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Rapid qualitative protease microassay (RPM)

S. Mohan [a], P.W.K. Ma [b], D.S. Luthe [a,*]

[a] *Department of Biochemistry and Molecular Biology, Mississippi State University, MS 39762, United States*
[b] *Department of Entomology, Mississippi State University, MS 39762, United States*

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Abstract

A rapid qualitative protease microassay (RPM) was developed as an alternative to conventional assays of cysteine protease activity in HPLC fractions. Using this technique protease activity in samples could be visually determined within 5 min. The method was sensitive to 3.3×10^{-7} U/mL of papain and detected cysteine protease activity in dilute HPLC fractions with activity of 5.4×10^{-5} U/mL. Because the method monitors the decolorization of Coomassie Brilliant Blue stained substrate, it can be modified to detect other classes of proteases.

Keywords: Rapid qualitative protease microassay; HPLC fractions; Protease

1. Introduction

Cysteine or thiol proteases are ubiquitous enzymes that participate in a multitude of cellular processes in both plants and animals. We have purified a novel 33-kD cysteine protease Mir1-CP from maize tissue culture cells [1]. This cysteine protease also accumulates in insect resistant maize plants in response to feeding by fall armyworm (*Spodoptera frugiperda*) and other lepidopterans [2]. When the gene *mir1* encoding Mir1-CP was expressed in transgenic maize cells, which was used in feeding bioassays, the protease retarded larval growth by approximately 70% and damaged the insect's

* Corresponding author. Tel.: +1 662 325 7733; fax: +1 662 325 8664.
E-mail address: dsluthe@ra.msstate.edu (D.S. Luthe).

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peritrophic matrix [1–3]. To obtain large quantities of the purified protease, we have expressed the Mir1-CP in a modified baculovirus-based expression system [4] in which recombinant baculovirus carrying *mir1* is inoculated into *S. frugiperda* larvae and the recombinant protein is collected from the hemolymph. In the past, activity of the protease was assessed by conventional enzyme assays [5–7], which could take hours for screening multiple column fractions, or days for in-gel activity assays [8]. In the process of developing an HPLC procedure for purifying Mir1-CP from the hemolymph, it was necessary to devise a rapid assay for detecting protease activity to monitor the numerous column fractions generated. Herein, we report on a rapid qualitative method for detecting cysteine protease activity that could be modified for use with other proteolytic enzymes.

2. Materials and methods

2.1. Chemicals

All reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Deionized water (18 MΩ) was used throughout the experiments.

2.2. HPLC

High-pressure liquid chromatography (HPLC), conducted on an HP (Hewlett Packard) 1050 instrument equipped with a TSK-Gel size-exclusion column (7.8 mm ID × 30 cm, 6 μm particle size; Phenomenex, Torrance, CA), was used to elute Mir1-CP from hemolymph. The column was previously calibrated using known molecular weight samples of 60–9 kD. Proteins of 33 kD were eluted with 1 M-phosphate buffer (pH 7.0 ± 0.2), at a flow rate of 0.75 mL/min. The absorbance at 214 nm of the eluent was monitored using a HITACHI 655A variable wavelength UV monitor.

2.3. Mir1-CP detection

HPLC fractions of 0.19 mL were collected manually at 15 s interval between 7 and 9 min (34–29 kD), based on column calibration, and tested for protease activity using an in-gel activity assay following protein separation by modified SDS-PAGE [8]. The 33-kD cysteine protease that eluted from the column was confirmed to be Mir1-CP by immunoblot analysis [4]. Mir1-CP activity in the pooled HPLC fractions was determined fluorometrically using Z-phe-arg-AMC as a substrate [6,7]. The protein concentration was determined using the Bradford method [9]. The Mir1-CP specific activity in the pooled HPLC fractions was 9 U/mg. The protein concentration was 0.6 mg/mL, resulting in a final concentration of 5.4 U/mL.

2.4. Rapid protease micro-assay (RPM)

Wet and semi-dry assays using gelatin (1 mg/mL in water) as the substrate were developed for rapidly testing protease activity. For the wet method, 10 μL of gelatin were

pipetted on to a clean glass microscope slide and mixed with an equal volume of 0.2% Coomassie Brilliant Blue forming a small circular droplet. Aliquots (0.5 to 1.0 μL) of papain (1 mg/mL of papain; positive control), water (negative control) or pooled HPLC fractions containing Mir1-CP were added to each substrate droplet at 29 °C. The specific activity of papain was 33 U/mg and was prepared as 1 mg/mL stock solution of 33 U/mL. The slide was incubated for 1 min with gentle swirling. Activity was scored by the appearance of white agglutinated material that formed in the presence of active protease. For the semi-dry method, a solution (1 mL) containing 250 μl gelatin (1 mg/mL), 500 μl of 0.2% Coomassie Brilliant Blue, 225 μl acrylamide (30 : 1, acrylamide : bisacrylamide), 11.25 μl 10% ammonium persulfate, 13.75 μl of 10% SDS and 1 μl of TEMED was prepared and 10 μl was pipetted into each well of a 24 well Teflon coated slide (Eric Scientific, NJ). After polymerization, 0.1–0.2 μl of sample was placed on the gel solution in each well and the slides were incubated at room temperature for 5–10 min. HPLC fractions pre-treated with varying concentrations of E64 [*N*-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide)] ranging from 0.5 to 2 mg/mL, were also tested by semi-dry RPM. A dissection microscope (1.5×) was used to visualize enzyme activity, which was scored as the disappearance of blue staining in the well.

Modified semi-dry RPM method was also performed by using *p*-hydroxybenzoyl-glycyl-L-phenylalanine (1.5 mg/mL) as substrate for carboxypeptidase A [10] and chymotrypsin substrate I (Suc-GGF-pNA, 2 mg/mL) as substrate [11] for chymotrypsin activity determination.

3. Results and discussion

Protease activity determination was instantaneous when the wet assay was used and took up to 5 min with the semi-dry RPM method. In the wet method, white agglutination appeared in the droplet of solution that was treated with the pooled HPLC fractions containing Mir1-CP (Fig. 1A). This was identical to the color change shown when papain was added. In the semi-dry method (Fig. 1B) the addition of either papain or the pooled HPLC fractions resulted in the disappearance of the blue color and production of a white sheen in the well, that were not apparent when water was added. Consequently, enzyme activity could be scored as the disappearance of the blue color from the wells on the slides.

To confirm that the activity visualized in the pooled HPLC fractions was due to the thiol protease activity of Mir1-CP, the pooled HPLC fractions were pretreated with E-64 (a specific inhibitor of thiol protease activity [12]) (Fig. 2). E-64 treatment inhibited protease activity in both the HPLC fractions containing Mir1-CP and papain. However, a higher concentration of E-64 was required for inactivation of Mir1-CP in the pooled fractions. The reason for this is currently unknown.

The sensitivity of the RPM was determined by assaying serial dilutions of papain (33 U/mL) and the Mir1-CP (5.4 U/mL) (Fig. 3). Activity was measured using semi-dry RPM and the in-gel activity assay [8]. Using the semi-dry RPM, enzyme activity was detected in the 10^{-8} diluted papain sample (Fig. 3A) and the 10^{-5} diluted Mir1-CP (Fig. 3B), whereas activity was detected only in the 10^{-1} dilution when Mir1-CP was analyzed using the in-gel activity assay (Fig. 3C). The in-gel activity assay may not be as sensitive as the

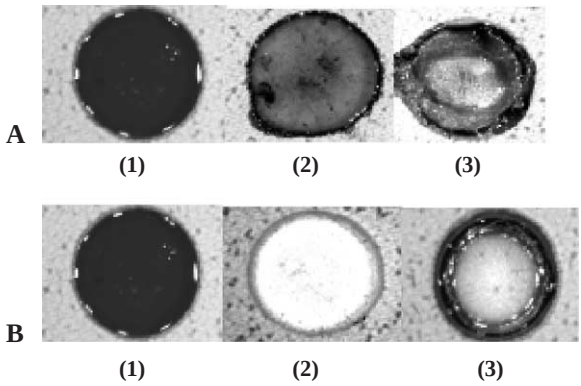

Fig. 1. RPM assay using the wet and semi-dry methods. A. Wet method with the substrate treated for 1 min with (1) water; (2) HPLC fraction containing Mir1-CP (5.4 U/mL); (3) papain (33 U/mL). B. Semi-dry method with substrate treated for 5 min with (1) water; (2) pooled HPLC fractions containing Mir1-CP (5.4 U/mL); and (3) papain (33 U/mL).

RPM because the gel is run under semi-denaturing conditions [8]. These results indicated that the RPM could detect papain activity with a specific activity as low as 3.3×10^{-7} U/mL and Mir1-CP with specific activity of 5.4×10^{-5} U/mL.

Decolorization of Coomassie Brilliant Blue stained gelatin occurred when it was treated with two other cysteine proteases, bromelain (diluted to 2.7×10^{-7} U/mL) and ficin (diluted to 2.9×10^{-7} U/mL) (Fig. 4A). The RPM was modified for detecting carboxypeptidase A activity by using *p*-hydroxybenzoyl-glycyl-L-phenylalanine (1.5 mg/mL) as substrate. When the substrate was treated with 10^{-8} diluted carboxypeptidase

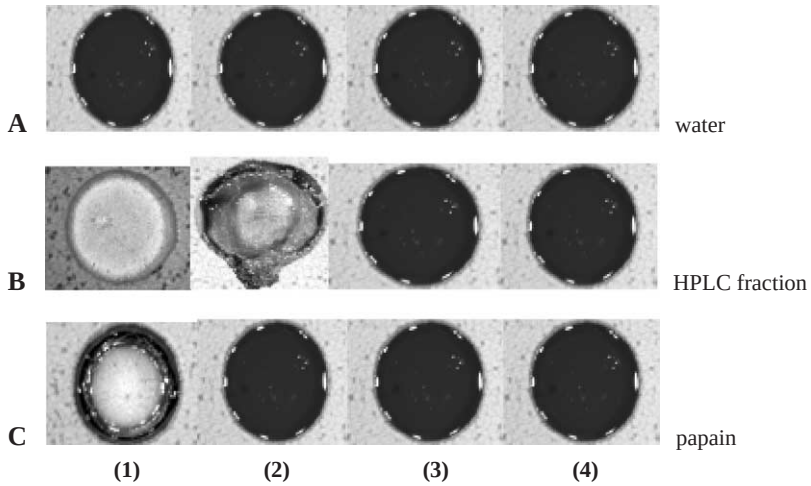

Fig. 2. Treatment of enzyme preparation with the cysteine protease inhibitor, E-64. Substrate was treated for 5 min with (A) water, (B) HPLC fraction containing Mir1-CP (5.4 U/mL), (C) papain (33 U/mL). Enzyme samples were treated with (1) no E64, (2) 0.5 mg/mL, (3) 1.0 mg/mL, or (4) or 2.0 mg/mL of E-64 (4) prior to their addition to substrate.

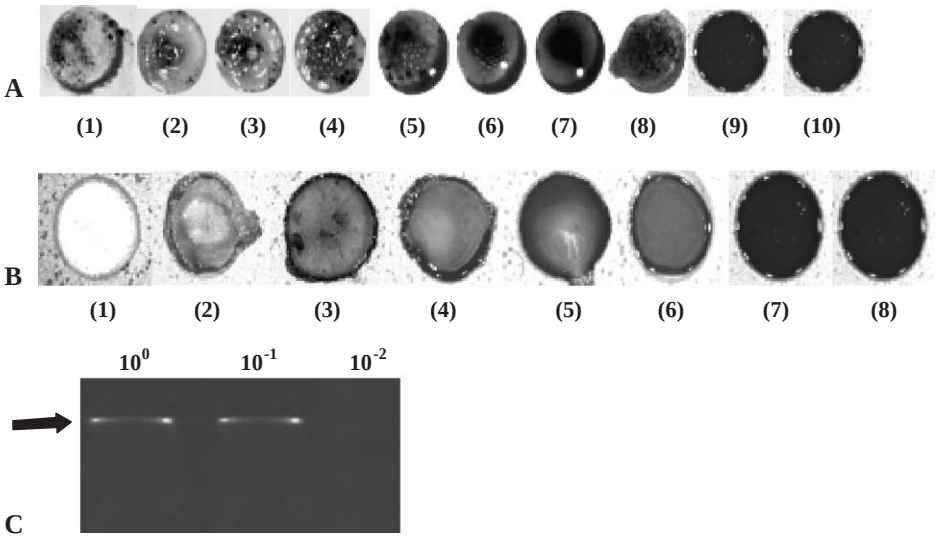

Fig. 3. Determination of RPM sensitivity using serially diluted papain and Mir1-CP. A. Standardization of semi-dry RPM using papain (33 units/mL) diluted (1) 10^{-1}, (2) 10^{-2}, (3) 10^{-3}, (4) 10^{-4}, (5) 10^{-5}, (6) 10^{-6}, (7) 10^{-7}, (8) 10^{-8}, (9) 10^{-9} or the water control (10). B. Semi-dry RPM used to determine protease activity of diluted Mir1-CP (5.4 U/mL) (1) 10^{0}, (2) 10^{-1}, (3) 10^{-2}, (4) 10^{-3}, (5) 10^{-4}, (6) 10^{-5}, (7) 10^{-6} or the water control (8). Results obtained in A and B were documented after 5 min exposure. C. In-gel activity assay with Mir1-CP (5.4 U/mL) diluted 10^{0}, 10^{-1} and 10^{-2}.

A (1 ng/mL, 2.8×10^{-7} U/mL) quinoneimine dye was produced which was visualized as color production (Fig. 4B). When Coomassie Brilliant Blue stained chymotrypsin substrate I (Suc-GGF-pNA, 2 mg/mL) was treated with 10^{-7} diluted chymotrypsin (1.2 ng/mL, 5.2×10^{-6} U/mL) the substrate was decolorized by the enzyme (Fig. 4C). This indicated the assay was highly sensitive for several different classes of proteases.

Determination of protease activity for a large number of HPLC fractions by conventional assay methods is unwieldy and time consuming, taking hours or several days depending on the assay method. We developed a rapid and sensitive qualitative method for detecting protease activity instantly. The method is well suited for rapidly determining the presence of protease activity in column fractions. Our preliminary results suggest that it may not be effective in determining protease activity unfractionated plant cell homogenates.

Although, other approaches for detecting cysteine protease activity [13,14] have been reported, they detected enzyme activity quantitatively by using either fluorogenic labeled dye conjugates or other substrates and required several complicated steps. The RPM method provided rapid qualitative enzyme activity measurement. The materials needed for the RPM were also simple and inexpensive. Further, enzyme activity was detected more rapidly with RPM than by other methods. Of the two assay methods, semi-dry method was preferred based on the ease of storage and documentation of results. The RPM method was relatively sensitive and detected activity in 5.4×10^{-5} U/mL of Mir1-CP and other commercially available proteases. By adding the appropriate inhibitors, the assay can also

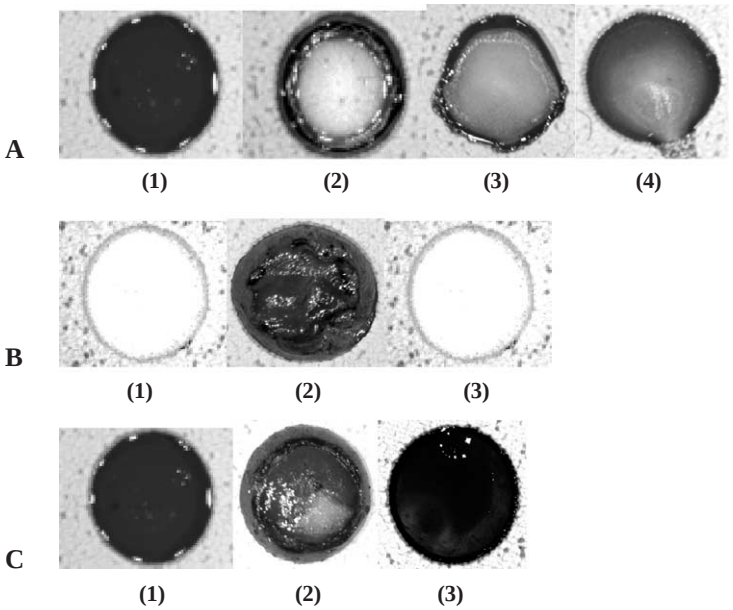

Fig. 4. Semi-dry RPM using commercially available proteases. A. Bromelain and ficin using Coomassie Brilliant Blue stained gelatin as substrate: (1) water, (2) papain (33 U/mL), (3) 10^{-8} diluted bromelain (2.7×10^{-7} U/mL) and (4) 10^{-7} diluted ficin (2.9×10^{-7} U/mL). B. Carboxypeptidase A using *p*-hydroxybenzoyl-glycyl-L-phenylalanine (1.5 mg/mL) as substrate: (1) water, (2) 10^{-8} diluted carboxypeptidase A (2.8×10^{-7} U/mL) and (3) papain (33 U/mL). C. Chymotrypsin using chymotrypsin substrate I (Suc-GGF-pNA, 2 mg/mL) as substrate: (1) water, (2) 10^{-7} diluted carboxypeptidase A (5.2×10^{-6} U/mL) and (3) papain (33 U/mL). All results were documented after 5 min exposure.

be used to determine the types of protease present in sample mixture. We have modified RPM method and successfully used it for determining chitinase activity (data not shown). Therefore the method does not confine itself to proteases, but could be the basis for activity determination of a variety of other hydrolytic enzymes.

Acknowledgements

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