Factors affecting the capillary electrophoresis of ricin, a toxic glycoprotein

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Abstract

Conditions for the analysis of ricin with capillary electrophoresis were investigated. Uncoated and coated columns were tested with a variety of different buffer combinations which included different principal components, pH, ionic strength, and additives. Of the combinations tested, uncoated columns used with either zwitterionic salts or putrescine gave the best results. Multiple peaks were resolved with these conditions. Coated columns generally yielded between 1000 and 5000 plates with several buffer combinations. Ricin may be analyzed faster and with greater resolution with capillary electrophoresis employing untreated fused-silica columns than by using other chromatographic techniques.

1. Introduction

Ricin (RCA 60) is a heterodimeric, glycoprotein phytotoxin with a molecular mass of approximately 66 000 and a pI of 7.1. The two protein chains, A and B, that comprise ricin are linked by a single disulfide bond. This toxin is produced by the castor bean plant, *Ricinus communis*, which grows in temperate climates, including California and the southern United States. Although ricin is found throughout the castor bean plant, it is concentrated in the seeds [1]. Up to 1.2 mg of ricin can be isolated from 100 g of seeds with lactamyl–Sepharose affinity chromatography [2]. Two basic forms of seeds and ricin are produced by castor bean plants from different regions of the world. The D form of ricin is found in large grain seeds, whereas small grain seeds contain the D and E forms. The two variants are distinguished by different pI values which reflect differences in amino acid composition of the B chain [3,4]. Regardless of form, ricin is highly toxic to eukaryotes with a reported mouse LD$_{50}$ as low as 0.1 μg per 25 g mouse [5]. Ricin's toxicity is based upon the ability of the A chain to inhibit cellular protein synthesis [6,7], which has prompted the incorporation of ricin or its individual chains into immunotoxins to treat different clinical conditions such as cancer [8–10]. The capability to separate and quantify ricin, or its subunits rapidly, is very important for monitoring the production and stability of

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ricin immunotoxins and for monitoring the potential health hazard posed by the plant within its indigenous areas.

In the past, ricin was purified for animal and cell toxicity studies. Generally, purification relied upon column chromatography [11–13]. Because the extracts were chemically complex, chromatographic techniques such as gel filtration, ion-exchange, and affinity chromatography had to be combined to obtain the required pure product. However, none of these preparative techniques provides the appropriate combination of speed, resolution, and simplicity needed for accurately screening many samples for ricin analytically. One analytical study on ricin included detection by capillary electrophoresis [14]. That report indicated that ricin eluted within 7 min as broad peaks, that the detection limit was 50 mg/ml, and that ricin was detectable in crude, acidic extracts of castor bean meal. A more complete study of the factors affecting ricin behavior in capillary electrophoresis columns was not conducted.

The previous study that included ricin detection emphasized one of the most attractive features of capillary electrophoresis (CE) for ricin analysis: its ability to resolve complex protein mixtures relatively rapidly. Ideally, ricin analysis by CE would be possible in one, relatively short step with the proper analysis conditions. However, discovering these conditions may require extensive investigation, as most protein separations require modifications to buffers or columns or both to suppress protein–column interactions. Reducing or preventing these interactions is essential for proper peak shape(s) and the required resolution. Many CE column and buffer variations have been developed to reduce protein–capillary interactions [15–27]. When planning a protein separation strategy for CE, it may be necessary to consider carefully many different approaches.

In this study, we evaluated the feasibility of using capillary electrophoretic analysis for ricin analyses, primarily as a function of column efficiency. We studied a number of different solutions and operating parameters to ascertain which would be optimal for efficiently screening samples for ricin.

2. Experimental

2.1. Materials and instrumentation

Affinity-purified ricin was purchased from Vector Laboratories (Burlingame, CA, USA) and was used without additional purification. Some ricin aliquots were desalted with 30 000 molecular mass-limit centrifuge filters (PGC Scientifics, Gaithersburg, MD, USA). Acidic extracts of castor bean meal were prepared according to the procedure described by Wannemacher et al. [14]. A modular CE unit from Spectrovision (Chelmsford, MA, USA) equipped with an ISCO CV^4 UV detector (Lincoln, NE, USA) and a Beckman P/ACE 2000 (Beckman Instruments, Palo Alto, CA, USA) CE unit were employed in this study. Untreated, fused-silica capillary columns were purchased from Polymicro Technologies (Phoenix, AZ, USA) and Beckman Instruments. Coated, fused-silica capillary columns were purchased from Supelco (Bellefonte, PA, USA). An aminopropyl-coated column was prepared according to the method of Mosely et al. [21]. All inner column dimensions were either 75 μm or 50 μm diameter. When listed, total column lengths are given in this report. The distance from the injection end to the detector is the total column length minus 7 cm. Buffer components were purchased from Sigma (St. Louis, MO, USA) and were the highest purity available. Accupure Z-1-methyl reagent (trimethylammoniumpropanesulfonic acid) was purchased from Waters Division of Millipore (Milford, MA, USA). Mesityl oxide, lysozyme, chymotrypsinogen A, and cytochrome c were purchased from Sigma. Phosphoric acid, glacial acetic acid, hydrochloric acid, sodium hydroxide, and putrescine were purchased from Aldrich (Milwaukee, WI, USA). Urea was purchased from Mallinkrodt (St. Louis, MO, USA). Deionized water (18 MΩ) was used in all experiments. All solutions were filtered with 0.22-μm filters (Gelman Science, Ann Arbor, MI, USA).

2.2. Column preparation and sample analysis

Uncoated columns were initially pretreated with the pressure feature of the Beckman P/ACE

2000 unit in the following manner: a water wash for 1 min, a 1.0 M NaOH wash for 5 min, a 0.1 M NaOH wash for 1 min, a 5-min water wash, and a 5-min wash with the running buffer, which varied depending upon the experiment. Between analyses, untreated columns were washed with 0.1 M NaOH for 0.5 min followed by a 2-min wash with running buffer. Before the first use, coated columns were cleaned with water for 1 min followed by a wash with 0.1 M NaOH for 1 min. Base treatment was followed by a 5-min water wash. Activation was concluded with a 5-min wash with running buffer. Between analyses, coated columns were cleansed with 0.1 M NaOH for 0.5 min followed by a 2-min wash with running buffer.

General operating parameters for the Beckman P/ACE 2000 CE unit included the following: 1 or 2 s hydrostatic, anodic sample injection; 20–30 kV applied voltage; 20°C coolant temperature; and either 200 or 214 nm detection wavelength.

Column efficiency was calculated using the following formula: $N = 5.54(t/t_{0.5})^2$, where N represents the theoretical plate number, t is the migration time of the peak and $t_{0.5}$ represents the peak width in time at half height. This formula did not compensate for various interactions, but it was adequate for assessing the variables we studied.

3. Results

Many variables were screened to determine which combination(s) would be optimal for analyzing ricin. A partial listing of buffers, additives, and column coatings is presented in Table 1. Variables fell into two general categories: (1) untreated columns with buffer modifications/additives, and (2) treated columns. Theoretical plate number values were used to evaluate the effectiveness of the analytical conditions. Of the variables tested, few combinations yielded satisfactory results.

Borate buffer was one of the first buffers tried due to its previous successful utility reported for other difficult proteins and glycoproteins [28,29]. By using alkaline 0.03 M borate buffer (pH 9) and a short, untreated column (35 cm × 50 μm), a single peak was obtained for ricin (injection from a 0.5 mg/ml solution). Fig. 1 depicts a representative electropherogram for that sample. These conditions provided reasonable results when theoretical plate number was considered ($N = 1550$; $N/m = 4429$). The presence of a broad, tailing peak indicated that either protein–column interaction occurred, that additional components were present, or both. To test for the presence of additional compounds, standard ricin was analyzed with a smaller inner diameter column. Two peaks were observed, although

Table 1
Representative selection of important buffers, additives, and inorganic salts used in this study

Organic buffers	Organic additives	Inorganic salts
CHES[a]	Ethylene glycol	Potassium chloride
Tricine[b]	Hexanesulfonic acid	Potassium sulfate
Tris[c]	Methanol	Potassium phosphate
	Putrescine[d]	Sodium acetate
	Sodium dodecylsulfate	Sodium borate
	Triethylamine	
	Trimethylammonium-propanesulfonic acid[e]	
	Urea	

[a] 2-[N-Cyclohexylamino]ethanesulfonic acid.
[b] N-Tris(hydroxymethyl)methylglycine.
[c] Tris(hydroxymethyl)aminoethane.
[d] 1,4-Diaminobutane.
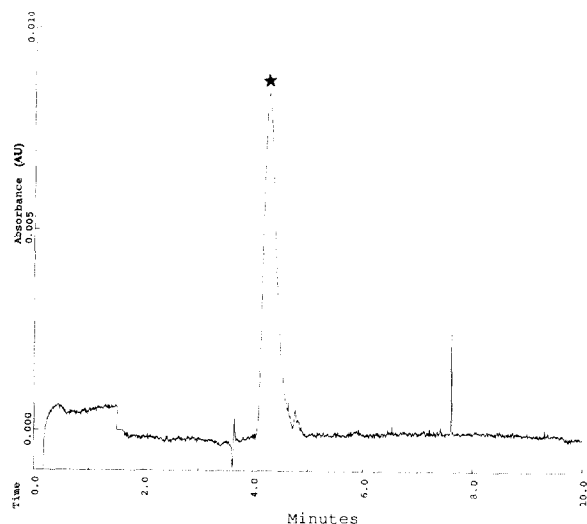[e] Accupure Z-1-methyl reagent (Millipore).

Fig. 1. Representative electropherogram of ricin (*) using an untreated 35 cm × 50 μm fused-silica capillary column. The running buffer was 0.03 M sodium borate, pH 8.5. A 3-s pressure injection (3447.4 Pa) of a 0.5 mg/ml solution of standard ricin was made. The applied voltage was 20 kV (571 V/cm). UV detection was accomplished at 200 nm.

resolution was poor (data not shown). This finding indicated that more than one component was present. To check for contaminants, ricin was analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Results showed that standard ricin contained only one component. Results acquired with sodium dodecylsulfate (SDS)-PAGE (data not shown) demonstrated that the A chain was heterogeneous, which may explain the appearance of at least two components in the borate electropherogram. Additional attempts with borate buffer to separate possible ricin components were unsuccessful. Other inorganic buffers such as potassium sulfate, potassium chloride, and potassium phosphate also had no utility. Additives such as ethylene glycol, hexanesulfonic acid, and triethylamine had little or no effect upon either column efficiency or resolution. No improvement was obtained when organic buffers such as 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) and Tris were used alone or combined with inorganic salts. Altering the pH of inorganic,

organic, and mixed buffers had no marked effect upon peak shape.

Zwitterionic and bifunctional buffers (or additives) were tested for their effect upon ricin's peak shape. These compounds do not increase Joule heating while providing pH stability and ionic strength [18]. Furthermore, zwitterionic buffers such as N-tris(hydroxymethyl)-methylglycine (Tricine) may interact with column silanol groups, as well as protein amine groups [18], which would enhance column efficiency and peak shape. When Tricine was used alone, there was no distinct improvement in theoretical plate number compared to borate buffer. However, by combining Tris and Tricine buffers and varying Tris–Tricine concentration ratios, more components of the ricin sample were resolved. Fig. 2 shows that, by holding the Tris concentration constant and increasing the quantity of Tricine, several additional peaks were detected (compare Fig. 2A to Fig. 2B). In Fig. 2A, we calculated the plate number to be 19 284 (N/m = 38 568), but this number could increase or decrease dramatically depending upon which peak was chosen. Tris concentration had little effect when it was increased and the Tricine level was held constant (data not shown). Doubling Tris–Tricine concentrations while maintaining a constant ratio compromised resolution, resulting from poor peak shape. The same was also true when Tris–Tricine concentrations were dropped to 0.005 M while maintaining a constant ratio. Altering the pH of the optimal Tris–Tricine buffer did not affect column efficiency and peak shape. SDS combined with methanol did not improve separations when added to the optimal Tris–Tricine buffer. Another zwitterionic compound, trimethylammoniumpropanesulfonate (Accupure Z-1-methyl reagent) was used as an additive to 0.1 M potassium phosphate buffer (pH 9.0). This additive reduced resolution, but improved overall peak shape, relative to the optimum Tris–Tricine buffer (Fig. 3). We calculated the plate number to be 42 405 (N/m = 74 395), but, again, the value was variable due to the double peak. This compromise between peak shape and resolution combined with a detection limit of approximately
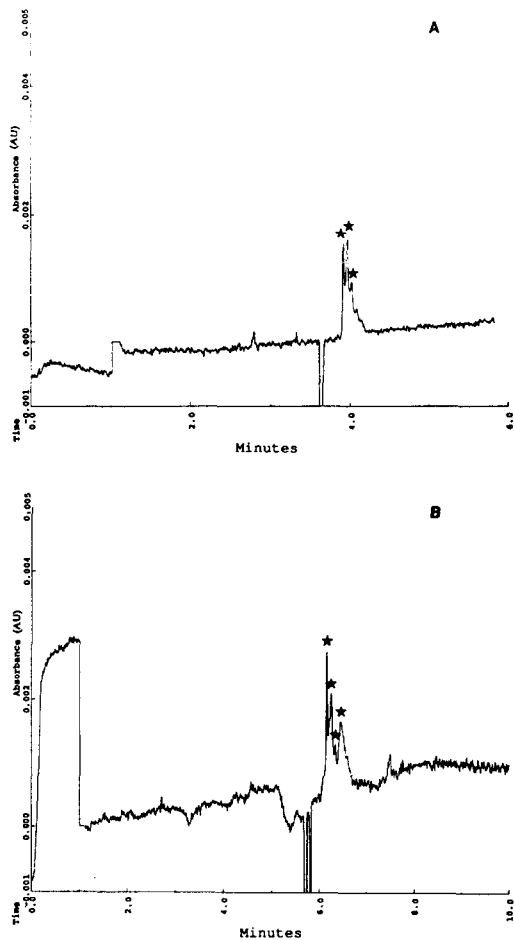
Fig. 2. Electropherogram of standard ricin (*) showing increased resolution of sample components when changes were made in buffer composition. (A) 0.01 M Tris, 0.01 M Tricine, pH 9.0. (B) 0.01 M Tris, 0.04 M Tricine, pH 9.0. An untreated 50 cm × 75 μm fused-silica column was used. Samples consisted of 0.5 mg/ml solutions of ricin and were injected for 1 s with pressure (3447.4 Pa). A voltage of 20 kV (400 V/cm) was applied to the column. UV detection was at 200 nm.

Fig. 3. Electropherogram of standard ricin (*) analyzed with 1 M trimethylammoniumpropanesulfonic acid (Accupure Z-1-methyl reagent) in 0.1 M KH$_2$PO$_4$ (pH 9.0). An untreated 57 cm × 50 μm fused-silica column was used. Pressure injections (3447.4 Pa) of 1 s were made for 0.5 mg/ml solutions of ricin. After injection, 25 kV were applied to the column (439 V/cm). A UV wavelength of 200 nm was used for detection.

10 mg/ml indicated that it may be possible to analyze a complex sample containing ricin under these conditions. Fig. 4 depicts a sample electropherogram. It was possible to tentatively identify ricin in this crude, acidic extract of castor bean meal. Ricin peaks were tentatively identified by comparing migration times to standard ricin.
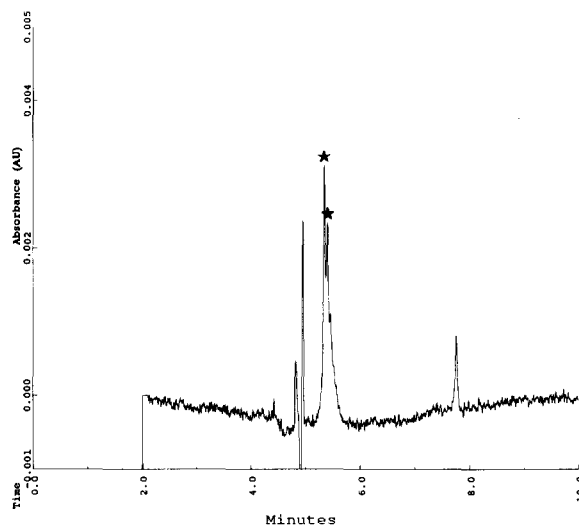
We also used a bifunctional compound as an additive. Putrescine (1,4-diaminobutane), added to potassium sulfate buffer [20], gave very good results for a standard mixture of three proteins: lysozyme, cytochrome c, and chymotrypsinogen A [theoretical plate numbers of 138 282 (N/m = 242 600), 32 000 (N/m = 56 140), and 170 717 (N/m = 299 504), respectively]. Each of these proteins is usually difficult to analyze with untreated columns. Fig. 5A is an electropherogram depicting the result of that analysis with putrescine. When ricin was analyzed under the same conditions, excellent resolution and peak shape were obtained (N = 64 300 for the largest peak; N/m = 112 807). More components were observed compared to the number of peaks obtained with Tris–Tricine (Fig. 2B).

To determine if the multiple peaks were aggregates, 0.03 M potassium phosphate buffer (pH 8.5) containing 6 M urea was used to analyze standard ricin. There was no evidence of aggre-
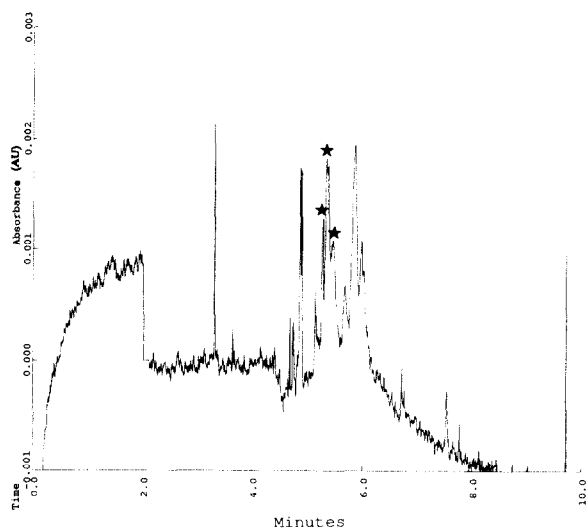
Fig. 4. Electropherogram of a crude, acidic extract of castor bean meal containing ricin (*). A 1 M Accupure Z-1-methyl reagent in 0.1 M KH$_2$PO$_4$, pH 9.0, buffer was used. The column was untreated, 57 cm × 50 μm fused-silica tubing. A 3-s pressure (3447.4 Pa) injection was used to load the solution. An applied voltage of 25 kV (439 V/cm) was used for analysis. The UV wavelength at 200 nm was used for detection.

Fig. 5. Electropherograms of (A) standard protein mixture containing 1 mg/ml each of chymotrypsinogen A (*), cytochrome c (+), and lysozyme (#); and (B) 0.5 mg/ml ricin (*) solution analyzed under the same conditions. The electrophoresis buffer contained 0.02 M K$_2$SO$_4$ and 0.03 M putrescine at pH 7.0. An untreated, 57 cm × 50 μm fused-silica column was used for both analyses. An applied voltage of 20 kV (351 V/cm) was used for analysis. The UV wavelength at 200 nm was used for detection.

gation (data not shown). Impurities or microheterogeneity seemed to be the best logical origin of the multiple peaks in the ricin samples

We investigated four coated columns in this study. As seen in Fig. 6, a column coated with a proprietary hydrophilic phase (Supelco P150) performed only slightly better than an untreated column with a borate buffer system. Plate numbers were 4546 (N/m = 7975) and 1550 (N/m = 4429) for the coated and uncoated columns, respectively. Attempts to enhance performance by changing buffers were unsuccessful. A C$_{18}$ column also performed poorly for ricin (Fig. 7; N = 494; N/m = 867). A C$_1$ column gave a relatively sharp peak (N = 12436; N/m = 21 878) with a 0.005 M disodium phosphate buffer (pH 6.0) containing 0.005 M SDS. A representative electropherogram is shown in Fig. 8. Equilibration times before analysis were typically 12 h or longer, which made it difficult to use this buffer routinely. Furthermore, the column was unable to resolve components of the ricin sample. Adequate results were obtained for peak charac-
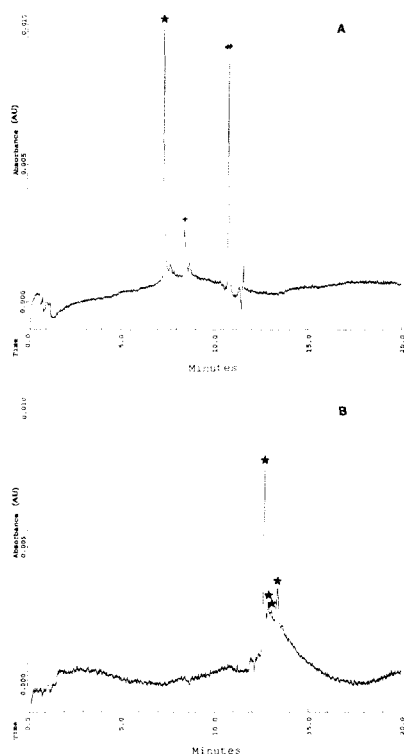
teristics with an aminopropyl column (N = 3638; N/m = 6382) prepared in this laboratory. However, the coating was unstable (Fig. 9), which ultimately rendered the column unusable after 3 days. The coating could be regenerated and used again satisfactorily, but the time required for regeneration made the coating an unlikely candidate for routine use.

4. Discussion

The results of this study indicated that relatively complex ricin samples may be rapidly analyzed in a single step with capillary electro-
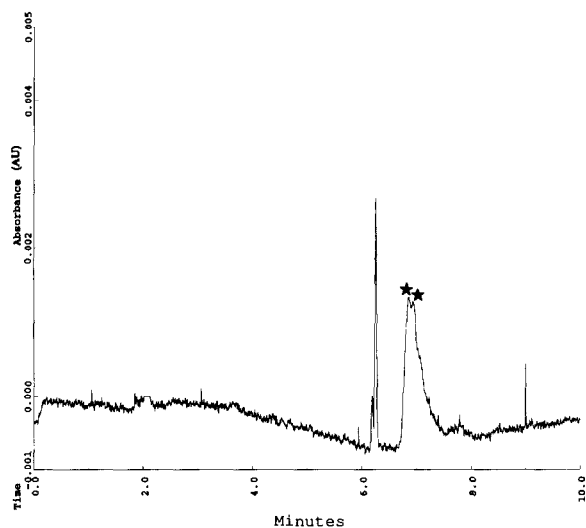
Fig. 6. A representative electropherogram of ricin (*) on a Supelco P150 column. A 1-s pressure injection (3447.4 Pa) of a 0.5 mg/ml ricin solution was made to load the sample. The electrophoresis buffer consisted of 0.01 M KH_2PO_4 at pH 8.6. The column dimensions were 57 cm × 50 μm. The applied voltage was 20 kV (351 V/cm). The UV wavelength at 200 nm was used for detection.
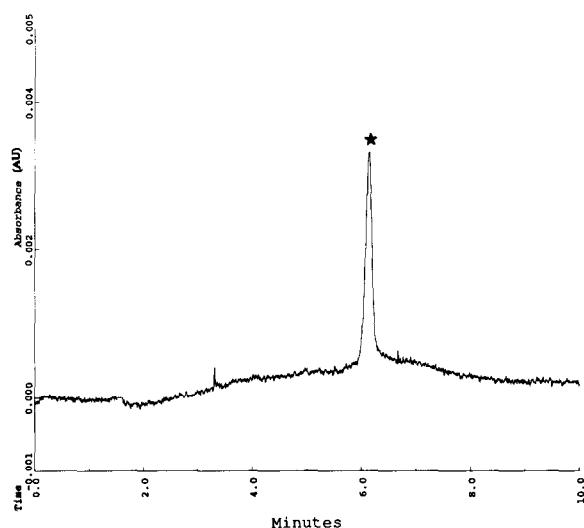

Fig. 8. Electropherogram of ricin analyzed on a Supelco C_1 column (57 cm × 50 μm). Pressure injections (3447.4 Pa) of 1 s were made to load the ricin sample (0.5 mg/ml). The electrophoresis buffer contained 0.005 M Na_2HPO_4 and 0.005 M SDS at pH 6.0. A voltage of 30 kV (526 V/cm) was applied to the column. UV detection was at 200 nm.
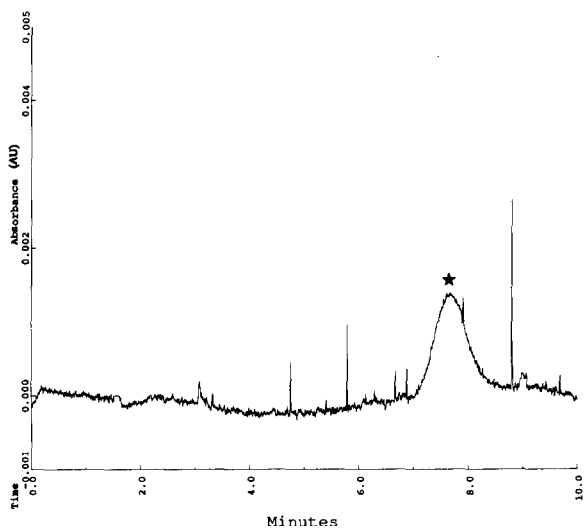

Fig. 7. Electropherogram of ricin using a 57 cm × 50 μm Supelco C_8 column. Injections of a 0.5 mg/ml solution of ricin were made with pressure (3447.4 Pa) for 1 s. The electrophoresis buffer consisted of 0.005 M Na_2HPO_4 and 0.005 M SDS at pH 6.0. The applied voltage was 30 kV (526 V/cm). UV detection was at 200 nm.

phoresis. Column efficiency, which influences resolution and sensitivity, was greatly affected by protein interactions. To compensate for protein–column binding, it was necessary to investigate several approaches. One of the first approaches utilized alkaline borate buffer. Several investigators report good separations with this buffer for difficult proteins, such as lysozyme and glycoproteins, for the following reasons: (1) induction of Coulombic repulsion among proteins and negatively charged silanol groups with alkaline conditions [15]; and (2) possible carbohydrate complexation by borate molecules [28,29]. Ricin appears to be an excellent candidate for this analysis profile as it is a glycoprotein with a pI of 7.1. While these conditions produced reasonable column efficiency for ricin, they were disappointing overall. It was necessary to shorten the column length from 50 to 35 cm to achieve a reasonable peak shape and efficiency ($N = 1550$). Attempts to improve these results with a 50 cm × 25 μm column yielded peak splitting, which indicated that peak shape was being influenced by the presence of unresolved components, as
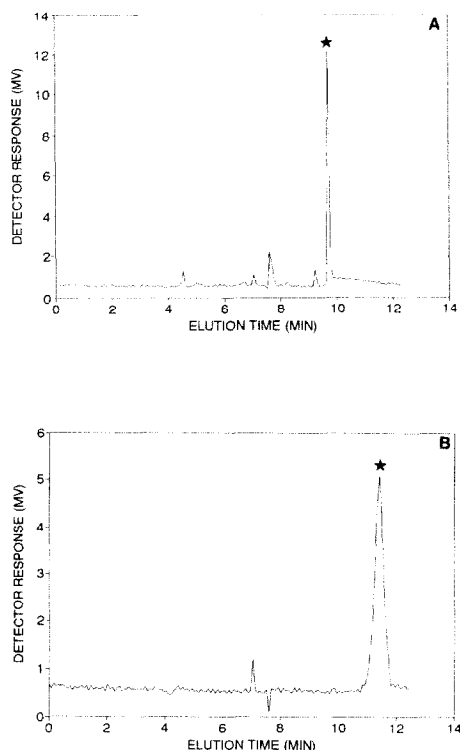
Fig. 9. Electropherograms of standard ricin analyzed on an aminopropyl-coated column (57 cm × 50 μm). (A) Freshly prepared column. (B) Same column after 3 days of use. For both analyses, 1-s pressure injections (3447.4 Pa) of a 0.5 mg/ml ricin solution were made to load the sample onto the column. The electrophoresis buffer consisted of 0.01 M sodium acetate at pH 3.5. A voltage of 20 kV (351 V/cm) was applied to the column. UV detection was at 200 nm.

well as protein interactions. We believed that protein interactions were the primary influence. Therefore, by using conditions to reduce protein interactions, we enhanced resolution by using other inorganic salts such as potassium sulfate, which revealed additional peaks. Alkali metal salts have been used to establish a neutral inner capillary surface through ion exchange with silanoate groups on the capillary's inner surface [17]. In the present study, this approach failed to improve the results obtained with alkaline borate buffer. We used insufficient ionic strength (0.02 to 0.03 M), compared to reported concentrations [17], for the potassium salt buffers to compete effectively against ricin for the cation-exchange

sites on the capillary surface. When the concentration was increased to the recommended 0.3 M potassium salt [17], the capillary current maximized, and poor peak shapes resulted, possibly due, in part, to lack of adequate capillary cooling. Decreasing the voltage did not markedly improve peak shape. Increasing pH of the lower ionic strength buffers did not positively influence the results, when compared to results with borate buffer. Most common organic buffers, used either singly or in combination with inorganic salts, failed to improve upon the results obtained with borate buffer.

Acidic buffers were ineffective for untreated, fused-silica columns. Generally, irreversible protein adsorption or loss occurred with most acidic buffers. It is reported that conformational changes occur in ricin at low pH [30,31]. Specifically, the ricin's hydrophobicity increases as the pH is lowered. This was determined with 1-anilino-8-naphthalene sulfonate binding [30,31]. An increase in hydrophobicity would increase the possibility for aggregation and protein loss, although we did not observe any precipitation in ricin solutions.

We used zwitterionic buffers and bifunctional additives because they are reported to compete for silanol groups on the inner surface of untreated capillary columns and active groups on the proteins, especially basic proteins [18,20]. For ricin, zwitterionic buffers enhanced peak shape, increased resolution, and resolved several peaks. Zwitterionic buffers and putrescine complicated analyses, however, due to the appearance of additional peaks in the electropherogram. Analyzing castor bean extracts demonstrated that the presence of additional ricin peaks may not prevent its identification in a complex matrix. Successful ricin analysis will depend upon the number of possible interfering substances in the sample, however. Nevertheless, ricin in a complex matrix would be difficult to quantify unless a peak was chosen to represent the ricin fraction. At best, only semi-quantitative results would be possible with such a restriction. In addition, the modest detection limit obtained with UV detection restricts application to samples containing relatively large

amounts of ricin. Because, ideally, a single, sharp peak is required for ricin analysis, we evaluated coated columns.

Coated columns tested in this study did not perform as well as the uncoated columns with the appropriate buffer or additives. Generally, a single peak was obtained for ricin for each of the columns tested, but, in each case, the peak was inadequate for analytical purposes due to its width. Attempts to optimize column performance by varying buffer characteristics were unsuccessful. Other coated columns must be explored for ricin analyses. It is especially important that columns and buffers be compatible with mass spectrometric detection. Acidic buffers, which are commonly used with coated columns, are also required for interfacing CE with electrospray ionization mass spectrometry. Molecular mass information on ricin samples could then be provided by electrospray ionization mass spectrometry. Of the coated columns we studied, the stability of the aminopropyl column requires improvement. Other investigators have been more successful using this type of column, even when it was interfaced with mass spectrometry [21]. Acrylamide-coated and gel-filled columns should also be studied.

There were several possible explanations for the appearance of multiple peaks in the ricin samples. First, contamination of the sample by individual A and B chains or other proteins may have occurred during the isolation/purification process. This possibility was eliminated because there were no individual chains or proteins detected by SDS-PAGE [14]. In addition, individual chains and extraneous proteins should have been resolved by CE due to differences in charge-to-mass ratios between ricin, its components, and, perhaps, other proteins. Second, possible aggregation of ricin presented another alternative, but analysis with 6 M urea did not reveal a change in the ricin's elution pattern. This indicated that no aggregation had occurred. A third possibility involved scrambling the A and B chains by dissociation then reassociating them at alkaline pH to give AA, AB, and BB recombinations. This seemed unlikely as no sulfhydryl compound was present to act as a proton donor

to cleave the disulfide bond that links the two chains; however, it cannot be totally eliminated.

Of the several possibilities, sample heterogeneity appears to represent the most likely explanation. Classical denaturing electrophoresis displayed the presence of a heterogeneous A chain, as has been previously reported [32–34]. In addition, there are subvariants of ricin [2]. One variation known to occur in ricin is glycosylation [35–37]. Glycoside microheterogeneity is restricted to neutral sugars, as charged sugars are not found in carbohydrates attached to ricin [37]. Consequently, glycoside variation has the greatest influence upon ricin's mass, not its charge. Other typical protein modifications such as phosphorylation, sulfation, methylation, and acetylation that could influence ricin's charge state have not been found [38–41]. On the other hand, the presence of small, but critical, differences in amino acid composition or sequence cannot be eliminated as a potential source of variation, which would affect the charge state of ricin. Such differences were reported for abrin, a related toxic glycoprotein [42]. Variations in amino acid composition could arise from post-translational processing of ricin precursors. Amino acid differences may also cause conformational changes in ricin which may be detectable with CE.

The present study demonstrated that it is possible to analyze ricin with CE. Our results indicated that untreated, fused-silica capillary columns and zwitterionic buffers produce the best peak shapes and column efficiencies for ricin. Analysis times were short and were accomplished in a single step. While detection limits were not evaluated in detail, 10 mg/ml ($S/N = 3$; optimal buffers) was the approximate detection limit when the largest peak was followed. This is less than the 50 mg/ml value reported previously for CE [14], which reflects improved analytical conditions. Nevertheless, the 10 mg/ml value for CE (UV detection) was much greater than the low ng/ml values reported for immunoassays [14] and will be a restriction for sample-limited analyses. In addition, it may be possible to resolve ricin variants and/or subvariants with capillary electrophoresis and buffers containing zwitterionic salts or diamino compounds. Addi-

tional studies to identify the extra peaks as ricin variants will be required in the future.

5. Acknowledgements

6. References

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