Analysis of Ricin Subunits by High-Resolution Acidic Native Gel Electrophoresis

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Abstract
Ricin is a potent toxic castor bean glycoprotein comprising of two subunits, A chain and B chain. We studied ricin subunits in an acidic electrophoresis system followed by electroblotting. To the best of our knowledge, there is no study reported for characterization of ricin subunits using acidic native PAGE. Sample preparation steps for acidic native PAGE were optimized and adequate electrophoretic conditions were developed for achieving a complete separation of ricin subunits. Ricin subunits were separated and characterized at acidic pH (2.9 and 4.3) and their immunological detection was made using polyclonal antibodies. Our findings showed that this method results in sharper band and therefore high resolution than SDS-PAGE and also the following protein transfer is highly efficient and rapid.

Keywords: Ricin, Acidic non-denaturing PAGE, Electroblotting, Resolution.

Introduction
Ricin is a 64 kDa a glycoprotein belonging to the type II group of ribosome activating protein (1, 2). It is a two chain variant protein that exists in slightly different isoforms in beans of different origin with differences in their biochemical properties (3). Ricin and it’s A chain has got therapeutic value as immunotoxins (4). Ricin has been extensively characterized using electrophoretic methods including SDS-PAGE and high resolution two-dimensional electrophoresis (2-DE) (5). In contrast, ricin subunits are poorly characterized in their native form by conventional electrophoresis. Ricin having molecular weight from 60 kDa to 65 kDa by SDS-PAGE technique has been reported under non-reduced conditions (6). Under reducing condition, ricin shows the presence of two subunits, corresponding to 30 kDa and 32 kDa (7).

In this study, we describe an additional electrophoretic method for rapid analysis of purity and homogeneity of the ricin subunits. The electrophoretic characterization of ricin has been done earlier at acidic pH 2.9 (8). To the best of our knowledge, there is no study reported for characterization of ricin subunits using acidic native PAGE. The aim of the present study was to optimize protocols for acidic non-denaturing PAGE of ricin subunits and their identification by blotting. We tested buffers of different pH for getting the best electrophoretic resolution. It was done at pH 2.9 and 4.3 with a gel concentration of 15% acrylamide.

Acidic native PAGE system without SDS enables the separation of proteins and peptides as a function of their combined charge and size. In particular acetic acid/KOH-PAGE system as described in the study provides excellent
resolution of many proteins and peptides that might not be resolved using SDS-PAGE. The method uses an acidic buffer to retain protein solubility and gives uniform gels to allow migration of proteins. The electrophoretic run is much shorter (90 min) than conventional SDS-PAGE.

Materials and Methods

**SDS-PAGE:** SDS-PAGE was performed under reduced and non-reduced conditions to assess the purity of ricin, and its subunits using Bio-RAD, USA electrophoretic apparatus. The SDS-PAGE was performed according to Laemmli (9).

**Acidic Native PAGE:** Acidic non-denaturing gel electrophoresis of all the above mentioned samples was performed according to Hames (10) with slight modifications. The 5% stacking gel was prepared in 120 mM KOH and 0.75% acetic acid (pH-5.9). Ammonium per sulfate (APS) and TEMED concentration was 0.7% and 0.06% respectively. 15% resolving gel was prepared in 30 mM KOH and 13.25% acetic acid (pH-2.9). The concentration of APS was similar to that of stacking gel. TEMED concentration was increased to 0.6%. Electrode buffer was 0.16% acetic acid containing 0.65% b-alanine, pH-2.9. Loading buffer contained 0.8% glycerol, 2% methylene blue, 120 mM KOH and 0.75% acetic acid (5.9). The acidic PAGE at pH 4.3 was carried out in a similar fashion but pH of all the buffers was kept 4.3. Samples were mixed with equal volumes of loading buffer for application onto the gel. Electrophoresis was performed in the cold (4°C) at 200V for 75 min. Gel was stained in coomassie blue stain (0.4% dye made in 50% methanol/10% acetic acid). Destaining was carried out in 30% methanol/10% acetic acid solution.

**Electro blotting:** Western blot analysis was performed as described by Caponi and Migliorini (11). After separation by acidic non-denaturing PAGE, proteins were transferred to PVDF membrane, (0.45 μm, pore size, Pierce Biotechnology, USA) using 0.7% acetic acid. Blotting was performed at constant voltage (80 V) for 2 h in Bio-Rad Trans-Blot apparatus. Temperature was kept low by using ice block supplied with the system.

For immunodetection, the blots were blocked overnight with 5% low fat milk powder in blocking buffer (5% milk powder, 0.05% Tween-20 in PBS) at 4°C. Blots were washed 3 x for 15 min each with PBST (PBS containing 0.05% Tween-20) and then incubated with primary antibody at 1:5000 dilution for 90 min at 37°C. The blots were incubated with a secondary antibody, goat anti-rabbit IgG-HRP conjugate (1:50,000) for 90 min at 37°C after washing thrice with PBST. After another three washes, the blots were developed using an enhanced chemiluminescent detection system (Super Signal West Pico Chemiluminescent Substrate, Pierce) according to manufacturer’s protocol and the images were taken on Pierce CL-XPosure TM X-ray film.

Results and Discussion

**Electrophoretic characterization of Ricin subunits:** The results of SDS–PAGE of ricin under non-reduced and reduced conditions are shown in Fig. 1A. Under non-reducing condition, ricin gave a single band in 60-65 kDa region. When ricin was treated with -mercaptoethanol, it gave two bands in the region of 30-34 kDa region which represent A and B subunits of the ricin. Ricin A subunit showed one major and one minor band in 30-34 kDa region which represent A and B subunits of the ricin. Ricin A subunit showed one major and one minor band in 30-34 kDa region. Ricin B subunit revealed one band (Mr » 33-34 kDa) compared to the molecular weight markers separated on the same gel.

The electrophoretic mobility of ricin and its subunits varied with pH of the buffer used in
native gel electrophoresis. We observed ricin and its A chain are highly charged at acidic pH 2.9 and moves faster in the gel. B chain carries insufficient charge at an acidic pH 2.9 and therefore is completely stacked in the gel (Fig. 1B). Both the chains of ricin have similar electrophoretic mobility and can be easily separated at pH 4.3 (Fig. 1C). The electrophoretic mobility of ricin is higher than its subunits at pH 4.3. Ricin, ricin B chain as well as A chain can be resolved at pH 4.3, so this pH is taken as reference standard for their characterization.

Immunological characterization of Ricin subunits: We also demonstrated electrophoretic transfer of acidic native PAGE separated proteins using acetic acid as transfer buffer onto a PVDF membrane for immunodetection. Transfer of acidic native PAGE separated proteins onto a PVDF membrane occurred with high efficiency (12). The western blot study demonstrated that this system of electrophoresis of ricin subunits is very useful for the identification of antibody specificities. We observed that the anti ricin A chain antibody is highly specific to A chain but anti ricin B chain antibody is cross reactive to A chain upon blotting (Fig. 2A and 2B).

Conclusion

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for the separation and characterization of proteins. Electrophoretic separation of glycoproteins like ricin results in broad and fuzzy bands in SDS PAGE. Here, we report a modified acidic non-denaturing PAGE for the study of ricin and its subunits. This system of acidic non-denaturing PAGE gives greater resolution for separation of ricin subunits than SDS-PAGE. In addition, protein transfer following acidic PAGE is also rapid and efficient. Compared to western blotting after SDS PAGE, for which a higher concentration of Tris/glycine buffer is used, the method employing a dilute acetic acid as transfer buffer is more economical.

In conclusion our results indicates that the high resolution acidic native PAGE followed by electro blotting may prove to be a valuable tool for biochemical characterization and
development of standard methods for identification and detection of ricin and its subunits.

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