Review Article

International Journal of Research in Pharmaceutical Sciences

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Zymography-types, advantages, uses and troubleshooting - A detailed review

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Article History:

Received on: 15 Jun 2021
Revised on: 19 Jul 2021
Accepted on: 23 Jul 2021

Keywords:

Zymography,
Matrix metalloproteinases,
In-Gel zymography (IGZ),
In-Situ zymography,
In-vivo zymography

Abstract

There are many methods for the study and quantification of enzymes in the body systems. Off-late, the method of zymography has assumed immense importance in diagnostic medicine. It is a method where enzymes are visualized using the substrate conversion technique. The product of reaction appears, or the substrate disappears and the mechanisms specific for the recognition of this measure the biochemical reaction. This procedure has many advantages, the most important ones being providing both qualitative and measurable data, such as molecular zymography methods for visualising hydrolytic enzymes and differentiating among whole molecules, degradation and complexes. There are three main types of zymography, namely- In-Gel zymography (IGZ), In-Situ zymography (ISZ) and In-vivo zymography (IVZ). There are many variations of this technique like transfer 2D and reverse zymography. This method is mainly used to study the expression of matrix metalloproteinases. Alternate to the conventional zymography techniques have been suggested, like usage of the new Ponceau S staining protocol that provides significant benefits in terms of assay usability and cost reduction and is comparatively faster and easier. Mild alterations in the actual procedure of zymography can take care of minor issues arising during the procedure.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v12i3.4833

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INTRODUCTION

There are many methods for the study and quantification of enzymes in the body systems. Off-late, the method of zymography has assumed immense importance in diagnostic medicine. It is a method where enzymes are visualized using the substrate conversion technique. It was introduced by Gross and Lapiére in 1962 when they first described the Matrix metalloproteinase -1 (MMP-1).

The product of reaction appears, or the substrate disappears and the mechanisms specific for the recognition of this measure the biochemical reaction (Vandooren *et al.*, 2013).

There are three main types of zymography-In-Gel, In-Situ and In-Vivo zymography and many variants of this technique which have found immense use in many fields of science. The main advantage of this method is that it provides both qualitative and quantitative data for analysis, unlike the other conventional techniques like microtitre plates.

This article provides an in-depth review about zymography, its basic protocol, advantages, types, variants, new modifications to the existing technique and troubleshooting aids useful in practical applications.

Basic Procedure

"Zymography" is a common electrophoretic method for determining proteolytic activities in a variety of samples. An appropriate protein substrate is copolymerized inside a sodium dodecyl sulfate-polyacrylamide gel in this process. Degradation of the substrate can be visualised using Coomassie blue staining after electrophoretic isolation of the samples containing protease and an appropriate incubation time. Proteolysis sites appear with a dark blue background in which white bands appear (Wechselberger *et al.*, 2019). It usually comes across as a reaction which is chromogenic or fluorogenic, accounting for enzyme visualisation on a gel, depending on the type of detection conducted (Wilkesman and Kurz, 2017). The following chemistry is used in some of the most important chromogenic reactions:

1. Tetrazolium salts are reduced.
2. Using diazonium salts in combination.
3. Changes in the pH of the local environment.
4. Orthophosphate or pyrophosphate development.
5. Formation of hydrogen peroxide (redox dye is used to detect this).
6. Carbon dioxide production.
7. The chromogenic substrates.

Fluorogenic recognition is an option in addition to chromogenic detection. Since the normal fluorochromes NADH and NADHP lose their fluorescence after oxidation, they are widely used (Wilkesman and Kurz, 2017).

Advantages

Unlike purely measurable test tubes or microtiter plate detecting modes for the transformation of the substrate, zymography provides both qualitative and measurable data, such as molecular zymography methods for visualising hydrolytic enzymes.

Zymography differentiates among whole molecules, degradation and complexes, unlike enzyme-linked immunosorbent assays (ELISAs), which cannot do so (Vandooren *et al.*, 2013).

This technique also has additional advantages of (a) being able to identify and measure several proteases operating on the same substrate in tandem, and (b) detecting proteolytically inactive pro-forms of proteinases since these are triggered during the renaturation process, and (c) As tissue inhibitors of metalloproteinases (TIMPs) are removed during electrophoresis, overall enzymatic activity in a given sample can be determined (Wechselberger *et al.*, 2019).

When studying unpurified enzyme preparations, zymography offers a range of merits, like the capacity to study an extensive array of biological samples, including cell and tissue samples, plasma, fluids of culture and also species as a whole (Wilkesman and Kurz, 2017).

1. Isozyme detection and recognition in extracts of cells in the crude form.
2. Characteristics of isozymes pertaining to their function, straight in the gel.
3. Measuring the enzyme's isoelectric point and molecular mass and gives quantitative and qualitative detail.
4. In diverse biological and medicinal samples, monitoring particular and non-specific enzymatic processes
5. Possible subunit composition determination
6. Discernment of enzymes with identical or conflicting substrate particulars

Zymography offers both general and detailed knowledge in the field of pure and applied biochemistry. Zymography has had a greater effect on clinical and diagnostic medicine. Normal zymography assays are used in procedures for tumors, cardiac and artery disorders, and disorders with an inflammatory or immunological basis. Animal research, as well as agrarian science, forensics and other fields, use zymography.

In contrast to other biotechnological methods, zymography remains a straightforward and effective method for partitioning, identifying, and characterising gene products. Provided its benefits, proteomic analysis is a powerful ally in zymography. Two-dimensional zymography (2DZ) provides useful material for further analysis by mass spectrometry by means of"Two-dimensional electrophoresis (2DE)" and "transition zymography". The amalgamation of modular enzyme assay practices and electrophoresis is a significant method for meeting the increasing need in protein manufacturing, genomics and combination chemistry. Considering the significance of zymography's effect, it is easy

to imagine why research into new and improved enzymatic analysis methodologies should be continued. (Wilkesman and Kurz, 2017)

Types

1. In-Gel zymography (IGZ)
2. In-Situ zymography (ISZ)
3. In-Vivo zymography (IVZ)

In-Gel zymography (IGZ)

Molecular weights of hydrolytic enzymes separate them and these are identified by their capacity to disintegrate a substrate in an IGZ experiment. This method can be used to examine any lysate, cell culture, or bodily secretion. Carbohydrates and proteins like chondroitin sulphate and collagen are major substrate groups (Vandooren *et al.*, 2013).

In the traditional IGZ process, the substrate is copolymerized with acrylamide in the Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) separating gel. After isolating the non-reduced SDS-PAGE, the SDS is substituted with nonionic detergent with a concentration of micelle that is less than the critical value. Enzymes will partly refold and conform to their active form during this process. The gel is immersed in a buffer containing basic cofactors which allows the enzymes to dissolve the copolymerized substrate. After staining, the proteolytic areas on this zymogram become clear. (Vandooren *et al.*, 2013)

In-Situ zymography (ISZ)

In tissue sections, ISZ can recognise and localise specific protease activities. ISZ, unlike IGZ, can estimate net enzymatic activity due to the presence of inactive proforms and enzyme-inhibitor complexes which are undetectable. Immuno histochemistry(IHC) is a commonly used tool for detecting immunoreactivity, but it can't always tell the difference between active and inactive enzymes, while ISZ confirms net functional activity. As a result, combining ISZ and IHC to analyse proteases in tissue samples is a powerful technique. ISZ allows for the development of a 2D map of proteolysis in vivo. A three-dimensional (3D) image can be reconstructed using serial segment analysis, theoretically allowing IVZ findings to be validated. (Vandooren *et al.*, 2013)

In-Vivo zymography (IVZ)

IVZ allows for the detection of lysis of protein in a living organism. The method is used to diagnose the remaining MMP behaviour in vital tissue as well as entire species like emerging humans, zebrafish or mouse embryos. As a result of these zebrafish experiments, it has become apparent that useful knowledge may vanish in the ISZ and IGZ as a result of processes involved in the preparation of samples. When tissue samples are homogenised in IGZ, the average distribution of enzyme activators is determined. Tissue sectioning in ISZ may be used to artificially combine enzymes and inhibitors. IVZ, on the other hand, has the ability to reveal information about the temporal and spatial (real-time 3D) dispersal of proteolytic behaviour through an entire organism.

IVZ necessitates a biocompatible fluorogenic substrate that can be detected after breakdown due to lysis of protein.

There are various methods available. One method is to use quenched Förster resonance energy transfer (FRET) peptides that have a group acting as a quencher on one end and a fluorophore on the other. There is an exchange of energy between the quencher and the fluorophore. The fluorescence is small as both groups are in a direct connection to the peptide, which is intact. The fluorescence is evident when the peptide cleaves and there is a scattering of quencher and fluorophore. There are a variety of pairs of quencher and fluorophore with various spectra of fluorescence visible. As a result, a combination of peptides that are FRET-quenched with various sequences is utilized to track several enzymes at the same time (Vandooren *et al.*, 2013).

Variations of the Technique

Transfer Zymography

Electrophoretic transfer zymography is a technique worth noting. First, the enzyme is resolved in a gel of polyacrylamide lacking protein substrate and then moved to the gel of receptor that has the substrate. This move avoids the problem of certain enzymes migrating in a substrate gel with a lower mobility value. The enzyme mobilities obtained by transfer zymography are equivalent to those acquired by SDS-PAGE that is non-reduced.

2D-Zymography

Two-dimensional zymography is another choice (2DZ). The sample that has to be analysed is primarily subjected to isoelectric focusing on Immobilized pH Gradient (IPG)-strips in this situation. After that, the strip is subjected to traditional IGZ, which adds an additional aspect to the study by separating proteins by molecular mass and by isoelectric point.

Reverse Zymography

Another variant of IGZ, known as reverse zymography that allows identification of inhibitors of the enzyme, is also significant. Copolymerized enzymes

and substrate are inside the matrix of the gel for this function. The copolymerized substrate is uniformly degraded after proper reactivation of the enzyme, barring certain spots in which the inhibitor is active, blocking the activity of the enzyme. Post staining, the location of the inhibitor can be visualized as bands of an intact substrate (Wilkesman and Kurz, 2017).

Uses

There are several uses of zymography, the most important of which is to study the expression of MMPs. Several methods for studying the expressions and behaviours of MMPs have been published in the literature like IHC, ELISA etc. Zymography is an important approach that uses SDS-PAGE gels copolymerized with a protein substrate to electrophorese (Lisboa *et al.*, 2013).

MMPs are identified using zymography, which measures the rate at which their preferred substrate degrades as well as their molecular weight (Vandooren *et al.*, 2013).

Gelatin zymography, a commonly used and sensitive procedure, and enamel proteins are used as MMP-20 substrates. Zymographic analysis can be used for the analysis of MMP-2, 3, 7, 9 and 10 (Boelen *et al.*, 2019).

This method can be used to decide whether the MMP is active or latent. MMPs must be found in tissue in order to be identified. In situ zymography may be used to locate MMPs in tissue sections. Reverse zymography, which is dependent on TIMPs' ability to inhibit MMPs, will detect them (van Beurden and den Hoff, 2005).

MMP-2 and MMP-9 proteolytic activity is measured using gelatin as a protein substrate, casein for MMP-3, MMP-7 and MMP-10, and collagen for MMP-1. Zymography enables the active and latent types of MMPs to be identified in a composite mixture such as cultured media or filtered preparations depending on molecular weight (Lisboa *et al.*, 2013).

The celiac disorder is triggered by gluten consumption in HLA-DQ2 or HLA-DQ8 carriers that are genetically predisposed. Multiple glutamines and proline residues in immunogenic gliadin epitopes make them immune to digestion by proteases. But microbes in saliva demonstrate"glutamine endoprotease activity", revealed to glutamine- and proline-rich proteins in saliva. The goal was to study if gliadins can act as substrates for enzymes of oral microbes. According to gliadin zymography performed, important enzymes have a molecular weight of 70 kD and are active at pH levels ranging from three – ten (Helmerhorst *et al.*, 2010).

"Dentine matrix gelatinases" are thought to be implicated in the degradation of decayed dentine after it has been demineralized by bacterial acids. Chlorhexidine has also been shown to obstruct the activity of these enzymes in cells of mammals at extremely low concentrations. The aim of this analysis was to see whether carious gelatinase activity could be measured. Zymography was used to assess gelatinolytic activity, and a Western blot was used to identify it. The enzymatic activity of softened decayed dentine samples was prevented by "chlorhexidine (CHX) at concentrations of 0.01 percent, 0.04 percent, 0.08 percent, and 1 percent" (Trufello *et al.*, 2014).

Another application of zymography was studied by Gu et al., "A chelate-and-rinse extrafibrillar calcium chelation" conception of adhesion of dentin was developed and tested for efficacy in "strengthening resin-dentin bonding by bridging the wet-dry dentin bonding" distance. Post-one-day"storage or thermomechanical cycling, operation inside the HL was investigated using in situ zymography and confocal laser scanning microscopy". Bonding was done with dry-bonding and wet-bonding strategies for each reagent (n = 10). A confocal laser-scanning microscope analyzed the adhesive-dentin interface, which was coated with fluorescein-conjugated gelatine." The strength of fluorescence produced by hydrolyzed fluorescein-conjugated gelatin was measured".

The activity of enzymes was only observed inside fully demineralized dentin etched with phosphoric acid after 24 hours of preservation, with dry bonding values being higher than wet bonding values (P 0.05. When dentin was primed with PAANs, practically nil signs of fluorescence were observed in the HL relative to the controls (P 0.05). "The phosphoric acid–conditioned, drying-bonding group's enzymatic activities improved dramatically after thermomechanical cycling relative to 24-hour storage (P 0.05)". And the main point was that ageing will still have HL. The retention of minerals within the fibrils inside the "dentin collagen matrix" may have resulted in the fossilisation of endogenous proteases (Gu *et al.*, 2018). See Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5.

"Dentin matrix metalloproteinases (MMPs)" are not well known in terms of their position and function, but they play a crucial part in the decay of dentin and the breakdown of a matrix of "resin-bonded dentin". "This study employed gelatin zymography to remove and separate the molecular types of gelatinolytic MMPs in demineralized mature sound dentin powder obtained from harvested human

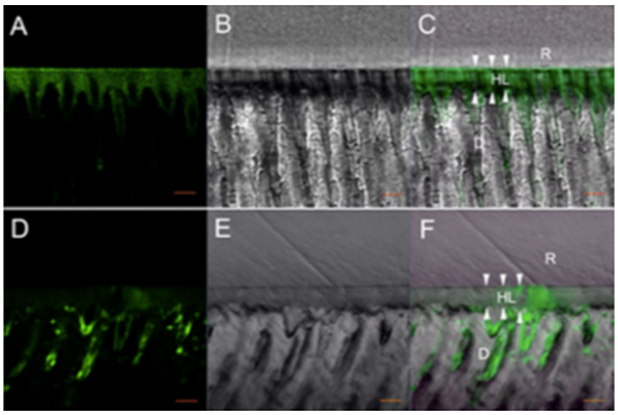

Figure 1: Resin-dentin interface conditioned with 15% phosphoric acid for 15 s, with adhesive bonded using the dry-bonding (A–C) or the wet-bonding (D–F) technique after storage for 24 h (T)

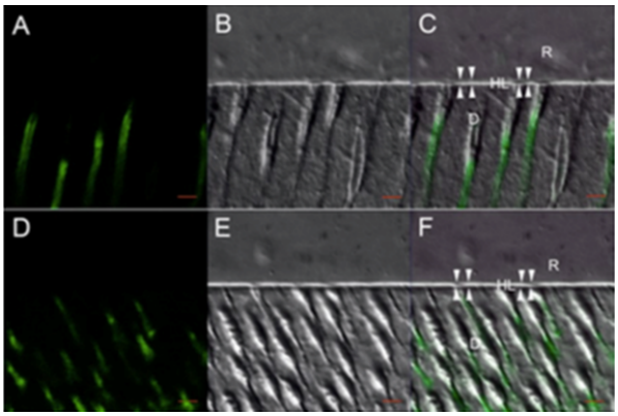

Figure 2: Resin-dentin interface conditioned with 15 wt% sodium salt of polyacrylic acid (PAAN)(similar features for the 2 PAANs of different molecular weights; Mw 225,000shown as representative) for 60 s and prepared using the dry-bonding (A–C) or the wet-bonding (D–F) technique after storage for 24 h(T0)

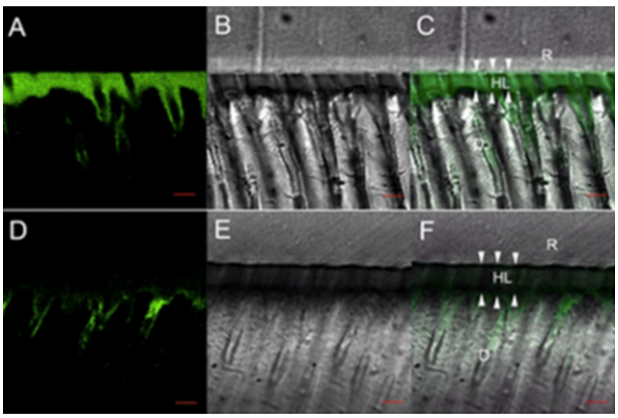

Figure 3: Resin-dentin interface conditioned with 15% phosphoric acid for 15 s and bonded using the dry-bonding (A–C) or wet-bonding (D–F) technique after thermomechanical cycling (T1)

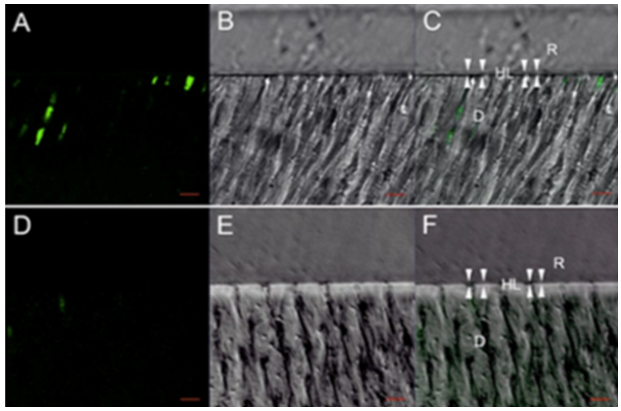

Figure 4: Resin-dentin interface conditioned with 15 wt% sodium salt of polyacrylic acid (PAAN)(similar features for the 2 PAANs of different molecular weights; Mw 225,000shown as representative) for 60 s and prepared using the dry-bonding (A–C) or wet-bonding (D–F) technique after thermomechanical cycling (T1)

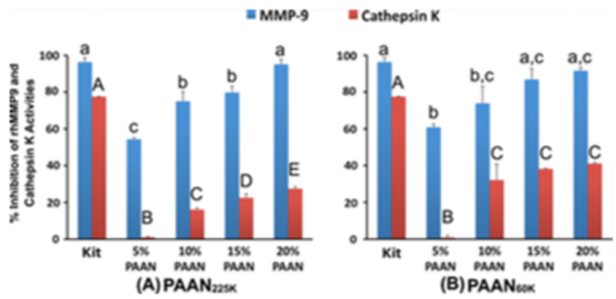

Figure 5: Percentage inhibition of rhMMP-9 and cathepsin K by increasing concentrations of PAANs of different average molecular weights (indicated by subscripts after PAAN), (A) $PAAN_{225k}$ and (B) $PAAN6_{0k}$

molars, and then used Western blotting to characterise and classify the enzymes".

MMP-2 and MMP-9 were identified as gelatinolytic MMPs in extracts of demineralized dentin matrix. MMP activity was 3-8 times higher in acidic extracts (pH 2.3) than in EDTA (pH 7.4). The consensus was that their activation can play a role in dentin matrix deterioration, which occurs as caries progresses and after resin bonding. MMP-2 and -9 proteolytic activity inhibitions can delay caries progression and improve resin-dentin bond durability (Mazzoni *et al.*, 2007).

In situ zymography is a critical tool for observing the unique spots of lysis of proteins in tissues or cells. It's mostly done on non-fixed frozen parts, and it's not done on calcified tissues very often. "The study by Porto et al. defined a technique for preserving proteolytic activity in fixed and decalcified parts obtained from traditional microtome and cryostat

sections after regular paraffin sectioning". Finally, they demonstrated that gelatinolytic activity and protein labelling with antibodies were colocalized and that this technique presented would enable ISZ reactions in fixed, decalcified and tissues impregnated with paraffin (Porto *et al.*, 2009).

The role of endogenous MMP-3 and where it is found in the human dentine are unknown. The aim of this research was to use biochemical and immunohistochemical assays to determine the occurrence and dispersal of MMP-3 within healthy human dentin. The methodology followed was that the dentin from human teeth was pulverized and either (a) partly demineralized with 1% H_3PO_4 (Phosphoric acid) for 10 minutes or (b) left untreated (control). A colorimetric assay method was used to determine the identity of MMP-3 (QuantiSirTM, Epigentek, USA). Under FEI-SEM (FEI-Scanning Electron Microscope), additional cryofractured dentine pieces were treated for immunohistochemical detection of MMP-3. MMP-3 activity was investigated using casein-zymography. The results showed that in demineralized dentine powder, casein zymography revealed confirmedlysis of protein by MMP-3. Thus, the existence and pattern of presence of MMP3 in healthy human dentine was clearly revealed by the study's findings. Although the involvement was confirmed, the position it played is still unknown. Future research is expected to look at MMP-3's role in the healthy and diseased conditions of the dentin and pulp tissues together (Mazzoni *et al.*, 2011).

The zymography techniques are also used in alternative medicine practices. This study reveals one such use of this technique in Homeopathy. The basis for this study is that inflammation is a physiological phenomenon that occurs when tissue is damaged by a microbial pathogen, inflammation, chemical irritation, or wounding. The anti-inflammatory action of four homoeopathic medicines, Calendula officinalis, Arnica Montana, Echinacea Angustifolia, and Hypericum perforatum, was investigated using Zymography. A gel electrophoresis instrument was used. The sample was prepared (10mg in 1ml Dimethyl Sulfoxide -DMSO) and the buffer was prepared (225μl D/W + 25μl stock solution). The apparatus was put together. The polyacrylamide gel was then cast and allowed to polymerize, after which the gel electrophoresis was run for at least one and a half hours before being removed and incubated. The gel was washed with zymogram buffer after electrophoresis. After that, it was stained with "Coomassie blue R-250" and then the de-staining solvent was applied. The results showed that the gel was stained with Coomassie blue, which showed sites of proteolysis as transparent bands on a dark blue backdrop. It found that the anti-inflammatory property of Arnica montana for MMP-2 was 85 percent and 80 percent for MMP-9, of Calendula officinalis was 80 percent for MMP-2 and 75 percent for MMP-9, of Echinacea Angustifolia was 75 percent for MMP-2 and 68 percent for MMP-9 and of Hypericum perforatum was 70 percent for MMP-2 and 60 percent for MMP-9. Thus, zymography was used to demonstrate the anti-inflammatory ability of all homoeopathic medicines used in the study (Yalgi and Bhat, 2020).

The NH2-end part of "dentin sialophosphoprotein (DSPP)", dentin sialoprotein (DSP), is required for dentin development and is additionally fragmented within "odontoblasts". Gelatinases (MMP9) cleave the DSP(P) in tooth structures. In teeth, MMP1 and MMP2 divide DSP(P). Gelatinases can divide DSP inside the cells within odontoblasts, according to the hypothesis of this analysis. "The expression and physical association of DSP and gelatinases were demonstrated in this analysis using double immunofluorescence and an in situ proximity ligation assay (PLA)". Gelatin zymography and in situ zymography were used to confirm gelatinases' intracellular enzymatic activity. "Lysates of wild-type (WT) odontoblastic cells treated with an MMP2 inhibitor, an MMP9 inhibitor, or an MMP general inhibitor, as well as Mmp 9-/- odontoblastic cells, were examined by western blotting to see if DSP was divided by active gelatinases within the cells". All of these classes had considerably greater proportions of increased molecular weight to reduced molecular weight band density than WT odontoblastic cells without inhibitor's presence. Double immunofluorescence and in situ PLA were used to confirm that FURIN was co-localized and physically interacted with MMP9. The findings suggested that gelatinases process DSP within the cells and that FURIN participated in the stimulation of proMMP9 inside the cells through the division of its $R^{96}TPR^{99}$ motif (Gou *et al.*, 2020).

The zymographic technique has been used to identify a method for determining the action of gelatinase of cultured fibroblasts present in human periodontium activated with Pam3 Cys and E. coli LPS, TLR2 and TLR4 ligands, and centrifugation of the cultures to simulate an orthodontic force. The study's aim was to show that zymography can be used to assess gelatinase production in cultured fibroblasts present in the human periodontium. To investigate MMP-2 behaviour, TLRs 2 and 4 ligands were added to primary cultured fibroblasts present in the human periodontium, and mechanical force was applied by centrifugation at 141 x g for 30 minutes. After 24 hours, the supernatant media was collected for protein quantification and zymography.

When cultures were co-activated with only TLRs 2 and 4 ligands or under mechanical force treatment, MMP-2 activity increased relative to baseline levels. Hence, it is clear that zymography is a very effective process for studying MMP activities (Lisboa *et al.*, 2013). See Figure 6.

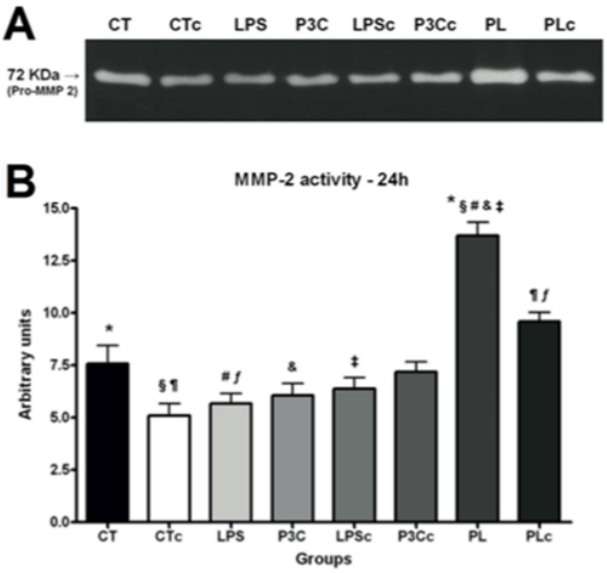

Figure 6: A –72 kDa MMP-2 activity seen in samples of culture medium

The following are some of the additional and advanced ways in which the different techniques of zymography have been used.

1. The collagen-IDZ is used in the treatment of disorders of eyes using MMP-2 as an inhibitor (Protease-MMP-2) and also in the analysis of catalytic fragments of many-domain serine proteases (MASP-1&-2-proteases).
2. IDZ is used to detect metalloprotease proteins (Protease-Metalloprotease INSP005 a and INSP005 b) (Wilkesman and Kurz, 2012).
3. Gelatin-IDZ is used in methods to hinder tumor growth and metastasis with p11 protein as protease, in the analysis of vectors with altered protease-dependent tropism with MMP-2 &-9 as proteases and to study Cathepsin L-like cysteine protease obtained from northern shrimp using Cathepsin L as protease.
4. Casein, gelatin or zein IDZ is utilized for detecting protease-activity-possessing polypeptides, and the former two are used for the reduction of protease action in hydrolysate in plants.
5. Zymography has also been used to describe the extract from plant Kesinai with protease activity (Wilkesman and Kurz, 2012).

Alternate to Conventional Zymographic Protocol

Alternate protocols to the traditional zymography have also been suggested. A new Ponceau S staining protocol provides significant benefits in terms of assay usability and cost reduction, especially when conducting large numbers of zymograms or in resource-constrained settings. The merits of this system are that it is comparatively faster and easier.

The Coomassie staining technique for protein gels is well-established and has remained virtually unchanged for nearly three decades. While this is a very sensitive and stable procedure, the resulting destaining steps necessitate large quantities of ethanol and acetic acid. This reality can have a significant impact on the final costs of analysing zymogram gels, particularly in high-throughput environments.

Protein staining with Ponceau, on the other hand, is a fast and low-cost approach that is currently primarily used to detect proteins on nitrocellulose membranes as a control for equivalent loading after transition blotting. This is particularly important in life science lab. Previous and recent research has focused on lowering dye concentrations for various staining applications ranging from zymography to western blotting, as well as lowering the concentrations of ethanol and acetic acid in Coomassie destaining solutions. Using the Ponceau staining protocol would reduce gel destaining costs by more than 70%.

Also, in contrast to the traditional Coomassie staining technique, this protocol showed reproducible zymogram staining and accurate quantitation of proteolytic operation. Thus, when reagent cost reduction, as well as waste minimization, has long been a priority, this new technique is a viable alternative. A Ponceau staining technique, which is used on histological slides to show some local gelatinolytic activity, is another application (Wechselberger *et al.*, 2019).

Troubleshooting

The preference of a particular zymographic approach can be influenced by a wide range of factors. Following a particular technique requires considering the interactions of the agents used with the enzyme and matrix, specificity, amount of protein, time-dependent procedures, measurement of effects, band consistency over time, and expenses. Beyond that, it's possible that zymography would struggle to have satisfactory results even after careful consideration. The following points go through those general scenarios where the general protocol doesn't work for any reason. Since there are so

many measures in the general zymography process, it's best to look at each one individually. (Wilkesman and Kurz, 2017)

Supporting media

"Enzyme" identification is seldom always reliant on the support medium used. Yet, the selection of support medium is critical since that determines the zymography's efficiency. Polyacrylamide is the most common; but, based on the enzyme studied, other media such as starch and cellulose acetate are also popular.

Gel staining with certain enzymes, there might be more than one procedure available. Choosing the most suitable one can be complicated, as it can be influenced by factors such as the procedure's duration, the chemicals used, the staining solution's delivery method, and the selection of suitable controls to monitor for normal and abnormal effects. Still, have enough of the staining solution on hand. The quantity of solution used alters depending on the dimension of the gel and the tray used for staining. This is especially important since certain staining procedures are very expensive (Wilkesman and Kurz, 2017).

An agar or filter paper immersed in the staining fluid/solution should be used as an alternative of immersing the gel in a tray holding the staining fluid/solution. Using a pippet to apply small amounts as droplets of the solution directly to the zymogram is also an alternative. (Wilkesman and Kurz, 2017)

Process of staining

Double-check whether the reaction needs a certain temperature (usually 37 °C) and whether light effects need to be evaded. It is important to note how long eggs have been incubating. In most cases, staining reactions take place within the first 10 minutes. In certain cases, however, a 24-48-hour incubation period is needed. This, in particular, indicates the enzyme's substrate specificity. "Coomassie blue is the most commonly used staining technique for proteases; nevertheless, alternatives can be utilized (protein silver, Congo red for cellulose, and Amido black). The spectrum of zymography applications is expanding thanks to the rising flexibility of gel scanners. New staining protocols, such as the new RAMA stain, are continuously being released.

Solution preparation

If possible, make an attempt to plan all solutions from scratch. It is true that stock solutions for gel polymerization can be prepared and stored in the freezer. However, after 14 days, these solutions, especially buffers, become contaminated by microorganisms.

Numerous staining fluids/solutions must be made from scratch, and solution reprocessing is seldom always recommended. Of course, each experimenter must decide if a determined solution can be reused or not, and if so, under what conditions it can be stored before it can be used further.

Running procedures

Pre-procedure gels can be beneficial in removing components that are not polymerized from the gel that can interfere with enzyme activation. The gel should be run at 4°C in general, particularly if the enzyme is susceptible to heat. It is better to run the gel at 20 mA per gel during electrophoresis. If it is set to a non-varying current, the voltage would rise over the period, gradually exceeding 200 V. If the power is tuned to operate at a steady voltage (usually 150 V), the starting current would be about 35 mA and would gradually decrease until it reaches 10 mA at the completion of the run.

In this case, it's best to use running conditions that put the enzyme under less heat, such as a lower current and longer run time.

pH conditions

This is a variable that hasn't had nearly enough attention. The amount of buffer and pH distribution must be closely regulated due to the difference in pH with temperature. The optimal pH of the catalyst, the pH of the stain reaction, that of the buffer, and the optimal pH of the coupled reaction, if any, must all be met by the ultimate staining solution pH.

Buffers

In addition to testing the buffer's pH at the actual temperature where it may be used, the abilities of the buffer can get harmed at a highly reduced concentration. To ensure adequate buffering conditions, the least concentration used should be 50 mM buffer.

Substrate

Since there is such a wide range of substrates, both natural and synthetic, it's a good idea to double-check their stability. Solubility can be a challenge at times. If the substrate is inserted into the matrix, it ought to be guaranteed that it will not drift under the influence of the field of electricity. The substrate should be given in a very pure condition, with the optimum concentration decided in advance. Gelatin, casein, collagen, and fibrin are common substrates for protease analysis (Wilkesman and Kurz, 2017).

Detergent removal

In IGZ, it's critical to choose the type of detergent to be used in the washing buffer. The removal of SDS

is influenced by the organization of the detergent's water-loving head group and its essential micelle concentration (CMC). "1–2.5 percent (v/v) Triton X-100 percent solution is used for 1 hour of incubation". Tween-20 or Tween-80, on the other hand, can be used successfully.

Reactivation stage

Since the SDS has been removed, the enzyme can fold itself again to regain natural function. Aside from testing pH and temperature, the reactivating buffer chosen ought to be also able to meet required cofactor requirements. Divalent cations (Ca2+, Mg2+), NAD+, and other cofactors vary depending on the enzyme.

Enzyme biological source

Homogenate preparation is a common source of errors. The following are some related questions to consider: how new is your sample? How long was it kept, and where was it kept? Has it been stored within the correct buffer? Does the amount suffice? Until electrophoresis, was an enzyme test, in parallel, in a test tube performed?.

The following are some often asked questions about process failure: – Does the staining solution have all the reagents? Examine the procedure and double-check every solution's readiness. Inaccuracy often happens in measuring or mixing solutes and solvents. Do reagents retain their stability over time? Both reagents should be tested for stability.

Any of them are light-sensitive and hydrolyze spontaneously when the pH is too high. Inadequate storage (left out of the freezer, open to the sun, or contaminated) is a possibility. Was there a change in pH? Compare the pH of the staining solution to the protocol's specifications. Check the pH for all. If necessary, make new solutions. Is it possible that the solutions contain enzyme inhibitors?

This is true, particularly if studies are carried out in the field. For experiments, always use pure water. It's possible that other enzyme inhibitors are found in the gel matrix. The enzyme could not reactivate after electrophoresis in some cases (Wilkesman and Kurz, 2017)

CONCLUSION

Thus, it is apt to say that zymography is a very important diagnostic method used in various fields. The new alterations to the conventional procedures and the variations in the methodology can be put to use in different ways. Further research can enhance its potential and improve the technique in such a way that it can be used for more diverse purposes.

Funding Support

The authors declare that they have no funding support for this study.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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