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Activity Gel Analysis of Endopeptidases in Rose Petals

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Abstract

Poor resolution for the identification of endopeptidase (EP) activity in activity gel assays is a critical issue in the analysis of the postharvest physiology of rose petals. In this paper, major factors influencing EP activity gel assays were evaluated. The results showed that a phosphate (NaH_2PO_4/Na_2HPO_4) buffer favors the detection of clear EP bands, as compared to Tris–HCl buffer. Removal of salts and pigments with Sephadex G-25 columns was vital to the measurement of EP activity in rose petal extracts. For optimal resolution of bands, we show that before electrophoresis, samples should be treated for 10 min at 40 °C. Additionally, electrophoresis should be done in 12% SDS–PAGE co-polymerized with 0.15% (w/v) gelatin. After electrophoresis, the optimal incubation temperature and pH are 42 °C and 7.0, respectively. Using our optimized assay, Rh-EP1, Rh-EP2, Rh-EP3, three proteases with molecular masses of 200, 123.5, and 97.4 kD, respectively, were detected in rose petals. Experiments using EP class-specific inhibitors revealed that Rh-EP2 and Rh-EP3 were both serine proteases, while Rh-EP1 was a metalloprotease. In this study, we also measured changes in EP activity during flower opening, senescence, and water deficit stress (WDS) using our optimized activity gel assay, and found that Rh-EP3 may be more relevant to senescence in roses compared with Rh-EP1 or Rh-EP2. Changes occurring to EPs after WDS were similar to those during the period from flower opening to senescence, and Rh-EP3 activities were greatly increased by WDS treatment. Collectively, our results suggest that significant increases in Rh-EPs activities, especially increases in Rh-EP3 activity, may contribute to the flower senescence induced under WDS treatment.

Keywords: rose; petal; endopeptidase; activity gel

1. Introduction

Endopeptidases (EPs) are proteases that degrade proteins by hydrolyzing internal peptide bonds. EPs are one of the best-characterized classes of cell death–related proteins in plants. These enzymes play important roles in organ senescence and programmed cell death (Schaller, 2004). EPs are classified based on the amino acid residues or metals required for their respective cleavage reactions, and include cysteine, serine, aspartic acid, and metalloproteases. An activity gel assay is defined as an electrophoresis technique that identifies the proteolytic activity of proteins under non-denaturing conditions. These assays allow for the identification of an enzyme of interest in a complex mixture of proteases, and an estimation of its molecular weight. This method was originally applied to detect urokinase activity using a gelatin substrate slab gel (Heussen and Dowdle, 1980). Since then, activity gel assays with gelatin have been applied to detect other plant EPs (Stephenson and Rubinstein, 1998; Jiang et al., 1999). The advantage of using activity gel assays is the superior detection limit over other methods such as enzyme-linked immunosorbent assays and Western blots (Kleiner and Stetlerstevenson, 1994). Some major factors known to affect EP activity gel assays include sample extraction methods and heat treatments prior to electrophoresis, SDS–polyacrylamide gel concentration, co-polymerized gelatin concentration in the gel, incubation temperature, and pH after electrophoresis.

Roses are highly economic important; they are within the top five ornamental plants worldwide in terms of economic impact. Rose has been established as the model plant for studies on the morphological development and physiological metabolism of

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petals (Debener and Linde, 2009). Several studies showed that senescence of roses was closely related with EP activity, which rises quickly in petals in conjunction with a drop in protein content (Liu et al., 2005; Zhao et al., 2005). Tripathi et al. (2009) also found that RbCP1, a cysteine protease, controls protein degradation during petal abscission in rose (*Rosa bourboniana*). It is difficult to determine EP activity by an activity gel assay because of interfering factors in rose petals such as salts and pigments. In order to understand the role of EPs in rose abscission and senescence, it will be important to optimize activity assays for these enzymes.

Our study aimed to optimize an activity gel assay for EP activity in rose petals. We then used the optimized assay to investigate the role of EP in accelerating rose flower senescence induced under WDS. Our intention here was to establish an optimum approach for the investigation of EP characteristics of rose petals and thereby deepen the understanding of the role of EPs in flower opening and senescence. Further, this study provides an empirical basis for additional research efforts aiming to understand how alteration of EPs may be able to prevent senescence of petals in vase life, such as by enabling the identification of inhibitors specific for EPs that may prolong the vase life of cut rose flowers.

2. Materials and methods

2.1. Plant material and treatments

Cut roses were harvested at stage 2 of flower opening from a local commercial grower (Beijing, China) and placed in water immediately. The schema for classification of the flower-opening stages was that of Ma et al. (2005): stage 0, unopened bud; stage 1, partially opened bud; stage 2, completely opened bud; stage 3, partially opened flower; stage 4, fully opened flower without anther appearance; stage 5, fully opened flower with anther appearance (yellow); stage 6, fully opened flower with anther appearance (black). The flowers were delivered to our laboratory within 1 h after harvest. Their stems were then cut to a length of 30 cm and flowers were then placed in distilled water for further processing. For the control treatments, flowers were placed in a vase full of distilled water until senescence (stage 5).

To induce water deficit stress (WDS), flower stems were exposed to air instead of distilled water. After a WDS treatment of 24 h, the bottom of each stem was re-cut by 1 cm, and then placed in distilled water to allow for a water recovery (WR) period. For repeated WDS treatments, flowers were re-exposed to air following WR. For all treatments, flowers were maintained in a climate-controlled room at 20 °C, 40%–60% relative humidity, and a 12 h/12 h light/dark photoperiod at an illumination of 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

During treatments, the middle ray petals (the fifth to eighth ray of petals from the outer to the inner layer) were collected, frozen in liquid nitrogen, and stored at −80 °C for later use.

2.2. Extraction of endopeptidases from rose petals

EP samples were extracted from petals (stage 5) as follows: three grams of petals were ground into fine powder in a mortar with liquid nitrogen, and proteins were extracted from the powder after 30 min of periodic vortexing in 15 mL of pre-cooled 50 $mmol \cdot L^{-1}$ NaH_2PO_4/Na_2HPO_4 (pH 7.0) buffer or 50 $mmol \cdot L^{-1}$ Tris–HCl (pH 7.0) buffer. Both of the extraction buffers contained 2 $mmol \cdot L^{-1}$ ascorbic acid, 1 $mmol \cdot L^{-1}$ dithiothreitol (Sigma, USA), 2 $mmol \cdot L^{-1}$ ethylenediamine tetraacetic acid (EDTA, Sigma, USA), and 1% (w/v) polyvinyl pyrrolidone. Extracts were centrifuged at 13 981 $r \cdot min^{-1}$ for 30 min at 4 °C, and the supernatants were either immediately used for measuring EP activity and activity gel assays or concentrated and purified to remove salts and pigments.

2.3. Gel filtration chromatography

To remove salts and pigments, supernatants were concentrated into 2 mL with a freeze-dryer (EZ-DRY, FTS systems, USA) and were then filtered by gel filtration using Sephadex G-25 medium (Pharmacia, USA). Eighteen fractions (1 mL per fraction) were collected at a flow rate of 1.0 $mL \cdot min^{-1}$. The soluble protein content and EP activity of each fraction were measured according to the method detailed below (Section 2.4). We then combined fractions that contained the same corresponding peaks of soluble protein content and EP activity, and re-concentrated them into 0.2 mL volumes with a freeze-dryer.

2.4. Determination of protein content and EP enzymatic activity

The soluble protein content was measured at 280 nm using a spectrophotometer (Shimadzu, Japan). Total EP activity was determined using azo-casein (Sigma, USA) as a substrate according to the protocol reported by Carrasco and Carbonell (1990) with slight modification. The assay mixture contained 0.1 mL of extract, 0.25 mL of 50 $mmol \cdot L^{-1}$ Tris–HCl buffer (pH 7.0), and 0.15 mL of a substrate solution (azo-casein, 10 $g \cdot L^{-1}$). This mixture was incubated at 37 °C for 3 h. The reaction was stopped by adding 1 mL of 10 $g \cdot L^{-1}$ trichloroacetic acid. After standing for 30 min at 4 °C, samples were centrifuged at 3 342 $r \cdot min^{-1}$ for 10 min, and the absorbance of the supernatants at 340 nm was measured. One unit of EP activity was defined as the quantity of enzyme that causes an increase of 0.1 in absorbance at 340 nm per hour. The EP activity gel assay was based on the protocol reported by Jiang et al. (1999), with some optimization of electrophoretic detection and incubation conditions. Protein (20 μg) of crude extracts or filtered and concentrated extracts from stage 5 petals were loaded, gels were washed with 2.5% Triton X-100 in 50 $mmol \cdot L^{-1}$ Tris–HCl buffer (pH 7.5) for 75 min to remove SDS after electrophoresis, then incubated in a Petri dish (containing activation buffer) for 16 h at 37 °C.

2.5. Electrophoresis conditions for optimizing the EP activity gel assay

Electrophoresis was performed in a mini-PROTEAN apparatus (DYCZ-24DN, Liuyi, China). The gel thickness was 1.0 mm. EP samples from stage 5 petals were used in the following tests.

Three key factors were tested: sample treatment temperature, separating gel concentration, and co-polymerized gelatin concentration in the gel. The sample treatment temperatures tested were 20, 30, 35, 40, 45, 50, 60, and 70 °C on 10% separating gels with 0.1% gelatin. The separating gel concentrations tested