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Characterization of Two Ricin Isoforms by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis and Capillary Isoelectric Focusing

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Sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) and capillary isoelectric focusing (CIEF) were employed to characterize and compare ricin E purified from the small grain seeds of *Ricinus communis* with ricin D isoform. During the purification of ricin E using ion-exchange and size-exclusion chromatography, SDS-CGE was found to be useful for monitoring the efficiencies of purification steps. SDS-CGE showed that the molecular size of ricin E was not significantly different from that of ricin D, which was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. CIEF was useful for discriminating ricin E from ricin D based on their isoelectric points (pI). The pI values of ricin E and D were 8.6-8.8 and 7.0-7.4, respectively. This study demonstrates the usefulness of SDS-CGE and CIEF for the characterization of ricin toxins.

Key Words : Ricin, Capillary electrophoresis, Capillary isoelectric focusing, Sodium dodecyl sulfate-capillary gel electrophoresis

Introduction

Ricin is a plant toxin obtained from the seeds of the castor bean (*Ricinus communis*).[1] It is a heterodimeric glycoprotein composed of a toxic A subunit linked by a disulfide bond to a galactose-specific lectin B subunit.[2] The B chain is responsible for binding to cells and entry of the toxin into cells by receptor-mediated endocytosis, and the A chain catalytically inactivates ribosome by hydrolyzing a specific bond between the base and the ribose of adenosine. The high cytotoxity of ricin has been widely exploited in the development of chemotherapeutic agent, such as immunotoxin, in which the toxin is coupled to a specific antigen-binding monoclonal antibody.[3,4]

There are two types of ricin, ricin D and ricin E, which have similar molecular mass of approximately 62 kDa but have different isoelectric points, amino acid compositions, affinity to Sepharose, and toxicity to some malignant cells.[5,6] Ricin D is found in large grain seeds, whereas small grain seeds contain both ricin D and E.[7,8] Ricin D is known to be a Sepharose-binding protein that exhibits higher cytotoxicity and has an isoelectric point at 7-8, whereas ricin E is a Sepharose-nonbinding protein with less cytotoxicity and isoelectric point near 9. Ricin D has been mainly used in immunotoxin studies concerning the developments of targeted chemotherapeutics, whereas relatively few studies have been conducted on the application of ricin E to immunotoxins because ricin E is more difficult to separate than ricin D and information concerning its physicochemical properties has been limited.[3,9,10]

Several analytical techniques have been devised to detect and characterize ricin, such as gel electrophoresis, capillary electrophoresis (CE), surface plasmon resonance, electrospray mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and immunoassay.[11-16] Among them, CE has received considerable attention because of its ability to separate proteins with high resolution and its applicability of various separation modes, such as sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE), capillary isoelectric focusing (CIEF), as well as capillary zone electrophoresis.[17,18]

The purpose of this study was to characterize and compare ricin E purified from the small grain seeds of *Ricinus communis* with ricin D isoform by CE methods (SDS-CGE and CIEF) and MALDI-TOF MS. Although several analytical methods have been reported for the analysis of ricin, there was no report on the comparison of two ricin isoforms (ricin D and E) by CE and mass spectrometric techniques. In this study, we purified ricin E from small grain seeds of *Ricinus communis* and characterized the electrophoretic properties of the purified ricin E and the commercially obtained ricin D using SDS-CGE and CIEF. The molecular weights of ricin E and D were determined by MALDI-TOF MS.

Experimental

Materials. Ricin D and sinapinic acid were purchased from Sigma (St. Louis, MO, USA). The CE-SDS Protein kit, CE-SDS Protein size standards (14-200 kDa), Bio-Lyte pH 3/10 ampholyte, and BioMark pI markers (pI 5.3-10.4 to 5.3) were purchased from Bio-Rad (Hercules, CA, USA).

Acetonitrile (HPLC grade) and trifluoroacetic acid were supplied by J.T. Baker (Philipsburg, NJ, USA) and Acros Organic (Pittsburgh, PA, USA), respectively. All other chemicals used were of analytical reagent grade.

Purification of Ricin E. Ricin E was purified from castor beans (small grain type) harvested from the Herbal Garden at SungKyunKwan University (Suwon, Korea). Decorticated castor beans (100 g) were homogenized in one liter of 10 mM phosphate buffered saline (PBS, pH 7.4) in a blending mixer for 30 min at room temperature. The homogenate was stirred overnight at 4 °C and centrifuged at 10,000 g for 30 min to separate it into a lipid and aqueous layers. The lipid layer was dismissed by ether extraction. The aqueous solution was dialyzed against 10 mM phosphate buffer (pH 6.5) and loaded onto a CM-Sepharose column (2.5 × 5.0 cm) pre-equilibrated with 10 mM phosphate buffer (pH 6.5). All unbound proteins were washed and bound proteins were eluted with 10 mM phosphate buffer (pH 6.5) containing 0.3 M sodium chloride. Eluted proteins were concentrated by ultrafiltration (membrane MWCO 10,000-12,000 Da), dialyzed against 20 mM Tris-HCl buffer (pH 7.5), and loaded onto a DEAE-Sephacel column (2.5 × 5.0 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Unbound proteins were collected and loaded onto Sephadex G-75 column equilibrated with 10 mM PBS (pH 7.4). The first eluted proteins were collected.

Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis (SDS-CGE). SDS-CGE was performed using a Bio-Rad BioFocus 3000 CE System equipped with an uncoated fused-silica capillary (50 μm I.D., 24 cm total length and 19.5 cm to the detector). Prior to sample injections, the capillary was rinsed with 0.1 M NaOH, 0.1 M HCl, and CE-SDS Run Buffer, for 120, 60, and 240 sec, respectively. Protein samples were dissolved in CE-SDS Protein Sample Buffer with the CE-SDS internal standard (benzoic acid). Samples were heated under non-reducing conditions at 80 °C in water bath for 10 min, cooled on ice for 3 min, and centrifugation. Sample injections were carried out in electrophoretic mode (30 sec at 10 kV). Separations were performed at a constant voltage of 15 kV and monitored on-column at UV 220 nm for 12 min. Both capillary and sample temperatures were maintained at 20 °C.

Capillary Isoelectric Focusing (CIEF). CIEF was performed with a BioCAP LPA coated fused-silica capillary (50 μm I.D., 24 cm total length and 19.5 cm to the detector). Samples were mixed with Bio-Lyte pH 3/10 ampholytes at a final ampholyte concentration of 2% and centrifuged for 10 sec at 5000 g. BioMark pI markers ranging from pI 5.3 to 10.4 were used as external standards to calibrate pI values. Samples mixed with ampholytes were injected by applying pressure for 20 sec at 100 psi. Focusing was performed at a constant voltage of 15 kV for 4 min using 20 mM phosphoric acid and 40 mM sodium hydroxide as anolyte and catholyte, respectively. Mobilization was performed by applying a constant voltage of 15 kV for another 21 min, and monitoring on-column at UV 280 nm. Sample and capillary temperatures were maintained at 20 and 27 °C, respectively.

MALDI-TOF MS. MALDI-TOF MS was performed using a Voyager-RP Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA). Linear and positive-ion TOF detections were performed using an acceleration voltage of 25 kV and a laser intensity of approximately 10% greater than threshold. Grid and guide wire voltages were chosen for each spectrum to achieve the optimal signal to noise ratios. Spectra were obtained by summing over 256 laser shots and smoothed with a 19-point Savitzky-Golay filter. A saturated solution of sinapinic acid in 70% acetonitrile containing 0.1% trifluoroacetic acid was used as a matrix solution and a mixture of cytochrome C and bovine serum albumin was used for external calibration. Sample-matrix solution was prepared at a ratio of 1:2. Mixtures were thoroughly vortexed and 1 μL aliquots of sample-matrix solutions were deposited onto the sample plate of the MALDI-TOF MS unit and dried by rapid vacuum evaporation.

Results and Discussion

SDS-CGE Monitoring of Ricin Purification. Ricin E was isolated and purified from small castor beans (*Ricinus communis*) by ion-exchange and size-exclusion chromato-

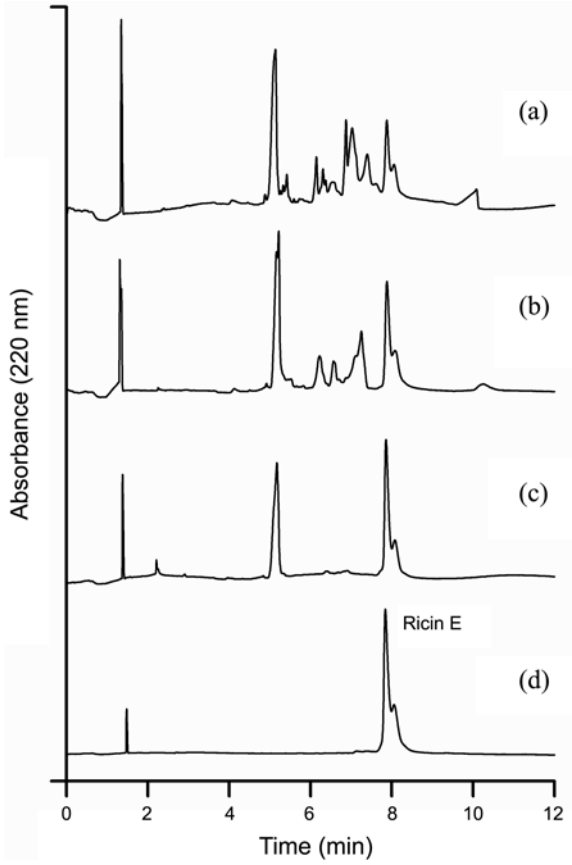

Figure 1. SDS-CGE electropherograms of castor bean extract (a), bound fraction of cation-exchange chromatography on CM-Sepharose column (b), unbound fraction of anion-exchange chromatography on DEAE-Sephacel column (c), and purified ricin E (d).

graphy. To optimize the purification process, the monitoring of ricin levels isolated in each purification step is necessary to ensure that the desired levels of purity have been achieved. For efficient monitoring, the analytical method needs to be easy, simple and speedy. Figure 1 shows SDS-CGE electropherograms of samples during the purification of ricin from castor beans. Several peaks observed in the crude extract (Figure 1(a)) were substantially removed by cation-exchange chromatography using a CM-Sepharose column (Figure 1(b)) and anion-exchange chromatography using a DEAE-Sephacel column (Figure 1(c)). As ricin E was known to be basic protein with pI near 9,[7,8] the ricin-containing fraction was found in the bound fraction of CM-Sepharose with phosphate buffer at pH 6.5 and the unbound fraction in DEAE-Sephacel with Tris-HCl buffer at pH 7.5. Finally, pure ricin was obtained by size-exclusion chromatography on a Sephadex G-75 column, and showed two partially separated peaks by SDS-CGE (Figure 1(d)). In ricin composed of an A chain (RTA) and a B chain (RTB) linked by an inter-chain disulfide bridge, RTA consists of two isoforms called RTA_1 and RTA_2, which have identical amino acid sequences but different carbohydrate contents.[19] The RTA_1 has a single complex-type oligosaccharide side-chain containing fucose and xylose residues, while the RTA_2 has an additional high-mannose oligosaccharide side-chain.[20] Therefore, the carbohydrate content of RTA_2 is more than twice that of RTA_1. In the SDS-CGE of purified ricin (Figure 1(d)), the first major peak was considered to be a ricin composed of RTA_1 and RTB, and the second minor peak was corresponded to ricin composed of RTA_2 and RTB. As a monitoring method for the ricin purification, SDS-CGE showed the advantages of speed (12 min of migration time per sample), high resolution, and automation compared with time-consuming slab gel technique, such as SDS-polyacylamide gel electrophoresis (SDS-PAGE).

SDS-CGE and MALDI-TOF MS of Ricin E and D. The gel electrophoretic mobilities of ricin E purified in this study and the commercially obtained ricin D were compared by SDS-CGE (Figure 2). The ricin E and D showed similar peak appearances and migration times (8.7 and 9.0 min for RTB-RTA_1 and RTB-RTA_2 isomers of ricin, respectively). Therefore, they were found to have similar molecular sizes based on their electrophoretic mobilities.

MALDI-TOF MS was used to determine the molecular masses of ricin E and D, because SDS-CGE was inappropriate for determining the molecular mass of ricin. As demonstrated in a previous study, the carbohydrate of ricin decreases charge-to-mass ratio when complexed to SDS than the non-glycosylated proteins used as molecular weight markers, which results in a slower migration rate and an overestimation of molecular mass.[13] Figure 3 shows the MALDI-TOF MS spectra of ricin E and D. MALDI-TOF MS could not resolve two peaks of ricin presented in SDS-CGE. In the molecular masses averaged with three times measurements, ricin E showed *m/z* 62445 (± 55) and ricin D showed *m/z* 62409 (± 106). These mass values were almost consistent with that of ricin D (*m/z* 62600) measured by

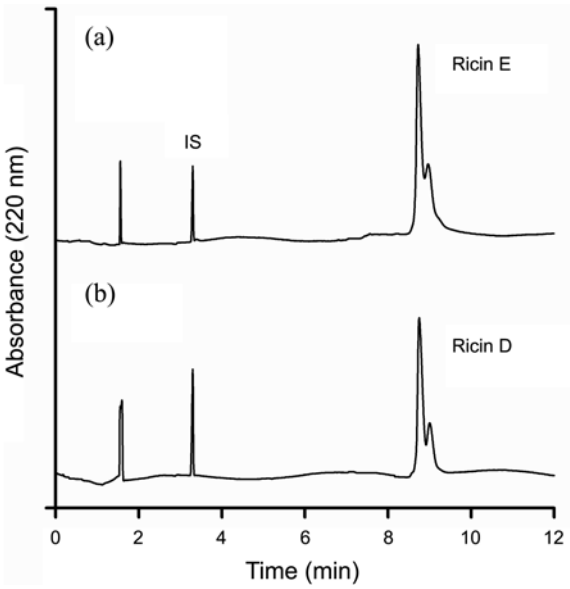

Figure 2. SDS-CGE electropherograms of ricin E (a) and ricin D (b). IS: internal standard (benzoic acid).

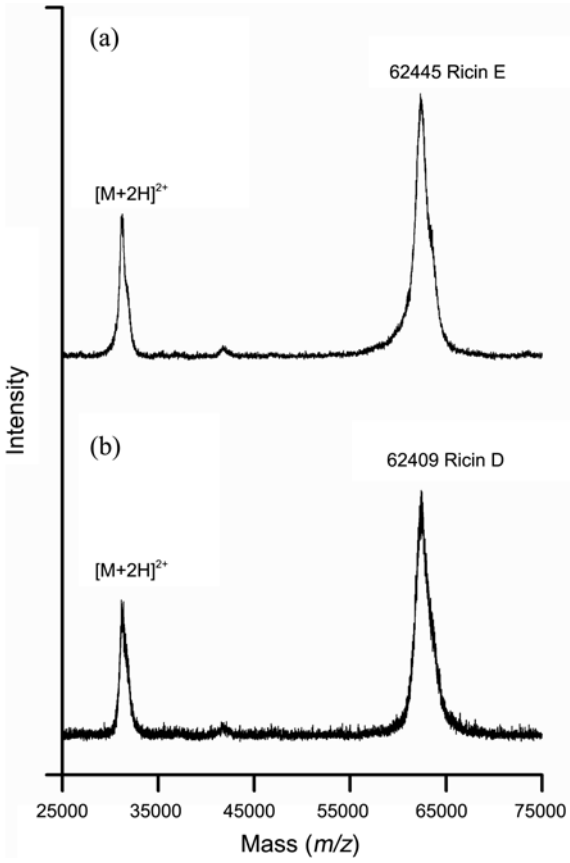

Figure 3. MALDI-TOF MS spectra of ricin E (a) and ricin D (b).

MALDI-TOF MS determination of other group.[21] Although ricin E seemed to have a slightly greater molecular weight than ricin D, this molecular weight difference was not significant.

CIEF of Ricin E and D. Figure 4 shows CIEF electropherograms of ricin E and D. The RTB-RTA_1 and RTB-

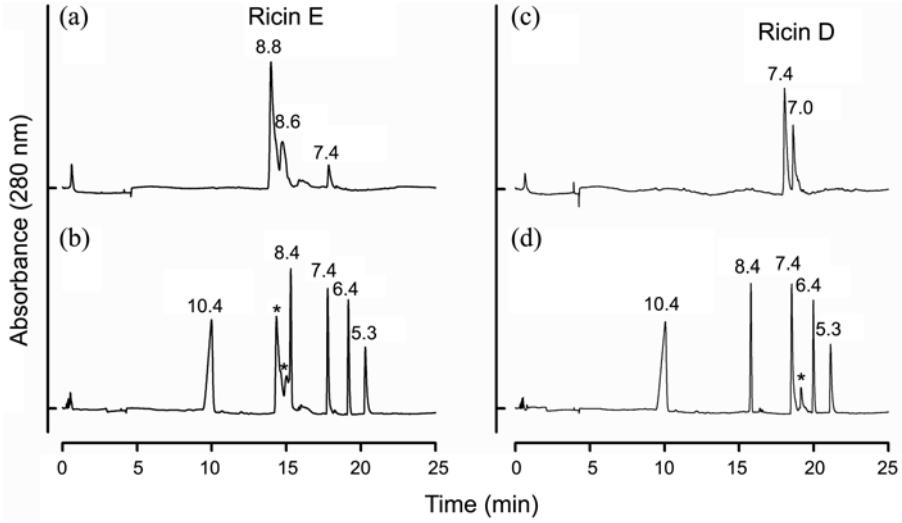

Figure 4. CIEF electropherograms of ricin E (a), ricin E-spiked pI marker (b), ricin D (c), and ricin D-spiked pI marker. The numbers on peaks are pI values. *indicates the peak corresponding to the spiked ricin. In (b) and (d), the peaks corresponding to the pI value of ricin were overlapped with the pI 7.4 marker peak.

RTA_2 isomers of ricin were better resolved than by SDS-CGE (Figure 4(a) and 4(c)). This indicates the resolving power of CIEF for the separation of ricin isomer is stronger than SDS-CGE. The CIEF is known to separate proteins with pI differences as small as 0.02 pI units.[22] The discrimination of the RTB-RTA_1 and RTB-RTA_2 isomers is important for the characterization of ricin because RTA_1 and RTA_2 showed different toxicity due to the different carbohydrate contents. RTA_1, which contains less carbohydrate with lower mannose content than RTA_2, accumulated less in the liver than did RTA_2 and cleared more slowly from the blood.[19] The toxicity of RTA_1 to mice was twice that of RTA_2.[23] To determine the pI values of ricin E and D, calibration curve with standard pI markers (pI 5.3-10.4) were constructed. CIEF of these markers showed a linear relationship between their known pI values and their average migration times, and relative standard deviations were between 2 and 5%. Based on this calibration curve, the pI values of ricin E and D were determined. These values were confirmed by spiking ricin samples with the standard pI markers (Figure 4(b) and 4(d)).

In the CIEF of ricin D, two peaks were well resolved and their pI values were determined to be 7.4 and 7.0 (Figure 4(c)). Based on the peak size of ricin isomers shown in SDS-CGE, the first major peak was considered to be ricin consisting of RTA_1 and RTB, and the second minor peak to be ricin of RTA_2 and RTB. In the sample spiked with pI marker, the peak corresponding to pI 7.4 of ricin D was overlapped by a pI 7.4 marker (Figure 4(d)). In CIEF of ricin E, the pI values of the partially separated two peaks were determined to be 8.8 and 8.6, respectively (Figure 4(a)). In addition, a small peak corresponding to pI 7.4 was also observed, which suggested that the purified ricin E contained a small amount of ricin D. In general, small grain seeds are known to contain both ricin D and E, whereas large grain seeds contain only ricin D.[7,8] Therefore, the ricin D observed in this study was originally contained in castor beans (small grain type) used. This result indicates that the ion-exchange chromatography conditions need to be more optimized in order to obtain pure ricin E. In cation-exchange chromatography on CM-Sepharose column, pH of the mobile phase (pH 7.5) was not enough for removing ricin D isomer with pI 7.4 from ricin E fraction. If the pH of mobile phase is elevated to 8.0, ricin D can be more eliminated in the ricin E fraction. However, the chance of ricin E loss must be also carefully considered in case of higher pH mobile phase.

Conclusions

SDS-CGE was found to be useful for monitoring the purification process of ricin E. SDS-CGE and MALDI-TOF MS were not able to differentiate ricin E and D by molecular size or weight. CIEF was found to be the most powerful technique for resolving ricin isomers (RTA_1-RTB and RTA_2-RTB) and differentiating ricin E and D isoforms based on their different pI values. The CIEF is recommended as a method for characterizing ricin isomers and checking purity of each ricin D and E isoform. However, the CIEF is more complicated method than SDS-CGE and its separation performance is usually degraded by the presence of salts in the sample.[24] As SDS-CGE is relatively tolerable to buffer salts and speedy, this method is more suitable for monitoring the presence of ricin in the purification process. This study illustrates the usefulness of complementary use of SDS-CGE and CIEF to characterize ricin toxins.

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