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# Zymogram: Study of an Active Enzyme with Electrophoresis

## Teachers Handbook

## (Cat. # BE-420)

## MATERIALS INCLUDED WITH THE KIT

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial Protein: Collagenase
- 1 vial PAGE: Gelatin
- 1 vial Collagenase Inactivation Buffer
- 1 bottle Folding Buffer (10X)
- 1 bottle PAGE: Reaction Buffer (10X)
- 1 bottle PAGE: LabSafe GelBlue
- 30 Centrifuge Tubes (1.5ml)

## SPECIAL HANDLING INSTRUCTIONS

- Store Collagenase at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

## ADDITIONAL EQUIPMENT REQUIRED

- Reagents and device for making and running (SDS) denaturing protein gel electrophoresis (SDS-PAGE)
- Gel staining tray
- Shaker
- 80-100°C waterbath

## TIME REQUIRED

- Day1: 2-3 hours
- Day2: 1-2 hours

## OBJECTIVES

- Study Zymogram – a protein electrophoresis technique.
- Use of electrophoresis for bioassay and enzyme studies.
- Study denaturation and renaturation of proteins.
- Study protein activation and folding.

## BACKGROUND

Proteins are crucial to every reaction and function of a living organism and as a result their expression and degradation has to be closely regulated. Basically, several processes exist that control the life cycle of a protein molecule. A protein's life cycle begins once its gene is turned on and its mRNA is transcribed and translated to produce the polypeptide strand.

This is the first crucial stage of the life cycle of a protein, the correct folding of a protein. As previously described in the "Protein Structure Analysis" experiment, a newly synthesized protein is folded into secondary, tertiary and sometimes quaternary structures. Correct folding is essential for a protein to be functional, whereas incorrect folding can have severe detrimental effects. For example, several known diseases are attributed to misfolded proteins, including bovine spongiform encephalopathy (BSE) and its human equivalent Creutzfeld-Jakob disease (CJD), Alzheimer's disease, Parkinson's disease, type II (non-insulin dependent) diabetes and some types of cancer. The symptoms of Mad Cow Disease (BSE) and Alzheimer's are a result of misfolded proteins aggregating and forming insoluble protein deposits in the brain.

As the protein is translated by a ribosome, the polypeptide chain lengthens and specialized proteins, known as chaperones, bind to the nascent polypeptide chain to prevent misfolding by association of hydrophobic amino acids. On completion of translation, the chaperone proteins are released, a step requiring ATP, and in most cases the polypeptide chain folds into its correct structure. In some cases, an additional protein complex, known as a chaperonin, is required. The nascent protein is bound inside the chaperonin and in the presence of ATP folds correctly and is then ejected from the chaperonin. An example of a protein using chaperonin is actin.

This experiment is designed to demonstrate that protein folding is crucial to a protein's function. Using a zymogram, an electrophoretic technique to study enzymes and isoenzymes, and the strong anionic detergent students will denature and refold the enzyme collagen. The zymogram will be used to visualize that the correctly folded protein has its biological function.

## TEACHER'S PRE EXPERIMENT SET UP


*Acrylamide/Bis-acrylamide is toxic. Always wear gloves and protective clothing when handling the chemicals.*

1. Make 2% gelatin stock solution: Transfer 0.2g gelatin in a 15ml tube and add 10ml distilled water. Incubate the tube in 80-100°C water and periodically invert the tube until the gelatin completely dissolves.

2. Add 1ml Collagenase Inactivation Buffer into the vial containing the collagenase. Gently vortex or invert the tube to completely dissolve the collagenase (denatured, collagenase concentration is ~0.05µg/µl). Aliquot 120µl to each student group.
3. Mix 100ml 10X Folding Buffer with 900ml distill water in a 1L container to make 1X Folding Buffer. Mix 50ml 10X Reaction buffer with 450ml distill water to 1X Reaction Buffer.
4. Prepare 10-12% polyacrylamide gel containing SDS (SDS-PAGE) and 0.1% gelatin. Each student group needs one 14-lane gel. If using G-Biosciences Protein Electrophoresis kit (Cat. # BE-406), follow the protocol provided in the kit. Add 2% gelatin stock solution in both running and stacking gel solutions to a final concentration of 0.1% gelatin. Mix well before casting the gel. *OPTIONAL: You can make this part of the protocol and have the students prepare their own gels.*
5. Aliquot reagents to each student group according to next section.

## MATERIALS FOR EACH GROUP

Each group is supplied with the following components.

- 1 polyacrylamide gel containing 0.1% gelatin
- 120µl Collagenase
- 150µl Collagenase Inactivation Buffer
- 100ml 1X Folding Buffer
- 50ml 1X Reaction Buffer
- 60ml LabSafe GelBlue
- 4 Centrifuge Tubes (1.5ml)

## PROCEDURE


*Wear gloves throughout the experiment.*

***I. Denaturation and Refolding of Collagenase***

OPTIONAL: Prepare 12% polyacrylamide gel containing SDS (SDS-PAGE) and 0.1% gelatin. Each group of 4 needs one 14-lane gel. Add 2% gelatin stock solution in both running and stacking gel solutions to a final concentration of 0.1% gelatin. Mix well before casting the gel.

1. Working in pairs, each pair labels 3 tubes with their names and number 1-3. Each group is provided with a denatured form of collagenase, denatured with SDS, and each pair will serially dilute the collagenase concentration in the following steps.
2. Transfer 30µl Collagenase Inactivation Buffer to tubes 2 and 3.

*Collagenase Inactivation Buffer contains the strong anionic detergent SDS to denature the protein.*

3. Pipette 30µl collagenase solution to tubes 1 and 2. Mix with the Collagenase Inactivation Buffer in tube 2 by pipetting up and down 2-3 times.
4. Transfer 30µl solution from tube 2 to tube 3 and mix with the Collagenase Inactivation Buffer in tube 3 by pipetting up and down 2-3 times.

5. Set up the polyacrylamide electrophoresis gel (SDS-PAGE) and load your samples, as follows.
6. Load 10µl of each sample onto the gelatin-SDS- polyacrylamide gel as shown below:

| **Lane #** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | - | 1 | 2 | 3 | 1 | 2 | 3 | - | 1 | 2 | 3 |
| | PAIR 1 | | | | | | | PAIR 2 | | | | | | |

7. Run the gel at 30mA for 60-90 minutes or until the blue dye front is close to the bottom of the gel.
8. Disassemble the gel carefully and cut the gel through the empty lane 4 and lane 11. Lanes 5-10 will be stained as the control. Lanes 1-3 and 12-14 will be used for the "Test Experiment".
9. Stain the "Control" gel, lanes 5-10, according to Section II. *The control gel contains denatured collagenase.*
10. Rinse the "Test Experiment" gel with distilled water twice, 5 minutes each wash. *To renature and correctly fold the collagenase into the enzymatically active enzyme form, the gel is washed with various buffers to remove the denaturing anionic SDS and allow the collagenase to refold.*
11. Add 30ml 1X Folding Buffer to the gel and gently shake the gel for 30 minutes. *Folding buffer contains a non-ionic detergent to wash away the anionic, denaturing SDS.*
12. Replace with 30ml fresh Folding Buffer and gently shake for a further 30 minutes. *Further washing is required to remove all the SDS.*
13. Rinse the "Test Experiment" gel with distilled water twice, 5 minutes each wash.
14. Add 30ml 1X Reaction Buffer to the gel and incubate it overnight at 37°C. Tightly seal the container to prevent evaporation. *The Reaction Buffer contains calcium ions that bind to collagenase and restore its native structure and enzyme activity.*
15. After overnight incubation, stain the gel according to Section II.

***II. Gel Staining***

1. Wash the gel twice in distilled water, five minutes each.
2. Remove all the free water from the gel.
3. Add 30ml LabSafe GelBlue stain to cover the gel. Gently shake the gel for 60 minutes at room temperature.

*Protein bands will start to appear after 10 minutes. Check gels at regular intervals to see the proteins appear.*

4. Decant the LabSafe Gel Blue stain and rinse the gel with distilled water. The gel can be stored in water. Longer destaining, such as overnight, in water will give a clearer view of the protein bands.

## RESULTS, ANALYSIS & ASSESSMENT

Compare and describe the "Control" and "Test Experiment" gels. *The protein stain will stain the "Control" gel completely blue, due to the presence of the gelatin in the gel. The "Test Experiment" gel will be mostly stained blue, except in the areas where the collagenase was renatured. These areas will be clear of gelatin, digested away by the enzyme and will be clear.*

Explain your results below.

*After treating the gel with Folding Buffer-I and -II, collagenase becomes active and it begins to digest gelatin embedded in the gel, creating gelatin free or protein depleted zones which do not stain with the protein stain, creating a pattern of negative staining.*

Q. Describe the denaturation and inactivation of collagenase.

*A. When treated with inactivation buffer, which contain SDS, collagenase structure is destroyed due to the unfolding of the collagenase protein molecule and coating the entire length of protein with negatively charged SDS.*

Q. Describe refolding and inactivation of the collagenase.

*A. During electrophoresis, collagenase remains unfolded and inactive. After electrophoresis, when the gel is washed in Folding Buffer-I, which contains non-ionic detergent, SDS bound with the collagenase is washed away and removed allowing refolding of the collagenase. When the gel is transferred into Folding Buffer-II, which contains calcium ions, calcium binds with collagenase and restores native collagenase structure and biological activity, i.e. the ability to digest proteins, gelatin.*

Q. Describe the pattern of bands seen after staining the gel.

*A. After treating gel with Folding Buffer-I, -II, collagenase becomes active and it begins to digest gelatin embedded in the gel, creating gelatin free or protein depleted zones which do not stain with protein stain, creating a pattern of negative staining.*


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## Student's Handbook

## (Cat. # BE-420)

## OBJECTIVES

- Study Zymogram – a protein electrophoresis technique.
- Use of electrophoresis for bioassay and enzyme studies.
- Study denaturation and renaturation of proteins.
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## BACKGROUND

Proteins are crucial to every reaction and function of a living organism and as a result their expression and degradation has to be closely regulated. Basically, several processes exist that control the life cycle of a protein molecule. A protein's life cycle begins once its gene is turned on and its mRNA is transcribed and translated to produce the polypeptide strand.

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## MATERIALS FOR EACH GROUP

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- 100ml 1X Folding Buffer
- 50ml 1X Reaction Buffer
- 60ml LabSafe GelBlue
- 4 Centrifuge Tubes (1.5ml)

## PROCEDURE


*Wear gloves throughout the experiment.*

***I. Denaturation and Refolding of Collagenase***

*OPTIONAL: Prepare 12% polyacrylamide gel containing SDS (SDS-PAGE) and 0.1% gelatin. Each group of 4 needs one 14-lane gel. Add 2% gelatin stock solution in both running and stacking gel solutions to a final concentration of 0.1% gelatin. Mix well before casting the gel.*

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   *Collagenase Inactivation Buffer contains the strong anionic detergent SDS to denature the protein.*
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6. Load 10µl of each sample onto the gelatin-SDS- polyacrylamide gel as shown below:

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| | 1 | 2 | 3 | - | 1 | 2 | 3 | 1 | 2 | 3 | - | 1 | 2 | 3 |
| | PAIR 1 | | | | | | | PAIR 2 | | | | | | |

Run the gel at 30mA for 60-90 minutes or until the blue dye front is close to the bottom of the gel.

1. Disassemble the gel carefully and cut the gel through the empty lane 4 and lane 11. Lanes 5-10 will be stained as the control. Lanes 1-3 and 12-14 will be used for the "Test Experiment".

2. Stain the “Control” gel, lanes 5-10, according to Section II.
   *The control gel contains denatured collagenase.*
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## RESULTS, ANALYSIS & ASSESSMENT

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Explain your results below.

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Q. Describe the denaturation and inactivation of collagenase.

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Q. Describe refolding and inactivation of the collagenase.

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Q. Describe the pattern of bands seen after staining the gel.

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