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Identification and characterization of a novel starch branching enzyme from the picoalgae *Ostreococcus tauri*

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ABSTRACT

Starch branching enzyme is a highly conserved protein from plants to algae. This enzyme participates in starch granule assembly by the addition of α-1,6-glucan branches to the α-1,4-polyglucans. This modification determines the structure of amylopectin thus arranging the final composition of the starch granule. Herein, we describe the function of the Ot01g03030 gene from the picoalgae *Ostreococcus tauri*. Although *in silico* analysis suggested that this gene codes for a starch debranching enzyme, our biochemical studies support that this gene encodes a branching enzyme (BE). The resulting 1058 amino acids protein has two *in tandem* carbohydrate binding domains (CBMs, from the CBM41 and CBM48 families) at the *N*-terminal (residues 64–403) followed by the *C*-terminal catalytic domain (residues 426–1058). Analysis of the BE truncated isoforms show that the CBMs bind differentially to whole starch, amylose or amylopectin. Furthermore, both CBMs seem to be essential for BE activity, as no catalytic activity was detected in the truncated enzyme comprising only by the catalytic domain. Our results suggest that the Ot01g03030 gene codifies for a functional BE containing two CBMs from CBM41 and CBM48 families which are critical for enzyme function and regulation.

1. Introduction

Starch is the major energy reserve polysaccharide in photosynthetic organisms, and is the most important source of energy in the human diet. The starch granule is composed of two glucose polymers: amylose (essentially an α-1,4-glucan chain) and amylopectin (composed by a backbone of a linear α-1,4-glucan chain branched with α-1,6-glycosidic bonds) [1,2]. In plants, this polysaccharide is a key component for the carbon balance during the light/dark cycle, acting as a carbon buffer [3]. It has been shown that mutants unable to synthesize starch cannot grow on short photoperiods because of carbon starvation [4,5]. The accumulation of starch is controlled by enzymes related to its synthesis [6]. Thus, ADP-glucose pyrophosphorylase (ADPGlc PPase) is the key enzyme in this process, being it responsible for the synthesis of ADPGlc, which is the glycosyl donor for starch build-up [4,7,8]. Granule bound (GBSS) and soluble (SSS) starch synthases catalyze the addition of ADPGlc to the non-reducing end of the α-1,4-linear glucan chain [7,9]. Besides, starch branching enzyme (BE) converts the linear α-1,4-polysaccharide into an α-1,4 α-1,6-branched product by cleaving α-1,4 bonds on amylose and amylopectin and transferring the released segment to the acceptor glucan, forming a new α-1,6-linkage [10,11]. There are two clases of BE (BEI and BEII) that differ in terms of the length of the chains transferred *in vitro*, where BEII transfers shorter chains than BEI [12]. In the other hand, Starch debranching enzymes (DBEs) hydrolyze α-(1,6) glycosidic linkages of α-polyglucans. Based on their substrate specificities, DBEs are classified into two types: isoamylase-type α-(1,6) glycohydrolase and pullulanase-type α-(1,6) glycohydrolase. Isoamylase-type DBEs can debranch glycogen, phytoglycogen and amylopectin [13] while pullulanase-type DBEs can degrade mainly pullulan and amylopectin [13]. Finally, the disproportionating enzyme (D-enzyme) catalyzes the transfer of α-(1,4) linked oligosaccharides from the end of one linear glucan chain to the end of another chain,

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although its function on starch biosynthesis is still unclear [14–17].

Most of the enzymes that catalyze reactions involving synthesis, degradation or modification of glycosidic bonds exhibit a modular organization that comprises a catalytic domain (CD) and one or more carbohydrate binding domain (CBM) usually connected by a linker region [18]. A CBM is defined as a non-catalytic module that has the capacity to bind carbohydrates and is adjacent to a CD of an enzyme involved in involved in carbohydrate metabolism [19]. CBMs are able to increase the rate of enzymatic reactions through a strong physical association with substrates. They can also act as an organizing subunit generating multienzymatic complexes [19]. According to their amino acid sequence, substrate binding specificity and structure, these modules can be classified to date into 81 families (CAZY http://www.cazy.org) [20,21].

CBMs could be found in proteins that recognize polysaccharides such as cellulose, starch, glycogen, xylan, chitin, pullulan, beta-glucans, inulin and many other different sugars. Occasionally, CBMs can also be found as a single protein (i.e.) a pollen protein from olive, Ole e 10 [22] and the non-catalytic chitin binding protein CBP21 from Serratia marcesens [23]. In carbohydrate-active enzymes (CAZYmes) like glycoside hydrolases o glycosyl transferases, CBMs can be localized at the *N*– or *C*-terminal end of these proteins, between two catalytic modules like in the *Paenibacillus polymyxa* αβ-amylase, or arranged in tandem [24,25]. In the latter case, the CBMs can be separated by intermediate regions rich in amino acids like serine or threonine which would increase the flexibility and mobility of the modules, or without them, generating a rigid structure [25].

Among CBMs, we can highlight the Starch Binding Domains (SBDs), which have acquired the evolutionary advantage of being capable of disrupting the surface of their substrate due to the presence of two binding sites [26–28]. These domains are distributed in twelve CBM families; 20, 21, 25, 26, 34, 41, 45, 48, 53, 58, 68 and 69 and they are found in archaea, bacteria, and eukaryotes [29] (http://www.cazy.org/).

The number of CBM families into the CAZY database is constantly growing as more data emerging from sequencing of new genomes are elucidated and evidence CBMs with different amino acid sequences and function. Among nuclear genomes sequenced in the last decade, some of them are from green algae including: *Volvox carteri* [30], *Micromonas pusilla* [31], *Chlorella variabilis* [32], *Chlamydomonas reinhardtii* [33], *Ostreococcus lucimarinus* [34], and *Ostreococcus tauri* [35,36]. As reported, *C. reinhardtii* and *O. tauri* are the best characterized [33–36]; albeit *C. reinhardtii* has been a model organism for years, *O. tauri* has gained importance from its first description as a photosynthetic organism relatively simple in terms of genetic components [37].

O. tauri is a species belonging to the ancient group Prasinophyceae [38] and is the tiniest free-living eukaryote known to date [37]. While this alga presents the smallest genome from a photosynthetic eukaryote know to date, it codes a large number of genes related to starch metabolism. For example, while many plants such as *Arabidopsis thaliana* presents only one gene coding for SSSIII enzyme, *O. tauri* presents at least three copies of SSSIII-like genes [39]. A similar example is observed for glucan water dikinase and phosphoglucan water dikinase enzymes [40]. Interestingly, this organism presents only one mitochondrion and one chloroplast [41], and also has a particular starch division and partitioning system where the only starch granule present in the chloroplast is elongated and divided into two daughter structures [42]. Ral et al. [42] have demonstrated that the protein encoded by Ot01g03030 in the alga is the second most abundant protein associated to starch, behind the GBSS [35,42]. It has been proposed that this finding would be of great significance to elucidate the partitioning mechanism that takes place in *O. tauri*.

The present investigation was conducted to biochemically characterize the enzyme coded by Ot01g03030 in order to better understand the starch metabolism in *Ostreococcus tauri*. We performed an *in silico* analysis of the coded protein to determine its evolutionary relation to other starch metabolism enzymes, and to propose a molecular model for this protein based on homology modeling. We have cloned and expressed the Ot01g03030 gene from *O. tauri* to produce recombinant wild type and different truncated isoforms of the protein in order to assay their catalytic activity and polysaccharide binding ability. To the best of our knowledge, this is the first characterization of this *O. tauri* BE (OsttaBE), which has been previously classified as a starch debranching enzyme based on computational prediction [43].

2. Materials and methods

2.1. Cloning, expression and purification of OsttaBE

The gene Ot01g03030 from *O. tauri* (coding for the Q6PYZ0 protein) was cloned from genomic DNA using primers containing *Not*I and *Nde*I restriction sites for subcloning into the vector pRSFDuet-1 (Novagen, EMD Biosciences Inc, Madison, WI, USA). CBM41 and CBM48 were cloned using *Bam*HI and *Hind*III; while CBM48CD and DC were cloned using *Not*I and *Nde*I restriction sites in the same vector. All the procedures were performed using standard molecular biology protocols and the following primers: BE-Fw, ATAGCGGCCGCAATGTCGGTCACCCAC (*Not*I site underlined); BEInt-Fw, CAGCAGAACTGGCGTGTGG; BE-Rev, GATCATATGCTATCGACACTGCACGAATAC (*Nde*I site underlined); BEInt-Rev, TGTGCTCGAACTCTCAGTG; BE-CBM41-Fw, AGAGGATCCGATCCTTCGAGTGCGTTAC (*Bam*HI site underlined); BE-CBM41-Rev, TCTAAGCTTCTACCCGCTCACCAACCACAC (*Hind*III site underlined); BE-CBM48-Fw, GAGGGATCCTCCTCTGGGCGCGGAATTTG (*Bam*HI site underlined); BE-CBM48-Rev, CTCAAGCTTCTACGCGTACGGATCGCTCGAG (*Hind*III site underlined); and BE-DC-Fw, ATAGCGGCCGCAGTGGACTGCGCGATCTAC (*Not*I site underlined).

The different constructions were transformed into *Escherichia coli* BL21 (DE3) strain (F^- *ompT gal dcm lon hsdSB*(rB^- mB^-) λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*])). Cells were grown at 37 °C to an $OD_{600} = 0.6$, the expression was induced with IPTG to a final concentration of 1 mM, and further incubated at 16 °C for 16 h. Cells were harvested by centrifugation at 6000x*g* for 10 min at 4 °C. Pellets were suspended in buffer containing 20 mM Tris-HCl (pH 7.4) and cells were disrupted by sonication and centrifuged at 13000x*g* for 10 min at 4 °C. The supernatant was loaded onto a HiTrap chelating HP column (GE Healthcare BioSciences, Little Chalfont, UK) equilibrated with binding buffer (20 mM Tris-HCl pH 7.4, 20 mM imidazole). The column was washed twice with 10 vol of binding buffer, and each protein was eluted using a linear gradient of elution buffer (20 mM Tris-HCl pH 7.4, 20–400 mM imidazole) [39]. The presence of the protein in the eluted fractions was confirmed by SDS–PAGE analysis and immunoblotting. The fractions containing the protein of interest were pooled, concentrated to more than 1 mg/ml and desalted using Vivaspin 6 3000 MWCO concentrators. Finally, glycerol was added to a final concentration of 20% (v/v) and the protein was stored at −20 °C until use. Under these final conditions the protein was stable for at least 6 months.

2.2. Gel electrophoresis and immunological studies

SDS-PAGE was performed using a Bio-Rad Mini Protean system as described by Laemmli [44] and using 8% and 12% (v/v) polyacrylamide/bisacrylamyde gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich, St. Louis, MO, USA) or transferred to nitrocellulose membranes

(BioRad, Hercules, CA, USA). Electroblotted membranes were incubated with antibodies against recombinant *E. coli* BE. The antigen–antibody complex was visualized with alkaline phosphatase-linked a-rabbit IgG, followed by staining with BCIP and NBT as described elsewhere [45].

2.3. Polysaccharide-binding assays

Purified proteins (approximately 20 μg each) were mixed with starch (Fluka-85649, St. Louis, MO, USA), amylose (Fluka-10130) or amylopectin (Fluka-10118) 10% (w/v) in 20 mM Tris-HCl (pH 7.5) at a final polysaccharide concentration of 5% (w/v). Binding assays were done at room temperature by orbital mixing for 30 min as reported previously with minor modifications. Briefly, the polysaccharides were pelleted by centrifugation at 12000xg for 10 min, and the supernatant removed and boiled in SDS loading buffer Tris-HCl 50 mM pH 6.8, SDS 2% (w/v), Glycerol 6% (v/v), Bromophenol blue 0.004% (w/v) and β-Mercaptoethanol 1% (v/v). The pellets were washed three times with 100 μl of 20 mM Tris-HCl (pH 7.5) by gentle vortexing and centrifugation, and then resuspended in an equal volume to the supernatant and finally boiled under the same conditions [39,46,47]. After SDS-PAGE, protein levels were determined by densitometric analysis of the protein bands stained with Coomassie Blue R-250 using the GelPro analyzer program (Media Cybernetics, Bethesda, MD, USA). The recombinant *Arabidopsis thaliana* frataxin protein was used as a negative control [48].

2.4. Homology modeling and sequence alignment

The sequence similarity search of OsttaBE (Genbank code: XP_003074364, 1–1058 amino acid residues) whole amino acid sequences was performed with PSI-BLAST [49] with the default parameters (inclusion threshold 0.005) until convergence, using no redundant databases. Sequences with an E value less than 1.10^{-5} were retrieved and aligned with the program CLUSTALW [50]. Alignment of amino acids sequences between homologous polypeptides was performed using the Unipro UGENE v.1.10.4 program [51] with default parameters. Phylogenetic tree was built by maximum likelihood using bootstrap analysis of 500 replicates in MEGA 7.0.14 version [52]. The tree was rooted by *Physcomitrella patens* predicted pullulanase.

A 3D structural model was obtained using the @TOME V2.2 program, which includes T.I.T.O. (Tool for Incremental Threading Optimization), Scwrl and Modeller [53]. OsttaBE structure was modeled using starch debranching enzyme from *Hordeum vulgare* (PDB code 4J3S) [54]. *Oryza sativa* and *E. coli* BE crystal structure's (PDB codes 3AMK and 5E6Z, respectively) were used to compare catalytic domains [55–57]. Alignment with the template was based on homology and secondary structure. Models were evaluated using Verify-3D [58] [59] and RAMPAGE [60] programs. Superposition of OsttaBE with 4J3S or 5E6Z were performed using the SuperPose server v 1.0 [61].

2.5. Circular dichroism

Far-UV circular dichroism spectra were obtained using a Jasco J810 spectropolarimeter (Jasco Inc., Easton, MD). The measurements were performed in a 0.1 cm quartz cuvette and the set-up was as follows: measurement rate 50 nm min^{-1}, bandwidth 1 nm, response time 2 s, data pitch 1 nm, and spectra accumulation of 10. The wavelength range used was from 250 nm to 200 nm, at 20 °C. All spectra were obtained in 20 mM Tris-HCl pH 7.4 buffer; and all data are reported as molar ellipticity $[\theta]_{molar,\ \lambda}$ (deg cm^2 $dmol^{-1}$) obtained after subtracting the baseline, and smoothing data.

2.6. Debranching activity assay

The debranching activity was measured using dinitrosalicylic acid reagent (DNSA) according to Bernfeld [62]. The activity was determined by quantifying the increase amounts of reducing sugars at different temperatures (20, 37 and 45 °C). The assay medium contained 20 mM Tris-HCl pH 7.4, amylopectin 5% (w/v) and different amounts of the recombinant purified proteins. Reaction was stopped by the addition of an equal volume of dinitrosalicilic acid reagent (50 mM dinitrosalicilic acid, 250 mM sodium hydroxide and 1 M potassium sodium tartrate) and boiled for 5 min. The increase of reducing power was determined by measuring DO at 575 nm. One unit of activity (U) is defined as the amount of enzyme that hydrolyzes the substrate to release 1 μmol of reducing sugar per minute.

2.7. Branching activity assay

Branching activity was assayed using a medium containing 20 mM Tris-HCl pH 7.4, amylopectin (Fluka #10118), final concentration 0–40 μg/μl, and different amounts of each recombinant enzyme: OsttaBE, CBM48CD or CD (0–2.5 μg) in a final volume of 33 μl. Assays were initiated by the addition of each enzyme, and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 100 μl of H_2O and 865 μl of iodine reagent (saturated $CaCl_2$, I_2 0.6 mM and KI 0.9 mM) as reported previously [63]. Enzyme activity was measured by the decrease of OD at 520 nm. Data is presented as ΔAbs against the water-iodine reagent blank. All the kinetic parameters are means of at least three determinations and are reproducible within ±10%. One unit of activity (U) is defined as the amount of enzyme that produces a decrease in absorbance of 1.0 per min at 520 nm.

3. Results and discussion

3.1. Sequence alignment and phylogenetic analysis of OsttaBE

Using PSI-Blast [49] we have performed similarity searches of the OsttaBE protein sequence, retrieving numerous results. This sequence is very similar to those of limit dextrinases (EC 3.2.1.142) and pullulanases (EC 3.2.1.41), both being starch debranching enzymes (DBEs) involved in (1–6)-α-D-glycosidic linkages hydrolysis [64]. In addition, both BEs and DBEs belong to the glycoside hydrolase family 13 (GH13) [65]. Probably these were the two reasons from which the OsttaBE analyzed in this work was previously classified as a DBE [43]. OsttaBE presents both, a CBM41 and CBM48 modules at its *N*-terminal region. CBMs from family 48 were found in BEs and DBEs; however, according to CAT (CAZymes Analysis Toolkit, http://mothra.ornl.gov/cgi-bin/cat/cat.cgi) analysis, the CBM41 family is only present in pullulanases and limit dextrinases [66]. This also agrees with the high similarity found between OsttaBE and several DBEs, leading to a misclassification.

The alignment of the amino acids sequence from OsttaBE with homologous polypeptides identified with PSI-Blast, allowed us to build a phylogenetic tree based on maximum likelihood (Fig. 1). In this tree we have included the sequence of BEs from both, monocots and dicots, in order to understand the relationship between OsttaBE and those BEs from higher plants. Fig. 1 shows two different groups of enzymes: DBEs (marked in light grey) and BEs (marked in grey), being the OsttaBE included in the first group. Thus, in order to confirm whether the OsttaBE (XP_003074364) is a functional BE, we decided to perform the gene molecular cloning, expression and production of the recombinant protein for biochemical characterization.

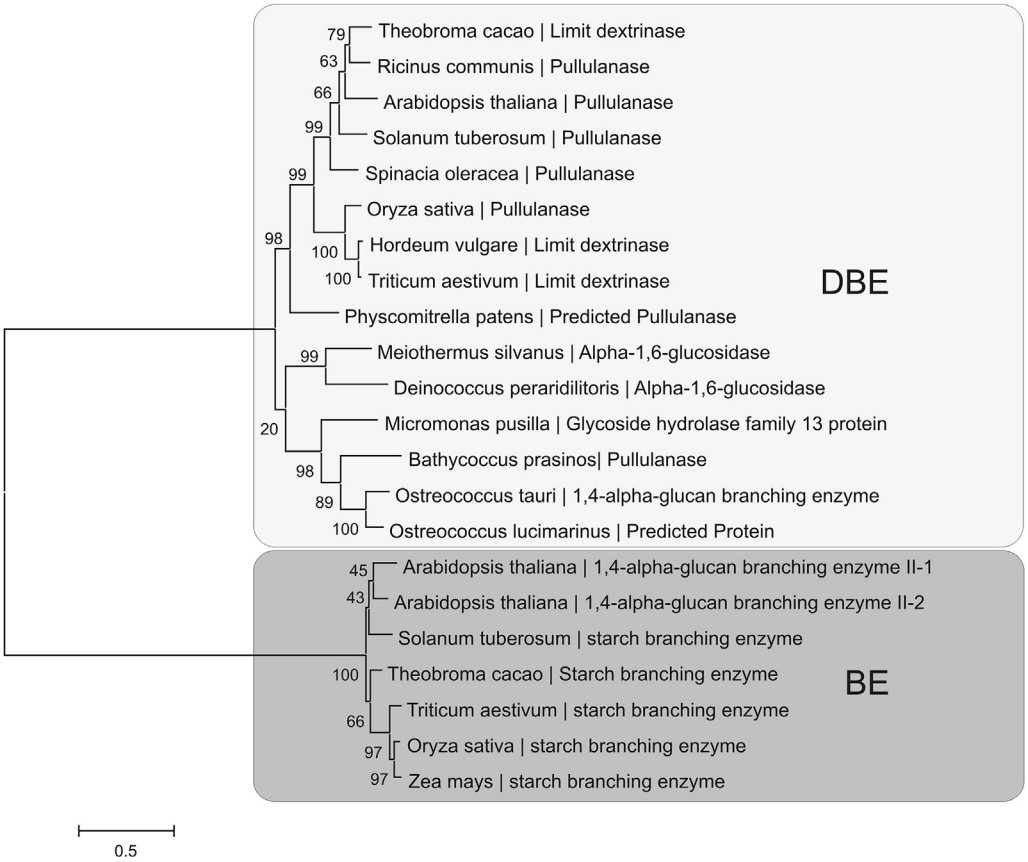

Fig. 1. Phylogenetic tree of DBEs and BEs. The tree is based on the alignment of OsttaBE with the amino acid sequences obtained with PSI-Blast. Accession numbers of the source proteins: *Ostreococcus tauri* 1,4-alpha-glucan branching enzyme XP_003074364, *Ostreococcus lucimarinus* predicted protein XP_001415537, *Bathycoccus prasinos* pullulanase XP_007511684, *Micromonas pusilla* glycoside hydrolase family 13 protein XP_003055653, *Physcomitrella patens* | predicted pullulanase XP_001771764, *Meiothermus silvanus* alpha-1,6-glucosidase WP_013157875, *Deinococcus peraridilitoris* alpha-1,6-glucosidase WP_015236161, *Theobroma cacao* limit dextrinase EOY18848, *Ricinus communis* pullulanase XP_015583098, *Arabidopsis thaliana* pullulanase AAO00771, *Solanum tuberosum* pullulanase XP_006361707.1, *Spinacia oleracea* pullulanase CAA58803.1 *Oryza sativa* pullulanase ACY56102.1, *Hordeum vulgare* limit dextrinase AAD34348, *Triticum aestivum* limit dextrinase ABL84490, *Arabidopsis thaliana* 1,4-alpha-glucan branching enzyme II-1 NP_181180, *Arabidopsis thaliana* 1,4-alpha-glucan branching enzyme II-2 Q9LZS3, *Solanum tuberosum* starch branching enzyme CAB40743.1, *Theobroma cacao* starch branching enzyme EOY08149.1, *Triticum aestivum* starch branching enzyme CAR95900.1, *Oryza sativa* starch branching enzyme BAA82828.1, *Zea mays* starch branching enzyme NP_001104920. The tree was built by Maximum Parsimony method in MEGA 7.0.14 version [52] and it is rooted to *Physcomitrella patens* predicted pullulanase. Phylogenies were determined by Bootstrap Analysis of 500 replicates in MEGA 7.0.14 version [52]. Branch lengths are proportional to distances. Bootstrap values are shown above branches. Abbreviation: DBE, debranching enzyme; BE, branching enzyme.

3.2. Sequence analysis and homology modeling of OsttaBE

Using the 3D structure of *Hordeum vulgare* DBE (HvLD, PDB 4J3S, 47% identity for residues 175–1058) as template, we built a homology model of the OsttaBE as described in the Methods section. The analysis of the structure evaluated with Verify 3D program showed 99.5% of the residues with a positive score, and 90.7% of them higher than 0.2 (average score 0.394). Moreover, the structure was also analyzed using Prosa II obtaining a Z-score of −8.89 [67,68]. According to these results, we conclude that OsttaBE model is of good quality and accurate to reach confidence in the molecular structure analysis.

The structure of OsttaBE presents a folding similar to 4J3S, being the α-helix and β-sheet secondary structure conserved along the sequence (Fig. 2A–B). The superimposition of the structures of both, 4J3S and OsttaBE shows that the 3D structure of the template and the proposed model for OsttaBE are very similar (Fig. 2 C). On the other hand, it has been shown that the catalytic mechanism of GH13 occurs *via* a general acid/base double-displacement [69]. In HvLD, Asp473 has been identified as the catalytic nucleophile, and Glu510 as the acid/base proton donor, while it has been postulated that Asp642 acts as a transition-state stabilizer [70]. Based on the amino acids sequence alignment, we have identified that such amino acids postulated to be involved in acid/base double-displacement are well conserved in OsttaBE: Asp647 (catalytic nucleophile), Glu684 (proton donor) and Asp816 (transition-state stabilizer, OsttaBE numbering). A closer view of the superposed positions of the mentioned amino acid residues is shown in Fig. 2 D. Results show that the three residues maintain a conserved spatial and rotational configuration in OsttaBE respect to HvLD.

The phylogenetic analysis of the catalytic domain of OsttaBE with BEs from microbial (*M. tuberculosis, E. coli*, and *A. tumefaciens*) and higher plants (*A. thaliana, O. sativa, T. aestivum* and *Z. mays*) showed that OsttaBE is more similar to the bacterial enzymes (Supplementary Fig. 1). Thus, we also compared the model of OsttaBE with the 3D structure of other BEs such as that from *E. coli* (PDB 5E6Z) and *Oryza sativa* (PDB 3AMK).

Glucan-branching enzymes play an essential role in the formation of branched polysaccharides. Only one type of BE, belonging to glycoside hydrolase family 13 (GH13), is found in eukaryotes, while two types of branching enzymes (GH13 and GH57) occur in prokaryotes (Bacteria and Archaea) [71]. Both, GH13 and GH57 BEs are multidomain proteins. GH13 BEs contains a CBM48, a central catalytic domain and a *C*-terminal domain; while GH57 BEs do not

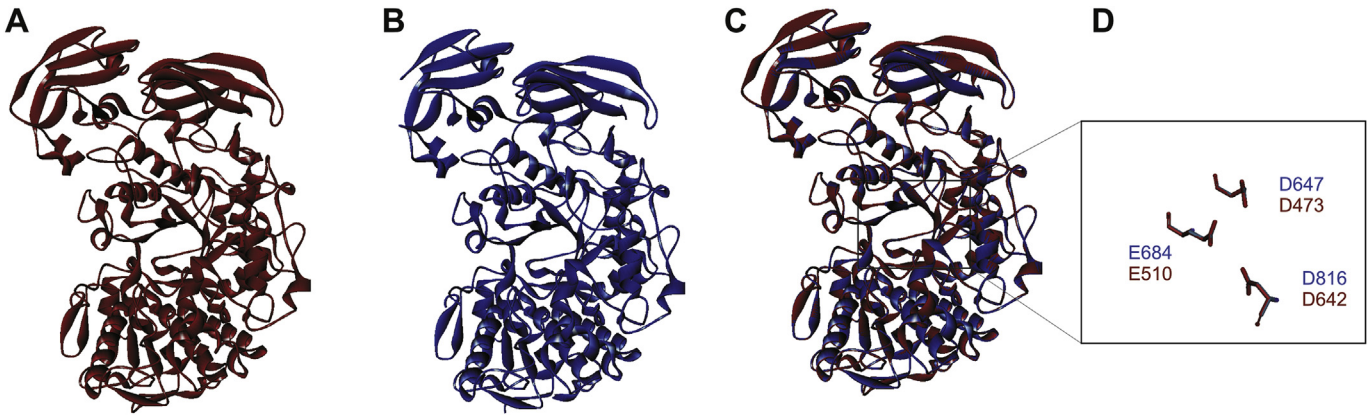

Fig. 2. Homology modeling of OsttaBE. A) 3D structure of barley limit dextrinase from *Hordeum vulgare*. (PDB code: 4J3S). B) Proposed model for OsttaBE. C) Superposition of 4J3S (red) and OsttaBE (blue) structures. Superposition of was performed using the SuperPose server v 1.0. D) Superposition between OsttaBE and 4J3S structures showing the amino acid residues of the catalytic site. OsttaBE residues are shown in blue and 4J3S residues are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

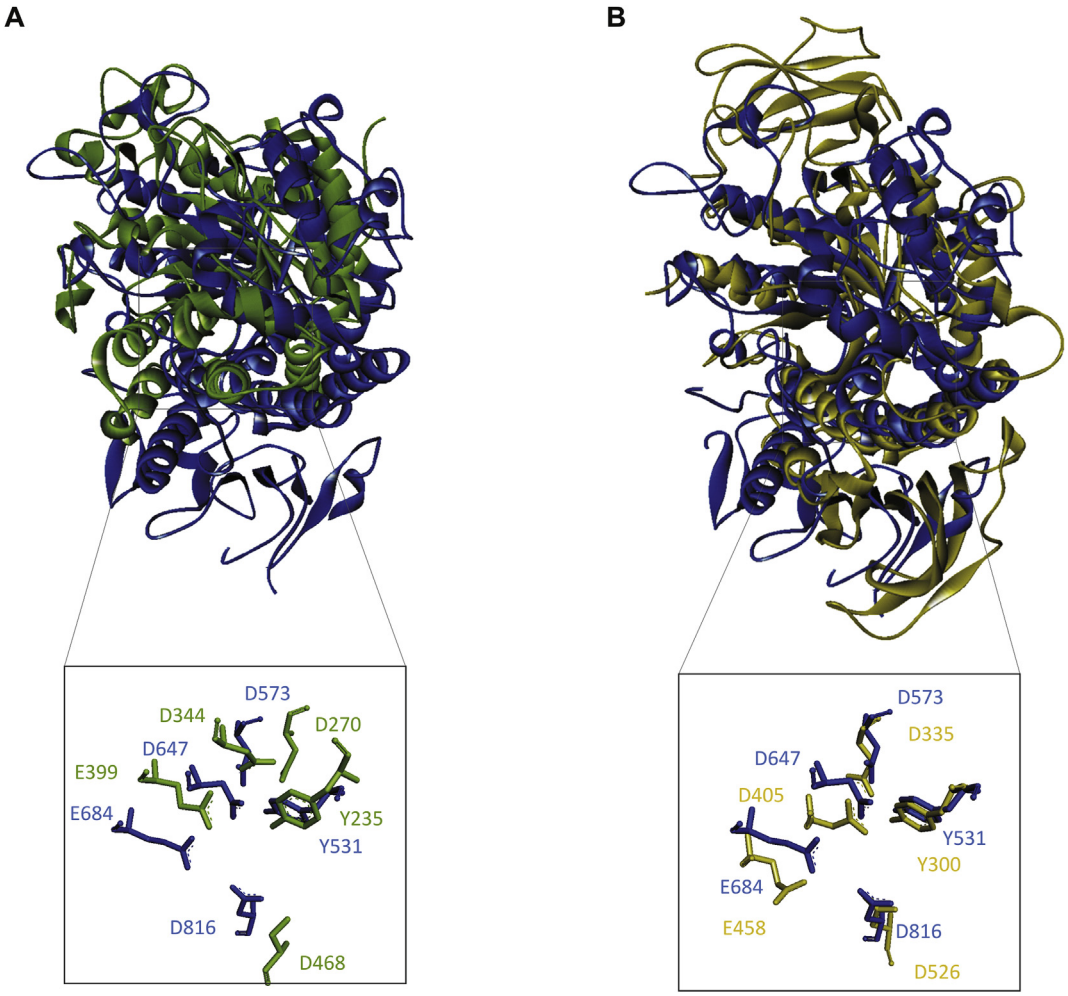

Fig. 3. Superposition of OsttaBE structure (blue) and starch BEs from (A) *Oryza sativa* (PDB 3AMK, green) and (B) *E. coli* (PDB 5E6Z, yellow). The insets show the amino acids involved in the active site in the BEs and OsttaBE. Superposition of was performed using the SuperPose server v 1.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have CBM48 and they are composed of three different domains named A, B and C. However, comparative biochemical characterization of BE across different families has not been extensively performed [71,72].

The comparison between the OsttaBE model of *O. sativa* and *E. coli* BEs showed that the global topology is almost the same (Fig. 3); however, a closer view of the superposed catalytic sites exhibit a high spatial conservation of the amino acids involved in

catalysis (Asp647, Glu684, and Asp816, OsttaBE numbering) with those from the bacterial enzyme (Fig. 3B). In addition, we also observed a conservation of other three essential residues involved in catalysis previously identified in the *E. coli* BE as Tyr300, Asp335 and Asp526 [57,73] at 531, 573 and 816 positions in OsttaBE, respectively (Fig. 3). The importance of Tyr300 for the stability and catalysis and in *E. coli* BE had already been described by MacGregor and Svensson [74] and Matsui et al. [75].

3.3. Cloning and expression of mature OsttaBE and truncated forms

The DNA fragments containing the OsttaBE full length sequence, the truncated CBM48DC, and DC forms were cloned in pRSF-Duet vector, containing a His_6-tag on the *N*-terminal region of each protein. The DNA fragment containing CBM41 and CBM48 were also cloned in pRSF-Duet vector, containing a His_6-tag on the *N*-terminal region (Fig. 4 A). The production of the different recombinant proteins was performed in *E. coli* BL21 (DE3) cells and purified using HiTrap chelating columns as described in the Methods section, and the purified concentrated fractions were analyzed by SDS-PAGE. In each case, a single protein band was eluted with 150–300 mM imidazole with the corresponding expected molecular mass: OsttaBE (120 kDa), CBM48CD (84 kDa), CD (70.0 kDa), CBM41 (11 kDa) and CBM48 (12 kDa) (Fig. 4B–C). Thus, we assume that all the recombinant proteins tested are homogeneous after this procedure. The presence of a protein band corresponding to OsttaBE was confirmed by western blotting using polyclonal antibodies against recombinant *E. coli* BE (Fig. 4 D).

3.4. Kinetic characterization of OsttaBE and the truncated forms CBM48CD and CD

As mentioned above, based on sequence data it has been predicted that OsttaBE was a DBE. Thus, we first evaluated the possibility that some of the proteins have debranching activity. Our results show that in the different assayed conditions neither OsttaBE nor the truncated forms CBM48CD and CD exhibited debranching activity when amylose, amylopectin, starch, or microcrystalline cellulose were used as substrates, suggesting that OsttaBE is not a DBE. Based on the fact that BEs and DBEs belongs to the glycoside hydrolase family 13 (GH13) [65] and also considering that (as described above) these proteins share a similar structure of the CD when compared with other BEs or bacterial GBEs (see Supplementary Fig. 2) [76], we decided to evaluate the starch branching activity of OsttaBE and the truncated protein forms. No enzymatic activity of OsttaBE, CBM48CD or CD was detected when amylose or starch were used as acceptor polysaccharides; however, OsttaBE showed starch branching activity when amylopectin was used as substrate. Whereas the full length OsttaBE is active under this condition, no detectable activity with amylopectin was measured for CBM48CD and CD alone (Table 1). The saturation plot shows that OsttaBE display Michaelis kinetics (n_H of 1.1), with a K_m value of 3.9 ± 0.5 mg/ml for amylopectin and a V_{max} of 0.53 ± 0.03 U/mg (Fig. 5, Table 1). It is important to mention that the obtained K_m and V_{max} parameters are similar to those values reported for BEs from other organisms such as rice BEI and II (K_m = 3.0 and 2.7 mg/ml, respectively) [77] and *Synechocystis* sp. BE (V_{max} = 1.0 U/mg) [78].

Due to the lack of activity of CBM48CD and CD in the presence of

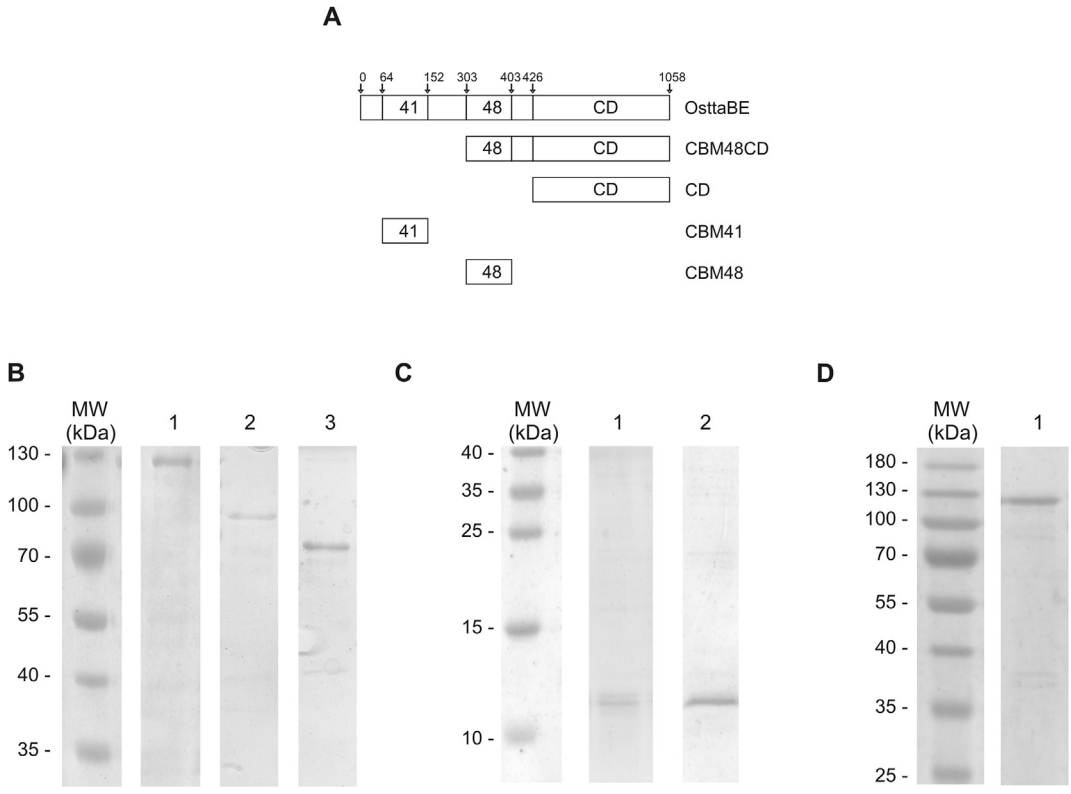

Fig. 4. A) Schematic representation of the recombinant peptides used in this study: OsttaBE, full length BE from *O. tauri*; CBM48CD, BE isoform lacking CBM41; CD, catalytic domain of BE; CBM41, carbohydrate binding module from family 41; CBM48 carbohydrate binding module from family 48. Numbers above each protein indicate the position of the amino acids residues. B) SDS-PAGE of recombinant OsttaBE enzymes: lane 1, OsttaBE full length enzyme; lane 2, CBM48CD; lane 3, CD. Numerals on the left indicate molecular mases of the standards (PageRuler prestained protein ladder, Thermo Scientific). C) SDS-PAGE of recombinant OsttaBE enzymes: lane 1, CBM48; lane 2, CBM41. The molecular mass standards used are the same as those used in panel A. D) Western blot detection of purified recombinant full length OsttaBE using polyclonal antibodies against recombinant *E. coli* BE.

Table 1
Kinetic parameters of OsttaBE full length and truncated proteins using saturating concentrations of amylopectin as substrate.

Enzyme isoform	K_m (mg/ml)	n_H	V_{max} (U/mg)
OsttaBE	3.9 ± 0.5	1.1 ± 0.2	0.53 ± 0.03
CBM48CD	n.a.		
CD	n.a.		

Ref: n.a.: no activity.

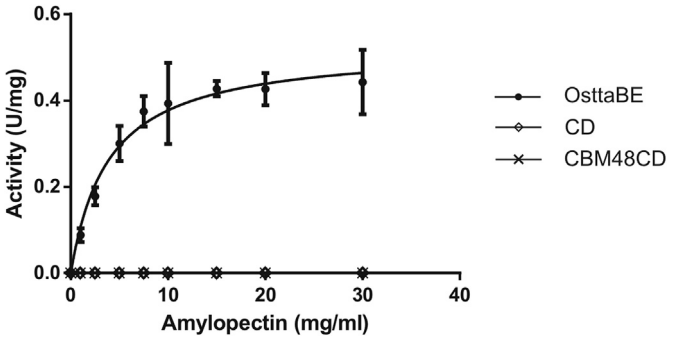

Fig. 5. Amylopectin saturation plots for OsttaBE (black circles), CBM48CD (crosses) and CD (diamonds) enzymes determined in the presence of varying concentrations of amylopectin (0–30 mg ml^{-1}). See Materials and Methods section for details.

amylose or amylopectin, we decided to analyze the correct folding of both proteins by the evaluation the respective secondary structure by circular dichroism (Fig. 6). OsttaBE exhibited a spectrum with a minimum of molar ellipticity at 208 nm. When the CBM41 is removed (CBM48CD spectrum), it can be observed that this minimum decreases from −4570.92 deg cm^2 $dmol^{-1}$ to −2944.68 deg cm^2 $dmol^{-1}$; while when both CBMs are removed the minimum of the circular dichroism spectrum decreases to −1532.79 deg cm^2 $dmol^{-1}$, in agreement with the decrease of β-sheet secondary when CBMs are removed. These results agree with previous reports on CBMs from family 41 and 48, where it was evidenced that this modules presents β-sandwich fold with seven or six β-strands [79,80]. Thus, the lack of catalytic activity of the CBM48CD and CD proteins should not be due to a misfolding, suggesting that the CBMs would be essential for the catalytic activity of OsttaBE.

3.5. Polysaccharide binding assays

CBMs are commonly associated to carbohydrate active enzymes, where they convey the catalytic domain of a protein into a prolonged and intimate association with the substrate [19]. Furthermore, CBMs can disrupt the surface of the substrates and act as a core for macromolecular aggregates [81,82]. These modules can also be found in non-hydrolytic proteins, where they act as scaffolding subunits and their deletion dramatically decreases the enzymatic activity [19].

Using bioinformatic tools we found that OsttaBE primary structure contains two potential CBMs belonging to two different families. CBM41 is located at the beginning of the *N*-terminal end of the protein and it is usually present in DBEs; while CBM48 is located downstream the former and is commonly found in both BEs and DBEs [79,83–85]. We have performed a homology model of the CBM41 from OsttaBE using the 3D structure of a pullulanase from *Klebsiella pneumoniae* as template (PDB 2FHF). It has been reported in this protein the presence of two tryptophan residues (W80 and W95, 2FHF numbering) that are involved in stacking interaction with the polysaccharide [86]. Based on the superposition of the homology model of CBM41 from OsttaBE with 2FHF we found that both tryptophan residues are well conserved (W84 and W93, OsttaBE numbering) (Supplementary Fig. 3). In addition, the alignment of CBM48 from different representative proteins shows that there is a high conservation of the amino acids involved in polysaccharide binding in the CBM48 region of OsttaBE [85]. Moreover, the essential tyrosine residue reported to be part of the starch binding 1 site is also well conserved (Y388, OsttaBE numbering) (Supplementary Fig. 4).

In order to confirm the ability of OsttaBE as well as both CBMs and the truncated proteins to bind to different polysaccharides, we carried out adsorption assays to different concentrations of amylose, amylopectin and starch, as described in the Methods section.

Fig. 7 shows the adsorption isotherms for the binding of OsttaBE to the different polysaccharides, showing a high K_{ad} value for starch (10.9 ± 0.8 ml g^{-1}), whereas it presents about 2- and 7-fold lower values for amylose and amylopectin, respectively (Fig. 7, Table 2). The CBM48CD protein presents about 2-fold decrease in the K_{ad} value for starch, whereas it show an increase of about 2-fold in the K_{ad} value for amylopectin respect to the full length enzyme. It is interesting to note that both, the CD and the CBM41 protein lack the ability to bind to the amylopectin fraction (Table 2), whereas the CBM48 binds to this polysaccharide, showing a K_{ad} value of 4.2 ± 0.6 ml g^{-1}. Our results suggest that CBM48 is essential for amylopectin binding, whereas both, CBM41 and 48 contribute to bind to starch and amylose.

The targeting and binding of starch metabolic enzymes to starch granules and the facilitation of enzyme activity and efficient catalysis depend on non-catalytic binding sites on the CBM

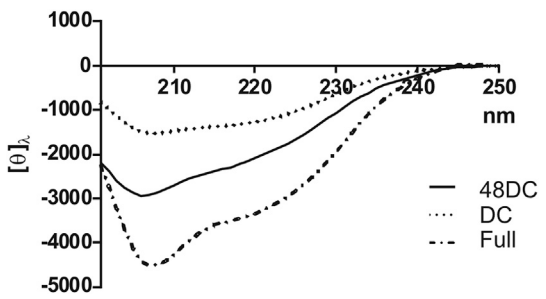

Fig. 6. Far-UV Circular dichroism spectra of recombinant OsttaBE. OsttaBE full length is represented by a dashed-dotted line; CBM48DC is represented by a solid line; CD is represented by a dotted line.

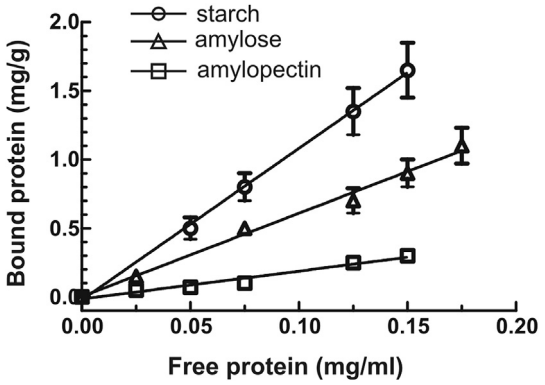

Fig. 7. Adsorption of OsttaBE to different polysaccharides: starch (circles), amylose (triangles) or amylopectin (squares). Linear adsorption isotherms indicate the apparent equilibrium distribution of the proteins between the solid (bound protein) and liquid phase (free protein) at different protein concentrations. K_{ad} (milliliters per gram of polysaccharide) values represent the slopes of each isotherm.

Table 2
Adsorption constants (K_{ad}, ml. g^{-1} of polysaccharide) of OsttaBE full length and truncated proteins to starch, amylase and amylopectin.

Protein	K_{ad} (ml. g^{-1} starch)	K_{ad} (ml. g^{-1} amylose)	K_{ad} (ml. g^{-1} amylopectin)
OsttaBE	10.9 ± 0.8	6.0 ± 0.5	1.6 ± 0.2
CBM48CD	4.6 ± 0.5	8.1 ± 0.9	3.1 ± 0.4
CD	1.3 ± 0.2	9.2 ± 1.0	n.d.
CBM41	2.0 ± 0.1	9.9 ± 1.4	n.d.
CBM48	12.6 ± 1.4	8.5 ± 1.1	4.2 ± 0.6

Ref: n.d.: no binding detected.

auxiliary domains (SBDs in this case), or on surface binding sites (SBSs) located on the CD [21,25,87,88]. It is probably that SBSs and CBMs have complementary function since about 25% of the enzymes known to have an SBS also contain a CBM [4] and around half of these SBS-containing enzymes belong to glycoside hydrolase family 13 (GH13; CAZy designation [21]) like OsttaBE. Therefore, we can not exclude that the higher catalytic activity observed for the enzyme OsttaBE with amylopectin, despite having the lowest K_{ad} for the substrate, could be due to the presence of SBS that collaborate and complement the role of CBMs.

On the other hand, substrate binding is typically one of the rate-limiting steps preceding enzymatic catalysis. It has been described that the non-productive binding of different enzymes such as cellulases with their substrates is an important factor limiting efficient catalysis [89]. However, after a thorough study where CBMs from 18 families were fussed to a multifunctional GH5 catalytic domain, which can hydrolyze numerous polysaccharides, it has been shown that the best catalysis was correlated with moderate binding affinity of the CBM [90]. Thus, the fact that the protein binds with high affinity to starch and amylose could also be the cause of the lack of activity on these polysaccharides.

In conclusion we have biochemically characterized an enzyme from *Ostreococcus tauri* that has branching activity. This enzyme presents a modular organization of two carbohydrate binding domains, from family 41 and 48, and a *C*-terminal CD. Our results shows that when CBM41 and CBM48 are removed from the polypeptide, the enzyme does not present enzymatic activity, which apparently is not a consequence of an incorrect folding of the protein, thus indicating that both CBMs are essential for the catalytic activity of OsttaBE. The polysaccharide binding assays show that the CBM48 is essential for amylopectin binding, whereas CBM41 and CBM48 cooperatively contribute to starch binding. In addition, we also made a molecular model that was accurate to identify the conserved catalytic amino acid residues in OsttaBE. Such residues seem to be spatially conserved according to the alignment with the BEs from *E. coli* and *O. sativa*, and the HvLD protein. Whereas the detailed structure differences between OsttaBE and BEs/DBEs still require further studies to reach a clearer picture, the data presented herein contribute to better understand the structural and functional properties of a BE from the model unicellular alga *Ostreococcus tauri*.

Author contributions

NH and JB performed experiments, analyzed data and wrote the paper. JB, DGC, AAI, MAB and MVB planned experiments, analyzed data and wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2017.02.005.

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