Method for determining gelatinolytic activity in tissue:

In situ gelatin zymography

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Running Head: In situ gelatin zymography

Abstract

To explore the physiological or pathological roles of proteases, it is important to be able to detect and precisely localise them in a tissue, to differentiate between inactive and active forms, as well as to quantify and determine the nature of the enzyme that degrade a given substrate. Here we will present an in situ gelatin zymography method that allows for a precise localization of active gelatin degrading enzymes in a tissue section. In this method, dye-quenched gelatin is put on top of a tissue section. During an incubation period, active gelatinolytic enzymes will degrade the substrate and fluorescent signals are emitted from the locations of these enzymes.

Key words

In situ gelatin zymography; tissue; gelatinase; proteases; matrix metalloproteases;

1 Introduction

Proteases cleave proteins and peptides either at the N- and C-terminal ends (exopeptidases) or within the polypeptide chain (endopeptidases). These enzymes exist in all living organisms and there is estimated to be more than 66000 different proteases [1, 2]. They are localized either within a cell, on or in the cell membrane, or secreted from the cell into the extracellular space [3]. Proteases are important for an organisms' survival [4, 5]. Dysregulation of one or several proteases in humans and other vertebrates is associated with disease, and proteases are often involved when micro-organisms invade a host [6, 7, 8, 9]. Hence, proteases are important targets for therapeutic intervention [10, 11].

Proteases are classified into eight different classes/clans based on the amino acid or prostetic group involved in the catalytic reaction. These classes are aspartic (A), cysteine (C), glutamic (G), metallo (M), asparagine (N), mixed (P), serine (S) and threonine (T) proteases (Merops database) [12].

There are more than 566 human and 644 murine proteases, of which 273/341 are secreted, 277/283 are intracellular and 16/16 are intramembraneous, respectively. The majority of human and murine proteases are of the metallo-, serine- and cysteine-type, where most of the metallo- and serine proteases are secreted while the cysteine proteases are mainly localized within the cell [3].

Proteases induce an irreversible change of the substrate they process. Hence, most proteases are tightly regulated at the transcriptional, post-transcriptional, translational and/or the post-translational level. These enzymes are synthesised in an inactive pro-form, and are either activated within the cell or in the extracellular space. Once activated, their activity is regulated by protease inhibitors that bind either reversibly or irreversibly to the enzyme [6, 9, 13, 14].

To explore the physiological or pathological roles of proteases, it is important to be able to detect and precisely localise them in a tissue, to differentiate between inactive and active forms, as well as to quantify and determine the nature of the enzyme that degrade a given substrate. Here, we present a protocol for the detection of active gelatinases in a tissue, i.e. in situ gelatin zymography. There are several enzymes that can degrade gelatin (denatured collagen), and two of them are the two matrix metalloproteases MMP-2 and MMP-9 [15]. Serine proteases like trypsin, plasmin and matriptase as well as cysteine proteases such as cathepsin L can also degrade gelatin [16, 17, 18, 19]. In this protocol we focus on proteases that function around a neutral pH.

In situ gelatin zymography allows for a precise localization of active gelatin degrading enzymes in a tissue section. Dye-quenched gelatin is put on top of a tissue section, and during an incubation period, active gelatinolytic enzymes will degrade the substrate and fluorescent signals are emitted from the locations of these enzymes. Originally, in situ zymography was only performed on frozen tissue sections because formalin, the most commonly used fixative for histology/histopathology practice, disrupts proteolytic activity. Compared to fixed, paraffin embedded tissue sections, frozen sections are generally thicker and the tissue morphology more difficult to interpret. This can hamper the precise localization of the enzyme activity by in situ zymography. We have demonstrated that some fixatives, including ethanol and a zinc-buffer based fixative (see Note 1), preserve the activity of gelatinolytic enzymes, allowing in situ zymographic analyses of fixed, paraffin embedded tissue with superior morphology [20]. Immunofluorescence is a frequently used method to localize specific proteases in a tissue and can be used along with in situ zymography to establish if a specific protease co-localizes with protease activity detected by in situ zymography. Another option is to extract proteases from the tissue used for in situ zymography, and analyse the extracted proteins by gelatin SDS-PAGE zymography (see protocol for “Method for

determining gelatinolytic activity in tissue extracts: Real-time gelatin zymography" by Hadler-Olsen and Winberg in this volume). For both in situ and SDS-PAGE gelatin zymography, various types of inhibitors can be used to determine the type of protease responsible for the substrate degradation (see Note 2). Figure 1 shows an example of our in situ zymography method, using zinc buffer fixed mouse mammary gland tissue.

"[Fig 1 near here]"

2. Materials

2.1 Reagents and buffers

1. Xylene or Histoclear (for paraffin embedded tissue).
2. 100% ,95% and 70% ethanol (for paraffin embedded tissue).
3. Adhesive/positively charged glass slides.
4. Formalin 10% (for frozen sections).
5. Fluorescent mounting media, cover slips
6. Optional: enzyme inhibitors such as EDTA, Galardin, Pefabloc.
7. Optional: nuclear stain such as DAPI or propidium iodide (PI). DAPI solution: DAPI stock (10 mg/mL) is diluted 1:10,000 in PBS.
8. Phosphate buffered saline (PBS): 10 mM phosphate, 2.7 mM KCl and 137 mM NaCl, pH 7.4
9. In situ zymography reaction buffer: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 mM sodium azide.
10. Dye-quenched (DQ) gelatin: Dissolve 1 mg DQ gelatin (1 vial) in 1.0 mL Milli-Q water. Protect the substrate from light. The dissolved substrate can be stored dark at 4 °C for some time (at least 2 months).

11. DQ gelatin working solution and control solutions: Dilute the dissolved substrate (DQ gelatin) 1:50 in the in situ zymography reaction buffer to make the working solution for in situ zymography. Calculate 250 µL of working solution per section. To evaluate the contribution of various enzyme classes to the gelatinolytic activity, working solutions containing both DQ gelatin and enzyme inhibitors can be used on control slides. See Note 2 regarding controls. To control for auto-fluorescence in the tissue, the in situ zymography reaction buffer without substrate (DQ gelatin) added can be used on control slides.

2.2 Equipment

1. Microtome (for paraffin embedded tissue) or cryostat (for frozen tissue).
2. Heating cabinet (37 °C and 58°C).
3. Humidity chamber (opaque).
4. Fluorescent microscope or confocal microscope with filters/lasers to detect FITC and DAPI (optional).

3 Methods

3.1 Cut tissue sections

Zinc-buffer fixed or ethanol-fixed, paraffin embedded tissue

1. Cut 4-5 µm thick tissue sections on a microtome.
2. Put a tissue section on an adhesive/positively charged glass slide.
3. Incubate in a heating cabinet set to 58°C for about 3 hours to let the section adhere to the slide and to remove most of the paraffin.

Frozen tissue

4. Cut about 10 µm thick sections
5. Put a tissue section on an adhesive/positively charged glass slide.
6. Let the tissue adhere to the glass slide by incubating it at room temperature for about 30 minutes.

3.2 Preparation of tissue sections

Deparaffinization and rehydration of fixed, paraffin-embedded sections

1. Deparaffinize the sections in xylene or histoclear (two baths, 10 minutes each).
2. Rehydrate the sections in graded alcohol baths (100% ethanol 5 min x 2, 95% ethanol 5 min x 2, 70 % ethanol 5 minutes x 2, water).
3. After rehydration, it is important to keep the sections humid.

Removal of traces of embedding medium such as OCT from frozen tissue

4. Rinse the sections in a PBS bath for 5 minutes.
5. Keep the sections humid during the remaining procedures.

3.3 Incubation of tissue sections with DQ gelatin and analysis

Incubate tissue sections with either DQ gelatin working solution or control solution. Remember to protect the sections from light in all steps below.

1. Pipet 250 µL of the DQ gelatin working solution or control solution (in situ zymography reaction buffer) to each tissue section.
2. Cover with a piece of parafilm, the size of the slide.
3. Incubate in a ***dark*** humidity chamber at 37°C. Incubation time must be optimized for the tissue of interest. It is wise to first run a pilot with different incubation times from 30 minutes to 6 hours.

4. Remove the parafilm gently.
5. Rinse off the substrate with Milli-Q water - don't direct the water flow straight on the tissue.
6. Rinse the sections further in PBS baths (5 min x 2).
7. If using frozen, unfixed sections: After step 6 above (method 3.3), fix the section by adding 500 µL of 10% formalin to the section or put in a formalin bath for 5 minutes (in a safety cabinet). Remove the formalin and rinse as above.
8. Optional (for both fixed, paraffin-embedded tissue and frozen tissue): To help orienting the tissue, you can counter-stain the nuclei with a fluorescent nuclear stain such as DAPI or PI. Some fluorescent mounting media contain DAPI or you can do a separate DAPI staining prior to mounting. If separate DAPI staining is performed, the following protocol may be used: sections are incubated with DAPI solution for 5 minutes. Rinse with PBS (5 minutes x 2) and then mount in a mounting medium for fluorescence.
9. Mount a cover glass over the tissue section and seal with nail polish.
10. Analyse in fluorescence microscope or confocal laser microscope. The DQ gelatin is labelled with (fluorescein isothiocyanate) FITC which has a λ_{ex} at 495 nm and λ_{em} at 517 nm. The DAPI has a λ_{ex} at 358 nm and λ_{em} at 461 nm. The fluorescence of FITC is pH dependent (see Note 3)

4. Notes

1. Tissue fixation: Formalin disrupts all gelatinolytic activity, whereas some fixatives preserves enzyme activity such as 70% ethanol or zinc-buffer fixative. The formula for the zinc-buffer fixative is as follows: Calcium acetate 0.5 g, Zinc chloride 5.0 g, Zinc

acetate 5.0 g, dissolve in 1 L 0.1 M Tris-HCl buffer, pH 7.4. Mix to dissolve. The final pH will be approximately 6.8 (6.5-7.0).

2. Control slides: A number of different enzymes can degrade gelatin. To evaluate the contribution of specific enzymes or classes of enzymes, inhibitors may be added to the in situ zymography reaction buffer. Make sure to add a sufficient concentration to inhibit the enzymes of interest. EDTA inhibits all metallo-dependent enzymes, Galardin inhibits matrix metalloproteases, Pefabloc inhibits serine proteases etc. Combinations of inhibitors can also be used.
3. The fluorescence of FITC is pH dependent with a plateau between pH 7-9. The fluorescence decreases drastically below pH 7 [21] and hence this DQ gelatin is not recommended for studies of gelatin degrading enzymes with a low pH optimum.

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