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Modified Blue Native Electrophoresis for better Resolution of Proteases

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Abstract

We report a modified blue native polyacrylamide gel electrophoresis (PAGE) protocol for better resolution of proteins in native form. Extracellular proteases from *Bacillus* sp., were resolved in their active form proved by Zymography.

Keywords: Proteases, *Bacillus* sp., PAGE, Zymography.

Introduction

Proteases are group of hydrolytic enzymes. Proteases catalyze the cleavage of peptide bonds in other proteins. Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals has conferred added biotechnological importance (Mala *et al*., 1998). Proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes.

Traditional native electrophoresis is limited in its applicability to native protein analysis because of high or low operative pH which may adversely affect proteins. Another drawback in native gel electrophoresis is the need for separate acidic and basic gels for resolution of enzymes. SDS confers net negative charge to proteins but sometimes it may dissociates protein complexes and irreversibly denature enzymes.

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a method for the isolation of intact protein complexes (Schagger and Jagow 1992). BN-PAGE preserves the biochemical activity of the components separated after electrophoresis. This technique uses an anionic dye, Coomassie Blue G250 which binds to surface-positive residues of proteins, this binding of a large number of negatively charged dye molecules to proteins facilitates the multiprotein complex migration without dissociating them. This protocol was used to overcome aggregation commonly observed in lipases (Saminathan *et al*., 2008). We have extended this protocol for the better resolution of proteases in their native form. To our knowledge, this is the first report on the use of BN-PAGE for protease studies.

Native polyacrylamide gel electrophoresis was performed using the discontinuous gel system of Davis (Davis, 1964). For BN–PAGE, the stacking and separating gels were prepared with 4% (w/v) and 10% (w/v) acrylamide, respectively, using 50 mM Tris–Cl, pH 7.5. Cathode buffer was prepared at pH 7.5 with 50 mM Tricine and 15 mM Tris and anode buffer was prepared with 50 mM Tris–Cl at pH 7.5. The cathode

buffer was supplemented with 0.002% (w/v) CBB G-250 dye. The 2X sample solubilizing buffer contained 1% (v/v) Triton X-100, 50 mM Tris–Cl, pH 7.5, 1% (w/v) CBB G-250, and 20% (v/v) glycerol. A protease secreted *by Bacillus* sp., (Eighteen-hour grown 1% *Bacillus* sp. culture was inoculated into 50 ml of nutrient broth and incubated for 20 h at 37°C at 160 rpm. Cells were pelleted at 5000g for 10 min and the cell-free culture supernatant was precipitated with 4 volumes of ice-cold acetone) was loaded in the gel. Protein sample was mixed with equal volume of sample solubilizing buffer and incubated for 0.5 h before loading; 20µg of the sample was loaded in each well. The gel was run at 20°C and 60 V. The gel was run for a period of about 6 h until the dye front reached the end of the gel.

One half of the gel was stained with Commassie Brilliant Blue R-250 to locate protein bands and the other half was used for activity staining. The gel was first equilibrated in 25 mM Tris–Cl, pH 7.5, 10 mM $CaCl_2$ for 0.5 h and then the gel was immersed in equilibration buffer containing 1%(w/v) gelatin, for 1h at 37°C in order to allow the substrate to diffuse into the gel. Then the gel was transferred to a clean petri plate and incubated at 37°C for 0.5h to allow the hydrolysis of diffused gelatin. Then stained with Commassie Brilliant Blue R-250. Extracellular proteases activity were observed as a clear zone against blue background (Fernando *et* al., 1993) (See Fig 1).

Results and Discussion

Extracellular secreted proteins were not resolved properly in native PAGE as seen in fig 1A and most of the protease active enzmes aggregated in the native PAGE. When then same extracellular proteins were loaded into modified BN-PAGE, proteins were resolved better and protease active bands were not aggregated in BN-PAGE.

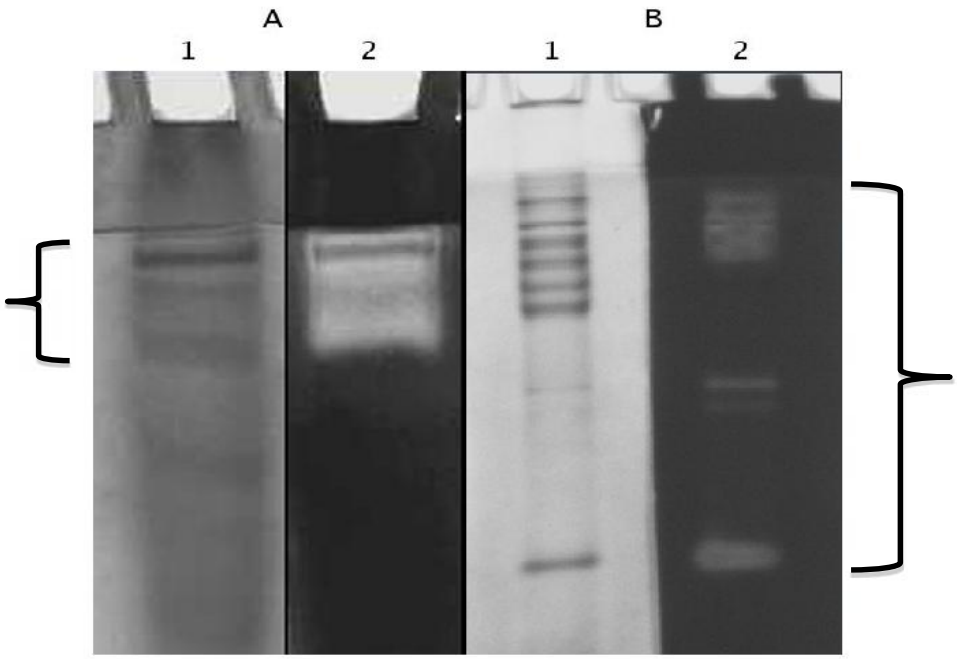

Fig 1: Protease activity staining after gel electrophoresis. A) Native gel electrophoresis, B) Blue Native gel electrophoresis. Lane 1, Coomassie stained *Bacillus* extracellular proteins (20µg total protein), Lane 2, activity stained *Bacillus* extracellular proteins (20µg total protein).

This protocol offers better resolution of proteins under native conditions. Buffers used for electrophoresis were near neutral (pH 7.5) which is well within the buffering range of both Tris and Tricine buffers. This provides gentle conditions for proteins and helps to keep the proteins intact upon solubilization and through electrophoresis. Oligomeric states of native proteins can be determined if BN-PAGE in first dimensional separation is combined with SDS-PAGE in second dimension. This novel electrophoretic technique can be employed for analysis of molecular mass, oligomeric state and

homogeneity of native proteins. (Niepmann and Zheng., 2006). BN-PAGE offers advantage for the separation of protein in second dimension to study the subunits of the enzymes in a manner analogous to the way BN–PAGE is currently used to study protein complexes (Brookes *et al.*, 2002). This simple protocol is easy to perform and offers better resolution for identifying extracellular protease multicomplex proteins without denaturing them in native conditions.

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