Research Article

Insights into the development of zymography from inception to current day - a discussion on innovations, challenges and solutions

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Zymography is a commonly used biochemical technique for detecting and analyzing enzyme activity using gel electrophoresis and substrate staining. This review traces the chronological advancements in zymography, from its origins with the pioneering fibrin-plate methods in the 1940s to the recent innovations in multi-substrate applications. It underscores early pivotal developments and acknowledges the overlooked contributors who laid the groundwork for zymography's current methodologies.

Current advancements, particularly in gel casting, are highlighted for their ability to facilitate the concurrent analysis of multiple substrates, thereby improving the efficiency, and reducing the environmental impact of proteolytic assays. These methodological enhancements offer a more detailed understanding of enzyme activity and specificity and represent a shift towards more economical laboratory practices.

Additionally, the review identifies persisting challenges such as substrate limitations and detection sensitivities, offering insights into potential research directions that could further enhance the technique's capabilities. The review concludes with a discussion on the necessity for standardized sensitivity and detection metrics across various zymographic methods and highlights the utility of zymography in proteomic research, with implications for the development of diagnostic and therapeutic applications.

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Historical development of zymography as a technique

Zymography, as performed today, is a biochemical technique that allows the semi-quantitative detection of proteases and other hydrolases following their electrophoretic separation in gel matrices. Almost every zymography protocol that is used in routine laboratory work or as a starting point in teaching courses, follows from Heussen & Dowdle's paper from 1980. But a thorough investigation into the origins of this technique takes us eight decades back to the 1940s.

In the realm of zymographic techniques, the foundational work of Permin, a graduate student training under Tage Astrup, as well as the subsequent methodological refinements by Astrup & Müllertz in 1952, mark pivotal milestones in the evolution of the fibrin-plate method for assessing fibrinolytic activity. Permin's initial approach, as detailed in his 1947 publication in Nature, involved the strategic placement of a plasmin-containing solution on a fibrinogen-thrombin matrix in a petri dish, leading to a discernible circular zone of fibrinolysis, with the radius being indicative of the plasmin concentration. This method provided an early, yet profound, quantitative measure of enzyme activity. Building upon this, Astrup & Müllertz's 1952 paper, titled 'The fibrin plate method for estimating fibrinolytic activity,' presented a more standardized and refined version of this technique, enhancing its reproducibility and accuracy. They meticulously adjusted variables such as fibrinogen concentration and incubation conditions, thereby optimising the assay for broader applications across various fibrinolytic enzymes.

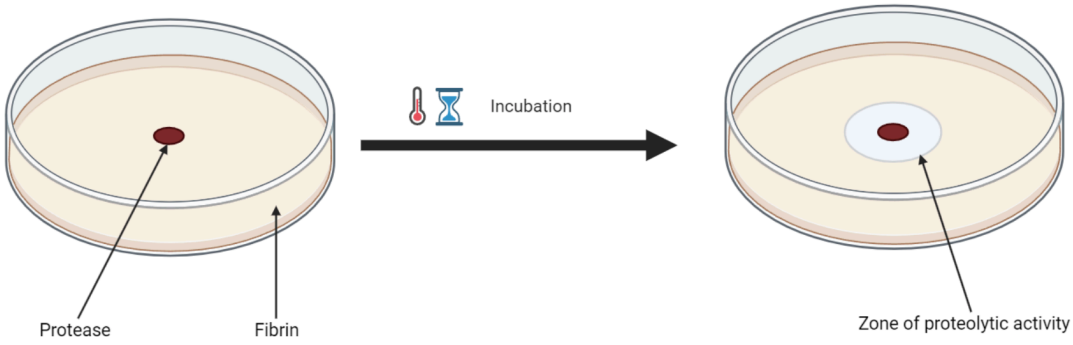

Figure 1. Fibrin Plate Method for Fibrinolytic Activity Assessment.
Illustration of the fibrin plate method as introduced by Permin in 1947 and standardised by Astrup & Müllertz in 1952. This technique involves the degradation of a fibrin clot by plasmin, where the extent of fibrinolysis is indicated by a clear zone, whose radius is proportional to the enzymatic activity present.

Concurrently, we see the development of ‘Immuno-electrophoretic-analysis’ where components of serum are electrophoretically resolved in an agar gel matrix and are then exposed to specific antibodies allowing the detection of serum components (Grabar & Williams, 1953). Further refinement of this method, particularly through J. J. Scheidegger's slide gel electrophoresis in 1955, catalysed the foundational concepts for modern zymography. This method involved the electrophoretic separation of serum components followed by the diffusion of specific antibodies against these antigens, leading to the formation of distinct ‘precipitate arcs’ within the gel. This technique not only enabled the localisation of antigens within a gel matrix but also allowed for the determination of their electrophoretic mobility.

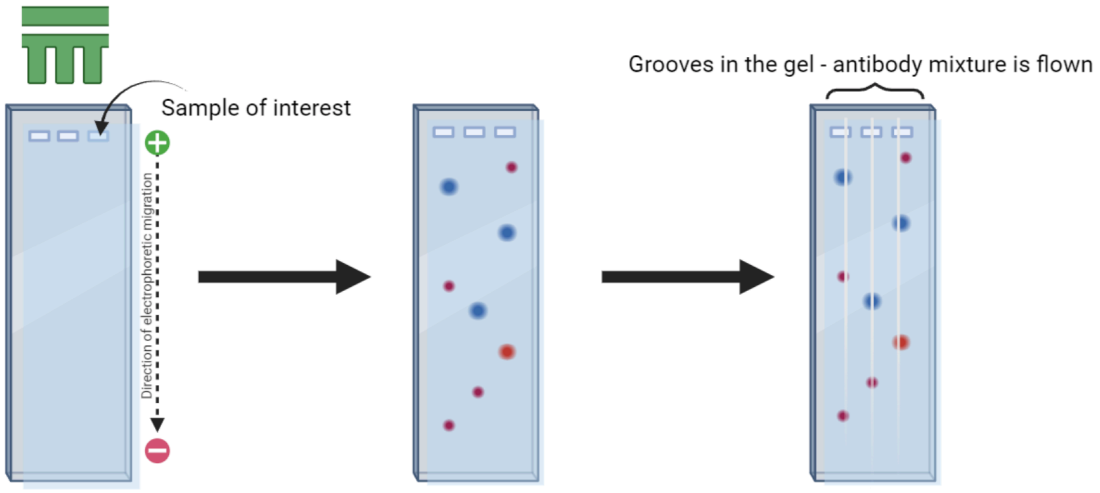

Figure 2. Immuno-electrophoretic-analysis, an Early Zymography Technique Utilising Slide Gel Electrophoresis.

A schematic representation of slide gel electrophoresis for analysing plasmin, as first described by Grabar & Williams and later Scheidegger. This method integrates the principles of fibrin plate methodology with electrophoretic separation, paving the way for modern zymography techniques.

Heimburger and Schwick’s seminal paper titled ‘*Die Fibrinagar-Elektrophorese*’ from 1962 on electrophoretically analysing plasmin, plasminogen, their activators and inhibitors, is the first instance of resemblance to modern day zymography. The authors allude to inspiration from Grabar & Williams (1953) and Astrup & Müllertz (1952). In their approach, fibrin is incorporated into a slide gel used for the electrophoretic separation of plasma. This method ingeniously allowed the observation of

clear zones of lysis post-incubation at specific locations corresponding to the presence of enzymes such as trypsin, chymotrypsin, and plasmin, identified by distance of migration, marking a pivotal advancement in detecting and analysing proteases and their inhibitors in human plasma.

In addressing technical challenges inherent to zymography, particularly in the context of substrate immobilisation within electrophoretic gel matrices, two principal questions arise. Firstly, the potential migration of a charged substrate under electrophoretic conditions could be a cause of concern. This issue is mitigated by the intrinsic properties of fibrin, which, when copolymerized with acrylamide and bisacrylamide, remains stationary even under an electric field. The resultant fibrin-acrylamide matrix, characterized by its opacity and insolubility, precludes ionic migration. Secondly, the challenge of preventing premature enzymatic activity during electrophoresis is addressed by conducting the process at a suboptimal temperature, specifically 4°C. This strategic temperature regulation effectively inhibits enzymatic action until the desired post-separation incubation at pH 8.0, a condition under which protease activity is reported to be optimal.

The term ‘zymogram’, and by extension zymography, however was coined in 1957 by Robertson and Wexler. Their method involved separation of a protein mixture in a starch gel and visualising enzymes through histochemical methods, successfully demonstrating enzyme activity retention within a hydrogel matrix, a concept foundational to zymography. (Hunter & Markert, 1957). The authors advocated it as a general method to study the enzymatic composition of tissues. Later this method was advocated as a method to discover new enzymes (Maravolo et al., 1967).

Consequent to the development of PAGE as a method of resolving proteins, Hochstraßer & Schorn (1974) incorporated gelatin and azocaesin, which would function as the protease substrate into a Native PAG. This approach, which employs negative protein staining post-electrophoresis, has significantly influenced contemporary zymographic techniques, closely resembling current practices in terms of substrate incorporation and staining methodology. Interestingly, the foundational work of Heimburger and Schwick in 1962, which demonstrated a similar principle in the analysis of plasmin, plasminogen, and their activators and inhibitors, was not cited in Hochstraßer and Schorn's paper. This omission is intriguing, given the similarity in the underlying principles of zymography between the two studies, suggesting a potential underappreciation of Heimburger and Schwick's contributions at the time.

Granelli-Piperno and Reich's 1978 exploration of extracellular proteolytic reactions using SDS-PAGE, later described as ‘overlay zymography,’ represents a significant leap in addressing the technical

challenges of zymography. Their innovation was grounded in the observation that some serine proteases could be reversibly inhibited by SDS, effectively addressing the necessity of running electrophoresis at low temperatures or using pH levels that deactivate enzymes. This approach allowed for the removal of SDS from the PAGE gel through anionic detergent washing, as described by Converse and Papermaster in 1975.

The advancements by Heussen and Dowdle in 1980 further solidified the role of SDS-PAGE in zymography. Their technique for detecting plasminogen-dependent and independent proteases in SDS-PAG, copolymerized with gelatin as a substrate, provided a refined approach to enzyme analysis. Notably, they regarded Reich's overlay method as complementary rather than substitutive, due to its limitations in real-time kinetic analysis of enzymatic activity.

The rapid and widespread acceptance of SDS-based transient inactivation of enzymes, combined with linearization and negative charge distribution correlating mobility with molecular weight, as detailed by Shapiro et al. in 1967, is unsurprising.

Further developments in zymography have been on the basis of this study, as described in the beginning of this section, which as of writing this review has 2455 citations well distributed over the years, testifying to its enduring impact and relevance in the field of biochemical research.

An overview of zymographic methods-

1. Regular Zymography-

This entails electrophoretic separation of enzymes in an SDS PAGE gel (or sometimes agarose gel) copolymerised with the substrate like fibrin, casein, gelatin etc. After enzyme refolding and activation, it can be visualised as a clear band against a stained gel, called a zymogram.

The size of clear bands in a zymogram corresponds to the amount of active enzyme present in the gel giving the assay its (semi) quantitative character. (Heimburger and Schwick, 1962)

In cases where the protease does not renature upon exposure to SDS or where multimeric enzymes are involved, reassembly is difficult after distant physical immobilisation of components owing to electrophoretic separation, a non denaturing gel is used.

White gelatin is the most commonly used substrate for zymography, studies involving incorporation of non proteinaceous substrates like carbohydrates and lipids demonstrate the suitability of this

technique for studying amylases and lipases (Ji et al., 2012)

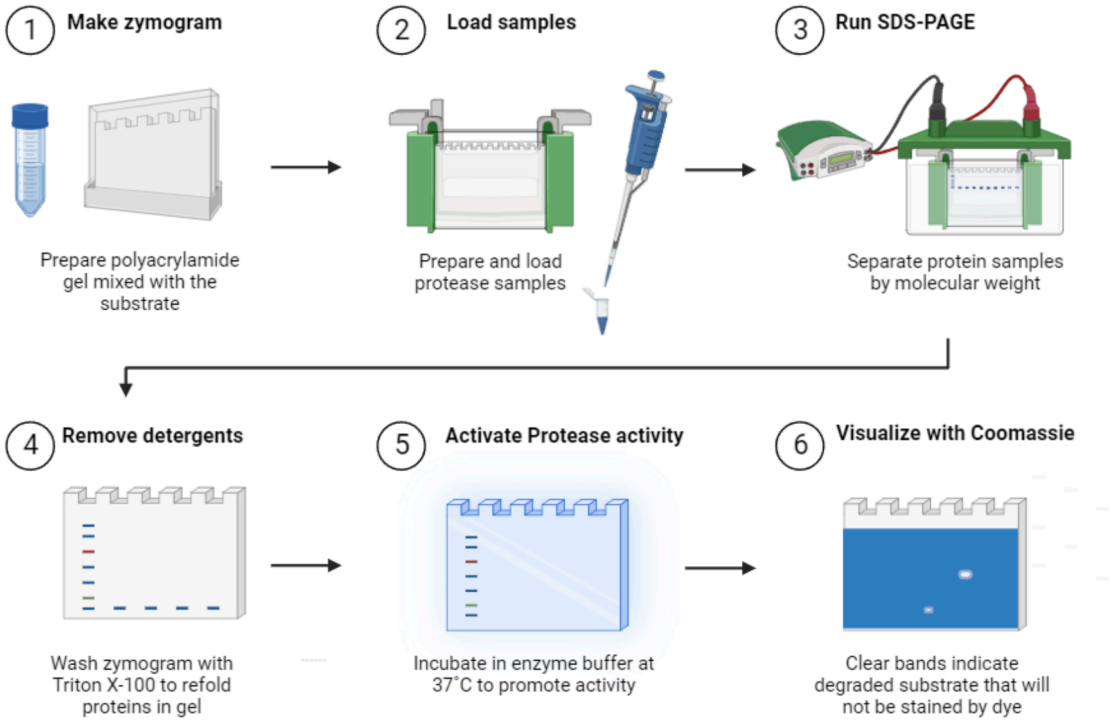

Figure 3. SDS-PAGE and Zymography Workflow for Protease Analysis.

A flow diagram outlining the steps from SDS-PAGE to zymography for the identification and characterization of proteases. The process includes gel preparation, electrophoresis, and subsequent analysis of proteolytic activity using specific substrates.

2. *Overlay Zymography-*

This differs from regular zymography in the sense that the gel on which the enzymes are electrophoretically separated is distinct from the one copolymerised with the substrate. The resolved gel is washed to remove SDS, constituent proteins are refolded and the gel is overlain on the substrate gel. Following incubation the substrate gel may be stained for visualising zones of enzymatic lysis.

A major advantage of this method is that when a substrate like fibrin is to be used, the substrate gel appears opaque and upon overlay of the separation gel, zones of enzymatic activity, seen by clearing up of the gel, can be visualised in real time without the need for staining. (Granelli-Piperno & Reich, 1978)

This method is also applicable when it is not desirable to expose the substrate to SDS.

(Lantz & Ciborowski, 1994) note that fibrin-agarose gels work better than fibrin-PAG for detecting V8 protease and pronase.

3. Multiple substrate zymography

This method initiated by (Choi et al., 2009) aims to detect multiple kinds of hydrolases – namely, lipases, cellulases and proteases in crude cell extracts using a single step of electrophoretic separation in an SDS-PAGE which is then washed for SDS removal and enzyme renaturing. Following which, 3 separate substrate gels are sandwiched, and the resolved proteins are perpendicularly transferred to all 3. Each substrate gel is processed as per standard, respective zymography protocol and developed.

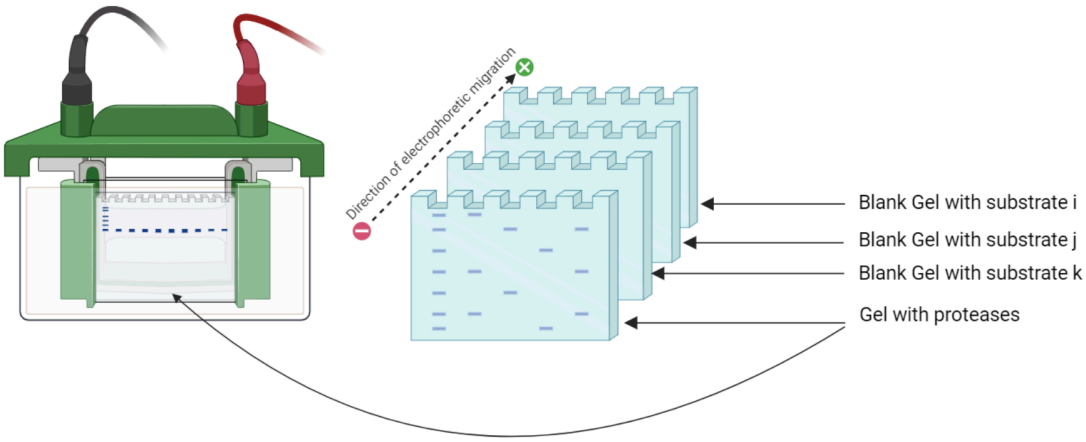

Figure 4. Multiple Zymography Workflow for Protease Analysis.

A flow diagram outlining the steps of multiple substrate zymography for the identification and characterization of proteases. The process includes regular gel preparation, electrophoresis of proteases, horizontal transfer to substrate containing gels, and subsequent analysis of proteolytic activity using the standard procedure.

4. In situ zymography

Overlay zymography paves the way for in-situ zymography. In in situ zymography the gel with enzymes is replaced by an unfixed biological tissue sample. The substrate gel may be autoradiographically labelled (Galis et al., 1994) or pre stained with a dye like amido black where the substrate is gelatin and loss of signal is indicative of spatiotemporal gelatinase activity.

(Frederiks & Mook, 2004) note that "sensitivity of reduction in staining intensity is less than that of formation of staining" and review methods developed to address this issue. A detailed discussion of those is beyond the scope of this review and the interested reader may refer to the cited review.

This assay has been developed to incorporate different labels for the substrate and has proven useful in cancer diagnostics and determining the role of proteinases in various physiological and pathological conditions. This has been extensively reviewed by (Frederiks & Mook, 2004).

5. Reverse zymography

In this technique, polyacrylamide gels containing copolymerized substrates are prepared for zymography, following the previously described procedure. However, instead of introducing enzyme samples, the gel is loaded with a protease inhibitor. During electrophoresis, the inhibitor migrates into the gel. Subsequently, the gel is incubated in a solution containing a protease and activators, as required. These components diffuse into the gel, leading to substrate lysis, except in the specific region of the gel where the inhibitor has migrated.

(Lantz & Ciborowski, 1994) notes that this method is suited to high molecular weight inhibitors. The reason being the possibility of lower molecular weight proteins diffusing out of the gel during the washing and refolding steps. **This may be overcome by using native gels, and gels of higher acrylamide percentages or tricine gels which can eliminate the need to wash and renature proteins and provide better resolution for small molecular weight inhibitors.**

Also worth noting is the fact that reverse zymography is prone to false positive results in cases where a kinetically stable; ie., protease resistant protein shows up as a dark band as reported by (Dutta & Bhattacharyya, 2013).

6. Real time zymography

In many cases the substrate upon incorporation into a PAG or an agarose gel is transparent. In those cases studying real time degradation of the substrate becomes difficult with overlay zymography. (Hattori et al., 2002)developed a zymographic method using FITC labelled denatured collagen to be used as copolymerisation substrate successfully demonstrating its use in regular as well as as reverse zymography.

A major advantage of this method is the ease of optimising incubation time as proteolytic activity can be detected as soon as the enzymes renature.

An important limitation of zymography is that only a limited number of substrates have been successfully copolymerized within the gel matrix. Out of all enzymes known today, zymographic visualisation schemes have been suggested only for 400 odd enzymes (Manchenko, 2003). The disparity in the numbers seems to have arisen from the relative difficulty in successfully incorporating and keeping active, substrates in the polyacrylamide matrix (Deshmukh et al., 2018).

A solution to this had been attempted by incorporating small protease site containing fluorescent peptides into the gel matrix. A challenge associated with this technique is the possibility of the small peptide getting diffused out of the gel (Yasothornsrikul & Hook, 2000).

To overcome this issue (Deshmukh, 2020) synthesised fluorescent peptides were synthesised using Fmoc solid-phase peptide synthesis and labelled with a quencher (dabcyl) and a fluorophore (fluorescein). A two-layer polyacrylamide gel was created, with the second layer containing an azido-maleimide crosslinker and the quenched fluorescent peptide, covalently linking the peptide to acrylamide.

This method achieves two goals – one of achieving substrate versatility and the other one of being able to monitor enzyme activity real-time.

The invisible ladder and other limitations

The methods discussed thus far have significantly advanced the field of zymography, addressing many of its prevailing challenges. However, a practical issue that has received comparatively less attention is the visualization of molecular weight markers, colloquially referred to as the 'ladder', in zymograms. This review briefly surveys existing methods before introducing a novel approach developed by the author.

In their 2012 study, Huang et al. tackled this issue in fibrin zymograms by meticulously optimizing substrate concentrations. They proposed a delicate balance between assay sensitivity, which is directly proportional to substrate concentration, and ladder visibility, inversely related to background staining. Huang recommended reducing the fibrin concentration in the gel to 0.05% w/v, a departure from the ~0.12% w/v typically used by others, like Park et al. (2017). This adjustment enabled Huang to achieve a sensitivity of 0.78 ng for urokinase, surpassing previous efforts that reached detection levels in the picogram range but at the expense of ladder clarity (Hattori et al., 2002).

Another strategy, as described by Krause & Goldring (2019), involves partial staining of the zymogram up to a point where the ladder is just visible, followed by photographing the gel. Subsequent continuation of staining and destaining reveals zones of enzymatic cleavage. This method, however, introduces the challenge of empirically matching molecular weight markers to commonly observed shrinkage in polyacrylamide gels.

In this review, a novel zymogram casting method by the author, is presented. This method, as detailed in Jorapur (2023), involves casting a polyacrylamide gel (PAG) with two distinct regions along the direction of electrophoresis. One section of the gel is devoid of the enzyme substrate, thereby facilitating clear visualisation of the molecular weight marker components in the enzyme mixture. This versatile casting approach offers a more streamlined and efficient alternative to the traditional multi-layer substrate zymography, where each gel section can be loaded with different enzyme substrates.

An exemplary application of this method is demonstrated in the visualization of fibrinolytic enzymes present in *Bothrops asper* venom. The results, reproduced with permission, underscore the efficacy of this innovative casting technique in enhancing the clarity and interpretability of zymograms, addressing a longstanding practical challenge in the field.

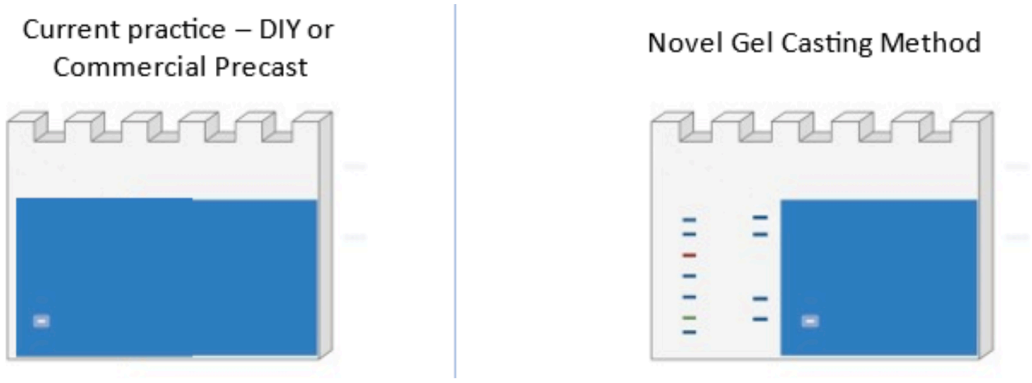

Figure 5. Comparison of Traditional and Novel Gel Casting Methods for Zymography.

A comparative illustration showing the traditional DIY or commercial precast approach versus a novel gel casting method for zymography, highlighting the integration of sample wells and fibrin substrate in the gel matrix.

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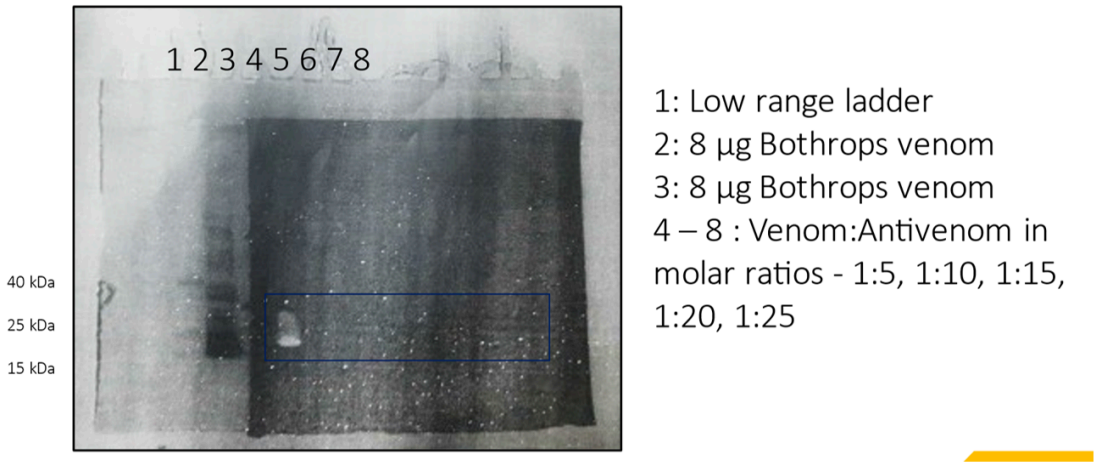

Figure 6. Fibrin Zymogram Demonstrating Fibrinolytic Enzyme Activity.

A fibrin zymogram depicting the fibrinolytic activity of enzymes in Bothrops asper venom, with varying venom:antivenom molar ratios. Zones of proteolytic activity are visualized against a low-range molecular weight ladder, demonstrating the inhibitory effect of scFvBaP1 on the venom's enzymatic function.

This approach also lends itself to multiple substrate zymography within a single gel, where each distinct substrate is incorporated directly into the initial SDS-PAG utilised for resolving the sample of interest. It offers a significant advantage in terms of efficiency and sustainability. By enabling the analysis of multiple substrates in one gel, it reduces the number of SDS-PAGE gels required, thereby making the process more economical and generating less waste. This adaptation not only streamlines the experimental workflow but also aligns with the increasing need for sustainable and environmentally conscious research practices.

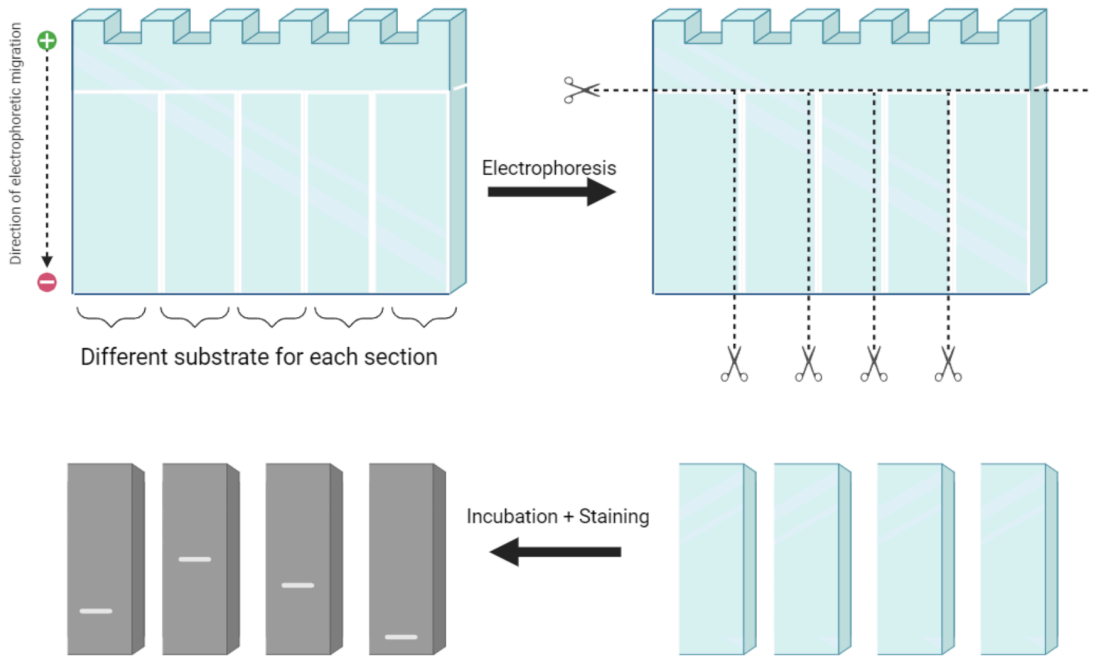

Figure 7. Multiple Substrate Zymography in a Single Gel.

This figure illustrates the process of multiple substrate zymography within a single SDS-PAGE gel. The gel is initially prepared with different substrates embedded within the resolving gel matrix. After electrophoresis, the gel is incubated and developed to reveal zones of hydrolytic activity, allowing for simultaneous visualisation of enzyme action on various substrates. This method enhances the economy and sustainability of the zymographic process by reducing the number of gels used, thereby minimising waste.

Conclusion

A systematic historiography of zymography as an analytical method has been undertaken in this review. It is observed that the majority of English language publications on zymography often attribute the foundational development of the technique, in its modern incarnation, to the works of Granelli-Piperno & Reich (1978) and Heussen & Dowdle (1980). However, this narrative overlooks the significant contributions of Heimburger and Schwick, along with their predecessors and contemporaries, within the English-speaking scientific community. This historical oversight, possibly due to language barriers or a lack of awareness, has led to a gap in recognizing the integral role these early researchers played in the evolution of zymography. This review seeks to address and rectify this oversight, highlighting the invaluable contributions of these overlooked pioneers.

The latter part of this review delves into the discussion of six contemporary advancements in zymography, addressing both challenges and potential solutions. The novel gel casting method by the author offers several advantages to current day techniques wherein not only is the molecular weight marker easily visible but also makes proteomic analysis of the enzymes of interest and performing multiple substrate assays more precise and sustainable, enhancing the analytical ability of this assay.

One notable gap in the current landscape of zymography is the lack of cohesive and standardized data regarding the sensitivity and limit of detection across various zymographic methods. A systematic experimental approach, employing a 'one enzyme-substrate system' and applying the protocols discussed in this review and elsewhere, would be instrumental in furthering knowledge and understanding in this field. Such studies would help in standardising methodologies and in developing a comprehensive framework for comparing and evaluating different zymographic techniques, ultimately contributing to the advancement of zymography as a robust and reliable analytical tool.

Glossary

1. **Zymography:**
    - Technique for detecting and analyzing enzyme activity using gel electrophoresis and substrate staining.
2. **Protease:**
    - Enzyme that breaks down proteins by cleaving peptide bonds.
3. **Substrate:**
    - Molecule acted upon by an enzyme, used for identifying and measuring enzyme activity.
4. **Hydrogel Matrix:**
    - Polymer network used as a medium in gel electrophoresis.
5. **Gel Electrophoresis:**
    - Method to separate macromolecules based on size and charge in a gel matrix.
6. **Electrophoretic Mobility:**
    - Speed of particle movement in a gel under an electric field.
7. **Polyacrylamide Gel:**
    - Gel used in electrophoresis, characterized by variable pore size.
8. **SDS-PAGE:**

- Technique to separate proteins by molecular weight using sodium dodecyl sulfate and polyacrylamide gel.

9. **Fibrin:**
   - Insoluble protein from fibrinogen, used in zymographic methods.
10. **Plasmin:**
    - Enzyme dissolving blood clot fibrin, derived from plasminogen.
11. **Fibrinolytic Activity:**
    - Process of breaking down fibrin in blood clots.
12. **Azocasein:**
    - Protein substrate in zymography for protease activity detection.
13. **Urokinase:**
    - Serine protease involved in fibrin degradation.
14. **Immunoelectrophoresis:**
    - Combines electrophoresis and immunoassay for protein characterization.
15. **Serine Proteases:**
    - Proteases with a serine residue at the active site.
16. **Negative Staining:**
    - Technique to visualize target structures by staining the background.
17. **Enzymatic Cleavage:**
    - Process of breaking down molecules by enzymes.
18. **Molecular Weight Marker:**
    - Standards in gel electrophoresis for estimating molecule size.
19. **Plasminogen Activators:**
    - Enzymes converting plasminogen to plasmin.
20. **Acrylamide:**
    - Compound for polyacrylamide gel formation in electrophoresis.
21. **Bisacrylamide:**
    - Crosslinking agent used with acrylamide in gel electrophoresis.
22. **Staining:**
    - Applying dyes to gels for visualizing molecules.
23. **Proteolytic Enzymes:**
    - Enzymes hydrolyzing peptide bonds in proteins.

24. **Cytosol:**
    - Liquid part of the cytoplasm, analyzed in cellular studies.
25. **Native PAGE:**
    - Polyacrylamide gel electrophoresis under non-denaturing conditions.
26. **Protease Substrate:**
    - Molecule acted upon by a protease.
27. **Renaturation:**
    - Restoring the native structure of proteins after denaturation.
28. **Zymogen:**
    - Inactive enzyme precursor requiring activation.
29. **Immunoassay:**
    - Technique using antigen-antibody reactions for detection and quantification.
30. **Protease Inhibitor:**
    - Molecule binding to a protease to decrease its activity.
31. **Gel Casting:**
    - Creating a gel matrix for electrophoresis.
32. **Overlay Zymography:**
    - Zymography variant with enzyme gel overlaid on a substrate gel.
33. **Detergent Washing:**
    - Using detergents to wash gels, often to remove SDS.
34. **Destaining:**
    - Removing excess stain to enhance band visibility in gels.
35. **Proteomic Analysis:**
    - Large-scale study of proteins and their characteristics.

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