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Zymography: Enzymes in Action

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ABSTRACT

Proteases are enzymes that cleave proteins by peptide bond hydrolysis. On the basis of their catalytic mechanism, proteases are further divided into five catalytic classes: aspartic, cysteine, metalloproteinase, serine and unclassified. The deregulation of the temporal and spatial expression and activation pattern of these different proteases can result in various pathologic conditions, such as neurodegenerative and cardiovascular diseases, arthritis and cancer. Consequently, there has been an increasing interest in the identification and functional characterization of proteases. In particular, several of these molecules and their substrates are considered attractive potential therapeutic targets by the pharmaceutical industry. Biochemical techniques have been developed to detect protease activity in tissue extracts. In particular, zymography also referred to as substrate zymography, is an established technique for the routine detection and quantitation of various protease activities, such as gelatinase activity. This technique provides reliable identification of proteases based on the molecular mass of their inactive and active forms after gel electrophoresis. Since 1978 to till date the technique base remains the same but the methodology and application has changed a lot. After 34 years, the Zymography has been transformed in to different forms like chromogenic substrate autography (substrate indicator gel), Reverse Zymography, 2D-Zymography and In situ Zymography. By considering the importance of zymography author made an attempt to colligate different forms of zymography, its uses in molecular biology and a glimpse of standardized protocol.

Key words: Protease, zymogen, chromogen, reverse zymography, gelatin, SDS-PAGE

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INTRODUCTION

Proteases are enzymes that cleave proteins by peptide bond hydrolysis. On the basis of their catalytic mechanism, proteases are further divided into five catalytic classes: aspartic, cysteine, metalloproteinase, serine and unclassified. There are more than 500 genes in the human genome that encode proteases or protease-like molecules, with the metalloproteinases and serine proteases comprising the largest classes. It has become increasingly recognized that highly specific hydrolysis of peptide bonds can regulate a wide range of biological processes in all living organisms. This highly specific and limited substrate cleavage is referred to as proteolytic processing: a process that regulates the activity and localization of many proteins. For instance, proteolytic processing controls the intracellular and extracellular localization of many proteins, activates or inactivates various enzymes, cytokines, growth factors or hormones, Through their catalytic activity, proteases regulate virtually all biologic processes, including, among others, cell proliferation and differentiation, cell migration, apoptosis, wound healing and angiogenesis[1]. Thus, deregulation of the temporal and spatial expression and activation pattern of these enzymes can result in various pathologic conditions, such as neurodegenerative and cardiovascular diseases, arthritis and cancer. Consequently, there has been an increasing interest in the identification and functional characterization of proteases. In particular, several of these molecules and their substrates are considered attractive potential therapeutic targets by the pharmaceutical industry. For instance, the matrix metalloproteinases (MMPs) have been identified as important targets for the treatment of various pathologic conditions, such as cancer, arthritis and cardiovascular diseases and several MMP inhibitors have already been tested in clinical trials. Several molecular techniques are available to identify and characterize proteases in cells and tissues. Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) can be used to quantitate protease messenger RNAs (mRNAs) in cell and tissue extracts, whereas in situ hybridization has been used to localize mRNA expression at the cellular level in tissue sections. A major limitation of evaluation of mRNA levels is that transcriptional activity does not necessarily reflect the amount and activity of the protein product of a particular gene. This is mostly due to variation in cellular location, regulation of translational activity, complex and versatile protein regulation mechanisms, such as context-dependent posttranslational phosphorylation, sulphation

and glycosylation. Conversely, immunohistochemistry and western blotting are used to evaluate expression at the protein level. However, these techniques do not provide any information on the activity of proteases in tissue because many enzymes are synthesized in an inactive or proenzyme form, called zymogen, which requires proteolytic processing for activation. In addition, ubiquitous protease inhibitors are present in tissues and can specifically or nonspecifically inhibit proteases in their active form. Biochemical techniques have been developed to detect protease activity in tissue extracts. In particular, zymography also referred to as substrate zymography, is an established technique for the routine detection and quantitation of various protease activities, such as gelatinase activity. This technique provides reliable identification of proteases based on the molecular mass of their inactive and active forms after gel electrophoresis.

Zymography is a fascinating technique in molecular biology especially in protein chemistry. You'll be fascinated each time you see your sample has one of nature's wonderful molecule -Protease, which is evident by the formation of colorless band on the gel. Zymography is the technique with many faces.

It was by Granelli-Piperno and Reich[2] who introduced this sensitive and at the same time efficient technique. Two years later the whole technique with necessary modifications put in front of the scientific community by Heussen and Dowdle[3] to analyze plasminogen activators in Sodium Dodecyl Sulphate Polyacrylamide gel. Soon its importance was noticed and applied to study Matrix metalo proteases (MMPs) whose expression is enhanced during tumor invasion and metastasis, the zymography gave very promising results since then it became a routine technique in Cancer research laborotaries around the world. The zymogram or activity stains are useful for visualizing the activity of enzymes in non denaturing protein gels. The sensitivity of zymography was shown by Kleiner and Stetlerstevenson[4] by quantifying gelatinases as less as pictogram level. Zymography is popular because of its flexible nature, it allows researcher to change substrate, reaction buffer and its pH, incorporation of inhibitors, effect of different agents (temperature, ion concentration, xenobiotics), necessity of co-factors etc.

Gelatin zymography as name indicates it has been used to evaluate gelatinase activity in various cancers and compared to normal tissues[1].

Zymography is used

- To analyze the proteases present in a biological tissue or cell or fluid etc.
- To study the substrate specificity of an enzyme (protease)
- To study the effect of different agents (temperature, ion concentration, pH, heavy metals, xenobiotics) on protease activity
- To identify the endogenous and exogenous inhibitors for protease
- To detect proteases from any source (from tissue or from single cell) or from any concentration as less as pictogram level (If the starting material is very small like invertebrate eggs, glands or brain tissue

Based on the type of substrate (natural or synthetic) used, it referred as gelatin zymography casein zymography, fibrin zymography, collagen zymography etc. Gelatin zymography has long been used as an assay for MMPs. It has been used to access the levels of these proteases in tissues and in biological fluids. It has the advantage of distinguishing different species of enzymes due to mobility difference. Casein zymography is useful in resolving μ and m-calpain (calcium activated neutral proteases) and studying some of the inhibitory agents of calpains. This technique can detect calpain activity from tissue or cultured cell samples even in the presence of endogenous calpastatin[5]. Fibrin is used as a substrate for assaying plasmin and plasmin like enzymes[6]. Collagen zymography is used to determine intestinal collagenases and it is proved to be more sensitive than gelatin and casein zymography and it can determine up to 0.1 pg of collagen[7]. Based on the methodology applied the zymography was classified in to following types Chromogenic substrate autography (substrate indicator gel), reverse zymography, fluorescence zymography, In situ zymography and 2D-zymography.

Chromogenic substrate autography: Wagner[8] modified the zymography, instead of routine protein substrates he used chromogenic substrate and named it as Chromogenic substrate autography (Fig. 1) by using this technique they characterized and quantified the serine proteases from crude systems after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The SDS-PAGE was over laid onto the agarose gel containing paranitroanilide as a substrate. The proteases present in the SDS-PAGE converted the paranitroanilide into yellow-colored zones which was quantified by densitometry at 405 nm. The technique proved suitable for determination of molecular weights, isoelectric points and quantitative measurements of amidolytic activities of urokinase, tissue plasminogen activator, tissue and plasma kallikrein and thrombin in biological fluids and purified preparations.

Reverse zymography: Reverse Zymography (RZ) is used for the detection of naturally occurring specific protease inhibitors found in cells or conditioned media. It was Herron[9] used RZ to demonstrate

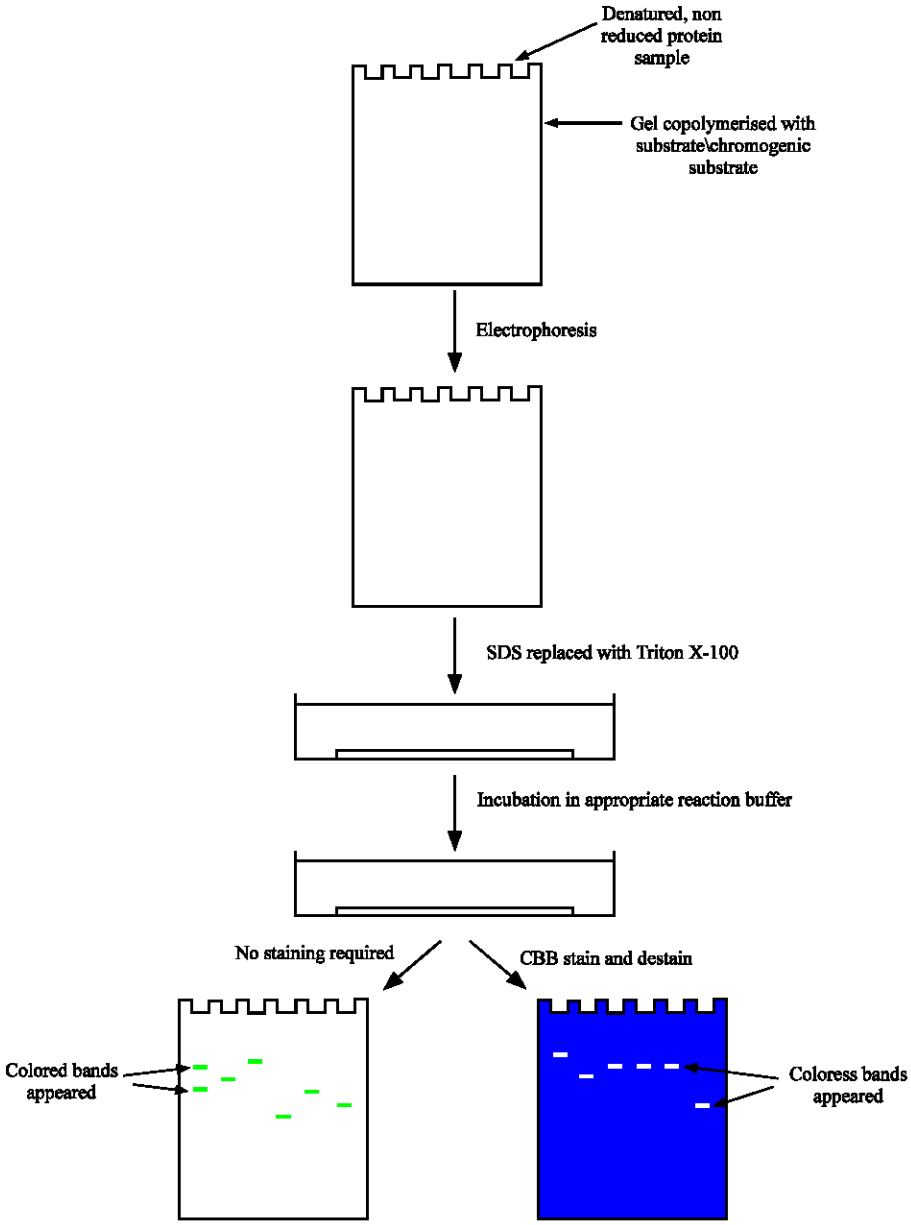

Fig. 1: Zymography and chromogenic substrate autography

metalloproteinase inhibitors. In general, the enzyme whose inhibitor is to be studied is added to the incubation buffer. After incubation, staining and followed by destaining, the inhibitors for particular enzyme remains as such without degradation and appears as dark blue band against a pale blue back ground (Fig. 2).

***In situ* zymography:** The limitations of immunohistochemistry to localize and quantify specific protease activities in tissue sections are most of the proteases are produced and secreted as zymogens, which require proteolytic cleavage of a propeptide domain to be activated. Most antibodies available for immunohistochemistry cannot discriminate between the active and inactive forms of these enzymes. In addition, these antibodies are species-specific and do not cross-react with all species. Moreover, tissue inhibitors of metalloproteinases (TIMPs) can prevent matrix degradation by MMPs, even if the enzymes are in an active form. Hence, to overcome the above mentioned

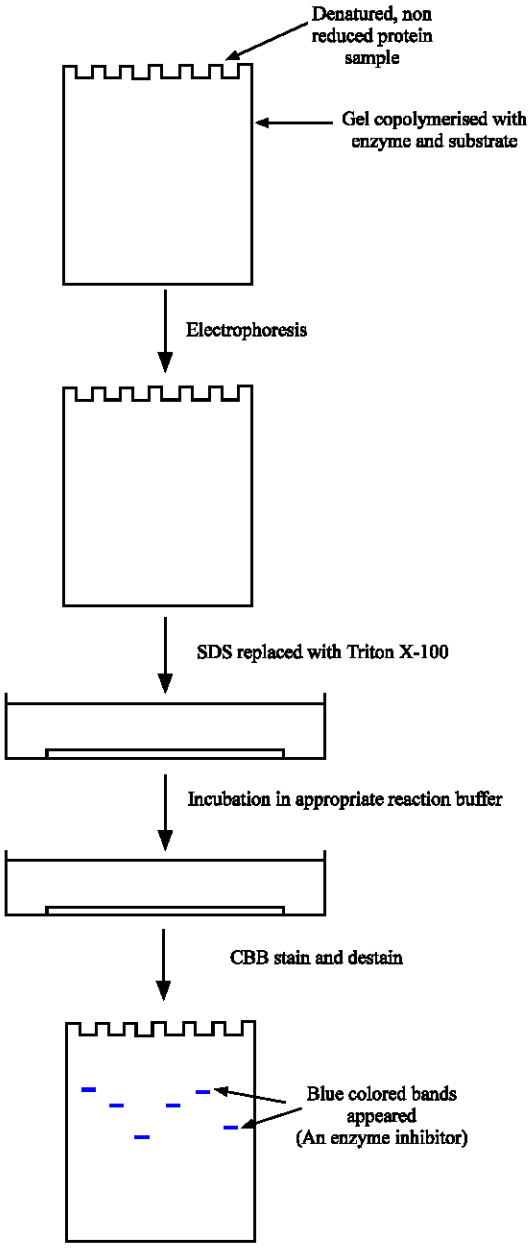

Fig. 2: Reverse zymography

limitations the In situ Zymography (ISZ) is the technique invented. ISZ visualizes localization of enzyme activities in a tissue using a cryosection adhered on a substrate thin membrane. Until now studies of in situ zymography have been performed on MMPs and plasminogen activators and casein, collagen or gelatin have been used as substrates. ISZ offers the ability to estimate various protease activities in combination with the localization of these activities in tissue sections.

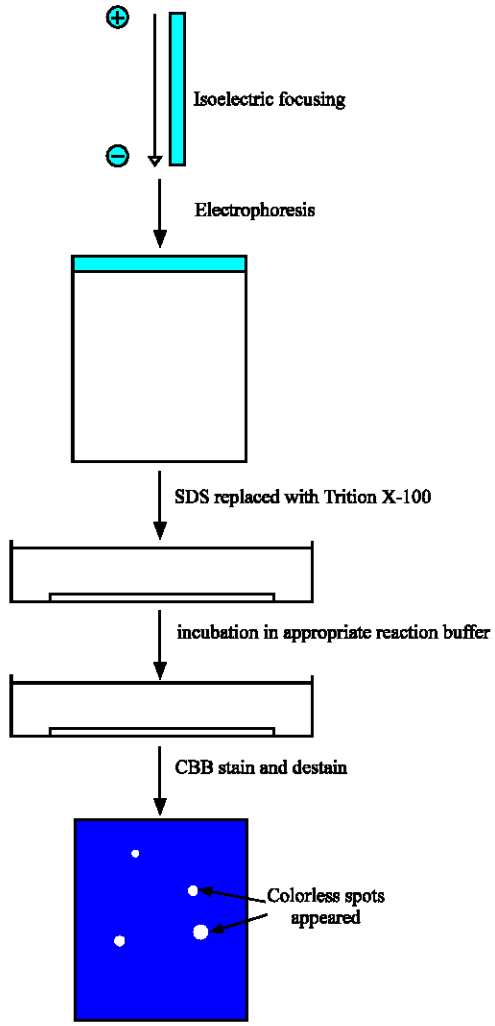

Fig. 3: 2D zymography

2D-Zymography: A combination of Isoelectric focusing (IEF) and zymography is referred as 2D-Zymography (2DZ) refer Fig. 3. 2DZ was applied for the characterization of proteases from marine sponges[10]. It is obvious that due to the harsh conditions imposed by IEF, proteolytic activity might be modified, (partially) inhibited or vanished. Inhibitors of MMPs were detected in muscle tissue from the Atlantic cod, *Gadus morhua*, by 2D reverse zymography[11].

Reader can refer the below mentioned protocol which was standardized by the author. The electrophoresed gel was rinsed twice in Triton X-100 (2.5%) at room temperature. In-gel protease activity was carried out by incubating the gel at 37°C for 18 h in reaction buffer (50 mM Tris-HCl, pH 6.8). The gel was stained with Coomassie brilliant blue R-250 in isopropanol (0.25%) followed by destaining in acetic acid

(7%). The protease activity was determined based on lytic bands in blue background and images were captured using any of the Gel Doc System[12].

DISCUSSION

Proteases play important role in modulating a wide range of cellular functions, in the regulation of biological processes and in the pathogenesis of various diseases. Several molecular techniques are available to identify and characterize proteases in cells and tissues. Most of these techniques do not provide information about the activity of proteases in tissue samples. Zymography is a relatively low cost technique that uses specific protease substrates to detect and localize specific protease activities in tissue extracts or tissue sections or from the single cell. Used in combination with other techniques, Zymography provides data that further helps in our understanding of the role of specific proteases in various pathologic and physiologic conditions.

Studies of in situ zymography with plasmin/plasminogen system were performed on tissue of rheumatic arthritis, colon adenoma and retina in humans, as well as on formation of corpus lutea in rats, formation of placenta and ovary in ovulation phase and normal kidneys in mice (animal models). On the other hand, studies with MMP in humans were the largest on circulation system such as atherosclerosis foci, aortic aneurysm, cerebral artery aneurysm and aortic occlusion and followed by studies on tumor tissue and skin normal and psoriasis. In animals (animal models), studies were made on horse hoofs, coronary artery occlusion in dogs and lung tissue in rats. In addition, there are studies with cultured tissues.

All of the aforementioned methods have been used for both qualitative and quantitative detection of enzymes. It is considered that relative comparison of enzyme activity would be possible even in different experiments and a unified evaluation of experimental results can be made by organs or by tissues, if the same substrate and the same experimental condition are employed.

Zymography and other forms of zymography are techniques used to analyze the activities of different enzymes in complex biological samples such as organs, tissues or cells. Zymography involves the electrophoretic separation of proteins under denaturing (SDS) but non reducing conditions through a poly acryl amide gel containing either natural proteinaceous or synthetic substrate. The resolved proteins are renatured by exchange of SDS with a nonionic detergent such as Triton X-100 and the gel is incubated in an appropriate buffer for particular enzyme under study. The gel is stained with Coomassie blue and enzymatic activities are detected as clear bands against blue background of un-degraded gelatin. The success of this technique, which was originally developed for the study of serine proteases, is based on the following observations: First, the substrate is retained in the gel during electrophoresis which was incorporated into the gel at the time of polymerization; it can therefore function as an in situ substrate. Second, proteolytic activity can be reversibly inhibited by SDS during electrophoresis and recovered by incubating the gel in aqueous Triton X-100; substrate degradation is thus postponed until the sample proteins have been resolved into bands. Finally, the separation of different complexes (ex:MMP:TIMP complex) by SDS-PAGE enables their activities to be determined independently of one another, which is not possible in solution assays. A particular advantage of this system is that both the proenzyme (zymogen) and active forms of enzyme, which can be distinguished on the basis of molecular weight, can be detected. This is possible because the proenzymes are activated in situ presumably by the denaturation/ renaturation process and autolytic cleavage. Before separating the proteins and performing the assay to localize the activity, it is necessary to consider the properties of the enzyme, pH optimum, cofactor requirements and inclusion of cations and co-substrates.

The aim of this mini-review is to outline the general principle employed in different types of Zymography, provide procedure to establish this technique in the laboratory, give an overview of its development and current research applications using specific examples from the literature and discuss possible future uses for this technique.

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