

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

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Potential competing interests: No potential competing interests to declare.

The topic is interesting, of practical value, and worth publishing after major revision. The review does not do justice to the title which has a broad scope. It is of limited scope as it only focuses on proteases though zymography has been used on enzymes from all classes. The review should discuss the advantages, limitations, various types, and most importantly its various Applications in a Table format.

1. Author statement: Statement: 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.

Reviewer Comment: Not necessarily limited to hydrolases but has been used for other classes like oxidoreductases, isomerases etc.

1. Describe the principle of Non-denaturing PAGE when SDS-PAGE fails for doing zymography. Point out that during native-gel, the enzymes run according to the charge/mass ratio and not based on subunit MW as in SDS-PAGE. For determining the native MW of enzymes, Fergusson plots using gels of various concentrations are used and zymography is performed to detect the specific enzyme.

2. Author statement: The invisible ladder and other limitations:

Reviewer Comment: This problem can be resolved by simply using prestained markers in the first and last lanes of the gel and subjecting the rest of the gel to zymography. Discuss.

1. Author statement: By enabling the analysis of multiple substrates in one gel,

Reviewer comment: It is not clear how different substrates can be incorporated in a SINGLE gel during gel casting. Explain.

1. The author discussed in vitro zymography but left out in vivo zymography.
2. The review on zymography is limited to proteases, especially to Fibrinolytic enzymes though the title indicates a much broader scope.

There are many interesting applications of zymography which have been omitted. The author is directed to the following references for discussion in the review.

- i. The active site is the least stable structure in the unfolding pathway of a multi-domain cold-adapted α -amylase. *J. Bacteriol.* 2005, 187 (17): 6197-6205

The above paper discusses the importance of zymography in Transverse-urea gradient PAGE for determining the stability of amylase.

- i. Two simple and rapid methods for the detection of polymer degrading enzymes on high resolution alkaline cold in situ native (HiRACIN)-PAGE and high resolution in situ inhibited native (HiRISIN)-PAGE. *Biotechnol. Lett.* 2000, 22(11) 957-960.

The above paper shows zymography on Native-PAGE using 3 different enzymes.

- i. Native Enzyme Mobility Shift Assay (NEMSA): A new method for monitoring the carboxyl group modification of carboxymethylcellulase from *Aspergillus niger*. *Biotechnol. Techniques*, 1997, 11:4, 245-247.

The above paper shows the application of zymography to monitor the chemical modification of an enzyme.

1. A key paper on zymography published in Nature has been omitted. Vandooren, J. et al. (2013). *Zymography methods for visualizing hydrolytic enzymes. Nature Methods*, 10(3), 211–220.