Zymography

Instrumentation and Biotechniques,
DSE-7, Sem VI

Zymogram Gels

- Zymogram Gels are excellent tools for detecting and characterizing proteases that utilize casein or gelatin as a substrate. Casein and gelatin are the most commonly used substrates for demonstrating the activity of proteases.
- Zymogram Gels can be used to analyze a variety of enzymes, including matrix metalloproteinases, lipases, and other proteases. Mostly Zymogram Gels are based on tris-glycine gel chemistry containing gelatin or casein as the substrate.

Zymogram PAGE

- Zymogram PAGE is used to detect and characterize collagenases and other proteases within the gel.
- Gels are cast with gelatin or casein, which acts as a substrate for the enzymes that are separated in the gel under nonreducing conditions. The proteins are run with denaturing SDS in order to separate by molecular weight. After renaturing the enzymes and then allowing them to break down the substrate, zymogram gels are stained with Coomassie (Brilliant) Blue R-250 stain, which stains the substrate while leaving clear areas around active proteases.

- Zymography is an electrophoretic technique, based on SDS PAGE for measuring enzyme activity
- The technique is particularly useful for analyzing the proteinase composition of complex biological samples
- Zymography is also widely used to study various aspects of matrix metalloproteinase (MMP) function

Types of zymography

- SUBSTRATE ZYMOGRAPHY:
  - Gelatin Zymography
  - Casein Zymography
  - Collagen Zymography
  - Heparin-Enhanced Substrate Zymography
- REVERSE ZYMOGRAPHY
- In Situ ZYMOGRAPHY

SUBSTRATE ZYMOGRAPHY

- when specific substrate is co-polymerized with the acrylamide
- In zymography, the proteins are separated by electrophoresis under denaturing [sodium dodecyl sulfate (SDS)], nonreducing conditions
- The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with the acrylamide

- SUBSTRATE ZYMOGRAPHY: gelatin or casein in the gel
- Gelatin Zymography –Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9
- Casein zymography –suitable for the detection of MMP-1, MMP-7, MMP-12, and MMP-1

- Collagen zymography – used for the detection of MMP-1 and MMP-13, but MMP-2 and MMP-9 can also be detected – The incorporation of native collagen fibers in polyacrylamide gels appears unsuitable for zymography because of their complicated structure, but SDS disrupts most of the fibrillar organization of the collagen, allowing proteins to run into the gel.

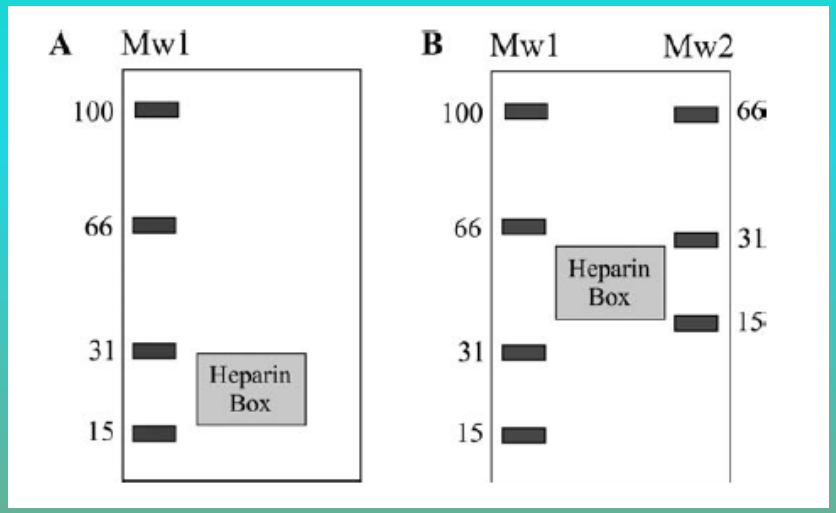
Heparin-Enhanced Substrate Zymography

It is known that the extraction of MMPs from tissue in the presence of heparin results in an enhancement of MMP activity.

The addition of heparin to the samples during or prior to electrophoresis also enhances MMP activity.

Used for MMP-7

The mechanisms by which heparin seem to enhance MMP-7 activity in zymography are: – (i) the induction of a conformational change – (ii) the facilitation of refolding – (iii) the reduction of autolysis – (iv) the increase of anchorage of the MMP in the gel



Advantages of substrate Zymography

- Expensive materials are not required (e.g., antibodies)
- Proteaseswith different molecular weights showing activity towards the same substrate can be detected and quantified on a single gel. – For example, MMPs are released from cells in a proteolytically inactive proform(zymogen) which is approximately 10 kDlarger than the activated form – Because the proform becomes activated during the process of denaturation and renaturation after gel electrophoresis, the active form and the originally inactive forms degrade gelatin, and both forms can therefore be detected on zymograms.

Advantages of substrate Zymography

- MMPs in solution are often associated with endogenous tissue inhibitors of metalloproteases (TIMPs). • During electrophoresis the inhibitors dissociate from the MMP and do not interfere with detection of the enzymatic activity. • On the other hand, sandwich ELISA can discriminate between MMP/TIMP complexes and free MMPs, resulting in determination of a potential active fraction (Zuckeret al. 1992; Ratnikovet al. 2002; Catteralland Cawston2003).
- On the basis of molecular weight markers, the molecular weight of the proteolytic band can be determined, and by comparison with recombinant proteins and the use of specific protease inhibitors the type of protease can be established

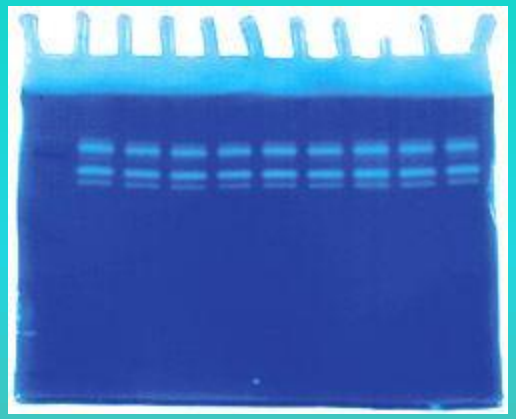
DISADVNATAGES of SZ

- Information on the localization of the proteolytic activity in cells or tissues cannot be obtained on the basis of zymography.

Main steps in gelatin substrate zymography:

- 1. Sample homogenization without a reducing agent or boiling in order for the enzyme to retain its native state (and therefore its proteolytic activity).
- 2. Preparation of a gel with a final concentration of acrylamide of 8%, containing 2 mg/mL gelatin
  3. Electrophoresis at 90 V constant voltage (no boiling, no use of reducing agents in order to preserve enzyme activity)
- 4. Gel washing in Triton X-100for 40 min at room temperature on anorbital shaker

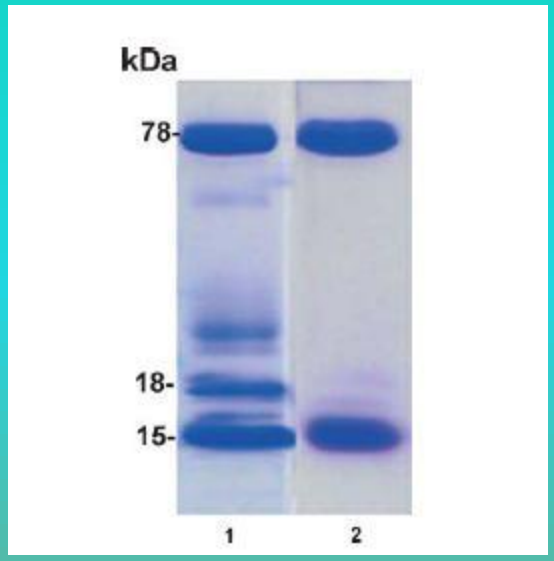
- 5. Gel washing in incubation buffer for 20 min • 6. Incubation of gels for 20 h at 37 °C 7.
- 7. Staining of the gels with CoomassieBlue for 30 min at room temperature. Areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme
- 8. Destaining with a methanol/acetic acid solution for 1 h at room temperature
- 9. Densitometry of gelatinolytic activity appearing as clear bands on the dark background



MMP-9
proMMP-2
MMP-2

Reverse Zymography

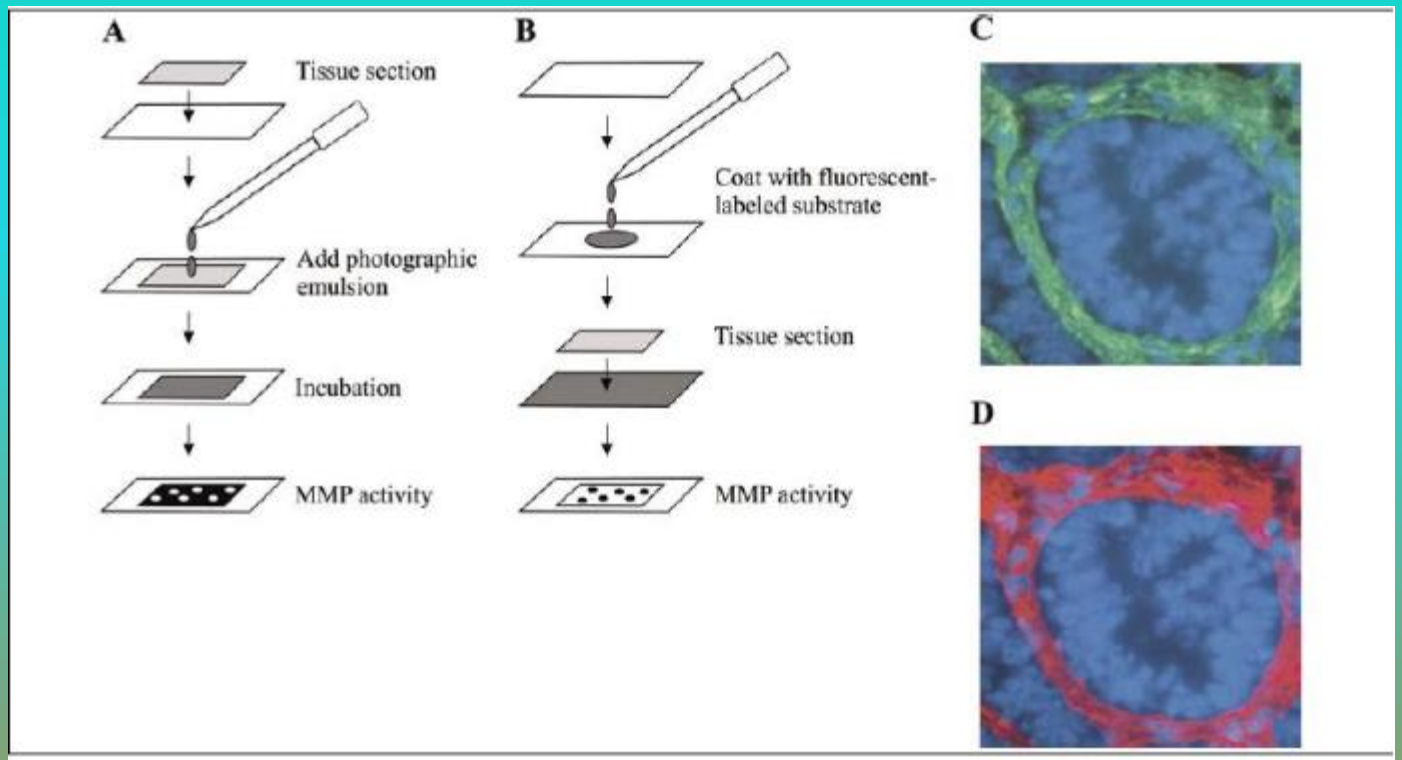
- TIMPscan be detected by reverse zymography, which is a modification of zymography for MMPs
- Besides gelatin, an MMP is also incorporated into the gel, usually MMP-2.
- During the activation step after electrophoresis, the MMP-2 only digests the gelatin in areas where TIMPs are absent.
- Thus, after staining, the gel will be colorless, except for the TIMP bands



In situ zymography

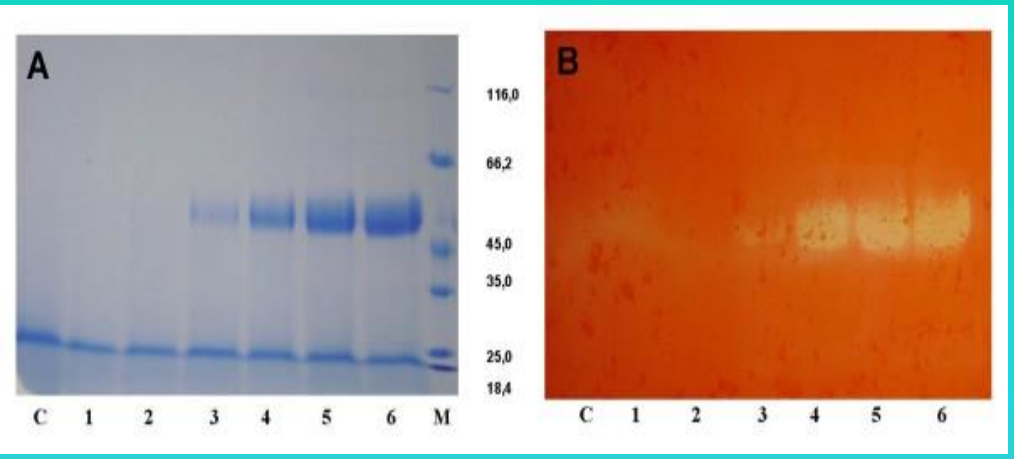
- In situ zymography allows the localization of MMPs in tissue sections
- in situ zymography uses a substrate that is deposited on or under a frozen section of an unfixed tissue sample
- During incubation, the substrate will be digested by the activated MMPs in a time-and dose-dependent manner
- The degradation of the substrate is detected by light microscopy or fluorescence microscopy, depending on the type of substrate.

In situ zymography

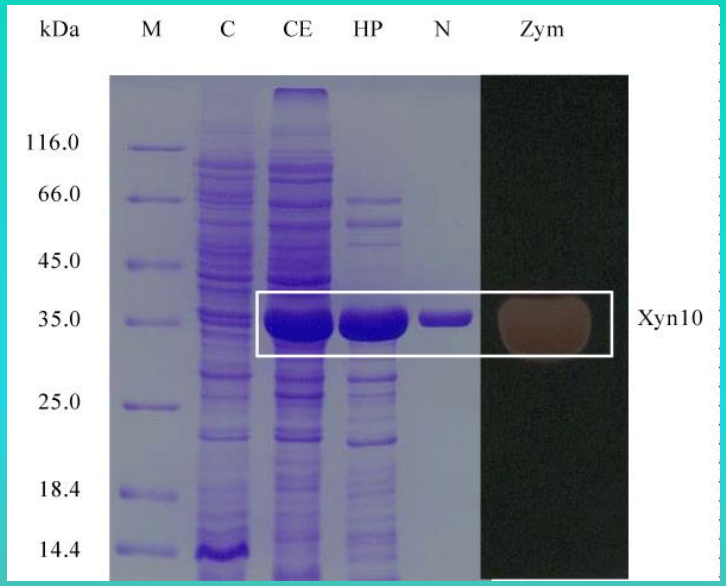


The red and green signal indicates the immunolocalization of MMP-2

examples



SDS page and Zymogram analysis of recombinat protein at different time points
C- control, 6, 24, 48, 36, 72 and 96 hours. Lane M ladder,



Purification process of Xyn10 and zymogram analysis. Proteins were separated via SDS-PAGE (gel 12 %) and stained with Coomassie brilliant blue. For zymogram analysis the gel was incubated for 1 h at 70 °C in a solution of beechwood xylan (1 %, w/v), stained with Congo red and decolorized in 1 M NaCl. kDa: kilo Dalton, M: Unstained Protein Molecular Weight Marker (Fermentas), C: control (crude extract of *E. coli* BL21 (DE3)/pQE-80L), CE: crude extract of *E. coli* BL21 (DE3)/pQE-80L::*xyn10*, HP: heat precipitation, N: Ni-NTA affinity chromatography, Zym: zymogram.