Biochimie 133 (2017) 37–44

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Identification of a novel starch synthase III from the picoalgae *Ostreococcus tauri*

Julieta Barchiesi [a], Nicolás Hedin [a], Alberto A. Iglesias [c], Diego F. Gomez-Casati [a], Miguel A. Ballicora [b], María V. Busi [a, *]

[a] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI-CONICET), Universidad Nacional de Rosario, Suipacha 531, Rosario, 2000, Argentina
[b] Department of Chemistry and Biochemistry, Loyola University Chicago, 405 Flanner Hall, 1068 W Sheridan Road, Chicago, IL 60660, USA
[c] Laboratorio de Enzimología Molecular, Instituto de Agrobiotecnología del Litoral (UNL-CONICET) & FBCB, Santa Fe, 3000, Argentina

ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form
14 November 2016
Accepted 8 December 2016
Available online 19 December 2016

Keywords:
O. tauri
Starch synthase
Protein activity

ABSTRACT

Hydrosoluble glycogen is the major energy storage compound in bacteria, archaea, fungi, and animal cells. In contrast, photosynthetic eukaryotes have evolved to build a highly organized semicrystalline granule of starch. Several enzymes are involved in polysaccharide synthesis, among which glycogen or starch synthase catalyze the elongation of the α-1,4-glucan chain. *Ostreococcus tauri*, accumulates a single starch granule and contains three starch synthase III (SSIII) isoforms, known as OsttaSSIII-A, OsttaSSIII-B and OsttaSSIII-C. After amino acids sequence analysis we found that OsttaSSIII-C lacks starch-binding domains, being 49% identical to the catalytic region of the SSIII from *Arabidopsis thaliana* and 32% identical to the entire *Escherichia coli* glycogen synthase. The recombinant, highly purified OsttaSSIII-C exhibited preference to use as a primer branched glycans (such as rabbit muscle glycogen and amylopectin), rather than amylose. Also, the enzyme displayed a high affinity toward ADP-glucose. We found a marked conservation of the amino acids located in the catalytic site, and specifically determined the role of residues R270, K275 and E352 by site-directed mutagenesis. Results show that these residues are important for OsttaSSIII-C activity, suggesting a strong similarity between the active site of the *O. tauri* SSIII-C isoform and other bacterial glycogen synthases.

1. Introduction

The synthesis of bacterial glycogen and plant starch exhibit several common features. Both involve the action of at least three enzymes, ADP-glucose (ADPGlc) pyrophosphorylase (EC 2.7.7.27), glycogen/starch synthase (EC 2.4.1.21), and starch branching enzyme (EC 2.4.1.18). Starch synthase (SS) catalyzes the elongation of α-1,4 glucans by the transfer of glucose units from a sugar-nucleotide donor to the non-reducing end of the growing polyglucan chain [1]. Photosynthetic eukaryotes SSs are GT-B-fold glycosyltransferases classified within the GT5 family in the CAZy database [2,3], being the archaeal and bacterial glycogen synthase (GS) their closest counterparts [4,5]. They use ADPGlc as the glycosyl donor. The catalytic region of SS, consisting of a conserved synthase catalytic domain (GT5) and a glycosyltransferase 1 domain (GT1), is encompassed by nearly the entire sequence of the ~60 kDa protein of bacterial GSs, but it corresponds only to the C-terminal portion of SSs from photosynthetic eukaryotes [2]. The N-terminal region of plant and algae SSs has a variable length as well as differences in amino acids sequence among the different isoenzymes. Such differences could be related to the specific functions attributed to each SS isoform.

Three SS subfamilies were reported in *O. tauri* consisting of one SSI, one SSII and three SSIIIs isoforms; all of them remaining uncharacterized to date. The conservation throughout the evolution of the three SSIII isoforms and the absence of an SSIV isoform could be related to the presence of a single starch granule in this alga, which has similar composition that those starch granules found in higher plants but with a particular partitioning and propagation mechanism [6,7]. In plants, the N-terminal region of

Abbreviations: GS, glycogen synthase; SS, starch synthase; SSIII, starch synthase III; ADPGlc, ADP-Glucose; SBD, starch-binding domain; CBM, carbohydrate-binding module; CD, catalytic domain; UDPGlc, UDP-Glucose; Ostta, *Ostreococcus tauri*.

* Corresponding author. Address: CEFOBI, Suipacha 531, 2000, Rosario, Argentina.
E-mail address: busi@cefobi-conicet.gov.ar (M.V. Busi).

http://dx.doi.org/10.1016/j.biochi.2016.12.003
0300-9084/

the SSIII isoform contains three *in tandem* starch binding domains (SBDs), belonging to the carbohydrate binding module 53 (CBM53) family [3,8]. These SBDs are non-catalytic modules that present starch binding ability and are involved in modulating the catalytic properties of the enzyme [8–13].

We have previously shown that SSIII isoforms from algae present a variable number of SBDs in their N-terminal region [6]. The model green algae *Chlamydomonas reinhardtii* for example, presents two SSIII isoforms, containing two and three SBDs respectively. Interestingly, the *O. tauri* SSIII-C isoform sequence stands for an atypical SSIII, as it contains the GT5 and GT1 domains but lacks of a SBD polypeptide. In this work we report on the molecular cloning and expression of the Ostta*SSIII-C* gene, and the production in a highly purified form and characterization of the recombinant protein. Our results show that *O. tauri* SSIII-C isoform comprises an active enzyme that preferably uses ADPGlc as substrate and shows a structure of the active site region conserved respect to the SSIII from *A. thaliana* or bacterial GSs, such as the enzymes from *E. coli* or *Agrobacterium tumefaciens*.

2. Materials and methods

2.1. Strains and culture media

Different commercial strains of *E. coli* from Stratagene (CA, USA) were used in this study. Specifically strains: XL1Blue (*endA*1, *gyrA*46, *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 (DE3) pLysS [F- *ompT gal dcm hsdS*$_B$(r$_B$- m$_B$-) λ(DE3) pLysS (Cm^r)]. All the cells were grown at 37 °C in LB medium containing the proper antibiotics.

2.2. Cloning, expression and purification of OsttaSSIII-C

The gene *OsttaSSIII-C* (Ot06g03410) was cloned from *O. tauri* genomic DNA (kindly provide for Dr. Evelyne Derelle) into *Eco*RI and *Hind*III sites of pRSET-B (Invitrogen CA, USA), using standard molecular biology procedures and the primers: OtSSIII-C Nt Fwd, ACGAATTCGGACCAGCGCCCATC and OtSSIII-C Ct Rev: ATGAAGCTTCGTGAGACTACCG (*Eco*RI and *Hind*III sites underlined).

The expression vector pRSET-B::OsttaSSIII-C (containing an N-terminal His-tag sequence) was used to transform *E. coli* BL21(DE3) pLysS strain. Cells were grown at 37 °C for 3 h, and then 1 mM IPTG was added and incubated at 30 °C for at least 4 h. Cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C. Pellet was suspended in buffer containing 20 mM Tris- HCl (pH 7.5). Cells were disrupted by sonication and centrifuged at 12000 × *g* for 15 min at 4 °C. The homogenate was loaded onto a HiTrap chelating HP column (GE Healthcare BioSciences, Uppsala, Sweden) equilibrated with binding buffer (20 mM Tris-HCl, pH 7.5, and 20 mM imidazole). The column was washed with 10–15 vol of binding buffer, and each protein was eluted using a linear gradient of binding buffer and elution buffer (20 mM Tris-HCl, pH 7.5, and 20–500 mM imidazole) [8]. The presence of OsttaSSIII-C (56.8 kDa) in the eluted fractions was monitored by SDS-PAGE analysis. Fractions containing the protein of interest were concentrated to >1 mg/ml using Vivaspin 6 10000 MWCO concentrators (GE Healthcare BioSciences, Uppsala, Sweden), desalted to 20 mM Tris-HCl, pH 7.5, and stored at −80 °C until use. Under these conditions the purified enzyme remained fully active during at least three months.

2.3. Bioinformatic analysis

Two approaches were used to detect domains in OsttaSSIII-C: the CD-search server [14] and the InterPRO resource [15]. Alignment of the OsttaSSIII-C, ArathSSIII-CD, AgrtuG, EsccoGS, ChlreSSIII1 CD and ChlreSSIII2 CD amino acids sequence were performed by using the Unipro UGENE v.1.10.4 program [16] with default parameters.

2.4. Homology modeling

The 3D structure modeling of OsttaSSIII-C and modified proteins, were performed with @TOME V2.2 server, which includes Scwrl, Modeller [17] and T.I.T.O. (Tool for Incremental Threading Optimisation). Structures were modeled using glycogen synthase from *E. coli* (PDB code 2QZS) as a template [18]. The alignment was based on homology and secondary structure. Models were evaluated using Verify-3D [19] and ProSA-web [20] programs.

2.5. Construction, expression and purification of site directed mutated proteins

The mutated OsttaSSIII-C proteins R270A, K275A and E352A were obtained using the QuickChange II site-directed mutagenesis kit (Stratagene, CA, USA). The pRSET-B::OsttaSSIII-C vector was used as the template for PCR amplification. The following primers (and their complements) were used (base substitutions in italics and underlined): OtC R270A, TGGCGTTGTCTCA*GC*CCTTACCGCTC; OtC K275A, CTTACCGCTCAA*GC*AGGTATCCATCTC and OtC E352A, CCGTCAATGTTTG*C*GCCGTGCGGTCTGAC. The mutated sequences were confirmed by DNA sequencing to control PCR fidelity (Macrogen, Korea). Recombinant plasmids named pRSET-B::OsttaSSIII-C R270A, pRSET-B::OsttaSSIII-C K275A and pRSET-B::OsttaSSIII-C E352A (containing an N-terminal His-tag sequence) were used to transform *E. coli* BL21(DE3) pLysS cells. Mutated recombinant proteins were produced, purified and conserved as described above in item 2.2 for the wild type enzyme.

2.6. Determination of starch synthase activity

Activity of *O. tauri* SSIII-C wild type and the mutated proteins was determined following the formation of Pi (after hydrolysis of generated ADP by alkaline phosphatase) by using a colorimetric assay adapted from Wu et al. [21]. The reaction medium (50 μl final volume) contained 50 mM Bicine, pH 8.0, 1 U of *E. coli* alkaline phosphatase (Sigma-Aldrich, MO, USA), 0–2 mg/ml of rabbit muscle glycogen or 0–4 mg/ml amylopectin, and 0–1 mM ADPGlc. When UDPGlc was used as the glycosyl donor, the assay conditions were identical, except that ADPGlc was replaced by UDPGlc in the range 0–10 mM. After incubation for 10 min at 35 °C the reaction was stopped by the addition of Malachite Green reagent. The complex formed with the released Pi was measured at 630 nm. Inorganic phosphate was used as standard. One unit of enzyme activity was defined as the amount of protein catalyzing the incorporation of 1 μmol of Glc from ADPGlc into glycogen per 1 min, under the specified conditions.

The kinetic data were plotted as initial velocity (μmol/min.mg protein) versus substrate concentration. The maximum catalytic rate and the Michaelis–Menten constants (K_m) for the binding of the substrates were determined from a non-linear fit of the Michaelis–Menten equation to the observed enzyme activities using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). All kinetic parameters are the mean of at least three determinations and the average values ± SE are reported. Total protein was determined using the Bradford method [22].

A pH profile of OsttaSSIII-C activity was conducted by measuring activity at 35 °C and pH values ranging from 6.0 to 10.0 in 50 mM

Bicine buffer. The temperature profile of OsttaSSIII-C activity was followed by measuring activity at pH 8.0 and temperature values ranging from 20 to 45 °C in 50 mM Bicine buffer. Both experiments were run with 2 mg/ml glycogen and 1 mM ADPGlc. Standard

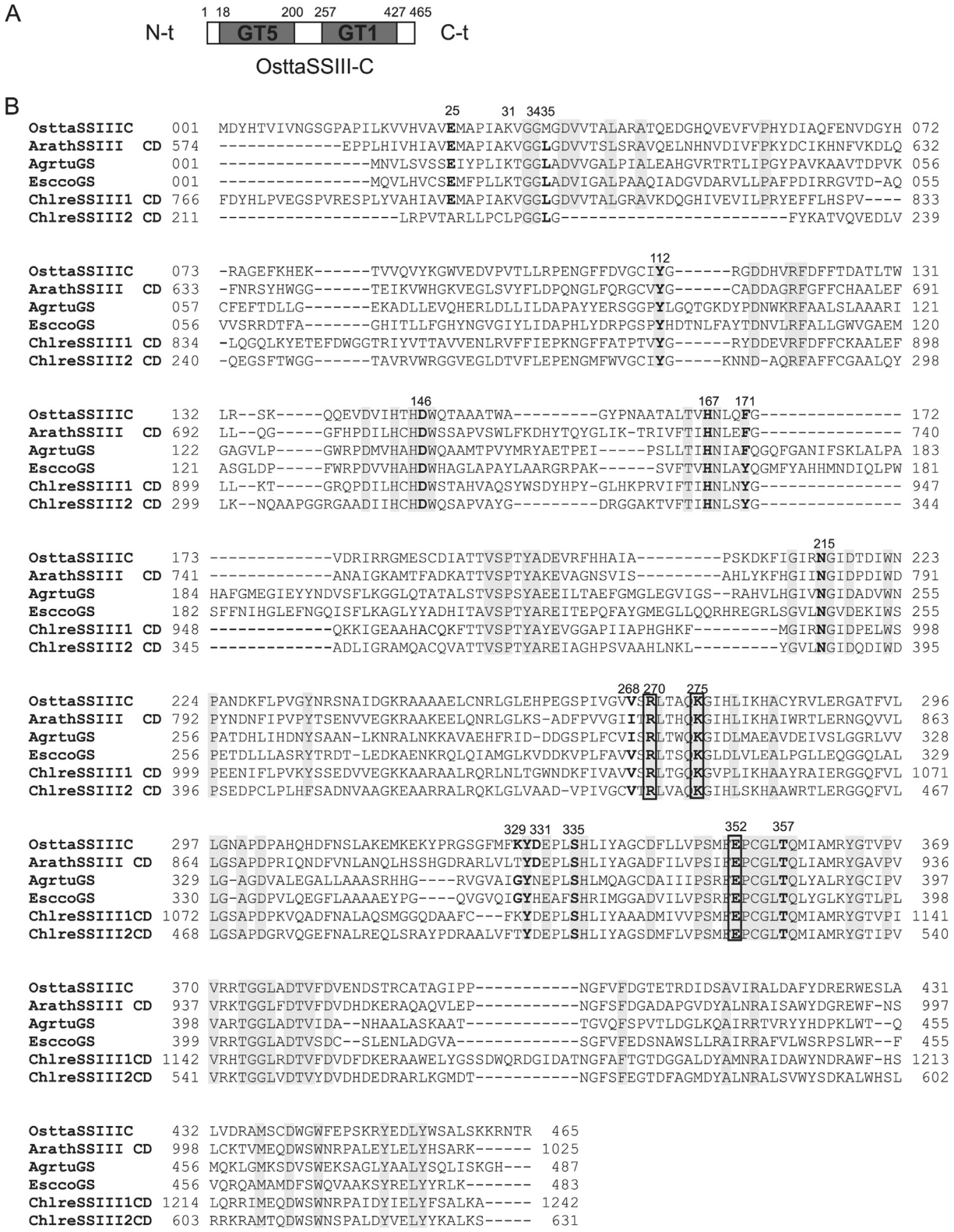

Fig. 1. (A) Schematic representation of OsttaSSIII-C. GT1 and GT5 domain are shown. (B) Alignment obtained using the Unipro UGENE v.1.10.4 program (with default parameters) between OsttaSSIII-C, ArathSSIII-CD (*Arabidopsis thaliana* SSIII catalytic domain), AgrtuGS (*Agrobacterium tumefaciens* glycogen synthase), EsccoGS (*Escherichia coli* glycogen synthase), ChlreSSIII1 CD and ChlreSSIII2 CD (*Chlamydomonas reinhardtii* SSIII 1 and 2, catalytic domains). OsttaSSIII-C amino acids residues R270, K275 and E352 are indicated by open boxes (corresponding to site active amino acids R300, K305 and E377 characterized in EsccoGS [18,26]). Other previously characterized catalytic residues are shown in bold. Grey boxes indicate identical residues.

kinetic parameters were determined at pH 8.0 and 35 °C.

2.7. Circular dichroism

Circular dichroism far-UV spectra were obtained using a Jasco J810 spectropolarimeter (Jasco Int. Co., Japan). Measurements were performed in a 0.1 cm quartz cuvette and the setting was as follows: measurement rate 40 nm min^{-1}, bandwidth 1 nm, response time 2 s, data pitch 1 nm, and spectra accumulation of 15. The range of wavelength used was from 250 nm to 200 nm, at 20 °C. All spectra data are reported as molar ellipticity [θ] molar, λ (deg cm^2 $dmol^{-1}$) obtained after subtracting the baseline, and smoothing data.

3. Results and discussion

3.1. Sequence analysis and homology modeling of OsttaSSIII-C

The catalytic activity of SSs and GSs is conducted by a highly conserved region common between these enzymes [23]. This 60 kDa "core" region is in the C-terminus of SSs from plant and algae, but it encompasses the entire protein sequence of bacterial GSs. An OsttaSSIII-C sequence analysis was done with CD-search and InterPro servers and the two typical SS domains, GT-5 (Pfam PF08323) and GT-1 (Pfam PF00534) were detected, with significantly low E-values (3.0×10^{-53} and 4.5×10^{-79}, respectively) (Fig. 1A) [14,15]. However, we could not identify any SBD characteristic of other SSIIIs within the amino acids sequence of the 56.8 kDa *O. tauri* protein, suggesting that OsttaSSIII-C has only a catalytic domain and it lacks of a SBD; which resembles the characteristic found in prokaryotic GSs.

Amino acid residues involved in substrates binding and catalysis has been identified in several SS and GS sequences. The alignment of OsttaSSIII-C, ArathSSIII-CD, EsccoGS and AgrtuGS amino acid sequences showed a high conservation of these residues in the *O. tauri* SSIII-C isoform (Fig. 1B, Tables 1 and 2). It has been reported that Arg299 and Lys304 from *A. tumefaciens* are involved in the catalytic activity of GS, specifically in the interaction with a phosphate group of ADPGlc [23–25]. Glu376 is also important, making a salt bridge with Lys304 and helps to stabilize the glucose bound to ADP. In addition, it was also reported that these groups are conserved in *E. coli* (Arg300, Lys305 and Glu375) [18,26] and *A. thaliana SSIII* (Arg837, Lys842 and Glu919) [27]. Accordingly, Fig. 1B showed a high conservation of these residues in the *O. tauri* SSIII-C isoform (Arg270, Lys275 and Glu352, OsttaSSIII-C numbering).

Using the 3D structure of the *E. coli* GS (PDB code 2QZS, 32% identity for residues 1–465) as template, we built a homology model of OsttaSSIII-C as described in the Materials and Methods section. The structure of OsttaSSIII-C presents a folding similar to 2QZS, being secondary structure conserved along the sequence (Fig. 2A and B) and exhibiting a spatial conservation of the position of the amino acids involved in catalysis (Arg300, Lys305 and Glu375 for 2QZS numbering; Arg270, Lys275 and Glu352, OsttaSSIII-C numbering, see Fig. 2), suggesting that also in this protein such residues would have an important role in catalysis.

3.2. Cloning, expression and purification of OsttaSSIII-C wt and modified proteins

Three genes coding for respective isoforms of SSIII have been identified in *O. tauri*. We focused our attention on OsttaSSIII-C, where the lack of an SBD makes it more similar to prokaryotic GSs. The DNA fragment coding for OsttaSSIII-C was cloned into pRSET-B vector to generate the pRSET-B:OsttaSSIII-C plasmid. This construct leads to the expression of OsttaSSIII-C with the His-tag at the N-terminal region of the protein. To test the importance of the

Table 1
Amino acid residues involved in ADPGlc binding in *E. coli* GS and OsttaSSIII-C. Ref: AthSSIII: *Arabidopsis thaliana* SSIII isoform; Adenine, ribose and Pi (phosphate) indicate which part of the Glc bound to ADP participates in the each interaction; O2 and O6: oxygen atoms in ribose; vdW: van der Waals interaction; KTGGM and KVGGL correspond to the conserved motifs in *E. coli* GS and OsttaSSIII-C enzymes, respectively.

E. coli GS	OsttaSSIII-C	AthSSIII	Function	Conservation
Lys15	Lys31	Lys591	ADPGlc binding (KTGGM/KVGGL)	+
Gly18	Gly34	Gly594	ADPGlc binding (KTGGM/KVGGL)	+
His161	His167	His736 H	bond with O6 ribose (380 loop)	+
Asn246	Asn215	Asn783	H bond with O6 ribose	+
Val297	Val268	Ile835	H bond with O2 ribose	+
Ser298	Ser269	Thr836	vdW with adenine	+
Arg300	Arg270	Arg837	ionic interaction with Pi	+
Lys305	Lys275	Lys842	ionic interaction with Pi	+
Gly354	Lys329	Thr896	interaction with adenine	–
Tyr355	Tyr330	Tyr897	stacking with adenine	+
His356	Asp331	Asp898	carbonyl interacts with adenine	–
Ser360	Ser335	Ser902	vdW with adenine	+
Glu377	Glu352	Glu919	ionic interaction with Pi	+
Thr382	Thr357	Thr924	H bond with O2 ribose	+

Table 2
Amino acid residues involved in glycogen binding in *E. coli* GS and OsttaSSIII-C. Ref: *Arabidopsis thaliana* SSIII isoform; Glc +1 and Glc +2 indicates which Glc in glycogen is involved in the interaction; OH2 and OH3 indicates OH groups of Glc +1 that interacts with the protein; KTGGL and KVGGM correspond to the conserved motifs in *E. coli* GS and OsttaSSIII-C enzymes, respectively.

E. coli GS	OsttaSSIII-C	AthSSIII	Function	Conservation
Glu9	Glu25	Glu585	binding of Glc + 1	+
Leu19	Met35	Leu595	binding of Glc + 1 (KTGGL/KVGGM)	–
Tyr 95	Tyr112	Tyr672	stacking with Glc + 2	+
Asp137	Asp146	Asp706	H bond with OH2 and OH3 Glc + 1	+
His161	His167	His736	H bond with O6 ribose (380 loop)	+
Tyr165	Phe171	Phe740	stacking with Glc + 2	+
Arg299	Arg270	Arg837	ionic interaction with Pi	+

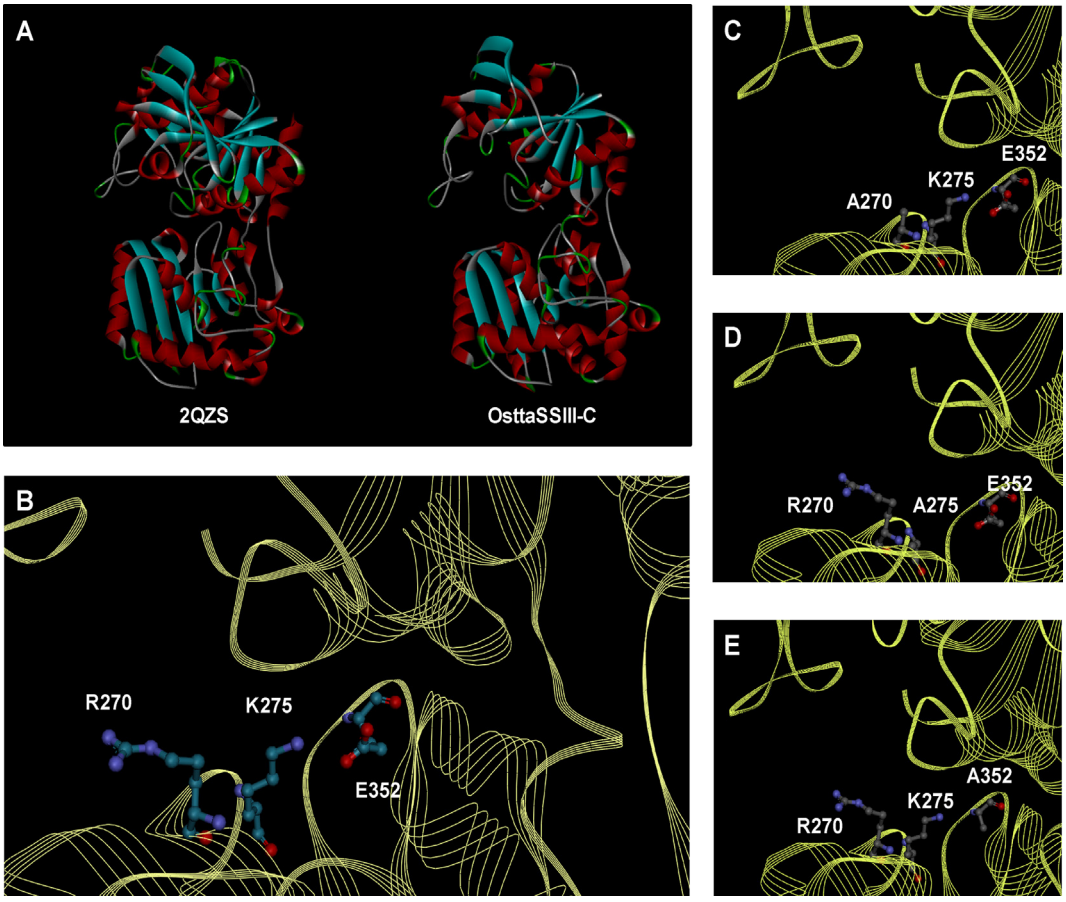

Fig. 2. Homology modeling of OsttaSSIII-C and mutants. (A) Structural model of *E. coli* GlgA (PDB 2QZS, left) and the proposed model for OsttaSSIII-C (right); (B) Close-up view of catalytic site of OsttaSSIII-C, showing the residues involved in the active site; (C, D, E) Close-up view of catalytic site of OsttaSSIII-C, showing the residues involved in the active site for the mutants R270, K275 and E352 respectively.

amino acid residues Arg270, Lys275 and Glu352 in OsttaSSIII-C catalysis, the modified proteins OsttaSSIII-C R270A, K275A and E352A were obtained after performing site directed mutagenesis on pRSET-B::OsttaSSIII-C (see Materials and Methods). The purification of OsttaSSIII-C wt and mutated proteins were carried out through a single step using HiTrapTM Chelating HP column equilibrated with 0.1 M $NiSO_4$. Using this procedure, we obtained about 1–1.5 mg of purified SSIII-C per gram of cells. Fig. 3 (lane 2) shows the SDS-PAGE profile of recombinant OsttaSSIII-C wt. The purification analysis of the modified proteins is shown in lanes 3 to 5. Results show a single protein band in all the lanes from the SDS-PAGE with an estimated molecular mass of ~57 kDa with no degradation products.

3.3. Kinetic characterization of OsttaSSIII-C

The kinetic parameters of OsttaSSIII-C were determined using the purified recombinant protein expressed in *E. coli* cells. Fig. 4 shows the saturation plots for the enzymatic activity of OsttaSSIII-C wt using ADPGlc as the sugar-nucleotide donor (Fig. 4A) and glycogen (Fig. 4B), or amylopectin (Fig. 4C) as the acceptor substrate. In all cases the OsttaSSIII-C enzyme displayed Michaelis kinetics, indicating a non-cooperative behavior. Table 3 shows the kinetic parameters of OsttaSSIII-C in the assayed conditions. The K_m values for the acceptor polysaccharide were 0.23 mg/ml for glycogen and 0.64 mg/ml for amylopectin; whereas the respective value for ADPGlc was 99 μM, using glycogen as the

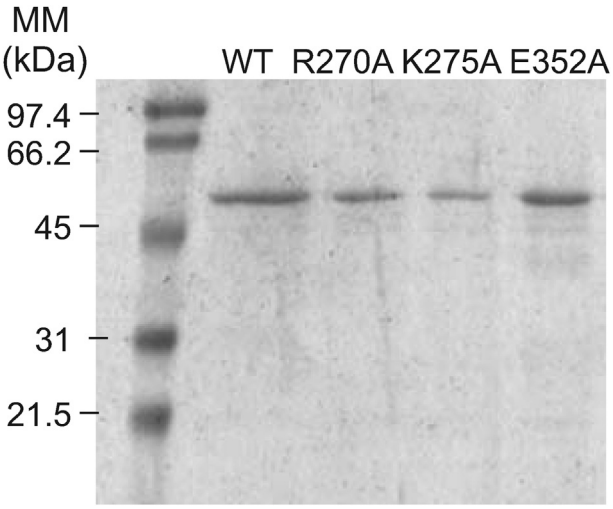

Fig. 3. (A) SDS-PAGE of recombinant enzymes. Lane 1: Molecular weight standard, lane 2, OsttaSSIII-C; lane 3, OsttaSSIII-C R270A; lane 4, OsttaSSIII-C K275A; and lane 5, OsttaSSIII-C E352A. Numerals indicate the molecular masses (MM) of the standards (Prestained SDS-PAGE Standards, Low Range, BioRad).

acceptor substrate. These K_m values are in the same range as those reported for SS isoenzymes from other plants and algae, including *Chlamydomonas reinhardtii* [8,28,29]. As mentioned above, *C. reinhardtii* presents two starch synthases III [30], containing two

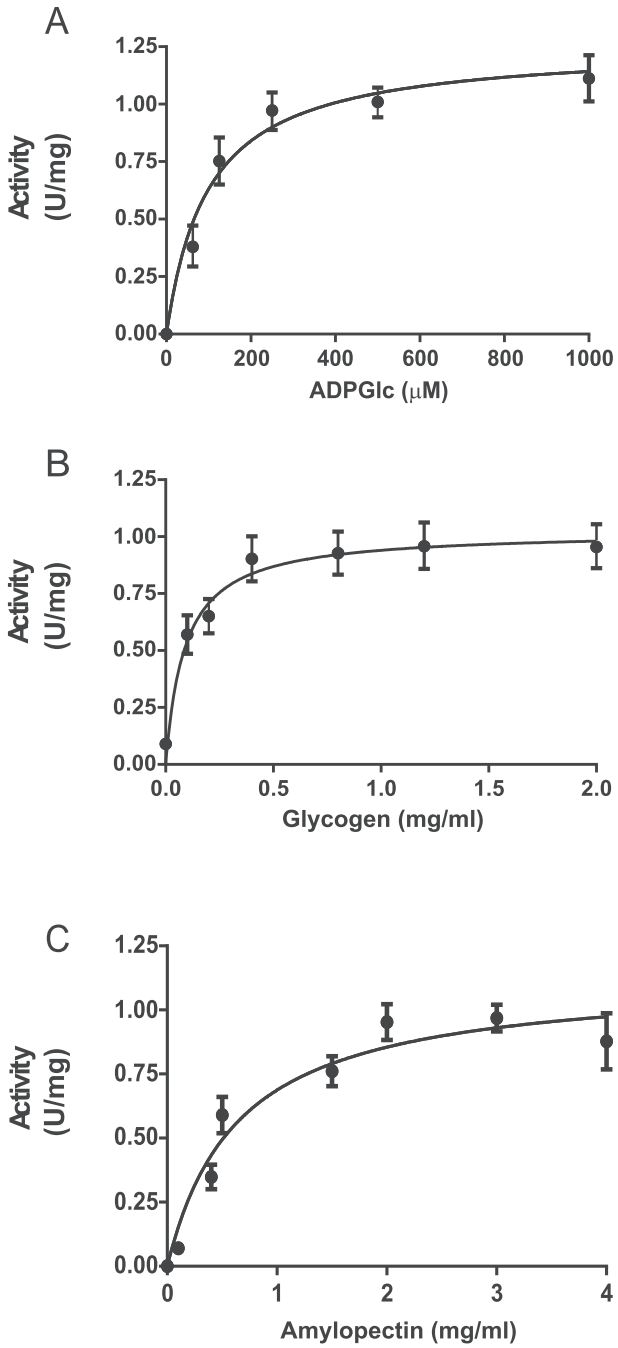

Fig. 4. ADPGlc (A), glycogen (B) and amylopectin (C) saturation plots for OsttaSSIII-C determined in the presence of 2 mg/ml glycogen (A) or 500 μM ADPGlc (B and C).

Table 3
Kinetic parameters of wild type OsttaSSIII-C.

	K_m (ADPGlc mM Polysacc mg/ml)	V_{max} (U/mg)
ADPGlc	0.099 ± 0.008	1.25 ± 0.03
Glycogen	0.23 ± 0.02	1.14 ± 0.06
Amylopectin	0.64 ± 0.04	1.09 ± 0.05
Amylose	n.a.	n.a.

n.a., no activity.

and three SBDs [6], that are structurally more similar to OsttaSSIII-A, OsttaSSIII-B, and ArathSSIII rather than OsttaSSIII-C. However, to date, only one *C. reinhardtii* SSIII isoform was kinetically

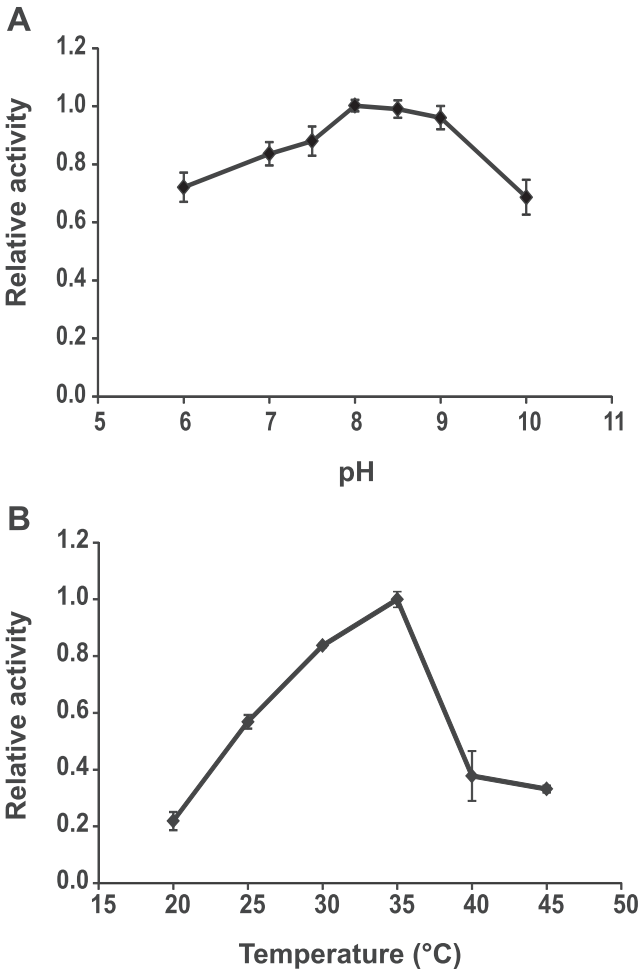

Fig. 5. (A) Effect of pH on the activity of OsttaSSIII-C. 50 mM Bicine buffer was used for pH 6.0–10.0. Assays were run with 2 mg/ml glycogen and 1 mM ADPGlc. Data are relative values compared with activity at pH 8. (B) Effect of temperature on the activity of OsttaSSIII-C. Assays were run with 2 mg/ml glycogen and 1 mM ADPGlc, in 50 mM Bicine buffer, with reaction temperatures of 20, 25, 30, 35, 40 and 45 °C. Data are relative values compared with activity at 35 °C.

characterized (ChlreSSIII1, Genbank AAY42381.1), showing kinetic parameters in the same range to those reported for OsttaSSIII-C [28].

Under the assayed conditions OsttaSSIII-C did not exhibit enzymatic activity when amylose and UDPGlc were used as acceptor and donor substrates, respectively. These results reinforced the previous statement that *O. tauri* synthesize starch through the ADPGlc pathway [7]. In addition, like other plants and algae starch synthases, OsttaSSIII-C showed an alkaline pH optimum close to 8, in Bicine buffer, and an optimum reaction temperature of 35 °C (Fig. 5A and B). On the other hand, we analyzed the effect of different metabolites such as citrate and malate. It was previously reported that both metabolites act as activators, increasing the SS activity in maize and rice [31,32]. However, no effect on OsttaSSIII-C activity was observed after the addition of different concentrations of malate or citrate (0–500 mM).

3.4. Effect of R270, K275 and E352 on OsttaSSIII-C catalytic activity

To test the importance of the amino acid residues Arg270, Lys275 and Glu352 in catalysis, the kinetic parameters of each isoform were determined as described in Materials and Methods.

Table 4
Kinetic parameters of wild type OsttaSSIII-C and modified proteins

	ADPGlc				glycogen			
	K_m (mM)	V_{max} ($mU \cdot mg^{-1}$)	V_{max}/K_m ($mU \cdot mg^{-1} \cdot mM^{-1}$)	Catalytic efficiency (%)	K_m ($mg \cdot ml^{-1}$)	V_{max} ($mU \cdot mg^{-1}$)	V_{max}/K_m ($mU \cdot mg^{-1}$).($mg \cdot ml^{-1}$)$^{-1}$	Catalytic efficiency (%)
WT	0.10 ± 0.01	1230 ± 110	12300 ± 2330	100	0.24 ± 0.03	1176 ± 97	4900.0 ± 612.6	100
R270A	0.13 ± 0.02	5.3 ± 0.4	40.8 ± 3.2	0.33	0.07 ± 0.01	6.6 ± 0.7	94.3 ± 13.6	1.9
K275A	0.67 ± 0.07	25.2 ± 3.1	37.6 ± 8.5	0.31	0.44 ± 0.05	29.2±3.1	66.4 ± 7.6	1.4
E352A	0.25 ± 0.04	21.0 ± 2.6	84.0 ± 10.6	0.68	0.04 ± 0.01	14.0 ± 2.3	350.0 ± 57.8	7.1

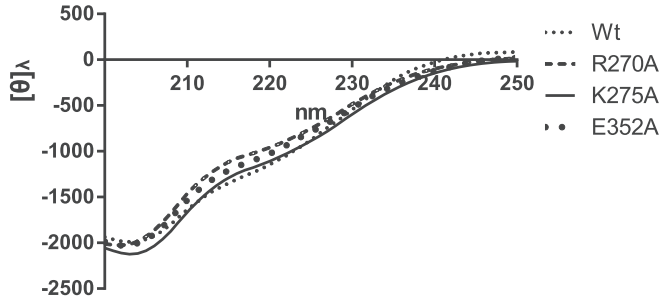

Fig. 6. Far-UV Circular Dichroism Spectra of recombinant and mutated OsttaSSIII-C isoforms. Wild type protein is represented by dotted lines. OsttaSSIII-C R270A, K275A and E352A are represented by dashed line, solid line and bold dotted line, respectively. Circular Dichroism spectra were obtained in 20 mM Tris-HCl buffer, pH: 7.5.

The results are shown in Table 4. All three residues mutated in this analysis were critical for catalysis, as the replacement of any of them decreased the specific activity several orders of magnitude. Replacement of Arg270 or Lys275 by Ala rendered an enzyme about 301/327- and 52/74-fold less active regards ADPGlc and glycogen, respectively. When Glu352 was mutated, the catalytic efficiency decreased 146- and 14-fold for ADPGlc and glycogen, respectively. We ruled out the possibility that the mutations generated misfolded forms of the enzyme, because their circular dichroism spectra were near identical (Fig. 6), suggesting that these mutations did not affect enzyme structure. Our results indicate that these residues play an important role in the catalytic reaction, and suggest that OsttaSSIII-C has a reaction mechanism similar to *E. coli* GS and other retaining GT-B glycosyltransferases [18,26].

4. Conclusions

In the last years, there has been an increasing demand for starch in many industrial processes, such as pharmaceutical, food, and bioethanol production. Therefore, a better understanding of starch metabolism, in particular the structure-function relationship of the enzymes involved in its biosynthesis, may provide a powerful instrument for the development of new strategies to increase plant biomass as well as to improve the quantity and quality of this polysaccharide [33,34]. *O. tauri*, a unicellular green alga of the Prasinophyceae family, has a very simple cellular organization and presents a single starch granule at the center of its unique chloroplast [7]. However, its starch metabolism exhibits a structural complexity comparable to higher plants, with multiple enzyme forms for each pathway step [35].

O. tauri possesses three SSIII isoforms, one similar to higher plants and *Chlamydomonas* SSIII, with three N-terminal SBDs (OsttaSSIII-B) and the other two isoforms, one with two central SBDs (OsttaSSIII-A) and the third one, lacking SBDs (OsttaSSIII-C) [6]. In this work we report the cloning, expression in *E. coli* cells and characterization of the SSIII-C isoform from this picoalga. We show that *in vitro* OsttaSSIII-C presents starch synthase activity. We evaluated its acceptor substrate specificity determining that OsttaSSIII-C displayed high ability to elongate branched glucans such as amylopecin and glycogen, whereas it showed inefficient to use amylose as the glucan acceptor. This agrees with the higher activity showed by rice SSI, SSIIa and SSIIIa [32], maize endosperm SSI, SSIIa, and SSIIb [36], potato tuber SSII [37], kidney bean SSIII [38] and pea embryo SSII [39], in the presence of glycogen and amylopectin with respect to maltohexaose. Furthermore, our results show that OsttaSSIII-C, which lacks SBDs, has the same substrate preference as the plant starch synthases containing SBDs. As previously reported, the main function of SBDs is related to the modulation of the biological activity of starch synthase III, rather than to determine the polysaccharide specificity [12].

On the other hand, OsttaSSIII-C displayed a high apparent affinity for ADPGlc, but it was not active when UDPGlc was used as the glucosyl donor substrate; agreeing a previous report that *O. tauri* starch metabolism is an ADPGlc based pathway [7]. The OsttaSSIII-C N-terminal region would be involved in the interaction with ADPGlc, through the Lys-X-Gly-Gly-Leu motif [40]. This motif is strictly conserved in all the members of the SSIII family, where the variable X position is occupied by Val (Val32 in *O. tauri*). The amino acid residue Lys as well as Gly are conserved in the whole group in agreement with the fact that these residues are very important for the binding activity of the motif [41]. Furthermore, the Leu residue present in *E. coli*, *A. tumefaciens* and *A. thaliana* glycogen/starch synthase enzymes is replaced by a methionine in a conservative substitution for binding to glycogen. This change is also found in other algae such as *Micromonas commoda*, *Micromonas pussilla, Volvox carteri* and *Ostreococcus lucimarinus* (data not shown).

OsttaSSIII-C sequence alignment with SSIII-CD and glycogen synthases previously characterized [18,24,26], identified conserved residues, and some of them were studied by site-directed mutagenesis. The kinetic characterization of the OsttaSSIII-C individual mutants in Arg270, Lys275, and Glu352 revealed the importance of the replaced residues. In all cases, the mutations significantly decreased the catalytic efficiency without affecting the protein folding. These results indicate that these residues play an important role in the catalytic reaction. Accordingly, this triad of residues has been described as critical for catalysis not only in glycogen/starch synthases but also in all other retaining GT-B glycosyltransferases such as the *E. coli* trehalose-6-phosphate synthase and maltodextrin phosphorylase [42] and also the sucrose synthases from *A. thaliana* and *N. europaea* [21,43]. The conservation of the mentioned residues in OsttaSSIII-C and both SSIII isoforms from *C. reinhardtii* respect to all other retaining GT-B glycosyltransferases suggests that these enzymes would have the same reaction mechanism (Fig. 1B).

In conclusion, our results support that the OsttaSSIII-C isoform is an active starch synthase enzyme without a N-terminal SBD. Further investigation is necessary to analyze the implications of the evolutionary conservation of this “bacterial like” starch synthase isoform in *O. tauri* and its physiological function in the picoalga

starch metabolism.

Funding

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 00134), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT RAICES 2011-0982; PICT Joven 2012-0981; PICT 2014-2436), and National Science Foundation (NSF MCB 1616851 and NSF MCB 1024945). NH is a doctoral fellow from ANPCyT. JB, AAI, MVB and DGC are research members from CONICET.

Acknowledgments

We thank Dr. Evelyne Derelle for kindly providing *O. tauri* genomic DNA.

References

[1] S.G. Ball, M.K. Morell, From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule, Annu. Rev. plant Biol. 54 (2003) 207–233.
[2] H. Liu, G. Yu, B. Wei, Y. Wang, J. Zhang, Y. Hu, Y. Liu, H. Zhang, Y. Huang, Identification and phylogenetic analysis of a novel starch synthase in Maize, Front. plant Sci. 6 (2015) 1013.
[3] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic acids Res. 42 (2014) D490–D495.
[4] S. Ball, C. Colleoni, U. Cenci, J.N. Raj, C. Tirtiaux, The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis, J. Exp. Bot. 62 (2011) 1775–1801.
[5] U. Cenci, F. Nitschke, M. Steup, B.A. Minassian, C. Colleoni, S.G. Ball, Transition from glycogen to starch metabolism in Archaeplastida, Trends plant Sci. 19 (2014) 18–28.
[6] J. Barchiesi, N. Hedin, D.F. Gomez-Casati, M.A. Ballicora, M.V. Busi, Functional demonstrations of starch binding domains present in Ostreococcus tauri starch synthases isoforms, BMC Res. Notes 8 (2015) 613.
[7] J.P. Ral, E. Derelle, C. Ferraz, F. Wattebled, B. Farinas, F. Corellou, A. Buleon, M.C. Slomianny, D. Delvalle, C. d'Hulst, S. Rombauts, H. Moreau, S. Ball, Starch division and partitioning. A mechanism for granule propagation and maintenance in the picophytoplanktonic green alga Ostreococcus tauri, Plant physiol. 136 (2004) 3333–3340.
[8] H.A. Valdez, M.V. Busi, N.Z. Wayllace, G. Parisi, R.A. Ugalde, D.F. Gomez-Casati, Role of the N-Terminal starch-binding domains in the kinetic properties of starch Synthase III from Arabidopsis thaliana†, Biochemistry 47 (2008) 3026–3032.
[9] M. Martin, N.Z. Wayllace, H.A. Valdez, D.F. Gomez-Casati, M.V. Busi, Improving the glycosyltransferase activity of Agrobacterium tumefaciens glycogen synthase by fusion of N-terminal starch binding domains (SBDs), Biochimie 95 (2013) 1865–1870.
[10] N. Palopoli, M.V. Busi, M.S. Fornasari, D. Gomez-Casati, R. Ugalde, G. Parisi, Starch-synthase III family encodes a tandem of three starch-binding domains, Proteins 65 (2006) 27–31.
[11] H.A. Valdez, D.A. Peralta, N.Z. Wayllace, M.J. Grisolía, D.F. Gomez-Casati, M.V. Busi, Preferential binding of SBD from Arabidopsis thaliana SSIII to polysaccharides: study of amino acid residues involved, Starch - Stärke 63 (2011) 451–460.
[12] N.Z. Wayllace, H.A. Valdez, R.A. Ugalde, M.V. Busi, D.F. Gomez-Casati, The starch-binding capacity of the noncatalytic SBD2 region and the interaction between the N- and C-terminal domains are involved in the modulation of the activity of starch synthase III from Arabidopsis thaliana, FEBS J. 277 (2010) 428–440.
[13] D.F. Gomez-Casati, M. Martin, M.V. Busi, Polysaccharide-synthesizing glycosyltransferases and carbohydrate binding modules: the case of starch synthase III, Protein Peptide Lett. 20 (2013) 856–863.
[14] A. Marchler-Bauer, S.H. Bryant, CD-Search: protein domain annotations on the fly, Nucleic acids Res. 32 (2004) W327–W331.
[15] S. Hunter, P. Jones, A. Mitchell, R. Apweiler, T.K. Attwood, A. Bateman, T. Bernard, D. Binns, P. Bork, S. Burge, E. de Castro, P. Coggill, M. Corbett, U. Das, L. Daugherty, L. Duquenne, R.D. Finn, M. Fraser, J. Gough, D. Haft, N. Hulo, D. Kahn, E. Kelly, I. Letunic, D. Lonsdale, R. Lopez, M. Madera, J. Maslen, C. McAnulla, J. McDowall, C. McMenamin, H. Mi, P. Mutowo-Muellenet, N. Mulder, D. Natale, C. Orengo, S. Pesseat, M. Punta, A.F. Quinn, C. Rivoire, A. Sangrador-Vegas, J.D. Selengut, C.J. Sigrist, M. Scheremetjew, J. Tate, M. Thimmajanarthanan, P.D. Thomas, C.H. Wu, C. Yeats, S.Y. Yong, InterPro in 2011: new developments in the family and domain prediction database, Nucleic acids Res. 40 (2012) D306–D312.
[16] K. Okonechnikov, O. Golosova, M. Fursov, Unipro UGENE: a unified bioinformatics toolkit, Bioinformatics. 28 (2012) 1166–1167.
[17] J.L. Pons, G. Labesse, @TOME-2: a new pipeline for comparative modeling of protein-ligand complexes, Nucleic acids Res. 37 (2009) W485–W491.
[18] F. Sheng, X. Jia, A. Yep, J. Preiss, J.H. Geiger, The crystal structures of the open and catalytically competent closed conformation of Escherichia coli glycogen synthase, J. Biol. Chem. 284 (2009) 17796–17807.
[19] R. Luthy, J.U. Bowie, D. Eisenberg, Assessment of protein models with three-dimensional profiles, Nature 356 (1992) 83–85.
[20] M. Wiederstein, M.J. Sippl, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins, Nucleic acids Res. 35 (2007) W407–W410.
[21] Z.L. Wu, C.M. Ethen, B. Prather, M. Machacek, W. Jiang, Universal phosphatase-coupled glycosyltransferase assay, Glycobiology. 21 (2011) 727–733.
[22] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[23] M. Leterrier, L.D. Holappa, K.E. Broglie, D.M. Beckles, Cloning, characterisation and comparative analysis of a starch synthase IV gene in wheat: functional and evolutionary implications, BMC plant Biol. 8 (2008) 98.
[24] A. Buschiazzo, J.E. Ugalde, M.E. Guerin, W. Shepard, R.A. Ugalde, P.M. Alzari, Crystal structure of glycogen synthase: homologous enzymes catalyze glycogen synthesis and degradation, EMBO J. 23 (2004) 3196–3205.
[25] M.V. Busi, J. Barchiesi, M. Martín, D.F. Gomez-Casati, Starch metabolism in green algae, Starch - Stärke 66 (2014) 28–40.
[26] A. Yep, M.A. Ballicora, J. Preiss, The active site of the Escherichia coli glycogen synthase is similar to the active site of retaining GT-B glycosyltransferases, Biochem. Biophys. Res. Commun. 316 (2004) 960–966.
[27] M.V. Busi, N. Palopoli, H.A. Valdez, M.S. Fornasari, N.Z. Wayllace, D.F. Gomez-Casati, G. Parisi, R.A. Ugalde, Functional and structural characterization of the catalytic domain of the starch synthase III from Arabidopsis thaliana, Proteins 70 (2008) 31–40.
[28] T. Fontaine, C. D'Hulst, M.L. Maddelein, F. Routier, T.M. Pepin, A. Decq, J.M. Wieruszeski, B. Delrue, N. Van den Koornhuyse, J.P. Bossu, et al., Toward an understanding of the biogenesis of the starch granule. Evidence that Chlamydomonas soluble starch synthase II controls the synthesis of intermediate size glucans of amylopectin, J. Biol. Chem. 268 (1993) 16223–16230.
[29] N. Szydlowski, P. Ragel, S. Raynaud, M.M. Lucas, I. Roldan, M. Montero, F.J. Munoz, M. Ovecka, A. Bahaji, V. Planchot, J. Pozueta-Romero, C. D'Hulst, A. Merida, Starch granule initiation in Arabidopsis requires the presence of either class IV or class III starch synthases, Plant Cell 21 (2009) 2443–2457.
[30] P. Deschamps, H. Moreau, A.Z. Worden, D. Dauvillee, S.G. Ball, Early gene duplication within chloroplastida and its correspondence with relocation of starch metabolism to chloroplasts, Genetics 178 (2008) 2373–2387.
[31] C.D. Boyer, J. Preiss, Properties of citrate-stimulated starch synthesis catalyzed by starch synthase i of developing Maize Kernels, Plant physiol. 64 (1979) 1039–1042.
[32] Y. Nakamura, S. Aihara, N. Crofts, T. Sawada, N. Fujita, In vitro studies of enzymatic properties of starch synthases and interactions between starch synthase I and starch branching enzymes from rice, Plant Sci. Int. J. Exp. plant Biol. 224 (2014) 1–8.
[33] S. Jobling, Improving starch for food and industrial applications, Curr. Opin. plant Biol. 7 (2004) 210–218.
[34] A.M. Smith, Prospects for increasing starch and sucrose yields for bioethanol production, Plant J. Cell Mol. Biol. 54 (2008) 546–558.
[35] O. Sorokina, F. Corellou, D. Dauvillee, A. Sorokin, I. Goryanin, S. Ball, F.Y. Bouget, A.J. Millar, Microarray data can predict diurnal changes of starch content in the picoalga Ostreococcus, BMC Syst. Biol. 5 (2011) 36.
[36] T.A. Hennen-Bierwagen, Q. Lin, F. Grimaud, V. Planchot, P.L. Keeling, M.G. James, A.M. Myers, Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts, Plant physiol. 149 (2009) 1541–1559.
[37] A. Edwards, A. Borthakur, S. Bornemann, J. Venail, K. Denyer, D. Waite, D. Fulton, A. Smith, C. Martin, Specificity of starch synthase isoforms from potato, Eur. J. Biochem./FEBS 266 (1999) 724–736.
[38] T. Senoura, N. Isono, M. Yoshikawa, A. Asao, S. Hamada, K. Watanabe, H. Ito, H. Matsui, Characterization of starch synthase I and II expressed in early developing seeds of kidney bean (Phaseolus vulgaris L.), Biosci. Biotechnol. Biochem. 68 (2004) 1949–1960.
[39] I. Damager, K. Denyer, M.S. Motawia, B.L. Moller, A. Blennow, The action of starch synthase II on 6"'-alpha-maltotriosyl-maltohexaose comprising the branch point of amylopectin, Eur. J. Biochem./FEBS 268 (2001) 4878–4884.
[40] K. Furukawa, M. Tagaya, M. Inouye, J. Preiss, T. Fukui, Identification of lysine 15 at the active site in Escherichia coli glycogen synthase. Conservation of Lys-X-Gly-Gly sequence in the bacterial and mammalian enzymes, J. Biol. Chem. 265 (1990) 2086–2090.
[41] K. Furukawa, M. Tagaya, K. Tanizawa, T. Fukui, Role of the conserved Lys-X-Gly-Gly sequence at the ADP-glucose-binding site in Escherichia coli glycogen synthase, J. Biol. Chem. 268 (1993) 23837–23842.
[42] R. Schinzel, P. Drueckes, The phosphate recognition site of Escherichia coli maltodextrin phosphorylase, FEBS Lett. 286 (1991) 125–128.
[43] Y. Zheng, S. Anderson, Y. Zhang, R.M. Garavito, The structure of sucrose synthase-1 from Arabidopsis thaliana and its functional implications, J. Biol. Chem. 286 (2011) 36108–36118.