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Research paper

Improving the glycosyltransferase activity of *Agrobacterium tumefaciens* glycogen synthase by fusion of N-terminal starch binding domains (SBDs)

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ABSTRACT

Glycogen and starch, the major storage carbohydrate in most living organisms, result mainly from the action of starch or glycogen synthases (SS or GS, respectively, EC 2.4.1.21).

SSIII from *Arabidopsis thaliana* is an SS isoform with a particular modular organization: the C-terminal highly conserved glycosyltransferase domain is preceded by a unique specific region (SSIII-SD) which contains three in tandem starch binding domains (SBDs, named D1, D2 and D3) characteristic of polysaccharide degrading enzymes. N-terminal SBDs have a probed regulatory role in SSIII activity, showing starch binding ability and modulating the catalytic properties of the enzyme. On the other hand, GS from *Agrobacterium tumefaciens* has a simple primary structure organization, characterized only by the highly conserved glycosyltransferase domain and lacking SBDs.

To further investigate the functional role of *A. thaliana* SSIII-SD, three chimeric proteins were constructed combining the SBDs from *A. thaliana* with the GS from *A. tumefaciens*. Recombinant proteins were expressed in and purified to homogeneity from *Escherichia coli* cells in order to be kinetically characterized. Furthermore, we tested the ability to restore *in vivo* glycogen biosynthesis in transformed *E. coli glgA*$^-$ cells, deficient in GS. Results show that the D3-GS chimeric enzyme showed increased capacity of glycogen synthesis *in vivo* with minor changes in its kinetics parameters compared to GS.

1. Introduction

Glycogen and starch are the major carbohydrate storage molecules in most living organisms ranging from archaea, bacteria, yeast, animals and plants [1]. Both polysaccharides are polymers of glucose molecules linked by α-1,4 glycosidic bonds and a varying degree of α-1,6 glycosidic branches. The final structure of these polysaccharides as well as the amount accumulated within the cells results from the coordinated regulation of the enzymes involved in their synthesis and degradation [1–4].

The synthesis of bacterial glycogen and plant starch involves mainly the action of three enzymes: ADP-glucose pyrophosphorylase (ADPGlc PPase, EC 2.7.7.27), glycogen (starch) synthase (GS or SS, EC 2.4.1.21), and branching enzyme (BE, EC 2.4.1.18) [1,3,5]. GSs or SSs catalyze the elongation of the α-1,4 glucans by adding glucose units from the sugar-nucleotide to the non-reducing end of the growing chain [1,3,5]. Bacterial GSs and plant SSs belong to the GT5 family of glycosyltransferases, using preferentially ADPGlc as glycosyl donor (http://www.CAZY.org) [1,6–8].

In plants it have been identified five different classes of SSs activities with a high sequence similarity in their catalytic C-terminal end. However, these proteins differ significantly in their N-terminal domain, so that it was not possible to find similarities between protein families [9]. Therefore, it could be postulated that such differences in the N-terminal region of SSs are related to the specific functions attributed to each isoform.

SSIII from *Arabidopsis thaliana* (At1g11720, GenBank accession number AEE28775.1) is a 1094-amino acid residue protein containing different domains: (i) a putative transit peptide of 89 amino acids, (ii) an SSIII N-terminal isoform specific domain (SSIII-SD,

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residues 90–660), and (iii) a C-terminal catalytic domain, which, as mentioned above, is common to all isoforms of starch synthases (SSIII-CD, residues 661–1094) [10,11]. It is worth mentioning that this structural arrangement of SSIII was also described in other plants [9,12–14].

We previously described that the specific region SSIII-SD contains three internal repeats, named D1, D2, and D3, that encode Starch Bindings Domains (SBDs) belonging to CBM 53 family [10]. The SBDs are non-catalytic modules related to the carbohydrate-binding module (CBM) family mainly found in microbial enzymes that degrade starch and glycogen. CBMs are structurally similar and their ability to bind carbohydrates can be attributed, at least partially, to several aromatic residues which assemble into a hydrophobic surface. Moreover, these modules have been found in hydrolytic and non-hydrolytic proteins as well. By driving the catalytic process through an intimate and prolonged physical association with substrates, CBMs would increase the rate of enzymatic reactions [15–19].

We recently proposed that the SSIII N-terminal SBDs, particularly the D23 domains, have a regulatory role in its activity, showing starch binding ability and modulating the catalytic properties of SSIII [10,11,20]. We further demonstrated that the interaction between two regions within the SBDs (D(385–413) in D2 and D(564–604) in D3) and the CD, as well as the full starch binding capacity of the D2 domain are requisites for the full catalytic activity of SSIII [11,20].

Besides the biochemical characterization, we had proposed a structural model of SSIII-CD that predicts a global structural similarity with the GS from *Agrobacterium tumefaciens* [21,22]. Particularly, a fully conservation of the ADP-binding residues was found: residues participating in the binding of ADPGlc are evolutionary conserved in plants and algae [22]. However, important divergences of the polysaccharide-binding domain were observed. In addition, GS has a simple primary structure organization, containing a highly conserved CD but lacking the N-terminal SBDs founded in plant SSIII enzymes. Thus, to further investigate the role of SBDs on glycosyltransferase activity, we constructed different chimeric proteins of *A. tumefaciens* GS that contain in its N-terminal region one, two or the three SBDs from *A. thaliana* SSIII.

2. Materials and methods

2.1. Strains and culture media

Commercial strains of *Escherichia coli* XL1Blue: *end*A1, *gyr*A46, *hsdR*17, *lac*$^-$, *rec*A1, *rel*A1, *supE*44, *thi*-1, F′ [*proAB*$^+$, lacI^q *lacZ*ΔM15, Tn*10*(tet^r)] and *E. coli* BL21-CodonPlus(DE3)-RIL strain [*E. coli* B F-*ompT hsdS*rB- mB-) *dcm* + Tetr *gal* (DE3) *end*A Hte [*argU ileY leuW* Cam^r] were used in this study (Stratagene, La Jolla, CA, USA). *E. coli glgA*$^-$ strain was also used [23]. All strains were grown, unless indicated, at 37 °C in LB medium containing the appropriate antibiotics.

2.2. Cloning, expression and purification of glycogen synthase chimeric proteins

Recombinant plasmid pHNAL32 was used as template to amplified the cDNA of *A. tumefaciens* GS with GSfwSBD (CCAGTCGTGGGAGGTATTTCAATGAATGTCCTTTCGGTTTCATCCG, bearing 25 bases complementary to the C-terminal end of D123 [24] and GSrv (AAACTCGAGATGGCCTTTCGAAAT AAGCTG) primers.

Plasmid pVAL15 was used as template to amplified D123 cDNA from *A. thaliana* SSIII with D123fw (AGAGCATATGATAGAAACGCTTGCA) and D3rv (CTGCTCGAGTGGTTCCTTTGAAAT, harboring 25 bases complementary to the N-terminal end of GS) [10].

Both cDNAs were mixed in equimolecular quantities, heated to 95 °C, cooled to room temperature and used as template for a *Pfu* PCR with D123fw and GSrv primers. The resulting chimeric cDNA (*sbds-gs*) was then used as template for cDNA amplification of D123-GS, D23-GS, and D3-GS chimeric enzymes with primers GSrv and D123fw or D23fw (AGAGCATATGATTAAGAAAGCTGTA) or D3fw (AGAGCATATGAGAAAACTTCAG) respectively. The resulting PCR products were cloned into pGEMT and XL1Blue *E. coli* cells were transformed with each construction. Positive clones were verified by DNA sequencing. The resulting inserts were then digested using restriction endonucleases *Nde*I and *Xho*I and cloned into pET32c vector (Novagen Inc., Madison, WI). Finally, recombinant plasmids named pNAL45, pNAL46, and pNAL50 (containing a C-terminal His-tag sequence, Fig. 1) were used to transform *E. coli* BL21 (DE3)-RIL cells for expression purposes. After growing the cells at 37C for 4 h (until $OD_{600} = 0.6$), 1 mM isopropyl-b-D-thiogalactoside (IPTG) was added to the culture medium and incubated at 28 °C for at least 4 h. Cells were harvested by centrifugation at 4000 *g* for 10 min at 4 °C. The pellet was washed and suspended in buffer containing 20 mM Tris–HCl, pH 8 (1 ml/g of wet cells) and 1 mM PMSF (phenylmethylsulphonyl fluoride). Cells were disrupted using an ultrasonicator (VCX130, Sonics and Materials Inc., Connecticut, USA) and centrifuged at 12,000*g* for 15 min at 4 °C. GS and SBDs-GS chimeric forms were purified using one step Ni-chelating chromatography as previously described [10]. Briefly, the homogenates were filtered through a 0.2 μm cellulose acetate membrane filter and loaded onto a HiTrapTM Chelating HP columns (GE Healthcare) equilibrated with binding buffer containing 20 mM potassium-phosphate, 0.3 M NaCl, 1 mM β-mercaptoethanol, 20 mM imidazole, pH 7.4. The column was washed at least with 10–15 volumes of binding buffer, and each protein was eluted using a linear gradient of binding buffer and elution buffer (20 mM potassium-phosphate, 0.3 M NaCl, 1 mM β-mercaptoethanol, 20–500 mM imidazole, pH 7.4). Recombinant proteins eluted in the fraction between 200 and 300 mM imidazole. The active fractions were pooled and concentrated up to 1 mg/ml. The concentrated proteins were desalted and used to determine enzyme activity or stored at −20 °C until use.

2.3. Determination of glycogen synthase activity

The activities of *A. tumefaciens* GS and the chimeric proteins were determined spectrophotometrically, using ADPGlc as the substrate as previously described [25]. The reaction medium contained 50 mM Bicine, pH 8.0, 80 mM MnCl2, 0.4 mM NADH, 1 mM phosphoenolpyruvate (PEP), 1 U lactate dehydrogenase (LDH) (Sigma–Aldrich), 1 U pyruvate kinase (PK) (Sigma–Aldrich), 10 mg of rabbit muscle glycogen and 3.5 mM of ADPGlc [25]. The production of ADP is coupled to NADH oxidation via pyruvate kinase (PK) and lactate dehydrogenase (LDH). Assays were initiated by the addition of the enzyme and also were performed at different enzyme concentrations to ensure steady-state conditions. The concentrations giving 50% maximal velocity ($S_{0,5}$) and Hill coefficients (n_H), calculated according to Brooks [26]. All kinetic parameters are means of at least three determinations and the average values ± SE are reported. Total protein was determined using the Bradford method as described [27].

2.4. Functional complementation of *E. coli glgA*$^-$ cells

Plasmids pET32c, pHNAL32, pNAL45, pNAL46 and pNAL50 were used to transform *E. coli glgA*$^-$ cells. Transformed cells were grown at 37 °C in M9 minimal medium [28] supplemented with 50 mM glucose, 250 μM $MgCl_2$ and 0.001% w/v thiamine and containing the appropriate antibiotic. Recombinant proteins were induced by addition of 1 mM IPTG when cells reached a D.O. 600 nm of 0.4–0.5.

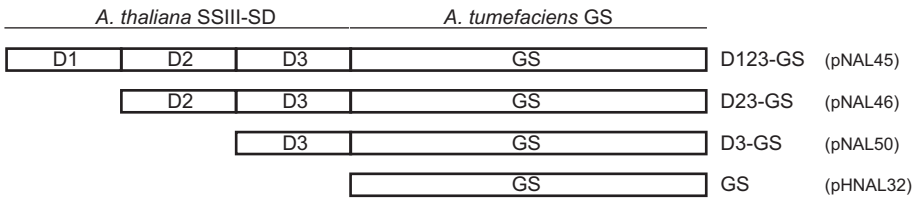

Fig. 1. Structure domain of GS from *A. tumefaciens* and chimeric proteins between GS and the SBDs of SSIII from *A. thaliana*. SSIII-SD, *A. thaliana* specific domain; GS, *A. tumefaciens* glycogen synthase; D1, D2 and D3, three in tandem modules containing the starch binding domains; D123-GS, full-length GS containing the three SBDs in its N-terminal end; D23-GS, full-length GS containing two SBDs in its N-terminal end; and, D3-GS, full-length GS containing only one SBD (D3) in its N-terminal end. The names of each plasmid construct are on the right side of the figure.

After 4 h, cells were collected for glycogen quantification. Glycogen content of different strains was quantified as described previously by Lugol or using an amyloglucosidase based assay [22].

2.5. Gel electrophoresis and immunological studies

SDS-PAGE was performed with the Bio-Rad Mini Protean system using 10% polyacrylamide/bisacrylamide gels as described [29]. Gels were developed by staining with Coomassie brilliant blue (R250, Sigma–Aldrich) or electroblotted onto nitrocellulose membranes (Trans-Blot Transfer Medium, Nitrocellulose Membrane, Bio-Rad). After electroblotting, membranes were treated with a-GS or a-SBD123 antibodies. The antigen–antibody complex was visualized after the incubation with a secondary antibody (a-rabbit IgG linked with alkaline phosphatase) followed by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) [30].

3. Results and discussion

3.1. Cloning, expression and purification of A. tumefaciens GS wild type and chimeric forms

To extend our knowledge on the functional role of starch binding domains, *A. tumefaciens* GS wild type, and chimeric constructs between *A. tumefaciens* GS and N-terminal SBDs from *A. thaliana* SSIII were cloned and expressed in *E. coli* cells. The generated chimeric proteins included D123-GS (containing the three N-terminal SBDs from *A. thaliana* SSIII), D23-GS (containing two SBDs), and D3-GS (containing only one SBD) (Fig. 1).

Each construct were generated by PCR as described in the Material and Methods section. The chimeric constructs were cloned in a pET-32c expression vector (Novagen) containing a C-terminal His_6 tag. Constructs pHNAL32 (encoding for GS, [20], pNAL45 (encoding for D123-GS), pNAL46 (encoding for D23-GS), and pNAL50 (encoding for D3-GS) were subjected to restriction mapping and DNA sequencing to control PCR fidelity.

All the constructs were expressed in BL21(DE3)-RIL cells and induced with IPTG. Proteins were purified to apparent homogeneity by a single purification step using a HiTrap chelating column (Amersham Biosciences). Thus, we obtained about 0.3, 0.8, 1.2 and 2.1 mg of D123-GS, D23-GS, D3-GS and GS proteins, respectively, per gram of cells. All the chimeric enzymes were soluble and stable since they did not suffer any kind of proteolysis. Fig. 2A show the SDS-PAGE analysis of recombinant GS (55.4 kDa), D3-GS (66.3 kDa), D23-GS (97.4 kDa) and D123-GS (116.3 kDa), respectively. A single protein band with the expected molecular mass, for GS and each chimeric form, was observed after the elution with the Imidazole gradient described above. Thus, we consider that all the enzymatic preparations are homogeneous after this purification procedure.

The identity of the recombinant proteins was confirmed by western blotting using antibodies raised against recombinant His_6-tagged GS (Fig. 2B) from *A. tumefaciens* and anti-SBD123 from *A. thaliana* SSIII (Fig. 2C). All the SBD-GS chimeric forms reacted with both antibodies confirming the identity of the recombinant proteins.

It is generally known that some hybrid proteins are unstable, and it is extremely difficult to combine the functions of each constituent [31]. However, some examples probed the contrary. The hybrid enzyme resulting from *Sacharomyces cerevisiae* glucoamylase and the SBD from *Aspergillus niger* glucoamylase was able to bind and hydrolyze insoluble starch, being this properties absent in the original yeast enzyme [32]. The same SBD in the chimeric enzyme AMY1-SBD confers to barley α-amylase (AMY1) an increased affinity for barley starch granules with increased catalytic activity respect to AMY1 [33]. Finally, the introduction of the SBD from *Bacillus* sp. cyclomaltodextrin glucanotransferase into *Bacillus subtilis* α-amylase confers raw starch binding and high starch-digestion capacity, both properties that are absent in the catalytic domain alone [34].

Other examples where a starch binding domain was fused to a catalytic core include the leucine aminopeptidase II from *Bacillus stearothermophilus*, where its C-terminal end has been fused to the SBD of *Bacillus* sp. alpha-amylase. The chimeric enzyme and

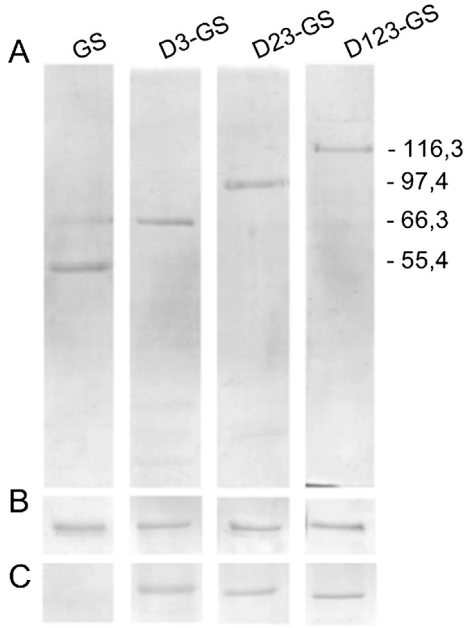

Fig. 2. (A) SDS-PAGE of recombinant enzymes. Lane 1, GS; lane 2, D3-GS; lane 3, D23GS; and lane 4, D123-GS. Numerals indicate molecular masses of the following standards: β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), BSA (66.3 kDa), and glutamic dehydrogenase (55.4 kDa). (B) and (C) Western blot detection of purified recombinant proteins shown in A using a-GS (B) or a-SBD123 antibodies (C).

adsorbed raw starch, showing a increase in thermostability and catalytic efficiency [35]; whereas another leucin aminopeptidase from *Bacillus kaustophilus* fused to an alpha-amylase SBD showed a decrease in catalytic efficiency due to the increase of the K_m value [36], indicating that in some cases, the chimeric proteins do not produce the functions expected from the original enzymes, possibly due to a different polypeptide-folding pattern [36]. Thus, although there are several examples to date of hybrid functional proteins between CBMs and a catalytic core, most of them involves CBMs from hydrolytic enzymes linked to bacterial xylanases or amylases catalytic domains [37–39]. Chimeric proteins bearing particularly SBDs are less exemplified [32,33], being this work the first report of an active bacterial GS fusion protein that contains an SBD from a plant starch biosynthetic enzyme.

3.2. Effect of SBDs on the kinetic parameters of GS for glycogen

As it is generally known, GS and SSIII use preferentially ADPGlc as glycosyl donor, although UDPGlc can also be used with less efficiency. Using glycogen as saturating substrate, and ADPGlc as the variable sugar-nucleotide donor, GS from *A. tumefaciens* display Michaelis kinetics, indicating a non-cooperative reaction. Table 1 shows the kinetic parameters of GS for glycogen and the chimeric forms of SBD-GS using ADPGlc as the sugar donor at saturating concentration. The $S_{0.5}$ values for the acceptor polysaccharide were 1.30, 1.05, 0.54, and 0.36 mg/mL for GS, D3-GS, D23-GS and D123-GS, respectively. These results indicated that the presence of the N-terminal SBD from *A. thaliana* SSIII increased the apparent affinity of *A. tumefaciens* GS for glycogen. The increment in the affinity caused by the presence of an SBD was also previously shown for *Solanum tuberosum* α-glucan, water dikinase (StGWD1), which possesses a CBM45 domain [40], StGWD3, which present a CBM20 family domain [41] and AMY1-SBD (for amylose DP17) [33]. It has been reported that the modules from the CBM45 and CMB20 family are present in some plant enzymes involved in starch metabolism and they bind starch with low-affinity, supporting the hypothesis that the low-affinity interaction with starch granules facilitates the dynamic regulation of polysaccharide metabolism [33,42,43].

On the other hand, all the chimeric enzymes showed lower V_{max} values than the wild type GS (see Table 1). While the V_{max} observed for D3-GS is only 34% lower respect to that from GS; we found about 10-fold lower V_{max} values for D23-GS, and D123-GS hybrid proteins. Thus, as the number of foreign SBDs increases, there is a drastic diminution of the V_{max}. However, we observed a similar catalytic efficiency for GS and D3-GS, whereas we found a decrease of about 74% and 67% in the catalytic efficiency for the D23-GS and the D123-GS enzymes, respectively, compared to GS (Table 1).

In summary, the results obtained show that the presence of SBDs increases the apparent affinity of the enzyme for glycogen, in detriment of the V_{max}. This was in contrast with what we previously described for SSIII from *A. thaliana*, where the apparent affinity and the V_{max} for the oligosaccharide acceptor substrate increased progressively with the increase in SBD number fused to SSIII-CD [10].

From the *in vitro* kinetic characterization we can conclude that the addition of one SBD (D3 domain) from *A. thaliana* SSIII, which consists of an independent and relatively small protein domain, represents a lesser perturbation of the functional properties of the GS from *A. tumefaciens* since the $V_{max}/S_{0.5}$ ratios are not significantly different.

3.3. Effect of the SBDs on the kinetic parameters of GS for ADPGlc

Table 2 lists the kinetic parameters for GS and the different SBDs-GS chimeric forms using ADPGlc as the sugar donor substrate and glycogen as the polysaccharide acceptor at a saturating concentration. While GS displays the lowest $S_{0.5}$ value for ADPGlc (0.37 mM), the full-length chimeric enzyme D123-GS shows an increase of about 5.4-fold of the $S_{0.5}$ value. The apparent affinity for ADPGlc of the two other variants of the enzyme ranged from 0.66 mM (for D3-GS) to 1.58 mM (for D23-GS) (Table 2). Thus, the presence of SBDs increases the $S_{0.5}$ for ADPGlc; just in opposition to what was observed for glycogen.

On the other hand, the change observed for the V_{max} is almost the same as the one observed for glycogen as the varying substrate. With ADPGlc, GS displayed the higher V_{max} value (167 U/mg), whereas D3-GS, D23-GS and D123-GS displayed a 1.2-fold, 7.2-fold and 8.9-fold decrease in the V_{max}, respectively. Moreover, we found a decrease of about 53%, 96% and 98% in the catalytic efficiency (Table 2).

As a consequence, we can conclude that the presence of a progressive number of SBDs in GS led to a decrease on the apparent affinity for ADPGlc as well as the V_{max}. It's worth mentioning that the same effect for the $S_{0.5}$ of ADPGlc was verified for the catalytic domain of SSIII from *A. thaliana*; the successive incorporation of the SBD to SSIII-CD resulted in an increase of the $S_{0.5}$ for the resulting enzyme [10]. Although the SSIII-CD protein displays the lowest $S_{0.5}$ value for ADPGlc (0.24 mM), the full-length enzyme has an $S_{0.5}$ value that is 18-fold higher.

The opposite effects on the catalytic efficiency showed by GS and SSIII-CD when added different number of SBDs could be due to structural differences between the two CDs, being the most important the lack of the 380s loop founded in the bacterial GS [22]. The 380s loop was initially described for *E. coli* maltodextrin phosphorylase [44]. This loop is a mobile region which responds to conformational changes after the binding of substrates, enabling a correct positioning of the polysaccharide molecule. It has been suggested that this 380s loop is involved in the communication between the catalytic site and the glycogen binding site [22,45–47].

3.4. Effect of the D3-GS enzymes on glycogen synthesis in vivo

The above results described the kinetics parameters of the engineered proteins purified to apparent homogeneity. Besides, to determine if the SBD-GS chimeric enzymes are also functional *in vivo* and to elucidate the role of SBDs, we evaluated the capacity of the chimeric proteins to accumulate glycogen *in vivo*, using an *E. coli glgA*$^-$ strain that is impaired in glycogen biosynthesis [23].

E. coli glgA$^-$ mutant cells were transformed with the different plasmids encoding GS, D123-GS, D23-GS or D3-GS chimeric enzymes (pHNAL32, pNAL45, pNAL46 or pNAL50 respectively) and transformants were selected on LB plates with the appropriate antibiotic. For the quantitative determination of GS activity,

Table 1
Kinetic parameters of recombinant proteins for glycogen.

Isoform	$S_{0.5}$ (mg/ml)	*n*H	V_{max} (U/mg)	$V_{max}/S_{0.5}$
D123-GS	0.36 ± 0.01	0.7 ± 0.1	15.8 ± 1.0	43.9 ± 2.5
D23-GS	0.54 ± 0.06	0.9 ± 0.1	18.5 ± 2.1	34.3 ± 4.2
D3-GS	1.05 ± 0.11	1.4 ± 0.1	112 ± 14	106 ± 24
GS	1.30 ± 0.20	1.1 ± 0.2	170 ± 21	131 ± 36

Table 2
Kinetic parameters for the substrate ADPGlc of GS and chimeric proteins.

Isoform	$S_{0.5}$ (mM)	*n*H	V_{max} (U/mg)	$V_{max}/S_{0.5}$
D123-GS	2.00 ± 0.29	1.0 ± 0.1	18.8 ± 1.9	9.40 ± 2.3
D23-GS	1.58 ± 0.20	1.1 ± 0.4	23.3 ± 2.1	14.7 ± 3.2
D3-GS	0.66 ± 0.05	1.1 ± 0.2	136 ± 11	206 ± 32
GS	0.37 ± 0.04	1.3 ± 0.4	167 ± 15	435 ± 87

transformed cells were grown in liquid M9 minimal medium until D.O. 600 nm of 0.4–0.5 and expression of recombinant proteins induced by addition of 1 mM IPTG. After 4 h of induction, GS activity was assayed and also the quantity of glycogen accumulated by each strain was analyzed. Fig. 3 shows the values of glycogen accumulated (mg/dl) respect to GS activity (U/mg). Results show that the strain expressing wild type GS or the hybrid enzymes D123-GS, D23-GS and D3-GS could successfully accumulate glycogen but to a different extent. The strain harboring D3-GS chimeric enzyme accumulates about 4-fold more glycogen than the one expressing *A. tumefaciens* GS wild type, whereas no significant differences in glycogen accumulation were observed for the strains expressing D123-Gs or D23-GS enzymes respect to the control.

From the *in vitro* and *in vivo* experiments, we can conclude that the presence of the SBD D3 from *A. thaliana* SSIII in the primary structure of GS from *A. tumefaciens*, greatly increases enzyme's capacity of glycogen synthesis *in vivo* (Fig. 3) with a $V_{max}/S_{0.5}$ ratio minor change for glycogen (Table 1) and a change of 53% for ADPGlc (Table 2), whereas the presence of additional SBDs in the D123-GS and D23-GS enzymes could result in major structural changes leading a steric effects that could prevent the proper modulation of the catalytic activity of the enzyme.

Thus, the results observed for D3-GS may arise from different reasons (acting separately or in combination): i) the high glycogen content observed for the *E. coli glgA*$^-$ cells expressing this chimeric protein may just be a consequence of macromolecular crowding in the cytosolic environment. It was reported that other enzymes involved in polysaccharide synthesis such as ADPGlc PPase increase significantly its catalytic activity in the presence of molecular crowding induced agents such as polyethyleneglycol or glycerol [48,49] or in the presence of polymerized microtubular proteins [50]; ii) we recently reported the existence of a protein–protein interaction between SSIII SBDs and the catalytic domain (CD) and we also postulate that this interaction is involved in the modulation of SSIII activity [20]. Although it is not clear the mechanism of regulation of the catalytic activity by the SBDs, it is possible to postulate that the protein–protein interaction plays an important function in the promotion of polysaccharide-binding to the CD, increasing its concentration in the active site. Taking into account the changes in kinetic parameters of GS and SBD-GS chimeric proteins, it is possible to postulate the existence of a similar mechanism between D3 and GS, where the presence of an SBD promotes glycogen binding to GS, subsequently increasing its concentration in the active site, and thus determining the catalytic efficiency of the enzyme. This is in agreement with the increase in the catalytic efficiency observed for the GS from yeast when multiple polysaccharide-binding sites are present [51].

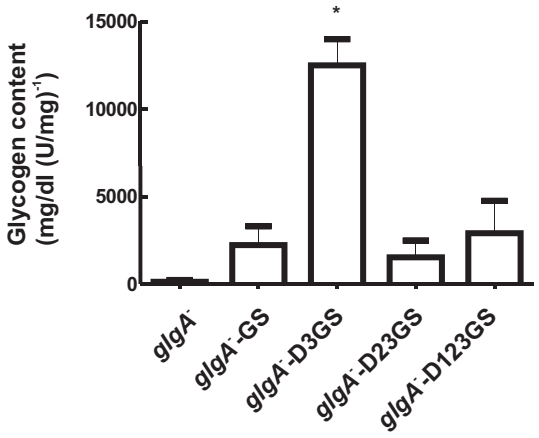

Fig. 3. Glycogen content of *E. coli glgA*$^-$ cells complemented with *A. tumefaciens* GS or *A. thaliana–A. tumefaciens* D3-GS, D23-GS or D123-GS chimeras. GS activity and the quantity of glycogen accumulated by each strain were analyzed. Bars represent values of glycogen accumulated (mg/dl) relative to GS activity (U/mg). The asterisk signals a statistically different result from the control value ($P < 0.05$).

4. Conclusions

Protein engineering by the addition of substrate binding domains is becoming an increasingly common approach to improve enzymes properties. Binding domains are often used to increase catalytic efficiency, as affinity tags that facilitate protein purification and for targeting a protein to specific cellular locations.

The addition of SBDs to GS caused opposite effects *in vitro* compared to those reported for the truncated SSIII proteins from *A. thaliana* [10]. While the ability of SBDs to bind glycogen increases with the number of SBDs [10,20], the enzyme loses catalytic efficiency. Despite of the similar catalytic efficiency of the D3-GS and GS proteins under ADPGlc saturating conditions, we observed an increase of about 4-fold in glycogen accumulation *in vivo*. This fact allows us to think that the addition of a new polysaccharide-binding site in GS is beneficial to increase the accumulation of polysaccharides in the cell. Baskaran et al. (2011) demonstrated that the existence of multiple glycogen binding sites in the GS from *S. cerevisiae* increase its catalytic efficiency [51]. The presence of a polysaccharide-binding site outside the active site of the enzyme would lead to improve the binding capacity through multiple contacts, increasing the local concentration of non-reducing ends in the active site and resulting in a greater processivity of the enzyme [52].

The addition of one SBD, D3, from *A. thaliana* SSIII to *A. tumefaciens* GS seems to confer higher capacity of glycogen biosynthesis demonstrating that the careful design of fusion proteins can led to the production of a fully active and conformationally stable molecule composed of domains that belong to different kingdoms, in this case, plants and bacteria. This approach could allow the development of a technology platform, where different SBDs from SSIII or other proteins (alone, in tandem and/or combined) can be fused to a large series of target proteins (with catalytic activity), to engineer other enzymes with novel and improved starch-synthesizing functionalities.

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