Mannitol metabolism in brown algae involves a new phosphatase family

Agnès Groisillier\textsuperscript{1,2,*}, Zhanru Shao\textsuperscript{1,2,3,*}, Gurvan Michel\textsuperscript{1,2}, Sophie Goulitquer\textsuperscript{4}, Patricia Bonin\textsuperscript{1,2}, Stefan Krahulec\textsuperscript{5}, Bernd Nidetzky\textsuperscript{5}, Delin Duan\textsuperscript{3}, Catherine Boyen\textsuperscript{1,2} and Thierry Tonon\textsuperscript{1,2,†}

\textsuperscript{1} UPMC Univ Paris 6, UMR 7139 Marine Plants and Biomolecules, Station Biologique, 29680, Roscoff, France
\textsuperscript{2} CNRS, UMR 7139 Marine Plants and Biomolecules, Station Biologique, 29680, Roscoff, France
\textsuperscript{3} Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, PR China
\textsuperscript{4} Plate-forme MetaboMER, CNRS and UPMC, FR2424, Station Biologique, 29680 Roscoff, France
\textsuperscript{5} Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12/1, 8010 Graz, Austria

\* These authors contributed equally to this work.
\† To whom correspondence should be addressed. E-mail: tonon@sb-roscoff.fr

Received 31 July 2013; Revised 25 October 2013; Accepted 4 November 2013

Abstract

Brown algae belong to a phylogenetic lineage distantly related to green plants and animals, and are found predominantly in the intertidal zone, a harsh and frequently changing environment. Because of their unique evolutionary history and of their habitat, brown algae feature several peculiarities in their metabolism. One of these is the mannitol cycle, which plays a central role in their physiology, as mannitol acts as carbon storage, osmoprotectant, and antioxidant. This polyol is derived directly from the photoassimilate fructose-6-phosphate via the action of a mannitol-1-phosphate dehydrogenase and a mannitol-1-phosphatase (M1Pase). Genome analysis of the brown algal model \textit{Ectocarpus siliculosus} allowed identification of genes potentially involved in the mannitol cycle. Among these, two genes coding for haloacid dehalogenase (HAD)-like enzymes were suggested to correspond to M1Pase activity, and thus were named \textit{EsM1Pase1} and \textit{EsM1Pase2}, respectively. To test this hypothesis, both genes were expressed in \textit{Escherichia coli}. Recombinant \textit{EsM1Pase2} was shown to hydrolyse the phosphate group from mannitol-1-phosphate to produce mannitol but was not active on the hexose monophosphates tested. Gene expression analysis showed that transcription of both \textit{E. siliculosus} genes was under the influence of the diurnal cycle. Sequence analysis and three-dimensional homology modelling indicated that \textit{EsM1Pases}, and their orthologues in Prasinophytes, should be seen as founding members of a new family of phosphatase with original substrate specificity within the HAD superfamily of proteins. This is the first report describing the characterization of a gene encoding M1Pase activity in photosynthetic organisms.

Key words: Carbon storage, \textit{Ectocarpus siliculosus}, HAD (haloacid dehalogenase) superfamily, mannitol, phosphatases, primary metabolism.

Introduction

Brown algae are multicellular, mostly marine organisms that have evolved independently from animals and land plants. They belong to the stramenopiles (Heterokonta), a phylum that diverged from the Archaeplastida and Opisthokonta 1 billion years ago (\textit{Yoon et al.}, 2004). Within the stramenopiles, brown algae are a relatively recent lineage that emerged
about 200 million years ago (Brown and Sorhannus 2010; Silberfeld et al., 2010). They feature a complex evolutionary history, with phaeoplasts that arose from the secondary endosymbiosis of an ancestral red alga (Reyes-Prieto et al., 2007), and a number of lateral gene transfers with bacteria that have shaped their extant chimaeric metabolism (Michel et al., 2010a,b). Most brown algae are found in the intertidal zone, a very harsh environment where they are under marine or terrestrial constraints according to the tides. Besides their interest as a distinct phylogenetic lineage and their ecological role in marine ecosystems, brown algae also represent a sustainable resource of valuable compounds for biotechnological applications (Wei et al., 2013), such as the cell-wall polysaccharides alginites and sulfated fucans (Popper et al., 2011), and the carbon storage compounds laminarin and mannitol (Michel et al., 2010a). Among brown algae, Ectocarpus siliculosus has been selected as a biological model to study physiological and ontogenic processes (Charrier et al., 2008), and its genome has been sequenced (Cock et al., 2010). This has allowed identification of the molecular bases for a number of peculiar metabolic pathways in these organisms.

Carbon storage is a key physiological process, and brown algae accumulate carbohydrates by producing laminarin (a vacuolar β-1,3-glucan with occasional β-1,6-linked branches) and mannitol. Depending on the species of brown algae examined, mannitol can represent up to 20–30% of their dry weight (Reed et al., 1985). Recent experiments in E. siliculosus have suggested that mannitol is likely to act as an osmoprotectant or local osmolyte (Michel et al., 2010a). In the same vein, this polyol constitutes a significant portion of the soluble carbohydrates in several families of vascular plants (including the Apiaceae, Fabiaceae, Oleaceae, Poaceae, and Rosaceae), where it represents one of the primary photosynthetic products and is important to increase tolerance to salt and osmotic stress (Stoop et al., 1996; Conde et al., 2011). Moreover, mannitol is also produced by a number of unicellular and pluricellular red algae (Karsten et al., 1992 and 1999), and by green microalgae belonging to the family Prasinophyceae (Richter and Kirst, 1987).

Different pathways have been identified for the synthesis and degradation of mannitol. In land plants, mannitol is produced from fructose-6-phosphate (fructose-6P) in source tissues via mannose-6-phosphate (mannose-6P) and mannitol-1-phosphate (mannitol-1P). It is then translocated to sink tissues where it is recycled to fructose-6P for use as a carbon and energy source (Stoop et al., 1996). In other organisms, including some bacteria, fungi, protozoan parasites, red micro- and macroalgae, and brown algae, the synthesis of mannitol involves two enzymatic steps: the direct reduction of fructose-6P into mannitol-1P, and the hydrolysis of the phosphoric ester of mannitol-1P to produce mannitol (Iwamoto and Shiraiwa, 2005) (Fig. 1). The first reaction is catalysed by a mannitol-1P dehydrogenase (M1PDH; EC1.1.1.17). Three putative EsM1PDH genes have been found in the E. siliculosus genome (Michel et al., 2010a), and among them, EsM1PDH1 was the most highly expressed throughout the diurnal cycle and under several abiotic stress conditions (Rousvoal et al., 2011). Moreover, analysis of recombinant EsM1PDH1 activity in bacterial cell-free extracts allowed the study of some of the biochemical characteristics of the algal enzyme. The second reaction for mannitol production is catalysed by a β-mannitol-1P phosphohydrolase also named mannitol-1-phosphatase (M1Pase; EC3.1.3.22). M1Pase activity has been measured in some plants known to accumulate mannitol, i.e. celery, rye, black gram, and apple (Grant and Rees, 1981; Rumpho et al., 1983), but the corresponding enzyme has not been characterized further in these organisms. In contrast, native M1Pases have been purified to homogeneity.

![Fig. 1. The mannitol cycle and its connection to central carbon metabolism in E. siliculosus. The arrow between mannitol and laminarin synthesis indicates that the structure of laminarin consists of two types of chains: in M-chains, mannitol is attached to the reducing ends, while in G-chains, glucose is attached to the reducing end.](https://academic.oup.com/jxb/article-abstract/65/2/559/486784)
from the red alga *Caloglossa continua* (Iwamoto et al., 2001) and from the protozoan parasite *Eimeria tenella* (Liberator et al., 1998). For this latter organism, the corresponding gene was expressed in *Escherichia coli*, and characterization of the native and recombinant protein showed that this enzyme was a new member of the phosphohistidine phosphotransferase family of phosphatases. For *Caloglossa*, only sequences of several peptides from the purified native enzyme are available so far, and they do not exhibit any similarity with the sequence of *Eimeria*. This suggests the existence of distinct types of M1Pases in these organisms. Interestingly, two genes encoding potential M1Pases were identified through annotation of the carbohydrate metabolism of *E. siliculosus* (Michels et al., 2013), which featured an appended C-terminal module belonging to the haloacid dehalogenase (HAD) superfamily. It was hypothesized that this M1PDH-HAD fusion protein was reminiscent of a bifunctional sucrose-phosphate synthase/sucrose-phosphate phosphatase, suggesting that the HAD module of MICPUN_62892 could be an M1Pase. The *E. siliculosus* genome displays two standalone homologues of M1PDH from *E. siliculosus* (MICPUN_62892), which bore a diurnal cycles (cultivation under a photoperiod of 10 h light phase, and snap frozen. Other samples used in this study were actively growing filaments were harvested from a 10 l culture and under a 12 h light phase, and snap frozen. Other samples used in this study corresponded to algae harvested at regular intervals through two diurnal cycles (cultivation under a photoperiod of 10 h light and 14 h dark), as described previously (Gravot et al., 2010; Rousvoal et al., 2011). For preparation of algal extracts, samples were ground in the presence of liquid nitrogen, and one part of powder was mixed with four parts (w/v) of extraction buffer (25 mM MOPS, pH 7.2, 15 mM EGTA, 15 mM MgCl2, 2 mM dithiothreitol, 0.5% polyvinylpyrrolidine, protease inhibitors). After homogenizing through a Wheaton tissue grinder and incubation for 30 min at 4 °C, extracts were sonicated for 10 s and centrifuged for 30 min at 15 000g. Protein concentration in the supernatants was measured by determining the absorbance at 280 nm with a Nanodrop 2000 spectrophotometer (Thermo Scientific), and extracts were stored at 4 °C before determination of enzymatic activity.

**Expression of EsM1Pase genes in *E. coli* and purification of recombinant EsM1Pase2**

The EsM1Pase1 open reading frame was amplified with primers EsM1Pase1for (5’-GGGGGGGAATCCGCGAT GAACCGGCCACATAACG-3’; BamHI restriction site underlined) and EsM1Pase1rev (5’-CCCCCCGAAATTCCTTATCCC ACACCGTCTTCCGTGC-3’; EcoRI restriction site underlined), and the EsM1Pase2 open reading frame with EsM1Pase2for (5’-GGGGGGGAATCCGAGAAGCCAAAGCATTC GACAAG-3’; BamHI restriction site underlined) and EsM1Pase2rev (5’-CCCCCCGAAATTCCTTATCCC ACACCGTCTTCCGTGC-3’; EcoRI restriction site underlined) using template plasmids containing full-length EsM1Pase1 and EsM1Pase2 from cDNA libraries prepared in the framework of the Ectocarpus Genome Project (Cock et al., 2010). Both genes were cloned in vector pFO4 (His-tag of six residues at the N terminus of the recombinant protein) as described previously (Groisillier et al., 2010). The recombinant plasmids pFESM1Pase1 and pFESM1Pase2 were transformed in *E. coli* BL21(DE3) and the integrity of their sequences was verified by sequencing.

To induce the production of recombinant *E. siliculosus* proteins, transformed *E. coli* were grown in 1 l of autoinducing ZYP 5052 medium (Studier, 2005) supplemented with 200 µg ml−1 of ampicillin (final concentration) at 20 °C and 200 r.p.m. After 3 days of incubation, cells were harvested by centrifugation at 4000g and 4 °C for 20 min.

Before large-scale purification, the presence of recombinant EsM1Pases was verified by applying a mini purification protocol described previously by Groisillier et al. (2010). After checking for the presence of proteins of interest, the pellet was resuspended in 30 ml of buffer A (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 10 mM imidazole) and disrupted using a Constant Cell Disruption System (Constant Systems), at a pressure of 1.6 bar. Anti-proteases (Novagen) were added to the extracts before centrifugation for 30 min at 13 800g and 4 °C. Quantification of proteins in extracts was performed by measuring the absorbance at 280 nm with a Nanodrop 2000 spectrophotometer (Thermo Scientific).

Recombinant protein was purified by Ni2+-affinity chromatography using the system ÄKTÁ Avant (GE Healthcare) equipped with a HisPrep™ FF 16/10 column (GE Healthcare). The column was equilibrated with 100 ml (5 column volumes) of buffer A at a flow rate of 5 ml min−1. Next, 30 ml of bacterial extract was injected at a rate of 1 ml min−1. Proteins were eluted with a linear increasing gradient of buffer B (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 500 mM imidazole) from 0 to 100% within 10 column volumes. Aliquots of collected fractions were separated by 12% SDS-PAGE to determine in which fractions the protein EsM1Pase2 was present. In parallel, M1Pase activity was checked in these fractions. All fractions exhibiting M1Pase activity were pooled and then centrifuged in a Centriprep Centrifugal Filter Device (Millipore) at 4500 g and 4 °C in the presence of 100 mM Tris/HCl to remove the imidazole. This procedure was applied in order to save time between purification and measurement of activity. The concentration of target protein was determined by considering a specific extinction coefficient ε of 18 700 M−1 cm−1 and a molecular weight of 36 kDa (calculated with the program ProtParam available at [http://web.expasy.org/protparam/](http://web.expasy.org/protparam/)). During the entire procedure, protein extracts were placed on ice. The identity of the purified proteins was confirmed by peptide mass fingerprinting analysis according to a procedure published previously.
(Thomas et al., 2012). Dynamic light scattering (DLS) analysis was conducted for protein samples with a minimal concentration of 1 mg ml\(^{-1}\) in a Zetasizer Nano S system (Malvern).

An estimation of the molecular mass of the native recombinant proteins was performed by size-exclusion chromatography. Eluted fractions from the Ni\(^{2+}\)-affinity chromatography were loaded at a flow rate of 1 ml min\(^{-1}\) on a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with buffer containing 20 mM Tris/HCl, pH 7.5, and 150 mM NaCl. Fractions were then eluted with the same buffer at a flow rate of 1 ml min\(^{-1}\).

Determination of M1Pase activity

For enzymatic characterization, six sugar and polyol phosphoesters were considered as potential substrates for E. siliculosus M1Pases were tested: mannitol-1P, mannose-6P, fructose-1-phosphate (fructose-1P), fructose-6P, glucose-1-phosphate (glucose-1P), and glucose-6-phosphate (glucose-6P). All substrates were obtained from Sigma-Aldrich, except for mannitol-1P, which was produced according to the procedure described by Krahulec et al. (2008).

M1Pase activity was determined in a reaction assay (80 µl) containing 100 mM Tris/HCl, pH 7.5, and 5 mM MgCl\(_2\) (final concentration), at 30°C. For measurements in all extracts, about 12.5 µg of proteins were used and incubation was carried out for 10 min. Individual algal extracts were prepared from three distinct samples, and three independent reaction mixtures were considered for each extract. To measure the enzymatic activity and determine the kinetic parameters of recombinant EsM1Pase2, a number of purified enzymes were tested. Measurements were done using 6–78 µg of purified protein and incubation was conducted for 12 min, with sampling every 3 min. The effect of pH on the enzymatic activity of purified EsM1Pase2 was determined from pH 5.8 to 9 in 100 mM Tris/HCl buffer. The influence of NaCl on this enzyme was assessed by testing final concentrations ranging from 0 to 1000 mM in the reaction mixture. Each condition was tested in three replicates. Boiled algal protein extract or boiled purified recombinant enzyme was used as a negative control. The reaction was initiated by adding substrate at a final concentration of 1 mM, except for the determination of enzymatic kinetic parameters for which final concentrations of mannitol-1P ranged from 0.08 to 0.6 mM (obtained by serial dilution of a stock solution at 25 mM), and stopped by adding 20 µl of malachite green reagent (Malachite Green Phosphate Assay Kit; Gentaur) for subsequent spectrophotometric measurements as described above, except that the final reaction volume was 0.5 ml and contained about 16 µg of recombinant protein. Each reaction was started by the addition of one sugar phosphate or one polyol phosphate at 0.5 mM final concentration, conducted for 10 min at 30°C, and stopped by placing the tube in liquid nitrogen. Samples were lyophilized and stored at –80°C. Freeze-dried samples were then resuspended in 100 µl of acetonitrile, and 100 µl of Sylon-BFT [99:1 bis(trimethylsilyl) trifluoroacetamide+trimethylchlorosilane; Supelco] were added to perform derivatization. After incubation for 1 h at 60°C, samples were evaporated under a gentle stream of nitrogen and resuspended in 100 µl of hexane. They were then analysed using an Agilent GC 6890+ equipped with a DB-5MS column (30 m length×0.25 mm internal diameter×0.25 µm film thickness; Agilent Technologies) coupled to a 5975 MS detector (Agilent Technologies) in the EI mode at 70 eV. The carrier gas was helium at 1 ml min\(^{-1}\). The temperatures of injection port and interface were 250 and 280°C, respectively. The oven temperature was set at 60°C for 5 min, rising to 170°C at a rate of 10°C min\(^{-1}\), then to 290°C at a rate of 3°C min\(^{-1}\), and held at 290°C for 5 min. The mass spectrometer ion source and the analyser were set to 250 and 100°C, respectively. The data were collected in both total ion current and single ion monitoring chromatograms. Quantification was performed using the peak area of m/z 319, referring to a calibration curve established with mannitol content ranging from 0 to 37.5 µg, and in the presence of mannitol-1P to make sure that there was no degradation of the substrate during the preparation of the GC samples.

Gene expression analysis

RNA samples used for gene expression experiments have already been used for similar analysis on E. siliculosus MIPDH genes (Rousvoal et al., 2011). They correspond to algae harvested every 6 h during two consecutive days. Synthesis of cDNA was performed starting from 0.8 µg of total RNA with an Improm-II\(^{TM}\) Reverse Transcription System (Promega) according to the manufacturer’s instructions. The quantitative PCR experiments were carried out as described by Le Bail et al. (2008). Specific primers designed for EsM1Pase1 were EsM1Pase1F (5'-ACCTCATCCCCCTGTGTGA-3') and EsM1Pase1R (5'-AGGCAGAAGTCTTCGCCC-3'), specific primers for EsM1Pase2 were EsM1Pase2F (5'-TCGGACTGAGTGTTGGATAC-3') and EsM1Pase2R (5'-GGCATGCGTCTCCTTTTTGT-3'). The expression levels of each gene were normalized by the expression of the gene coding for elongation factor 1α (EsEF1α0378_0021) in the Ectocarpus genome database; http://bioinformaticps.psb.ugent. be/orca/overview/Ectsiels.html), which was assessed using the primers EsEF1αF (5'-GCAAGGGGCCCTCAGCTCTG-3') and EsEF1αR (5'-ACAAGGGCGTCTGGTATGTTTAGC-3'). Normalized expression values were then log\(_2\) transformed and subjected to a repeated measures analysis of variance carried out in Statistica version 7 and using 'time of the day', 'day', and 'time x day' as predictors; a P value of <0.01 was considered sufficient for rejection of the null hypothesis.

Sequence analysis and retrieval

The theoretical isoelectric point (pI) of proteins coded by EsM1Pase genes was calculated using the Compute pI/MW tool at the ExPASy website (http://web.expasy.org/compute_pi/). Predictions for transmembrane helices in proteins were performed at the TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Hydrophobic cluster analysis (HCA) was done at http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA#forms:HCA.

EsM1Pase2 was used for blastp analysis at http://www.uniprot.org/blast/ on the UniProtKB/Swiss-Prot database to retrieve the closest biochemically characterized homologues and on the Protein Data Bank (PDB) to identify most similar proteins of known 3D structure. Potential EsM1Pase2 homologues in genomes of land plants were searched for within the Phytozone database version 9.1 available at http://www.phytozone.net/, which provided access to annotated and sequenced land plant genomes, including those for plants (or closely related species) where M1Pase activity has been measured. Proteins identified were then aligned individually with EsM1Pase2 using MUSCADIN available at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html to determine the percentage identity over the entire sequences.

For 3D homology modelling, a multiple sequence alignment was generated using MAFFT version 7 with the iterative refinement method and the scoring matrix Blossum62 (Katoh et al., 2002), and manually refined, using BioEdit, on the basis of the crystal structure of the haloc acid dehalogenase-like protein ATU0790 from Agrobacterium tumefaciens strain CS8 (PDB code 2FDR). This structure was also used to generate a 3D model of the protein EsM1Pase2.
Results and discussion

Measurement of algal endogenous M1Pase activity

In order to assess M1Pase activity in *E. siliculosus*, cell-free extracts were obtained from algae harvested during the second half of the light period. A value of $9.26 \pm 0.57$ nmol min$^{-1}$ mg protein$^{-1}$ ($0.15 \pm 0.01$ nkat mg protein$^{-1}$) was determined in the presence of mannitol-1P. In addition, a lower activity of $1.57 \pm 1.02$ nmol min$^{-1}$ mg protein$^{-1}$ ($0.03 \pm 0.02$ nkat mg protein$^{-1}$) was calculated in the presence of fructose-1P. No phosphatase activity was detected with the other substrates tested. To complete these observations, M1Pase activity was also measured in algal samples harvested at different times of the diurnal cycle. The activity ranged from $8.92 \pm 2.42$ to $15.67 \pm 5.38$ nmol min$^{-1}$ mg protein$^{-1}$ ($0.15 \pm 0.04$ and $0.26 \pm 0.09$ nkat mg protein$^{-1}$) (Fig. 2A), and variations observed between the light and dark phases of the diurnal cycle were not statistically significant (analysis of variance, $P=0.09$). Furthermore, there

![Fig. 2.](https://academic.oup.com/jxb/article-abstract/65/2/559/486784)
was no correlation between the measured M1Pase activity and the mannitol content determined for the same samples (Fig. 2B).

Phosphatase activity on mannitol-1P has previously been quantified in cell-free extracts from red (Dixoniella and Caloglossa) and brown (Spatoglossum, Dictyota, and Laminaria) algae (Ikawa et al., 1972; Grant and Rees, 1981; Karsten et al., 1997; Eggert et al., 2006), and the results are summarized in Supplementary Table S1 at JXB online. Interestingly, in each case, phosphatase activity was also detected for at least one of the other sugar or polyol phosphates tested. However, values were always lower than for mannitol-1P, except for Dixoniella grisea, which exhibited the highest phosphatase activity in the presence of glucose-1P.

Identification of an E. siliculosus M1Pase

The most striking difference between EsM1Pase1 and EsM1Pase2 (Supplementary Fig. S1A at JXB online) was the presence of an N-terminal extension of 85 aa in EsM1Pase1, as revealed by comparison of HCA plots. This N-terminal module did not exhibit similarities to the module of unknown function identified previously in the N-terminal end of the EsM1PDH1 and EsM1PDH2 proteins (Rousvoal et al., 2011), nor to any sequence in the nr database of GenBank. After removing this module, both EsM1Pases featured 52% identity and 70% similarity (Supplementary Fig. S1B). Interestingly, no signal peptide was found in EsM1Pase2, indicating that this enzyme is likely to be cytosolic, whereas EsM1Pase1 has been predicted to be localized in the chloroplast by the Program HECTAR (Gschloessl et al., 2008).

To functionally characterize these EsM1Pases, the full-length coding regions of the two genes were cloned separately in the expression vector pFO4. Despite several attempts to improve the conditions of induction for EsM1Pase1, and cloning of a truncated form (N-terminal extension deleted), no expression of the corresponding protein was observed. This problem also persisted with other expression vectors and with different E. coli host cells. Therefore, we focused our attention on the characterization of EsM1Pase2.

Recombinant His-tagged EsM1Pase2 was purified by Ni\(^{2+}\)-affinity chromatography (Fig. 3A). Aliquots of the purified proteins were analysed by DLS. The distribution of the protein was monodisperse, suggesting that a homogeneous pure protein was present in the samples tested (data not shown). However, after 12% SDS-PAGE, despite the presence of a main band consistent with the predicted theoretical mass of 36 kDa for the purified recombinant EsM1Pase2, several other bands of different sizes were observed (Fig. 3A, inset). To check their identity, several were excised from the gel and analysed by MS. All peptides produced corresponded to EsM1Pase2, suggesting the presence, in the gel, of recombinant proteins not completely denatured and of several cleaved forms. The apparent molecular mass of the native recombinant EsM1Pase2 was then estimated by gel filtration. Two peaks were observed, indicating that the purified recombinant enzyme in solution was present in two forms (Fig. 3B). Estimation of their molecular mass was consistent with the occurrence of a homotetrameric (~155 kDa) and a homodimeric (~72 kDa) quaternary structure for the protein of interest (Fig. 3B, inset). M1Pase activity was only detected in the fraction corresponding to the homotrimer. This is different from the structure observed for the only other M1Pase purified from algae, i.e. the enzyme from the red alga C. continua, which was shown to be monomeric (Iwamoto et al., 2001).

Specific activity of the recombinant EsM1Pase2 was compared after Ni\(^{2+}\)-affinity chromatography with and without subsequent gel filtration, and no significant difference was observed. This indicated that the Superdex 200 column did not improve the level of purity of EsM1Pase2. In addition, no enzymatic activity was detected in fractions produced by both types of chromatography after storage at 4 °C for 16 h, suggesting that the recombinant EsM1Pase2 was not stable in the purification conditions tested. Therefore, to limit the time of the purification procedure, the kinetic parameters and the influence of several chemicals were studied using EsM1Pase2 purified by a single step of affinity chromatography.

To investigate the substrate specificity of EsM1Pase2, enzymatic reactions were performed in the presence of individual sugar phosphates or mannitol-1P, and the products of the hydrolysis reaction were analysed by GC-MS. Among the six compounds tested, significant production of carbohydrates was observed only in the presence of mannitol-1P (Fig. 4A). The identity of the product as mannitol was confirmed by MS analysis. A low amount of mannitol was also present in the mannitol-1P used for the test, as indicated by the barely detectable quantity determined under the control condition; however, the quantity of polyol was 10-fold higher in the assay containing the active recombinant Ectocarpus protein, and this difference was statistically significant \((t\text{-test, } P<0.01)\). A very low quantity of glucose was detected in presence of glucose-1P, but the difference between the control and test condition was not statistically significant. These observations suggested that EsM1Pase2 is highly specific to mannitol-1P when compared with other substrates tested, i.e. hexose monophosphates, and validated the putative function assigned previously to EsM1Pase2 (Michel et al., 2010a).

Biochemical characterization of EsM1Pase2

To determine the kinetic parameters of the purified recombinant His–EsM1Pase2, specific enzymatic activities were calculated in the presence of concentrations of mannitol-1P ranging from 0.08 to 0.6 mM. At pH 7.5 and in the presence of 5 mM MgCl\(_2\), this protein exhibited Michaelis–Menten kinetics with respect to mannitol-1P concentration (Fig. 4B). The Michaelis–Menten constant \((K_m)\) value for this substrate was 0.67 mM, and the maximum enzyme velocity \((V_m)\) value was 25.25 nmol min\(^{-1}\) mg protein\(^{-1}\) (0.42 nkat mg protein\(^{-1}\)). This latter value was in the same range as the activity determined for the hydrolysis of phosphate groups from mannitol-1P in

---

**Fig. 2B**

**Fig. 3A**

**Fig. 3B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**

**Fig. 4A**

**Fig. 4B**

**Fig. S1B**
algal extracts (0.15±0.01 nkat mg protein⁻¹). The turnover \(k_{\text{cat}}\) value was 0.015 s⁻¹ and \(k_{\text{cat}}/K_m\) was 22.39 M⁻¹ s⁻¹. The \(K_m\) determined for the EsM1Pase2 was intermediate between values obtained for the native red alga *C. continua* M1Pase (0.41 mM; Iwamoto *et al.*, 2001) and for the partially purified enzyme of the brown alga *Dictyota dichotoma* (0.83 mM; Ikawa *et al.*, 1972).

The pH dependence was assessed in Tris/HCl buffer. The optimal pH was determined to be 7.0, and 80% of the enzyme activity remained for pH values ranging from...
5.8 to 7.5 (Supplementary Fig. S2A at JXB online). This was in agreement with the pH optimum determined for M1Pase activities in different brown and red algae, which is between 7.0 and 7.5 (Eggert et al., 2006). The influence of various compounds was also tested on the activity of recombinant EsM1Pase2. A final concentration of 1 mM NaF, an inhibitor of phosphatase activity, or 0.1 mM p-hydroxymercuribenzoate, an inhibitor of SH enzymes, completely abolished the EsM1Pase2 activity. This latter result suggested that some or all of the four cysteine residues present in the protein are important for the hydrolysis reaction catalysed by this enzyme. Similar results were observed for the purified enzyme from the red alga C. continua (Iwamoto et al., 2001) and for brown algal M1Pase activities (Ikawa et al., 1972). Additional assays conducted to assess the influence of NaCl concentration on the activity of the recombinant Ectocarpus protein showed a dose-dependent effect, at least for the first four concentrations tested (Supplementary Fig. S2B). Indeed, a linear decrease down to 33% of the initial phosphatase activity was observed in the presence of NaCl up to 400 mM. This decrease was less marked at higher concentrations, and only 15% of the initial activity remained in the presence of 1 M NaCl in the enzyme reaction mixture. Similar observations were obtained for the M1Pase enzyme purified from C. continua (Iwamoto et al., 2001).
**Ectocarpus siliculosus**

**mannitol-1-phosphatase**

Gene expression analysis

A transcriptomic study using microarrays to assess the short-term response under hyposaline, hypersaline, and oxidative conditions in *E. siliculosus* ([Dittami et al., 2009](#)) demonstrated that, under these stresses, only *EsM1Pase1* exhibited marked and statistically supported (*P*<0.01) variations. This gene was highly down-regulated under hyposaline and oxidative stress (108- and 9-fold change, respectively). To complete this analysis, changes in gene expression of both *EsM1Pase* genes were monitored during two consecutive diurnal cycles. Statistical analysis of the results showed that significant changes in the quantity of *EsM1Pase1* transcripts occurred during the diurnal cycle, and these variations followed a similar trend during the two consecutive days (Supplementary Fig. S3 at *JXB* online). For *EsM1Pase2*, levels of expression were significantly altered during the diurnal cycle, but also between the two days. This was in part due to an increase in the quantity of transcripts during the second day of experiments. Further experiments, e.g. alteration of expression of *EsM1Pase2*, will be necessary to establish if there are cause–effect relationships between changes in expression of this gene, *E. siliculosus* M1Pase activity, and changes in the mannitol content.

**EsM1Pases as founding members of a new family of multiple helical C1 cap proteins within the HAD superfamily**

A search for characterized homologues of *EsM1Pase2* retrieved bacterial enzymes identified as phosphatases acting on different organic acids and carbohydrates but not mannitol. However, the sequence identities were very low (between 13 and 19%), indicating that *EsM1Pase2* is the first characterized member of a new family of proteins. The protein of known structure most similar to *EsM1Pase2* was the

![Fig. 5. Structure-based sequence alignment of the crystalized HAD-like protein ATU0790 from *A. tumefaciens* strain C58 (PDB code 2FDR) with orthologues of M1Pases in *E. siliculosus* and *Micromonas* species. The secondary structure of ATU0790 is shown above the alignment. Conserved amino acids in white on a red background are identical and those in red on white background are similar. Alpha-helices are represented as helices symbols and β-turns are indicated as TT. Motifs I to IV, defined according to Burroughs et al. (2006), are indicated by a blue bar below the alignment, and conserved residues involved in the catalytic machinery and the Mg²⁺ co-factor binding site are indicated by green triangles.](https://academic.oup.com/jxb/article-abstract/65/2/559/486784)
HAD-like protein ATU0790 from \textit{A. tumefaciens} strain C58 (PDB code 2FDR, 29\% identity over 227 residues). This protein was included in a multiple sequence alignment considering both \textit{E. siliculosus} sequences and their homologues identified in \textit{Micromonas} species (Fig. 5). This comparison confirmed the conservation of all the residues that form the catalytic machinery and the Mg\textsuperscript{2+} co-factor binding site of ATU0790 in EsM1Pase2 and its orthologues. Together, they are referred to as motifs I to IV in the HAD superfamily by Burroughs et al. (2006). The level of sequence identity was also sufficient to produce a reliable 3D model for the most conserved parts of EsM1Pase2, which essentially corresponded to the main secondary elements of ATU0790 (Fig. 6A). Based on this model, EsM1Pase2 adopted a Rossmann-like fold for its catalytic domain with a six-stranded parallel \(\beta\)-sheet, in the order \(\beta_3-\beta_2-\beta_1-\beta_4-\beta_5-\beta_6\), flanked by six \(\alpha\)-helices (\(\alpha_5-\alpha_{11}\)) (Fig. 6B, shown in cyan). After the ‘squiggle’ domain within motif I, EsM1Pase2 displayed a C1 cap domain that replaces the FLAP motif (a \(\beta\)-turn) found in archetypal HAD proteins such as the deoxy-o-mannose-ocululosonate-8-phosphate phosphatase 8-phosphate phosphatase from \textit{Haemophilus influenzae} (8KDO; Burroughs et al., 2006). The four \(\alpha\)-helices forming the C1 cap of Atu0790 (Fig. 6A, shown in brown) were conserved in EsM1Pase2. However, this latter protein and its orthologues in microalgae featured a 42 aa insertion between the helices \(\alpha_3\) and \(\alpha_4\) of ATU0790 (Fig. 5). In our prediction, this large insertion was modelled as an additional domain comprising at least one \(\alpha\)-helix (Fig. 6B, shown in magenta). Although the exact 3D structure of this insertion remained uncertain, our model allowed us to at least predict the localization of this additional domain in the vicinity of the active cleft. This large insertion contained a conserved signature VxxxRxxxxGL (Fig. 5), unique to algal sequences, and thus could be related to new substrate specificity, including towards mannitol-1P. Our 3D model also confirmed that all the conserved residues constituting the motifs I to IV clustered together spatially, similarly to what was observed in the structure of ATU0790 (data not shown). Considering that EsM1Pase2 and its orthologues do not feature a FLAP motif but instead a C1 cap domain predicted to contain at least four \(\alpha\)-helices, we propose that these sequences constitute a new family within the C1 cap assemblage of the HAD superfamily of proteins, with new substrate specificity.

According to these observations, EsM1Pase2 differs from the M1Pase characterized from the chicken parasite \textit{Eimeria tenella}, which belongs to the phosphohistidine phosphotransferase family (Liberator et al., 1998). Although brown algae and this apicomplexa share some similarities in the organization of their mannitol cycle (Schmatz, 1997), their M1Pases emerged from convergent evolution. When using both sequences to search for homologues in genomes representative of land plants where M1Pase activity has been determined, no hit was found with the parasite M1Pase, while EsM1Pase2 provided hits corresponding to proteins predicted as HAD-like hydrolases but not biochemically characterized (Supplementary Table S2 at JXB online). The percentage of identity between EsM1Pase2 and these latter sequences was low, ranging between 13 and 17\%. Furthermore, members of the HAD superfamily have been characterized as dx-glycerol-3-phosphatases and phosphosugar phosphatase in \textit{Arabidopsis thaliana} (Caparrós-Martin et al., 2013), and featured 12–15\% identity with EsM1Pase2. Despite the low level of identity between the plant sequences and \textit{E. siliculosus} M1Pase, the characterization of EsM1Pase2 suggests that proteins of the HAD superfamily may be interesting candidates for M1Pases in land plants.

**Supplementary data**

Supplementary data are available at JXB online.

\begin{itemize}
  \item **Supplementary Fig. S1.** \textit{E. siliculosus} mannitol-1-phosphatase genes and proteins.
  \item **Supplementary Fig. S2.** Influence of pH (A) and NaCl concentration (B) on recombinant EsM1Pase2 activity.
  \item **Supplementary Fig. S3.** Changes in expression of the genes \textit{EsM1Pase1} and \textit{EsM1Pase2} during two consecutive diurnal cycles.
  \item **Supplementary Table S1.** Summary of M1Pase activity determined in red and brown algae.
\end{itemize}
Supplementary Table S2. Results of blastp analysis using EsM1Pase2 as query in Phytozome v9.1 (http://www.phytozome.net/) for plants representative of those for which M1Pase activity has been measured.

Acknowledgements

The authors thank Laurence Dartevelle for cultivation of *E. siliculosus*, Dr Fanny Gaillard for MS analyses within the METABOMER facilities, Sylvie Rousvoal and Dr Simon M. Dittami for providing *E. siliculosus* diurnal samples, and the latter for critical reading of the manuscript. ZS was hosted at the Station Biologique de Roscoff within the framework of the Joint Doctoral Promotion Programme CNRS-CAS 2010. PB received a PhD grant by the Émergence-UPMC 2011 research programme. This work also benefited from the support of the project IDEALG (ANR-10-BTBR-04) ‘Investissements d’avenir, Biotechnologies-Bioresources’.

References


Ridder IS, Dijkstra BW. 1999. Identification of the Mg$^{2+}$-binding site in the P-type ATPase and phosphatase members of the HAD (haloacid dehalogenase) superfamily by structural similarity to the response regulator protein CheY. *Biochemical Journal* 339, 223–226.


