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Research article

Biochemical characterization of the triticale TsPAP1, a new type of plant prolyl aminopeptidase, and its impact on proline content and flowering time in transgenic Arabidopsis plants

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ABSTRACT

Proline aminopeptidase (PAP, EC 3.4.11.5) is the only enzyme that effectively releases proline from the N-termini of peptides. The amino acid sequence of the PAP from *Triticosecale*, TsPAP1, comprises conserved regions, characteristic of the monomeric forms of PAP found in bacteria but not yet identified in plants. Therefore, we aimed to obtain and biochemically characterize the TsPAP1 protein. The recombinant TsPAP1 protein was received through heterologous expression of the *TsPAP1* coding sequence in a bacterial expression system and purified with affinity chromatography. Gel filtration chromatography and SDS electrophoresis revealed that TsPAP1 is a monomer with a molecular mass of 37.5 kDa. TsPAP1 prefers substrates with proline at the N-terminus but is also capable of hydrolyzing β-naphthylamides of hydroxyproline and alanine. Among the peptides tested, the most preferred were di- and tripeptides, especially those with glycine in the Y position. The use of diagnostic inhibitors indicated that TsPAP1 is a serine peptidase; however, further characterization revealed that the SH residues are also important for maintaining its activity. To examine the role of TsPAP1 under physiological conditions, we developed transgenic Arabidopsis plants overexpressing *TsPAP1*. Compared with wild-type plants, the transgenic lines accumulated more proline, flowered an average of 3.5 days earlier, and developed more siliques than did untransformed controls. Our paper is the first to describe the biochemical properties of a novel monomeric plant PAP and contributes to the functional characterization of PAP proteins in plants.

1. Introduction

Plants are equipped with a large proteolytic machinery, which is not only necessary for the general turnover of nonfunctional proteins but also participates in the mobilization of storage proteins, mobilization of carbon and nitrogen reserves from senescing organs, and, by limited proteolysis, controls the activity of enzymes, regulatory proteins and peptides (van der Hoorn and Jones, 2004; Schaller, 2004). Thus, proteases are involved in all stages of plant development ranging from germination, differentiation and morphogenesis to senescence and programmed cell death. They also participate in defense responses induced by a variety of abiotic and biotic stress factors (van der Hoorn and Jones, 2004).

Proline is an amino acid with a unique heterocyclic structure. The peptide bonds adjacent to proline are resistant to cleavage by most peptidases; thus, the presence of proline may lead to restriction of protein and peptide degradation. Among the enzymes releasing N-terminal amino acids, only proline aminopeptidase (PAP, EC 3.4.11.5) has the ability to effectively liberate proline or hydroxyproline from short peptides (Cunningham and O'Connor, 1997). This enzyme has a very narrow substrate specificity as other N-terminal amino acids are not liberated by this enzyme or are removed with a 10–20-fold lower yield (Szawłowska et al., 2011). PAPs function as monomers with a low molecular mass (30–35 kDa; the S33.001 subfamily in MEROPS database) or as multimeric enzymes (100–370 kDa; the S33.008 subfamily) consisting of two to six subunits with molecular weights of 50 ± 5 kDa each (Albertson and Koomey, 1993; Kitazono et al., 1994b; Matsushita-Morita et al., 2010). Multimeric forms of PAPs have

Abbreviations: DFP, diisopropyl fluorophosphate; E-64, (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane); β-NA, β-naphthylamide; PAP, prolyl aminopeptidase; PMSF, phenylmethylsulfonylfluoride; TsPAP1, *Triticosecale* prolyl aminopeptidase 1.

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been detected so far in bacteria, fungi and plants while the monomeric forms have been detected only in bacteria.

Plant PAP proteins, which have been previously purified and biochemically characterized from apricot, triticale, rye, peanut, and cabbage, are homotetramers with a molecular weight of approximately 200 kDa (Marinova et al., 2008; Ninomiya et al., 1982; Ovando et al., 2004; Szawłowska et al., 2006, 2011). There is no information related to the existence of monomeric PAP proteins in plant tissues, although the presence of other aminopeptidases, such as aromatic aminopeptidases, which function as monomers, has been reported (Pyrzyna et al., 2011). Recently; however, some plant nucleotide sequences have been identified, which encode amino acid sequences with a relatively high percent identity to the monomeric PAPs from bacteria and contain regions specific for these monomeric forms of the enzyme (Szawłowska et al., 2012; Sun et al., 2013; Wang et al., 2015). The amino acid sequence of *Triticosecale prolyl aminopeptidase 1* (*TsPAP1*), which was previously suggested to encode a monomeric form of PAP, shares approximately 55% identity with small monomeric PAP proteins from *Serratia marcescens*, *Neisseria gonorrhoeae*, and *Xanthomonas axonopodis*, and only 15% identity with the amino acid sequences of multimeric PAPs (Szawłowska et al., 2012). The specific GXSXG motif, which contains a nucleophylic serine, consists of the GGSWG amino acid sequence ($G^{180}G^{181}S^{182}W^{183}G^{184}$ w TsPAP1) in monomeric PAPs while in the multimeric forms is composed of GQSFG residues (Li et al., 2010; Szawłowska et al., 2012). Bacterial and fungal PAPs are considered serine peptidases, which have a catalytic triad composed of Ser, His and Asp (Asp^{338}-Ser^{182}-His^{356} in TsPAP1). The positions of Ser and Asp in the catalytic triad are conserved between monomeric and multimeric PAPs; however, His is located in a different position in the monomeric compared with multimeric proteins (Szawłowska et al., 2012). Thus, it is interesting to investigate whether the sequence isolated previously from triticale encodes a catalytically active PAP protein, and if this protein, according to the analysis of the primary structure, will belong to a group of monomeric PAPs. Understanding the biochemical properties of the TsPAP1 protein and clarifying its biological function should also be a priority.

In a few recent studies, an increase of the PAP transcript and the total iminopeptidase activity in response to various abiotic stress factors, such as drought, salinity, and heavy metals, was observed (Szawłowska et al., 2011, 2012; Sun et al., 2013; Wang et al., 2015). These studies suggest that plant PAP might be involved in the response of plants to stress factors most likely by contributing to an increased accumulation of proline. Proline, apart from being one of the most widespread compatible osmolytes, may also function as a signaling molecule and is thought to be essential for embryo development and also influences flowering time and programmed cell death (Kishor et al., 2005; Szabados and Savouré, 2010; Trovato et al., 2008). In plants, proline is synthesized mainly from glutamate, which is reduced to glutamate-semialdehyde by pyrroline-5-carboxylate synthetase (P5CS), then spontaneously converted to pyrroline-5-carboxylate (P5C), which is reduced to proline by P5C reductase (Kishor et al., 2005). Overexpression of the genes involved in proline biosynthesis enhances the tolerance of transgenic plants to various stress factors but also leads to early flowering, influences inflorescence architecture and root biomass (Kishor et al., 1995, 2005; Mattioli et al., 2008).

In the present manuscript, the biochemical characterization of the first plant PAP, TsPAP1, of which the amino acid sequence displays the characteristics of monomeric proteins functioning in bacteria, is presented. The impact of *TsPAP1* overexpression on flowering time and the number of siliques due to the enhanced accumulation of proline in transgenic Arabidopsis plants, is also described.

2. Materials and methods

2.1. Bioinformatics analysis of the TsPAP1 protein sequence

The alignment of the amino acid sequences of proline aminopeptidases of the S33.001 subfamily was performed using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html). The ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) servers were used to predict the chloroplast transit peptide.

2.2. Expression and purification of the recombinant TsPAP1 protein in E. coli

The coding region of the *Triticosecale* prolyl aminopeptidase 1 (*TsPAP1*; GenBank ID JN808306), without the signal peptide coding sequence, was amplified using the forward primer (5′-TATTAGCTAGCATGGATCCCGCCGCGCGGAAGGATC-3′), which introduced a *Nhe*I restriction site at the 5′ end of the amplified *TsPAP1*, and the reverse primer (5′-TATTACTCGAGTCATTTTTTCAACATGCTTTTAAGCTTTTCGGTAGC-3′), which generated an *Xho*I restriction site at the 3′ end. PCR was performed with a Long PCR Enzyme Mix (Thermo Scientific) under the following conditions: 2 min at 94 °C, 40 cycles of 20 s at 94 °C, 15 s at 58 °C, 1 min at 68 °C, and a final elongation for 5 min at 68 °C. The correct *TsPAP1* sequence was then subcloned into the pET-28a expression vector (Novagen), previously digested with *Nhe*I and *Xho*I endonucleases. The resulting recombinant vector was then amplified in the *E. coli* strain NovaBlue (Novagen), then transformed into the *E. coli* expression strain BL21(DE3)/LysE (Novagen). The recombinant *E. coli* cells were grown at 37 °C in LB medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml). When the culture OD at 600 nm reached 0.8, the expression was induced by addition of 1 mM IPTG. After 3 h, the cultures were centrifuged at 4 °C, 4000 × g for 15 min. The bacterial cells were lysed by 4 cycles of freezing (liquid nitrogen) and thawing (37 °C) in lysis buffer: 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM imidazole, and 1% Triton X-100. The lysates were centrifuged at 4 °C, 16 000 × g for 30 min, and the resulting supernatants were used to purify the recombinant TsPAP1 protein by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) resin (ProBond Purification System, Invitrogen) according to the manufacturer's instructions. The recombinant TsPAP1 protein was eluted in buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 250 mM imidazole. The eluted fractions were desalted by ultrafiltration using UltraFree centrifuge filters (Millipore) and were used for molecular and biochemical analyses.

2.3. Estimation of molecular weight

The molecular weight of the native TsPAP1 was determined by gel filtration on a Superdex 200 column (1.6 × 60 cm; GE Healthcare), which was equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0,5 mM DTT. The column was calibrated with β-amylase (200 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa) and carbonic anhydrase (29 kDa) as molecular weight standards. The molecular weight of TsPAP1 under denaturing conditions was estimated by SDS-PAGE on 10% polyacrylamide gels (Laemmli, 1970). Proteins in the gel were stained with the colloidal Coomassie stain Roti Blue (Carl Roth).

2.4. Western blot analysis

Proteins were separated by SDS-PAGE on 10% gels (Laemmli,