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Purification, characterization and identification of a senescence related serine protease in dark-induced senescent wheat leaves

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ABSTRACT

Senescence-related proteases play important roles in leaf senescence by regulating protein degradation and nutrient recycling. A 98.9 kDa senescence-related protease EP3 in wheat leaves was purified by ammonium sulfate precipitation, Q-Sepharose fast flow anion exchange chromatography and gel slicing after gel electrophoresis. Due to its relatively high thermal stability, its protease activity did not decrease after incubation at 40 °C for 1-h. EP3 protease was suggested to be a metal-dependent serine protease, because its activity was inhibited by serine protease inhibitors PMSF and AEBSF and metal related protease inhibitor EGTA. It was identified as a subtilisin-like serine protease of the S8A family based on data from both mass spectrometry and the cloned cDNA sequence. Therefore, these data suggest that a serine protease of the S8A subfamily with specific biochemical properties is involved in senescence-associated protein degradation.

1. Introduction

Leaf senescence is a genetically controlled process involving decline of photosynthetic activity, disintegration of chloroplasts, breakdown of biomolecules, and loss of chlorophyll (Nooden, 1988; Buchanan-Wollaston, 1997; Rui and Xu, 2003, 2004; Krupinska and Humbeck, 2004; Zhang et al., 2005, 2007). Breakdown of protein is one of the most significant processes by providing a large pool of cellular nitrogen for recycling during leaf senescence, because remobilization and translocation of amino acids are very important for reproductive organs including seeds and fruits (Makino and Osmond, 1991). Induction of protease activities has been considered a key characteristic for senescence in higher plants (Chen et al., 2004). Nevertheless, molecular mechanisms explaining the induction of senescence-inducible proteases are still largely unclear. In addition, it is usually not easy to purify candidate senescence-associated proteases because of relatively high concentrations of proteases already present in vacuoles of developing leaves, and further studies on characteristics of the purified senescence-related proteases are necessary (Buchanan-Wollaston, 1997; Beers et al., 2000).

In previous studies, the use of some techniques, such as subtractive hybridization and differential screening, allowed identification of genes with altered expression levels during senescence in several plant species (Buchanan-Wollaston and Ainsworth, 1997; Ueda et al., 2000; Gepstein et al., 2003; Guo et al., 2004; Beyene et al., 2006). Interestingly, many identified genes were predicted to encode different types of proteases. Among the identified proteases associated with leaf senescence, most belong to cysteine proteases (Ueda et al., 2000; Chen et al., 2002, 2006, 2010; Xu et al., 2003; Beyene et al., 2006; Martinez et al., 2007; Prins et al., 2008; Parrott et al., 2010). For example, *SPG31* gene, encoding a sweet potato cysteine protease, was specifically induced in senescing leaves but not in other organs (Chen et al., 2002). The cysteine protease SPCP3 was enhanced significantly in senescent leaves and in dark- and ethephon-induced senescent leaves of sweet potato (Chen et al., 2006). *NtCP1* gene, encoding a cysteine protease, was expressed only in senescent tobacco leaves and not induced in mature green leaves upon exposure to drought or heat (Beyene et al., 2006). However, even for cysteine proteases, it is still necessary to identify and characterize more proteases with specific properties in order to elucidate the molecular mechanisms of protein degradation in leaf senescence and to raise effective methods to delay leaf senescence and to improve quality and output of the crops.

Abbreviations: EP, endopeptidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; AEBSF, 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis-(2-amino ether) tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; TPCK, N-*p*-toluenesulfonyl benzene propionyl chloride methyl ketone; MS, mass spectrometry; PVP, polyvinyl pyrrolidone.

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In a previous study, to identify specific proteases involved in leaf senescence, a gel activity assay was employed to detect protease activity, and six senescence-related endopeptidase (EP) isoenzymes (EP 1–6) was found in dark-induced senescent wheat leaves (Rui and Xu, 2003). Among these six EPs, EP3 was identified as a stable protease because its activity was detected at a wider range of pH and temperature conditions compared with those of the other five (Rui and Xu, 2003). Although the leaf senescence induced by dark treatment is somewhat different from natural leaf senescence (Oh et al., 1996; Sperotto et al., 2009), this stable protease EP3 exists in both the dark-induced and naturally senescing wheat leaves, and its activity was remarkably increased during senescence of wheat leaves, implying that the EP3 protease is important for both natural and dark-induced senescence (Rui and Xu, 2002, 2003). Moreover, this EP3 protease existed in both excised and intact senescent leaves (Rui and Xu, 2002, 2003). However, its characterization and identification was not performed in wheat. Therefore, in the current study, the purification, identification and characterization of this EP3 protease was carried out, where it was established that the encoding gene is a member of the subtilisin-like S8A serine protease family in wheat.

2. Results

2.1. Purification of the EP3 enzyme

In a previous study, several leaf senescence-associated protease isoenzymes were reported, including protease EP3 with a quite stable property to temperature, in senescing wheat leaves (Rui and Xu, 2003). This EP3 enzyme was further purified from the 48-h dark treatment-induced wheat leaves according to the purification procedure described in Section 5. Crude enzyme extracts were first incubated at 33 °C for 1-h, at 50 °C for 30-min, or at 50 °C for 1-h. Incubation of the crude enzyme at 33 °C for 1-h did not significantly alter the content either of Rubisco or the activity of EP3 enzyme; however, incubation of the crude enzyme at 50 °C for 30-min or for 1-h noticeably decreased the Rubisco content (data not shown). EP3 protease activity did not decrease after incubation at 50 °C for 30-min (data not shown). Thus, crude enzyme extracts were incubated at 50 °C for 30-min to remove other proteins including Rubisco, and then the targeted protease was concentrated by ammonium sulfate precipitation. Most of the EP3 protease could be precipitated by 50–70% $(NH_4)_2SO_4$ treatment, and the Rubisco was nearly undetectable in samples treated with 50–70% $(NH_4)_2SO_4$ (Fig. 1). In contrast, only very limited EP3 protease activity and nearly undetectable EP3 protease activity were observed in samples treated with 30–50% and 0–30% $(NH_4)_2SO_4$, respectively (Fig. 1).

$(NH_4)_2SO_4$ was removed by dialysis, and EP3 was purified by a Q-Sepharose fast flow anion exchange chromatography (Fig. 2a). A total of 11 peaks were eluted by the Q-Sepharose fast flow anion exchange chromatography (Fig. 2a). Assay of the EP3 activity indicated that most of the EP3 activity existed in the peak 8 (Fig. 2a and b).

Again, the fraction in peak 8 from Q-Sepharose column was separated simultaneously by natural gradient polyacrylamide gel electrophoresis (PAGE) and natural gradient polyacrylamide gel electrophoresis with gelatin in the gel. Four possible candidate proteins (Fig. 3a, fraction I–IV) were then found after comparing gel patterns obtained by protein electrophoresis with protease activity assay by electrophoresis (Fig. 3a and b). The next step was thus to further effectively separate these 4 possible candidate proteins by natural gradient-PAGE (Fig. 3c) and gelatin-gradient-PAGE. The results herein indicated that only fraction II had the anticipated protease activity (Fig. 3a, lanes 5 and 6), implying that this purified fraction had the targeted protease activity.

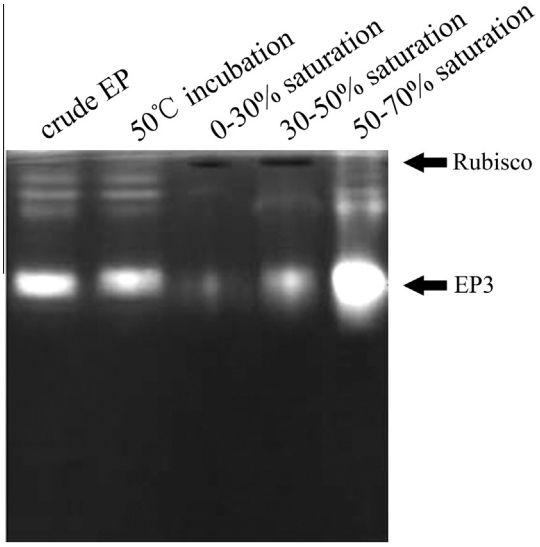

Fig. 1. EP3 protease activity of each fraction after $(NH_4)_2SO_4$ precipitation. The precipitant was dissolved, centrifuged and desalted by dialysis.

2.2. Characterization of the EP3 enzyme

The molecular weight of EP3 protease (Fig. 3a) was determined as 98.9 kDa based on a natural gradient-PAGE analysis.

Moreover, the effects of different environmental factors were examined on EP3 protease activity. First this included determining the effects of temperature on EP3 protease activity by incubating gel slices at different temperatures (4, 22, 30, 40, 50, 60, 70, and 80 °C) in Tris–HCl buffer (pH 7.0) for 2 h. As shown in Fig. 4, after assay of the EP3 protease activity, it was found that the optimum temperature for EP3 protease was about 50 °C. The EP3 protease showed an activity over a broad range (4–80 °C), and even retained 40% of its maximal activity at 80 °C.

Secondly, the effects of incubating the gels in buffer solutions were investigated with various pH values (pH 3, 4, 5, 6, 7, 8, 9 and 10) at 50 °C for 2 h on EP3 protease activity. As shown in Fig. 5, it was observed that the EP3 protease was highly active between pH 5.0 and pH 8.0, and the optimum pH value for EP3 protease activity was around pH 5.0–7.0. The relative activities of EP3 protease at pH 5.0 and 8.0 were about 94% and 66%, respectively, of that at pH 7.0. The EP3 protease activity decreased significantly above pH 8.0 or below pH 5.0. Nevertheless, there were still 10% and 30% of the EP3 protease activity at pH 4.0 and 9.0, respectively.

Thirdly, the effects of different temperatures on the EP3 protease stability were examined by pre-incubating aliquots of purified enzyme at 4, 20, 30, 40, 50, 60, 70 and 80 °C for 1 h prior to electrophoresis. As shown in Fig. 6, after assay of EP3 protease activity by electrophoresis with gelatin in the gel, pre-incubation at 20 °C, 30 °C and 40 °C led to relatively high EP3 protease activity. In contrast, EP3 protease activity was strongly reduced at temperatures above 50 °C.

Fourthly, the effects of some chemicals, including detergents, reducing agents and organic solvents, on EP3 protease activity were investigated by adding the particular chemical to the extracts to pre-incubate on ice for 1 h prior to electrophoresis. A sample with buffer was included as a control. As shown in Fig. 7, the EP3 protease was highly stable in the presence of 1% sodium dodecylsulfate (SDS), 1 M urea, 25% ethanol, 25% dimethyl sulfoxide (DMSO), 0.2% Tween 80, or 0.2% Triton X-100. It was found to be able to retain more than 40% of its activity in 25% isopropanol. In contrast, 80 mM dithiothreitol (DTT) and 25% methanol inhibited most of the EP3 protease activity.

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