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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

sample buffer (Invitrogen). Each lane was carefully inserted into the long well of a 10–20% Tricine 2-D SDS-PAGE gel (Invitrogen), with consistent orientation of the pH gradient, using a filter paper strip cut to the dimensions of the gel strip to ensure contact with the polyacrylamide gel. Molecular mass markers (8 μl) were loaded in the appropriate well (MultiMark, Invitrogen). Following electrophoresis at 4 °C and 125 V, one gel was stained with CBB. The remaining gels were electrophoretically transferred to nitrocellulose as previously described, except for a 1 h transfer time.

2.4. Activity blot assays

Nitrocellulose blots were incubated with individual substrate solutions, BApNA, to detect trypsin-like proteinases, SAAPFpNA, for chymotrypsin-like proteinases, or LpNA for aminopeptidases, as indicated. All substrates were made as stock solutions in organic solvents as per the manufacturer's protocol and were diluted 0.5 mg/ml in 20 mM Tris, pH 8.0. The substrate solution was conserved by placing the blots in an EconoBlot tray (LabLogix, Belmont, CA), layering 5 ml of substrate solution over the blot, and covering with an EconoBlot plastic sheet. The blots were gently rotated at 37 °C until a faint yellow color was observed, approximately 15 min. The blots were processed for visualization by diazotization of the released nitroaniline by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% *N*-(1-naphthyl)-ethylenediamine in 47.5% ethanol.

Blots were stored wet on a plastic Gel-Bond sheet (FMC, Rockland, ME) in heat-sealed bags at −20 °C. Stained gels were digitized using the Odyssey gel documentation system (LiCor, Lincoln, NB), and activity blots were digitized using a scanner and PhotoPaint software (Corel, Ottawa, Ontario). 2-DE activity blots were analyzed by Melanie (Swiss Institute of Bioinformatics, Geneva, Switzerland).

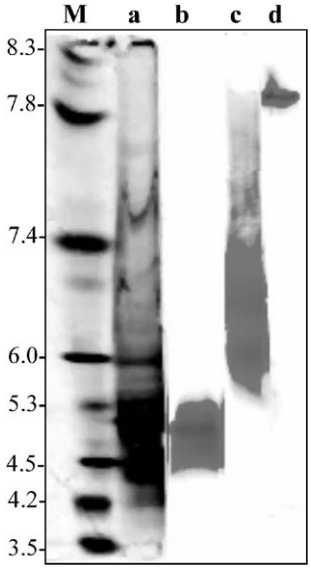

Fig. 1. *P. interpunctella* soluble gut proteins separated by IEF, with different treatments post-focusing. Lane a, CBB-stained; lane b, activity blot with the substrate BApNA; lane c, activity blot with the substrate SAAPFpNA; lane d, activity blot with the substrate LpNA. M: IEF markers, as notated on the left.

3. Results

3.1. IEF separation and analysis

Soluble gut proteins from *P. interpunctella* larvae were separated in the 1st dimension by charge using IEF (Fig. 1). Gels containing CBB-stained proteins indicated that a majority of gut proteins were acidic (Fig. 1, lane a). In corresponding activity blots of IEF-separated proteins and specific ρ-nitroanilide substrates, a major trypsin-like activity was detected with a pI of approximately 5.0, probably consisting of multiple trypsin isoforms due to the broad activity band (Fig. 1, lane b). Chymotrypsin-like proteinase activities ranged from acidic to slightly alkaline pI values (Fig. 1, lane c). An aminopeptidase-like activity was detected in the soluble extract with a pI of ~ 8.0 (Fig. 1, lane d).

3.2. SDS-PAGE and analysis

P. interpunctella proteins separated in the second dimension by SDS-PAGE were stained for protein and contained mostly small molecular mass proteins, as well as a number of peptides in the dye front (Fig. 2). These degraded proteins likely were due to the hydrolysis of proteins during the analysis procedure, as the mixture of luminal proteins contained active proteinases, because precautions were taken to preserve proteinase activity by not heating samples prior to electrophoresis. In addition, *P. interpunctella* proteinases have been demonstrated to be active in SDS buffers [7].

2-DE activity blot analysis with the substrate BApNA indicated the presence of three groups of soluble trypsin-like activities in *P. interpunctella* luminal extracts (Fig. 3a). These activities corresponded to molecular masses of 25–27, 40–41 and 289 kDa (Table 1), and were similar to previously described trypsin-like activities in *P. interpunctella* that were separated by 1D electrophoresis [7,8]. All trypsin-like proteinases had acidic pI values in the range of 4.7–5.5. Since the spots of trypsin-like activity were broad and diffuse, it was difficult to determine the

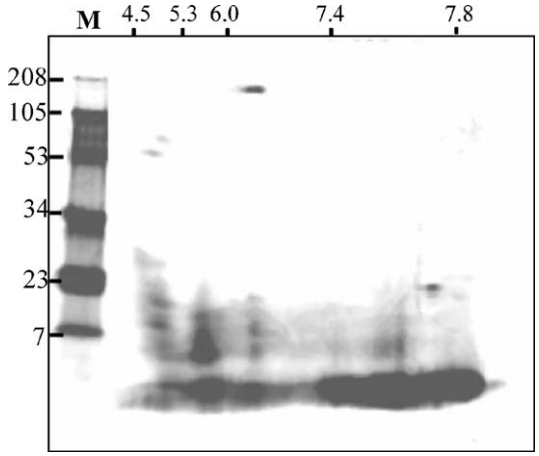

Fig. 2. CBB-stained 2-DE analysis of *P. interpunctella* soluble gut proteins. Molecular mass markers (M) are notated on the left, and IEF markers are notated at the top.

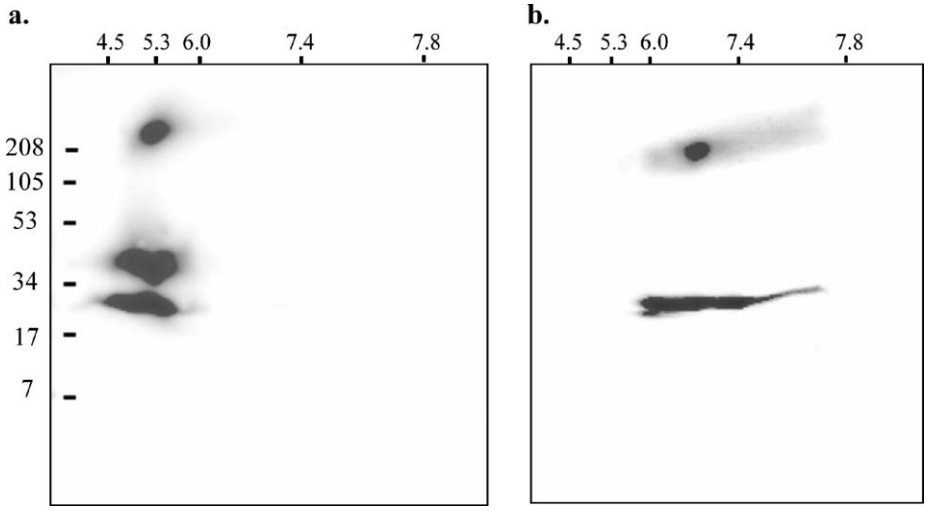

Fig. 3. Activity blot analysis of *P. interpunctella* soluble larval gut proteinases separated by 2-DE and incubated with (a) BApNA or (b) SAAPFpNA. Molecular mass markers are indicated on the left of (a) for both blots, and IEF markers are notated at the top of each blot.

exact number of trypsin-like proteinases in the extract. However, gel documentation analysis indicated the presence of at least six isoforms (Table 1).

In a 2-DE activity blot analysis with the substrate SAAPFpNA, two major groups of chymotrypsin-like proteinase activities were detected (Fig. 3b). Chymotrypsin-like activities consisted of proteinases with molecular masses of 28 and 220 kDa (Table 1), also similar to previously described chymotrypsin-like proteinases in *P. interpunctella* [7,9]. The pI of the 28 kDa chymotrypsin-like proteinases ranged from 6.1 to 7.3, consisting of at least four isoforms. A larger molecular mass chymotrypsin-like activity also was observed, with an estimated pI of 6.7. This activity pattern was similar to a previous report of chymotrypsin-like activity associated with multiple protein alterations in toxin-resistant insects [9].

Table 1
Some physical properties of serine proteinases obtained from the analysis of 2-DE activity blots using either BApNA (to detect trypsin-like proteinases) or SAAPFpNA (for chymotrypsin-like proteinases) substrates

Proteinase activity	pI[a]	Mm[a]
Trypsin-like	4.7	27
	5.1	27
	5.4	25
	5.0	40
	5.5	41
	5.3	289
Chymotrypsin-like	6.1	28
	6.4	28
	6.8	28
	7.3	28
	6.7	220

[a] Relative pI and Mm (molecular mass) was estimated by Melanie analysis of 2-DE activity blots.

No activity was detected on the 2-DE activity blot incubated with LpNA (data not shown). Probably this was due to the instability of *P. interpunctella* aminopeptidase proteinases in SDS buffers.

4. Discussion

Development of the 2-DE activity blot technique has permitted the identification of distinct populations of proteinase activities in luminal extracts from individual insect larvae. With this method, at least six trypsin-like and five chymotrypsin-like proteinase activities were identified in soluble gut extracts from *P. interpunctella* larvae. It is likely that the lower molecular mass activities represented multiple isoforms, as these molecular masses (25–28 kDa) were similar to previously reported trypsins and chymotrypsins in other organisms. However, the larger molecular mass activities were probably the result of the oligomerization of multiple trypsin and chymotrypsin molecules that formed in the relatively gentle conditions that were maintained to preserve enzyme activity. Oligomers have been reported for serine proteinases in other insects under certain conditions [10–12].

For the successful detection of enzyme activity following denaturing steps during electrophoresis, samples must be either recalcitrant to denaturation, or capable of regaining function after denaturation through the removal of detergents or similar chaotropes. Another major limitation of the technique is the necessity of enzyme stability during the entire process, approximately 6 h. While electrophoresis is performed in a cooling chamber to preserve activity that might be lost to excessive heating, some activity is undoubtedly lost during the initial IEF step. However, for enzymes that are relatively stable under these conditions, the technique is economical and convenient, because the use of IEF gels that fit standard electrophoresis equipment avoided the more expensive isoelectric focusing equipment. In addition, the use of the EconoBlot system reduced the amount of substrate required for the analysis.

The 2-DE activity blot method was used to the identify proteinase activity profiles in complex mixtures and can be used to complement other proteomic techniques. This approach has permitted the comparison of related physiological differences in various subpopulations of insects [9]. The technique also is applicable to comparisons of proteinase activities in different insect species, as well as other organisms, provided the enzymes are stable under the analysis conditions. With comparatively minor time and monetary investments, information can be obtained on the relative number and physical properties of proteinases in a mixture without prior fractionation.

5. Simplified description of the method and future applications

A method, 2-DE activity blot analysis, was developed to identify serine proteinase activities in complex mixtures.

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References

[1] Khan A, Williams K, Molloy MP, Nevalainen H. Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. Protein Expr Purif 2003;32:210–20.
[2] Serrano SMT, Shannon JD, Wang D, Camargo ACM, Fox JW. A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. Proteomics 2005;5:501–10.
[3] Lødemel JB, Egge-Jacobsen W, Olsen RL. Detection of TIMP-2-like protein in Atlantic cod (*Gadus morhua*) muscle using two-dimensional real-time reverse zymography. Comp Biochem Physiol 2004;139B:253–9.
[4] Giri AP, Harsulkar AM, Ku MSB, Gupta VS, Deshpande VV, Ranjekar PK, et al. Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds. Phytochemistry 2003;63:523–32.
[5] Ohlsson BG, Westrom BR, Karlsson BW. Enzymoblotting: a method for localizing proteinases and their zymogens using para-nitroanilide substrates after agarose gel electrophoresis and transfer to nitrocellulose. Analyt Biochem 1986;152:239–44.
[6] Oppert B, Kramer KJ. Economical use of substrates in enzyme activity blots. Am Biotechnol Lab 1998;16:20.
[7] Oppert B, Kramer KJ, Johnson D, Upton SJ, McGaughey WH. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem Mol Biol 1996;26:571–83.
[8] Oppert B, Kramer KJ, Beeman RW, Johnson D, McGaughey WH. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J Biol Chem 1997;272:23473–6.
[9] Candas M, Loseva O, Oppert B, Kosaraju P, Bulla Jr LA. Insect resistance to *Bacillus thuringiensis*: alterations in the Indianmeal moth larval gut proteome. Mol Cell Proteom 2003;2:19–28.
[10] Terra WR, Ferreira C, Bastos F. Phylogenetic consideration of insect digestion. Disaccharidases and the spatial organization of digestion in the *Tenebrio molitor* larvae. Insect Biochem 1985;15:443–9.
[11] Brito LO, Lopes AR, Parra JRP, Terra WR, Silva-Filho MC. Adaptation of tobacco budworm *Heloithis virescens* to proteinase inhibitors may be mediated by the synthesis of the new proteinases. Comp Biochem Physiol 2001;128B:365–75.
[12] Wagner W, Möhrlen F, Schnetter W. Characterization of the proteolytic enzymes in the midgut of the European Cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae). Insect Biochem Mol Biol 2002;32:803–14.