as anchoring zones for the growing dextran chains during their elongation.

To better characterize the role of the C-terminal domain of DSR-S, the aim of this study was to identify the minimal determinants responsible for dextran binding. For this purpose, several truncated forms of the DSR-S C-terminal domain were generated, along with N-, C- and both C- plus N-terminal truncations. Their observed glucan-binding properties are discussed with regard to their primary structures. Finally, the usefulness of the shortest glucan-binding motif (GBD-7) for affinity chromatography onto cross-linked dextran resins was evaluated and is discussed.

2. Materials and methods

2.1. Construction and expression of GBD truncated genes

Full-length (1014 bp) and various truncated *gbd* genes (Table 3) were amplified from *L. mesenteroides* NRRL B-512F genomic DNA (Genbank accession number I09598) using the Expand High Fidelity PCR system (Roche) and the primers described in Table 1 (given in $5' \rightarrow 3'$ sense). Amplicons were then cloned into pBAD/TOPO Thiofusion vectors, in fusion with *Thioredoxin* at the N-terminal and polyhistidine tag (*His*) encoding tag at the C-terminal extremity (*Thioredoxin*–GBD–*His* variants). All the constructions were sequenced (Millegen, France or Macrogen, Korea). For sake of clarity, the presence of the *Thioredoxin* and *His* tags are not always mentioned, although they are present in the all constructions.

Escherichia coli TOP10 (Invitrogen) was used as host for both cloning and recombinant protein expression and purification. Transformation and cultivation of *E. coli* transformants were performed as described before [12]. Cells were concentrated to an $OD_{600\ nm}$ of 80 in 50 mM potassium phosphate buffer pH 7.2 supplemented with 0.05 g/l of $CaCl_2$ and 1 mM PMSF, and then sonicated. Cell debris was eliminated by centrifugation ($7500 \times g$, 15 min).

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Abbreviations: DSR-S, *Leuconostoc mesenteroides* NRRL B512-F dextransucrase; GBD, glucan-binding domain; GH, glycoside-hydrolase; His, polyhistidine tag

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The recombinant wild-type GBD and truncated variant proteins were separated by electrophoresis using 10% (w/v) Bis-Tris SDS–PAGE gels (Invitrogen) under denaturing conditions. After migration, the gels were either stained with Colloidal Blue (Invitrogen) or used for Western-blot (Nu-Page system, Invitrogen) using anti-*His* or anti-*Thioredoxin* as primary antibodies and anti-mouse secondary antibody coupled to alkaline phosphatase. The protein expression levels were equivalent for all the variants.

2.2. Glucan-binding assays

Glucan-binding assays were performed using the method developed by Shah et al. [10], involving the use of *His*-tagged proteins and Ni-NTA coated 96-well HisSorb plates. *Thioredoxin*–GBD–*His* recombinant proteins were first bound onto the immobilized Ni-NTA molecules to form the immobilized capture phase and then washed in PBST (PBS buffer, pH 7.2 containing 0.05% (v/v) of Tween 20) to remove excess non-bound proteins. Next, 100 μg/ml of biotin-labeled dextran (70 kDa) ligand was added in PBSA (PBS buffer, pH 7.2 containing 0.2% (w/v) of BSA), incubated 10 min. Four washes were performed with PBST to remove non-specifically bound and unbound biotin. The bound GBD:biotin–dextran complexes were detected using Extravidin-alkaline phosphatase. Four independent assays were performed for the all constructs.

To determine the dissociation constants K_d, different amounts of biotin–dextran ranging from 0.14 nM to 143 nM were added to the GBD extracts fixed into the Ni-NTA wells. K_d values were determined using the one-site saturation ligand-binding equation $\mathrm{Abs}_{405\ nm} = \frac{B_{max} \times [\text{Biotin–dextran}]}{K_d + [\text{Biotin–dextran}]}$ with SigmaPlot 10.0 software. B_{max} corresponds to the maximum absorbance value obtained after complete saturation of the binding site.

2.3. GBD-7 purification onto Ni-NTA matrix

One volume of Ni-NTA slurry (Qiagen) was added to 4 vol of GBD extracts for 2 h at 4 °C. Washes were performed with 4 vol of buffer A (50 mM PBS pH 7.2, 0.05 g/l $CaCl_2$, 1 mM PMSF) supplemented with 1 M NaCl and 20 mM imidazole. The bound proteins were then eluted using 8 vol of buffer A containing 250 mM imidazole and 0.3 M NaCl. The fractions of interest were dialyzed overnight against 50 mM PBS buffer pH 7.2. The protein content was quantified by Microbradford assay using BSA as standard.

2.4. GBD-7 purification onto Sephacryl® gel

One volume Sephadex®, Sephacryl® or Superdex® resins (Amersham Biosciences) were rinsed with 10 vol of buffer *A* + 0.3 M NaCl to remove ethanol traces and recovered by centrifugation (5000 × *g*, 2 min). Ten volumes of GBD-7 extracts (297 μg/ml) were added to the washed resin, mixed and allowed to stand for 2 h at 4 °C. Both GBD-7 extracts previously purified onto Ni-NTA supports and crude extracts for the case of purification of the Thioredoxin fused GBD-7 were used in order to determine the best support matrix. After incubation the unbound protein solution was removed, and the resins washed four times with 10 volumes of buffer A + 0.3 M NaCl each time. The Thioredoxin tagged GBD-7 was then eluted using 14 vol of 50 mM PBS pH 7.2 buffer containing 0.1–50 g/l of commercial dextrans. Several sizes of dextrans were used. They range from 0.4 to 2000 kDa (Sigma). The protein content of the eluate was measured using microbradford assay with BSA as the standard, whilst the Thioredoxin content was measured using Holmgren's method [13].

3. Results and discussion

3.1. Construction of GBD truncated forms

One full length and 14 different N- and C- terminal truncated *gbd* encoding protein contructs (Table 3) were generated by PCR using the primers shown in Table 1. SDS–PAGE and Western-blot using anti-*Thioredoxin* and anti-*His* antibodies showed that all the recombinant proteins had the expected molecular weight and were not degraded (data not shown). It was also verified that they were able to bind via their *His* tag onto the Ni-NTA molecules adsorbed on the HisSorb plates.

Table 1
Primers designed for PCR based amplification of full length and truncated GBD variants

Primer name	Sequence (5′–3′)
ForGBD	3568-ACCATGGATAATAACTATTACTATTTTGAT-3591[1]
RevGBD	4581-GGCTGACACAGCATTTCCATTATTATCAAA-4552
GBD1F	3577-TATTACTATTTTGATAAAACAGGTCATT-3604
GBD3R	3966-TTGATTCTTACTATTTTGCACATAAC-3941
GBD5F	3967-TGGTTCTATTTTGATGGTAATG-3988
GBD7R	4269-TTGCAATACAAACCCTGTTTTCGCACG-4243
GBD9F	4270-GATGGTGTACTAAGATACTTCGATCA-4295
GBD10F	4063-GGTGAATTCATTGATGCAGACGGGGATA-4090
GBD11F	4189-AATCAGTATTATCAATTAGCAGATGGTAAA-4218
GBD12R	4545-AACCTTACCCTGAGCACTTATTAAAG-4519
GBD13R	4431-AGCATCTGTTAAATACCATTTACCTTGATA-4402
GBD14F	4090-ACTTTCTATACGAGTGCCACTGATGGTCGC-4119
GBD15R	4326-AATGATAGCATCTTTCACTTGCTCACCGTT-4297
GBD16R	4389-ATTTTTTACAGCGACACCTTGTGTTGCATT-4360
GBD17F	4195-TATTATCAATTAGCAGATGGTAAATATATG-4224
GBD18R	4470-GTCGTCAACTGCTTTAAAACCTTTGATAAG-4441

Table 2
Sequences of the repeats identified in DSR-S C-terminal domain, following the consensus YG motive defined by Giffard and Jacques [3]

Motive	Sequence
Consensus	-**ND**G**YYF**xxx**G**xx**H°**x**(G/N)**x**H°H°H°**
YG_1	**-N**NY**YYF**DKT–**G**HL**V**T-**GL**QK**I**
YG_2	-**N**HT**YFF**LPN–**GIEL**VKSF**L**Q
YG_3	**D**GTIV**YF**DKK–**G**HQ**V**FDQY**I**T
YG_4	**N**GNA**YYF**DDA–**G**VM**L**KS**GL**AT**I**
YG_5	**D**GHQQ**YF**DQN–**G**VQ**V**KDKF**VI**G
YG_6	-**N**QW**FYF**DGN–**G**HA**V**T-**G**FQT**I**
YG_7	**N**GKKQ**YF**YND–**G**HQSK
YG_8	-**D**GDT**FY**TSATD**G**RL**V**T-**GV**QK**I**
YG_9	**N**GIT**YAF**DNT–**G**NL**I**
YG_{10}	T**N**-Q**YY**QLAD–**GKYM**LLDDSGR
YG_{11}	**D**GVLR**YF**DQN–**G**EQ**V**KDA**IIV**D
YG_{12}	**DTNL**S**YY**FNATQ**G**VA**V**
YG_{13}	–KND**YF**EYQ–**G**NWY
YG_{14}	T**D**-AN**Y**QLIK–**G**FKAVDD
YG_{15}	A**Q**GKV**Y**QFDNN-**G**NA**V**SA

Boldface type indicates conserved amino acids, *x* corresponds to non-conserved amino acids, and H° to hydrophobic residues.

The GBD of DSR-S is composed of various YG repeated motives as shown in Table 2. The positions of the truncatures were chosen so as to avoid cutting YG motifs (Table 3). The glucan-binding ability of the entire C-terminal domain (GBD-0) was first compared to that of the whole recombinant DSR-S. As shown in Fig. 1, both proteins bound to biotin–dextran. In the work reported by Shah et al., the native dextransucrase from *L. mesenteroides* NRRL B-512F did not reveal dextran ability [10]. However, their binding assay was carried out with the native enzyme that was associated to its natural polymer film. This latter probably interfered during the assay.

Of the 14 truncated proteins, only four proteins (GBD-5, GBD-6, GBD-7 and GBD-8) revealed significant dextran binding properties. All the other truncated proteins have lost their glucan-binding ability (Table 3 and Fig. 1). However, we cannot assert that these fragments are correctly folded. Indeed, the progressive truncations may have altered the second-

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