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Two-dimensional analysis of proteinase activity

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Abstract

A method was developed to separate proteinases in a complex mixture in two dimensions followed by activity detection using class specific substrates. Using this method, serine proteinase activity was evaluated in gut extracts from a stored-product pest, *Plodia interpunctella*. With the substrate N-α-benzoyl-L-arginine ρ-nitroanilide, three major groups of at least six trypsin-like activities were identified, consisting of proteinases with estimated molecular masses of 25–27, 40–41, and 289 kDa, and all with an acidic pI of 4.7–5.5. With the substrate, N-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide, two groups of at least five chymotrypsin-like activities were detected, with estimated molecular masses of 28 and 192 kDa and pI values ranging from 6.1 to 7.3. Using the 2-DE activity blot method, information was obtained on the relative number and physical properties of serine proteinases in a mixture of insect gut proteinases without prior fractionation.
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Keywords: 2-D electrophoresis; Proteinase activity; Proteomics

1. Introduction

With the advent of proteomic techniques to study subsets of proteins in organisms, new techniques are being developed to identify specific enzyme activities and inhibitors in complex samples. Examples include the detection of chitinase activity in a filamentous fungus, *Paecilomyces lilacinus*, following the separation of fungal proteins by two-dimensional electrophoresis (2-DE) [1], and viperid snake venom proteinases by 2-DE gelatin zymography

Abbreviations: 2-DE, two-dimensional electrophoresis; BApNA, N-α-benzoyl-L-arginine ρ-nitroanilide; CBB, Coomassie brilliant blue; LpNA, leucine ρ-nitroanilide; IEF, isoelectric focusing; SAAPFpNA, N-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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[2]. Inhibitors of metalloproteinases were detected in muscle tissue from the Atlantic cod, *Gadus morhua*, by 2-DE reverse zymography [3]. Similarly, trypsin inhibitors in the winged bean, *Psophocarpus tetragonolobus*, were identified following 2-DE gel electrophoresis and exposure to gelationous X-ray film [4].

Previously, methods were described whereby insect gut proteins were separated by one-dimensional electrophoresis, and proteinase activity was visualized by incubation with ρ-nitroanilide substrates and subsequent processing of the released nitroaniline product to detect enzyme activity [5,6]. To further resolve the differences in serine proteinase activity patterns in the Indianmeal moth, *Plodia interpunctella*, a 2-DE activity blot assay was developed.

2. Materials and methods

2.1. Chemicals

Gels were stained with Coomassie brilliant blue (CBB) (Simply Blue SafeStain, Invitrogen, Carlsbad, CA). All other reagents, including the substrates N-α-benzoyl-L-arginine ρ-nitroanilide (BApNA), N-succinyl-ala-ala-pro-phenylalanine ρ-nitroanilide (SAAPFpNA), and leucine ρ-nitroanilide (LpNA) were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose was BA-S 83 (Optitran, 0.2 μm, Schleicher and Schuell, Keene, NH).

2.2. Insect dissection

Larvae were selected from a laboratory colony of *P. interpunctella*, colony 688s. Whole guts were extracted from late 4th instar larvae by anaesthetizing on ice and clipping the posterior and anterior ends, and pulling the gut from one end with forceps. The gut was placed in 25 μl of deionized water and stored frozen at −20 °C. Immediately prior to analysis, samples were thawed, spun at 12,000 ×*g* for 5 min, and the supernatant was collected.

2.3. Protein separation

Soluble proteins were separated in the first dimension by loading 15 μl of gut extract in each well of eight wells total of a 10-well isoelectric focusing (IEF) gel (pH 3–10, Invitrogen), using the manufacturer's recommended protocol and sample, anode, and cathode buffers. One of the remaining wells was loaded with 5 μl of IEF markers (3–10, Invitrogen). The gel was subjected to 100 V (constant) for 1 h, 200 V for 1 h, and 500 V for 30 min. Lanes were excised with a new razor. To detect protein, one of the gel lanes was fixed in 3.5% sulphosalicylic and 11.5% trichloroacetic acid for 30 min and was stained with CBB.

For activity detection, three lanes of IEF-separated *P. interpunctella* gut proteins were transferred to nitrocellulose at 30 V for 30 min using Tris–Glycine transfer buffer (Invitrogen). Each lane was incubated with a different substrate and processed for proteinase activity, as described below.

The remaining four IEF lanes containing *P. interpunctella* gut proteins were separated in the 2nd dimension by adapting the manufacturer's protocol for two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) (Invitrogen). In this procedure, excised IEF gel lanes are stained and destained and then incubated for 10 min in 20% ethanol, followed by incubation in a 2× sample buffer and 20% ethanol for 3–5 min. However, to preserve enzyme activity, gel slices were incubated only for 5 min in 1× Tricine