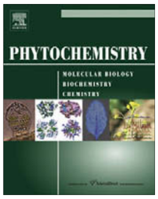
Phytochemistry 71 (2010) 515–523

Contents lists available at ScienceDirect

Phytochemistry

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



Structure–function characterization of the recombinant aspartic proteinase A1 from *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 24 November 2009
Available online 14 January 2010

Keywords:
Arabidopsis thaliana
Cruciferae
Plant aspartic proteinase
Substrate specificity
Thermal stability
pH stability
Plant specific insert
Proteases

ABSTRACT

Aspartic proteinases (APs) are involved in several physiological processes in plants, including protein processing, senescence, and stress response and share many structural and functional features with mammalian and microbial APs. The heterodimeric aspartic proteinase A1 from *Arabidopsis thaliana* (AtAP A1) was the first acid protease identified in this model plant, however, little information exists regarding its structure function characteristics. Circular dichroism analysis indicated that recombinant AtAP A1 contained an higher α-helical content than most APs which was attributed to the presence of a sequence known as the plant specific insert in the mature enzyme. rAtAP A1 was stable over a broad pH range (pH 3–8) with the highest stability at pH 5–6, where 70–80% of the activity was retained after 1 month at 37 °C. Using calorimetry, a melting point of 79.6 °C was observed at pH 5.3. Cleavage profiles of insulin β-chain indicated that the enzyme exhibited a higher specificity as compared to other plant APs, with a high preference for the Leu_{15}–Tyr_{16} peptide bond. Molecular modeling of AtAP A1 indicated that exposed histidine residues and their interaction with nearby charged groups may explain the pH stability of rAtAP A1.

1. Introduction

Aspartic proteinases (APs) (EC 3.4.23) are a family of proteolytic enzymes that are widely distributed in nature and perform a number of vital biological processes. They are characterized by two aspartic acid residues at the catalytic center, have a low optimal pH for activity, and are inhibited by pepstatin (Davies, 1990). They share some common physicochemical characteristics in terms of their primary, secondary and tertiary structures, and catalytic mechanism; however, some differences exist in their catalytic properties, cellular localization and biological functions (Chen et al., 2002; Kervinen et al., 1999).

Plant genomes contain multiple genes encoding for APs and the expression of this proteases class has been detected in various tissues (Faro and Gal, 2005). The great diversity of APs expressed via both single and multiple co-expression in the same plant or tissue (Brodelius et al., 2005; Tamura et al., 2007) suggests different roles exist for each AP and may be related to specificity, catalytic efficiency, and localization (Athauda et al., 2004; Chen et al., 2002; Mutlu and Gal, 1999; Simoes and Faro, 2004; Simoes et al., 2007). Various plant aspartic proteinases have been used in food processing, particularly to alter the sensory properties (e.g., cacao fermentation) and/or used as a processing aid (milk coagulant for cheese production). Due to their expanded use as milk-clotting agents, the identification and isolation of plant APs from various sources has increased (Llorente et al., 2004). However, only cardosins from cardoon, a vegetal coagulant used traditionally in the Iberian Peninsula for cheesemaking, and phytepsin from barley have been extensively characterized (Frazao et al., 1999; Kervinen et al., 1999). It is believed that plant APs participate in processing and degradation of storage-protein necessary for seed germination (Glathe et al., 1998; Mutlu et al., 1998,1999; Pereira et al., 2008) and have also been implicated in defense mechanisms against pathogens in tobacco, tomato and potato leaves (Guevara et al., 2002,2005; Rodrigo et al., 1991). Furthermore, their presence in flowers suggests that they may be involved in sexual reproduction, senescence and cell death (Mutlu and Gal, 1999; Ramalho-Santos et al., 1998; Duarte et al., 2006). However, the function for the majority of plant APs is still speculative in contrast to those from mammalian animals and viruses (Simoes and Faro, 2004).

Plant APs are similar in structure to non-plant APs, however, they contain a unique domain known as the plant specific insert (PSI) which consists of approximately 100 residues positioned internally in the enzyme sequence. The physiological, structural and functional relevance of this domain is unknown (Guevara et al., 2002). It is thought that the PSI is not critical for enzymatic

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doi:10.1016/j.phytochem.2009.12.005

activity (Asakura et al., 2000), however, roles such as protein targeting to the vacuole, proper folding, vesicle leakage, and structure–function have been identified (Tormakangas et al., 2001; Egas et al., 2000; Payie et al., 2003; Simoes and Faro, 2004; Terauchi et al., 2006). The PSI was found to undergo proteolytic cleavage resulting in its partial or total removal during activation of the enzyme to produce a single-chain or a two-chain mature form or a complex mixture of products (Mutlu and Gal, 1999; Simoes and Faro, 2004). Previously, we hypothesized that the processing of AtAP A1 into the mature form proceeded via two steps: the cleavage of the prosegment followed by a cleavage in the PSI (Mazorra-Manzano and Yada, 2008).

AtAP A1, the first AP identified from *Arabidopsis thaliana*, has been found in various tissues, including flowers, stems, leaves, roots and predominantly in seeds (Chen et al., 2002; Mutlu et al., 1998), but specifically in the protein processing vacuoles (Mutlu et al., 1998; Otegui et al., 2006).

Recently, we reported the recombinant expression and purification of functional AtAP A1 (Mazorra-Manzano and Yada, 2008). The heterodimeric enzyme was glycosylated, retained most of the PSI in its structure and exhibited most plant AP characteristics with respect to pH activity and inhibition with pepstatin. The present study describes the structure–function characterization of rAtAP as a function of pH and temperature as well as the cleavage specificity against insulin β-chain, stability and structural properties.

2. Results and discussion

2.1. Cleavage specificity of rAtAP A1 against oxidized insulin β-chain

Oxidized insulin β-chain has been used as a substrate to determine the specificity of various aspartic proteinases due to its high content of hydrophobic amino acids and the preference of APs for peptide bonds formed by hydrophobic amino acids (Athauda et al., 2004; Bleukx and Delcour, 2000; Bleukx et al., 1998; Guevara et al., 2004; Kervinen et al., 1993; Park et al., 2000; Payie et al., 2003; Simoes et al., 2007; Verissimo et al., 1995). Fig. 1 indicates the cleavage sites for rAtAp A1 in comparison to other plant APs. Analysis of the peptide fragments generated from the oxidized insulin β-chain by action of rAtAP A1 indicated preferential cleavage at the Leu^{15}–Tyr^{16} bond, followed by hydrolysis at the positions Phe^{24}–Phe^{25} and Phe^{25}–Tyr^{26} during prolonged incubation time (24 h). This pattern of specificity was similar to that reported for sunflower AP (Park et al., 2000) and phytepsin (Kervinen et al., 1993), with the exception that phytepsin also cleaved Ala^{14}–Leu^{15} and Leu^{11}–Val^{12} (Kervinen et al., 1993). Cardosin A and potato StAP1 also displayed a similar cleavage profile with the exception that Glu^{13}–Ala^{14} and Leu^{17}–Val^{18} were hydrolyzed in place of Phe^{24}–Phe^{25} (Guevara et al., 2004; Verissimo et al., 1995). In contrast, potato StAP3 exhibited greater specificity where only Leu^{15}–Tyr^{16} and Phe^{24}–Phe^{25} were hydrolyzed but not Phe^{25}–Tyr^{26} (Guevara et al., 2004). The "atypical" AP CDR1 from Arabidopsis (Simoes et al., 2007) and the monomeric AP (67 kDa) from wheat gluten (G1AP 2) (Bleukx and Delcour, 2000), which retains the PSI in its mature form, were found to be the most specific cleaving only Leu^{15}–Tyr^{16}. Nephentesin I, cardosin B and wheat G1AP have the broadest specificity (Athauda et al., 2004; Bleukx and Delcour, 2000; Bleukx et al., 1998). It has been hypothesized that the PSI has a functional role in defining substrate specificity since the insertion of the PSI in pepsin resulted in a modified cleavage profile with a greater specificity compared to wild-type pepsin (Payie et al., 2003).

2.2. Effect of pH and temperature on the stability of rAtAP A1

Factors such as pH and temperature affect the structural stability of proteins which impacts on enzyme function, thereby, helping to define possible industrial uses/processes. In general, the pH optima for plant APs are found in the acidic pH range, e.g., the maximum for rAtAP A1 was observed at pH 4.0 (Mazorra-Manzano and Yada, 2008). In the present study rAtAP A1 retained 50% and 70% activity after 7 days of incubation at pH 3.5 and 4.0, respectively. However, higher stabilities were observed at pH 5.0 and 6.0 where 70% and 80% of the original activity was retained, respectively, after 30 days of incubation at 37 °C (Fig. 2). The above results were consistent with the fact that plant APs are relatively stable at pH levels slightly higher than the acidic catalytic pH optima where autolysis is more pronounced (Lee et al., 1998).

Acidic optimal pH and stability under acidic conditions may result from its subcellular localization in the plant since normal

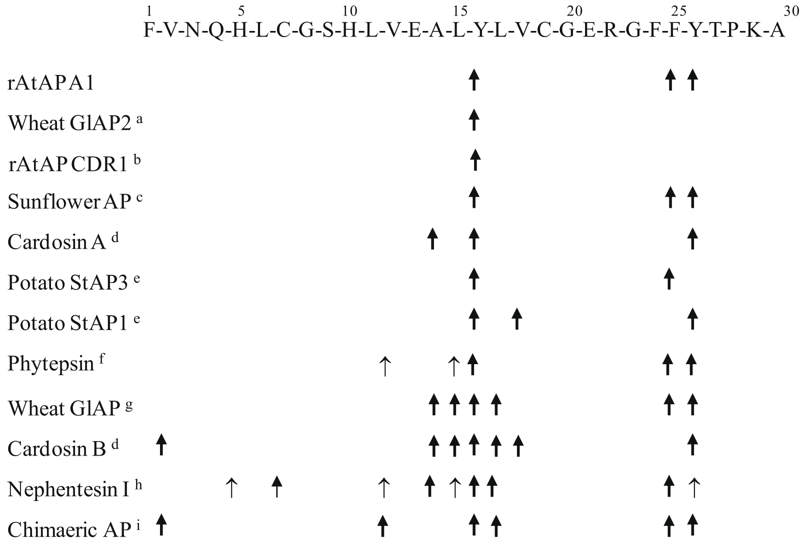

Fig. 1. Sites of oxidized insulin β-chain cleavage by rAtAP A1 and other plant APs. Arrows indicate cleavage sites, with bold arrows indicating the sites of highest preference for each enzyme. Cleavage preference for AtAP A1 was determined by HPLC with amino acid analysis. [a]As reported by Bleukx and Delcour (2000). [b]As reported by Park et al. (2000). [c]As reported by Simoes et al. (2007). [d]As reported by Verissimo et al. (1995). [e]As reported by Guevara et al. (2004). [f]As reported by Kervinen et al. (1993). [g]As reported by Bleukx et al. (1998). [h]As reported by Athauda et al. (2004). [i]As reported by Payie et al. (2003).