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Direct detection of cysteine peptidases for MALDI-TOF MS analysis using fluorogenic substrates

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ABSTRACT

A method is described for the direct detection of unstable cysteine peptidase activity in polyacrylamide gels after native electrophoresis using new selective fluorogenic peptide substrates, pyroglutamyl-phenylalanyl-alanyl-4-amino-7-methylcoumaride (Glp-Phe-Ala-AMC) and pyroglutamyl-phenylalanyl-alanyl-4-amino-7-trifluoromethyl-coumaride (Glp-Phe-Ala-AFC). The detection limit of the model enzyme papain was 17 pmol (0.29 μg) for Glp-Phe-Ala-AMC and 43 pmol (0.74 μg) for Glp-Phe-Ala-AFC, with increased sensitivity and selectivity compared to the traditional method of protein determination with Coomassie G-250 staining or detection of activity using chromogenic substrates. Using this method, we easily identified the target digestive peptidases of *Tenebrio molitor* larvae by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis. The method offers simplicity, high sensitivity, and selectivity compared to traditional methods for improved identification of unstable cysteine peptidases in multi-component biological samples.

1. Introduction

Peptidases are involved in many biological processes [1]. The detection and estimation of proteolytic activity commonly involves different substrates, such as proteins like casein, gelatin, hemoglobin, albumin and azo-derivatives of these proteins [2]. However, peptides or amino acids with marker chromogenic groups, such as *p*-nitroanilide and derivatives [3–6] or a *p*-nitrophenyl group [7], and fluorogenic groups based on β-naphtylamide (βNA) [3,8], 4-methoxy-βNA [9] and methylcoumarin derivatives, AMC [9], and AFC [10], can provide increased sensitivity and specificity.

Molecular identification of peptidases in biological material often includes multistage purification. However, purification procedures, particularly applied to endopeptidases with broad specificity, result in a significant loss of activity due to high levels of autolysis. These problems can be mitigated by post-electrophoretic detection and characterization of peptidases. This technique, referred to as zymography, offers significant advantages over conventional enzyme purification with regard to convenience, sensitivity, speed, simplicity and the ability to specifically detect active peptidase forms in crude extracts [11,12]. The method is based on the electrophoretic separation of peptidases, preferably in a native gel, followed by detection and visualization of activity with protein or peptide chromogenic and fluorogenic substrates.

There are two main techniques that are used for postelectrophoretic detection of proteolytic activity: (1) transfer zymography, where the

Abbreviations: AMC, 4-amino-7-methylcoumaride; AFC, 4-amino-7-trifluoromethyl-coumaride; βNA, β-naphtylamide; Bz, benzoyl; Cys II, fraction of cysteine peptidase II; Cys III, fraction of cysteine peptidase III; DMF, dimethylformamide; DTT, dithiothreitol; E−64, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide; Glp, pyroglutamyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MALDI-TOF MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TmCysII, *Tenebrio molitor* cysteine peptidase II; TmCysIII, *Tenebrio molitor* cysteine peptidase III; UB, universal buffer; Z, benzyloxycarbonyl

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activity is visualized using an overlaid indicator gel, or a membrane containing a peptidase substrate [13–16]; and (2) in-gel detection, using substrates incorporated in the gel, which is easier, but can be problematic if enzymes are active during the separation phase [17–19]. Compared to protein substrates, peptide substrates offer advantages in mass spectrometry (MS) analysis, as products of the reaction as well as remaining substrates are easily and quickly removed after documentation, and the detected enzyme that has been freed of impurities can be used for further MS analysis.

The choice of the method of electrophoretic separation is also important for correct determination of proteolytic activity. The most popular method is electrophoresis in a denaturing gel, introduced in 1970 by Laemmli [20]. The advantage of this approach is the ability to determine the molecular mass of the enzyme with high resolution. However, the Laemmli method has a number of restrictions in the study of unstable enzymes that require a renaturation step. A classical approach employs sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under mildly denaturing, nonreducing conditions followed by a refolding step in a renaturation buffer, allowing both estimation of molecular mass and identification of peptidase activity. However, this method is not applicable for unstable peptidases where activity is not regained after renaturation, such as cysteine peptidases [16]. With these enzymes, native PAGE is preferable as no renaturation step is needed. However, direct in-gel detection of proteolytic activity after native PAGE has been described only for exopeptidases, namely aminopeptidases [21], which are not characterized by high levels of autolysis.

Native PAGE offers the following advantages for enzyme detection: (1) quick detection and estimation of enzyme activity due to the lack of a renaturation step; (2) maximum retention of activity of unstable enzymes [16]; and (3) applicability to detection of enzymes consisting of several subunits or noncovalent complexes of several proteins, especially in complex biological samples. Evaluation of the true proteolytic activity in samples by native PAGE can be more reliable than SDS–PAGE because of (a) the lack of possible activation of proenzymes by denaturation, and (b) the lack of dissociation of native enzyme-inhibitor complexes with noncovalently bound inhibitor [12].

Previously, we described a series of original fluorogenic substrates for detection of activity of cysteine peptidases from the papain family [22,23]. In this work, we describe a quick and efficient method of direct detection of activity of papain C1 family peptidases and demonstrate that the application allows the molecular identification of cysteine peptidases found in a multicomponent natural mixture – the insect larval gut – after only one purification step.

2. Materials and methods

Papain (EC 3.4.22.2) was obtained from Sigma–Aldrich (USA). Chromogenic substrates Bz-Arg-pNA (where Bz = benzoyl), Z-Arg-Arg-pNA (where Z = benzyloxycarbonyl), and Z-Phe-Arg-pNA were obtained from Bachem (Switzerland). Substrates Glp-Phe-Ala-pNA, Glp-Phe-Ala-AMC and Glp-Phe-Ala-AFC were synthesized as described in Refs. [22–24].

2.1. Fractionation of Tenebrio molitor larvae midgut peptidases

T. molitor larvae rearing, isolation of midgut protein extracts, and fractionation of larval digestive peptidases were performed as described earlier [25,26]. Briefly, the anterior midgut (AM) from 200 larval guts was homogenized in a glass Downce homogenizer, centrifuged for 5 min at 10,000 × *g* and applied to a Sephadex G-100 column (2.5 × 124 cm) equilibrated with 500 mM NaCl in 10 mM phosphate buffer, pH 5.6, containing 0.02% NaN_3. Fractions of 9.0 mL were collected and analyzed for protein content and enzymatic activity, with the chromogenic peptide substrate Glp-Phe-Ala-pNA (pH 5.6 with 1 mM dithiothreitol (DTT)) in 50 μL aliquots by a standard enzymatic activity assay, which measures the release of *p*-nitroaniline at A_{405}, as described in Ref. [26].

2.2. Determination of protein concentration

Concentration of protein was determined by measuring the optical density (O.D.) of protein solutions at 280 nm. The proportion of active enzyme in a commercial preparation of papain was determined by active site titration, using the inhibitor N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E − 64) as described in Ref. [23].

2.3. Electrophoresis

2.3.1. Native PAGE

As the reference protein for determining the sensitivity of the proposed method, papain was used with O.D. of 0.05, 0.10, 0.25, 0.50, and 1.00 optical units at 280 nm. Gel filtration fractions from the two major peaks of cysteine peptidase activity from the *T. molitor* larval gut [26], Cys II and Cys III, were pooled and lyophilized. Aliquots contained 1 mg of Cys II or 3 mg Cys III fraction in 20 μL of gel buffer. All samples were in 20 μL gel buffer (35 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 43 mM imidazole gel buffer at pH 7.4 according to Ref. [27]) and were subjected to native PAGE in 4% acrylamide and 0.2% bis-acrylamide stacking (1 × 8.3 × 0.1 cm) and 12% acrylamide and 0.6% bisacrylamide (5 × 8.3 × 0.1 cm) separating polyacrylamide gels, with electrophoresis towards the anode at a constant current of 10 mA for 40 min at 4 °C. For protein detection, the gel was stained in 0.1% Coomassie Brilliant Blue G-250 in 30% ethanol and 10% acetic acid. Gels were destained in the same solution without dye and photographed.

2.3.2. SDS-PAGE

Bands of proteolytic activity in native gels were excised and ground in 20 μL of lysis buffer (62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 6 M urea, 20% glycerol, 0.02% bromophenol blue), heated at 99 °C for 5 min and incubated at 4 °C for 12 h. The resulting solution was subjected to electrophoretic analysis under non-reducing conditions in 15% (acrylamide:bisacrylamide = 33:1) separating (5 × 8.3 × 0.1 cm) and 8% (acrylamide:bisacrylamide = 33:1) stacking (1 × 8.3 × 0.1 cm) SDS-PAGE according to Laemmli [20]. Electrophoresis was toward the anode at a voltage of 200 V for 60 min. To visualize the protein bands, silver staining was performed according to the manufacturer's recommendations (BioRad, Hercules, CA USA).

2.4. Post-electrophoretic detection of proteolytic activity

2.4.1. Post-electrophoretic detection with chromogenic substrate Glp-Phe-Ala-pNA

Post-electrophoretic detection of proteolytic activity using the cysteine peptidase-specific *p*-nitroanilide substrate Glp-Phe-Ala-pNA was performed by overlaying a nitrocellulose membrane impregnated with the substrate onto native gels as described in Ref. [25].

2.4.2. Post-electrophoretic detection with fluorogenic substrates Glp-Phe-Ala-AMC and Glp-Phe-Ala-AFC

After electrophoresis, native gels were placed in 10 mL of 0.1 M acetate-phosphate-borate Universal Buffer (UB, [28]), pH 5.6, containing 1 mM DTT, 2.5% dimethylformamide (DMF) and 125 μL of 10 mM solution of either fluorogenic substrate Glp-Phe-Ala-AMC or Glp-Phe-Ala-AF in DMF, and incubated for 10 min at 37 °C. Localization of proteolytic activity was visualized under UV light at 366 nm.

2.5. Identification of peptidases by mass-spectrometry

Fluorescent bands of peptidase activity in the gels after native PAGE were excised and subjected to in-gel tryptic hydrolysis. The tryptic peptides were subjected to MALDI-TOF MS and MS/MS analyzes, as described previously [29].

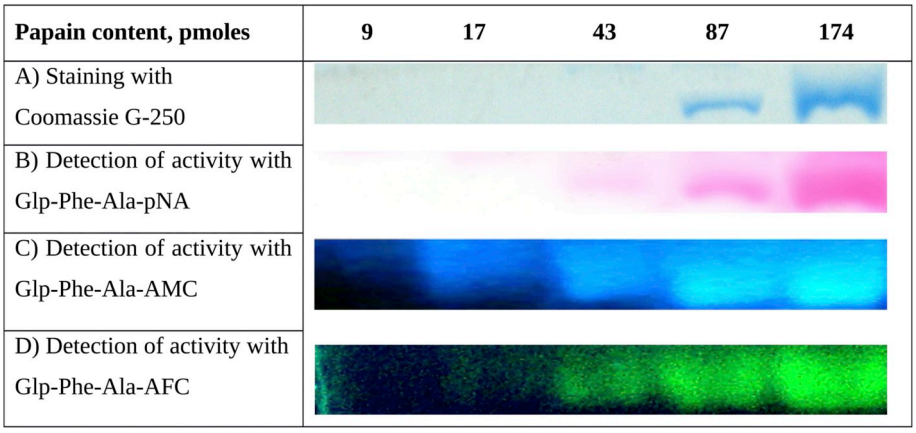

Fig. 1. Different methods of papain detection after native PAGE.

3. Results

3.1. Comparison of different zymography methods for papain detection

In developing the protocol in this study, we compared the detection of the model enzyme papain ranging from 9 to 174 pmoles after native PAGE using different approaches: protein visualization in the gel stained with Coomassie Brilliant Blue G-250 (Fig. 1A), detection of papain activity with the chromogenic substrate Glp-Phe-Ala-pNA by overlaying a membrane impregnated with the substrate (Fig. 1B), and visualization of papain activity directly in gels soaked in buffer containing fluorogenic substrates Glp-Phe-Ala-AMC (Fig. 1C) or Glp-Phe-Ala-AFC (Fig. 1D). Detection of papain activity with fluorogenic substrates was about 5-fold more sensitive than with a chromogenic substrate, and about 10-fold more sensitive than with protein stain Coomassie G-250.

3.2. Identification of cysteine peptidases in Tenebrio molitor

Selective fluorogenic substrates were used to provide the identification of major digestive cysteine peptidases in the gut of *T. molitor* larvae among at least 29 predicted by a *T. molitor* gut transcriptome study [30]. A distinctive feature of digestive cysteine peptidases in *T. molitor* is their high instability caused by autolysis and the presence of a large number of active serine peptidases in the larval midgut [25,26]. Therefore, the correct identification of digestive cysteine peptidases in *T. molitor* and other biological systems is possible only in a narrow window of time after larvae dissection.

In this study, we used gel chromatography fractions from the extract of the anterior midgut of *T. molitor* larvae [26]. Fractions containing active cysteine peptidases were identified with Glp-Phe-Ala-pNA and the major activity fractions were pooled and lyophilized (corresponding to peaks Cys II and Cys III in Ref. [26]). However, further attempts to purify cysteine peptidases from the very heterogeneous Cys II peak proved ineffective due to a significant loss of enzymatic activity and the inability to separate the cysteine peptidases from serine peptidases (data not shown). Therefore, Cys II and Cys III preparations were fractionated by native PAGE, and peptidase activity was detected in the gel by cleavage of fluorogenic substrate Glp-Phe-Ala-AMC (Fig. 2). We

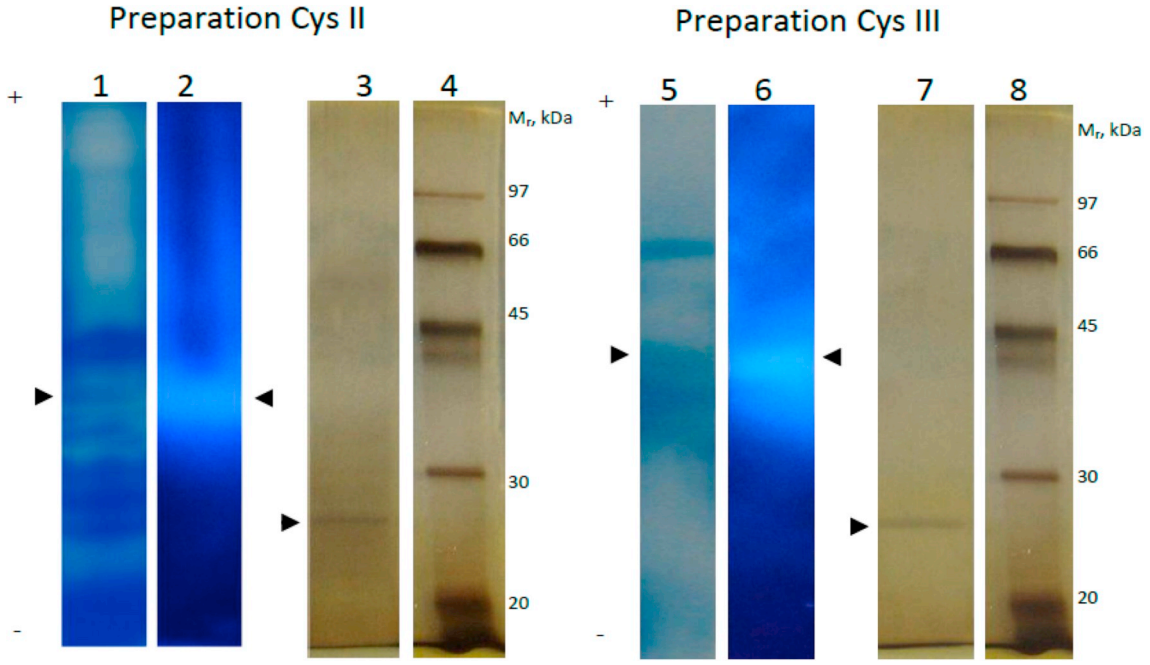

Fig. 2. Electrophoretic analysis of Cys II and Cys III, where TmCysII and TmCysIII are shown by arrows. After native electrophoresis, lanes 1 and 5 were stained with Coomassie G-250 for protein visualization, and lanes 2 and 6 are the activity of cysteine peptidases with Glp-Phe-Ala-AMC. Lanes 3 and 7 are from electrophoresis in denaturing conditions of TmCysII and TmCysIII fraction bands cut from lane 2 and 6, and silver stained; lanes 4 and 8 are molecular mass markers.

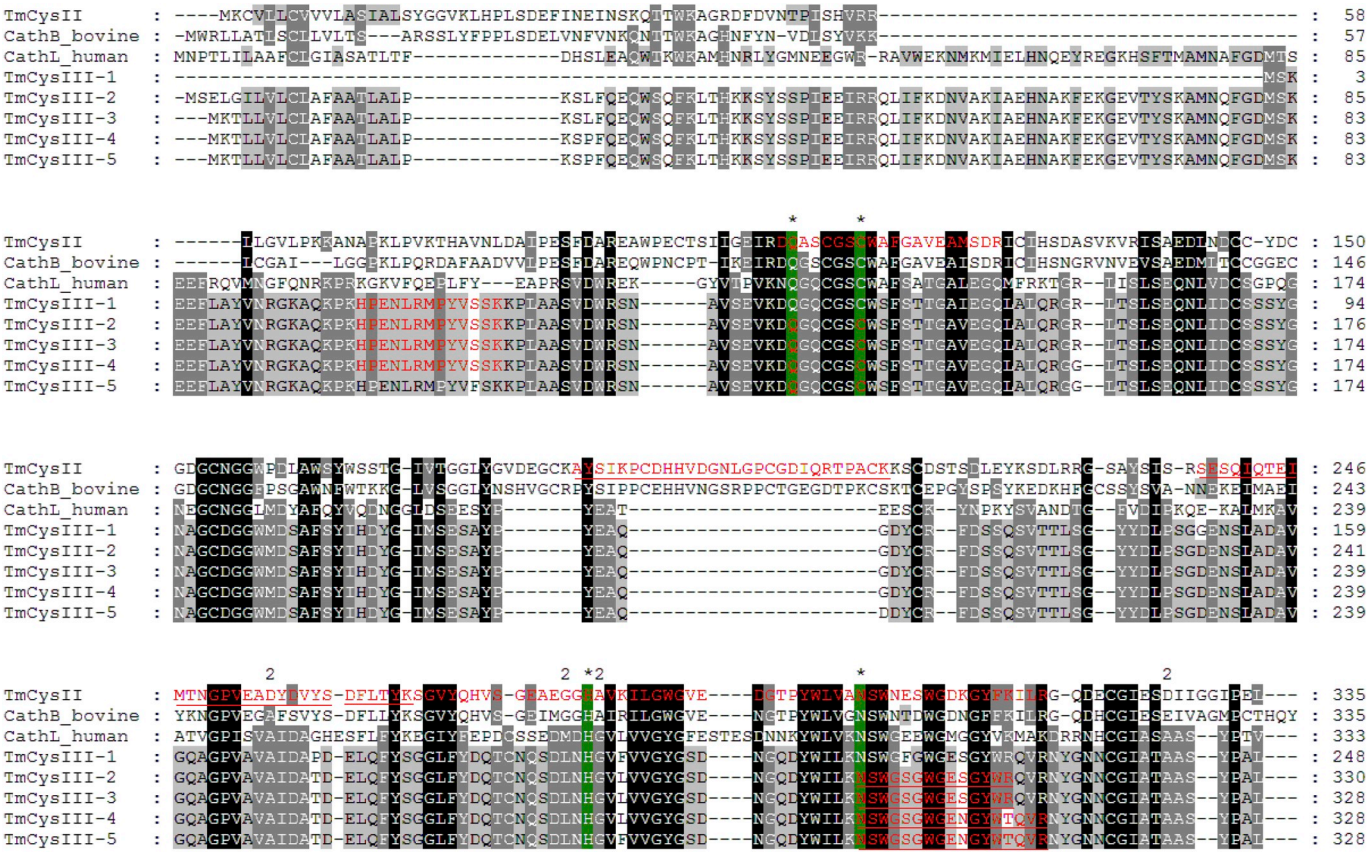

Fig. 3. Alignment of amino acid sequences corresponding to cysteine digestive peptidases from *T. molitor* larvae: TmCysII (AJF94901), TmCysIII-1 (ABC88770), TmCysIII-2 (AAP94048), TmCysIII-3 (ABC88769), TmCysIII-4 (AJF94885) and TmCysIII-5 (ABC88768), compared to bovine cathepsin B (NP_776456) and human cathepsin L1 (NP_001903). Peptides found by peptide fingerprint with MALDI-TOF MS are marked red, and those determined by MS/MS are underlined. Residues Q, C, H and N of the active site are indicated by "*" above the sequences and marked green, amino acid residues in the S2 substrate binding subsite are marked as "2". (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared the total protein in the fractions, visualized by Coomassie G-250 staining (lanes 1, 5), to cysteine peptidase activity visualized by cleavage of Glp-Phe-Ala-AMC (lane 2, 6). Each lane contained one fluorescent band representing TmCysII and TmCysIII activities, which were excised from the gel and subjected to SDS-PAGE and silver staining to demonstrate the purity of cysteine peptidases (lanes 3, 7).

Tryptic hydrolysates of bands containing TmCysII and TmCysIII after native electrophoresis were investigated by MALDI-TOF MS and MS/MS, and peptide sequences were queried in NCBI and transcriptome databases [30] (Fig. 3). TmCysII was identified as a cathepsin B-like peptidase (AJF94901). This enzyme is the major digestive cathepsin B in *T. molitor* larvae, encoded by the TmB33 transcript with the highest level of cathepsin B gene expression in the larval gut [30]. TmCysIII corresponded to a group of cathepsin L-like peptidase isoforms that differ by a few amino acids (ABC88770, AAP94048, ABC88769, AJF94885 and ABC88768, designated as TmCysIII-1 – TmCysIII-5 in Fig. 3, respectively) and encoded by TmL13, which has many SNPs, and has the highest level of expression among cysteine cathepsins in the larval gut [30]. One of the variations in the conserved C-terminus was identified by MS/MS analysis. The alignment of *T. molitor* peptidases with those from mammals indicates that the sequences of the identified peptidases contain conserved motifs characteristic of C1 family peptidases.

4. Discussion

Previous studies have described detection of various peptidases using protein (gelatin) substrates, either individual [18], or in different biological systems including plant tissue extracts [19] or supernatants of cells and tissues [31–36]. Some techniques apply in-gel detection after electrophoresis in mild denaturing conditions, using SDS or lithium dodecyl sulfate (LDS) gels copolymerized with gelatin. After electrophoresis the gel is washed to remove detergent, incubated in the activity buffer and stained with Coomassie stain. The gelatin zymography is a time-consuming method and can cause smearing in activity zones if enzymes are active in the presence of denaturants. The method also is inappropriate for further MS analysis.

Most post-electrophoretic visualization techniques are based on chromogenic reactions coupled with diazotization of the released product, either using overlays of a nitrocellulose membrane [13,16,25,26] or agarose gel [15], or direct detection of activity in the gel after native PAGE [37]. We developed a technique to evaluate the enzymatic activity of a number of serine and cysteine peptidases after native PAGE using chromogenic *p*-nitroanilide substrates, which involved overlaying of a nitrocellulose membrane impregnated with a specific substrate on a gel, followed by diazotization of the released *p*-nitroaniline [16]. The method allowed detection of various proteolytic activities with high sensitivity, so that a thorough analysis of *T. molitor* larvae digestive peptidases was possible [25,26]. Post-electrophoretic detection was successfully used to characterize *T. molitor* larval digestive peptidases [25,26].

Replacement of chromogenic with fluorogenic substrates in post-electrophoretic detection of peptidase activity provides increased sensitivity, and the substrate can either be incorporated into the gel prior to electrophoresis [19,38] or added to a buffer for incubation with the gel after electrophoresis [39]. Peptidase activity also may be visualized by overlay of agarose impregnated with a fluorogenic substrate on the gel after electrophoresis [40]. However, all previous techniques

separated proteins in denaturing conditions, and thus were not applicable for enzymes that cannot be renatured to regain activity. In-gel peptidase detection after native PAGE has been used only for aminopeptidases to determine substrate specificities, content and pH profiles in an unfractionated tissue extract [21,37]. The activity of peptidases was visualized by exposure to UV light, following incubation of the gel in a solution of appropriate fluorogenic substrate.

In our previous work [22,23] we reported new selective fluorogenic substrates for detection of cysteine peptidase activity. Fluorogenic substrates Glp-Phe-Ala-AMC and Glp-Phe-Ala-AFC have greater sensitivity and selectivity compared to the previous chromogenic substrate Glp-Phe-Ala-pNA [24–26]. These substrates provided improved identification of cysteine peptidases after native PAGE due to the ability to quickly identify activity at low levels, and because the fluorescent gel band containing the cysteine peptidase protein was available for downstream analysis.

In this study, we demonstrated that the coupling of our fluorescent substrates with native PAGE provided the ability to distinguish the activities of the two major digestive cysteine peptidases in *T. molitor* larvae, which were then identified by MALDI-TOF MS and MS/MS as cathepsin B and cathepsin L. Previously Cristofoletti et al. [41] identified the major digestive cysteine peptidase of *T. molitor* larvae as cathepsin L (CAL2, AJF94877) using N-terminal sequencing of isolated enzymes after five stages of purification. A second cathepsin L (CAL3, AJF94885) was described as a minor digestive peptidase. The data in this study, together with the data on the corresponding mRNA expression levels of AJF94877 and AJF94885, 1357 and 19,727 RPKM units [30] respectively, indicate that the major digestive cathepsin L in *T. molitor* larvae is instead AJF94885, in agreement with another study [42].

5. Concluding remarks

In this study, we have demonstrated that new fluorogenic peptide substrates provide a selective method for direct detection of cysteine peptidases in complex enzyme mixtures after native PAGE, which can be used to facilitate the identification of peptidases by mass spectrometry. Given the critical role of cysteine peptidases in biological systems, the technique provides new tools for their efficient identification in complex mixtures.

Potential conflict of interest

No conflict.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2018.12.001.

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