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Analysis of snake venoms by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and two-dimensional electrophoresis

T. Marshall* and K.M. Williams

School of Health Sciences, The University of Sunderland, Fleming Building, Sunderland SR2 7EE (UK)

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ABSTRACT

The protein composition of snake venom has been analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and high-resolution two-dimensional electrophoresis (2-DE). SDS-PAGE suggests differences between species and similarities between related species. As expected, silver staining greatly enhanced detection sensitivity whilst 2-DE dramatically improved separation to reveal multiple strings of spots indicative of molecular heterogeneity. It is recommended that similarities (or differences) in the SDS-PAGE patterns of venoms from the same (or different) species of snake should be further characterized by 2-DE.

INTRODUCTION

The pharmacological/toxicological properties of snake venom are mainly associated with proteins particularly enzymes [1–3]. The protein composition of venom has been studied for the purpose of species comparison/development of anti-venoms [3] and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) has been widely applied [4–10]. Recently, Mendoza *et al.* [10] compared the SDS-PAGE profiles of 21 snake venoms and demonstrated individual patterns with shared characteristics. We have investigated additional factors affecting electrophoretic analysis of snake venoms and have extended previous work to include the application of high-resolution two-dimensional electrophoresis (2-DE).

* Corresponding author.

EXPERIMENTAL

Sample preparation

The following snake venoms were supplied by Sigma (Poole, UK): *Agkistrodon contortrix mokason* (Northern Copperhead), *Bothrops jararacussu* (Jararacussu river snake), *Crotalus molossus molossus* (Black-tailed rattlesnake), *Crotalus horridus horridus* (Timber rattlesnake), *Crotalus ruber* (Red diamond rattlesnake), *Bitis arietans* (Puff adder), *Naja haje* (Egyptian cobra), *Enhydrina schistosa* (Common sea snake) and *Dispholidus typus* (Boomslang). The lyophilized venoms were dissolved (final concentration 5–10 mg/ml) by carefully adding an appropriate volume of 0.0625 *M* Tris–HCl pH 6.8 containing 2% SDS and 20% glycerol. 2-Mercaptoethanol (final concentration 5%) was added to an aliquot of each solubilized venom and the sample mixtures (both reduced and non-reduced) heated at 95°C for 10 min. In subsequent experiments selected venoms were di-

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luted to 0.2–5.0 mg/ml for Coomassie blue staining and 0.002–0.10 mg/ml for silver staining.

Electrophoresis

SDS-PAGE. The denatured venoms (5 μl) were loaded in agarose wells precast on the upper surface of 6–20% (w/v) polyacrylamide gradient gels (75 × 75 × 3 mm) and electrophoresed at 70 mA/gel for 1 h in precooled (4°C) 0.025 *M* Tris, 0.2 *M* glycine containing 0.1% (w/v) SDS [11]. M_r was estimated by co-electrophoresis of Pharmacia-LKB calibration proteins (2.5 μl).

2-DE. The denatured venoms (200 μg for Coomassie blue staining; 10 μg for silver staining) were analysed by the simplified method [12] of high-resolution 2-DE [13]. Isoelectric focusing (IEF, first dimension) in 4% (w/v) polyacrylamide gel rods containing 9 *M* urea, 0.5% Nonidet P-40 and 2% ampholine (pH 2.5–4.0, pH 5.0–7.0, pH 3.5–10.0; 2:3:6, v/v/v) was followed by SDS-PAGE (second dimension) as described above. Co-electrophoresis of a human serum and a serum/venom mixture were used for calibration of p*I* and M_r.

Staining

Coomassie blue. The SDS-PAGE gels were fixed in aqueous 20% (w/v) trichloroacetic acid (TCA) and the proteins stained at 60°C for 30 min in methanol–acetic acid–water (50:10:40, v/v/v) containing 0.1% (w/v) Serva Blue R prior to destaining in methanol–acetic acid–water (10:10:80, v/v/v) [11]. The 2-DE gels were fixed in methanol–acetic acid–water (50:10:40, v/v/v) prior to staining as above —this eliminates "shadow effects" associated with the staining of ampholytes.

Silver. The SDS-PAGE and 2-DE gels were fixed in methanol–acetic acid–water (50:10:40, v/v/v) prior to silver staining as previously described [14] and outlined in Table I.

RESULTS

Fig. 1 shows the SDS-PAGE patterns of a range of venoms following sample preparation in the presence (Fig. 1A) or absence (Fig. 1B) of 2-mercaptoethanol ("reducing" and "non-reducing" conditions, respectively). The optimal sample load for CBB staining was 50 μg. Higher amounts (100 μg) resulted in smearing but lower

TABLE I

THE METHYLAMINE-INCORPORATING SILVER STAIN

(1) Wash in two changes of water (10 min each)[a].
(2) Incubate in aqueous 0.1% (w/v) formaldehyde (30 min); cool in water (20°C, 10 min).
(3) Incubate in silver methylamine[b] (10 min).
(4) Quickly rinse in water (2 changes) and developer [formaldehyde, 0.02% (w/v) containing 0.005% (w/v) citric acid]. Change developer at 5-min intervals till gel blackens (*ca.* 30 min).
(5) Rinse in three changes of water (10 min each).
(6) Incubate in destaining solution[c] till gel background is golden yellow (*ca.* 1–4 min).
(7) Rinse in water, incubate in aqueous 2.5% (w/v) Kodak hypo clearing agent[d] (30 min) and wash in three changes of water (10 min each).

[a] Perform all steps with gentle shaking in a fume cupboard; steps 1, 2 at 60°C (with reagent volume of 200 ml/gel) and steps 3–7 at room temperature (100 ml/gel).
[b] For 100 ml (1 gel): mix commercial methylamine solution (30%) with 0.36% (w/v) sodium hydroxide (1:5, v/v), add (*ca.* 10 ml) to 4 ml of a stirring solution of 20% (w/v) silver nitrate till it just clears and dilute to 100 ml with water.
[c] For 800 ml (8 gels): solution A, dissolve 11.1 g sodium chloride and 11.1 g cupric sulphate in 285 ml water and add ammonia solution (25%) till the precipitate clears to a deep blue solution (final volume *ca.* 300 ml). Solution B, dissolve 44 g sodium thiosulfate pentahydrate in 85 ml water (final volume 100 ml). Mix solutions A and B (3:1, v/v) and dilute to 800 ml with water.
[d] Gels may be photographed at this stage.

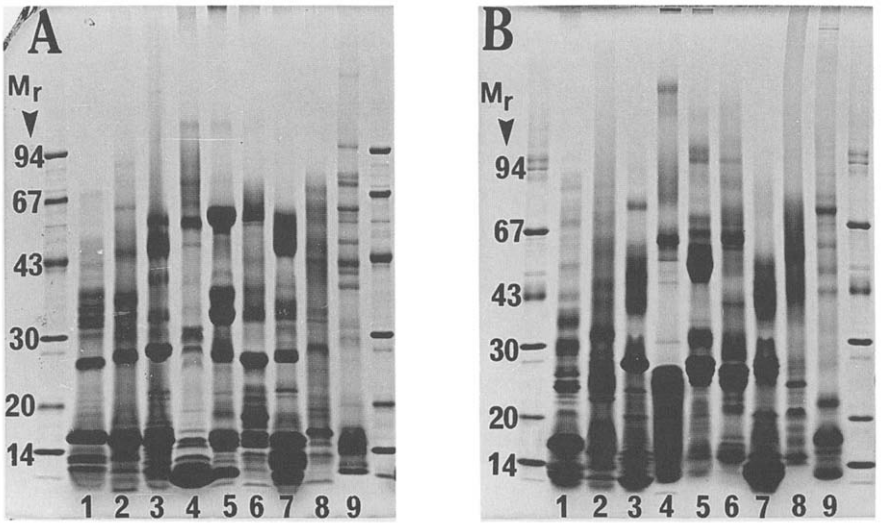

Fig. 1. SDS-PAGE of snake venom (50 μg) analyzed following sample preparation in the presence (A) or absence (B) of 2-mercaptoethanol ("reducing" and "non-reducing" conditions, respectively). Venoms: 1 = *Agkistrodon contortrix mokason*; 2 = *Bothrops jararacussu*; 3 = *Crotalus molossus molossus*; 4 = *Naja haje*; 5 = *Crotalus horridus horridus*; 6 = *Bitis arietans*; 7 = *Crotalus ruber*; 8 = *Dispholidus typus*; 9 = *Enhydrina schistosa*. M_r indicates relative molecular mass × 10^{-3}. Protein was detected with Coomassie brilliant blue.

loads (5–10 μg) were useful for improving the resolution of the major protein constituents. The lower-molecular-mass proteins showed a tendency to diffuse upon destaining —this was minimized by fixing the gels in aqueous 20% TCA [rather than methanol–acetic acid–water (50:10:40)] and destaining in methanol–acetic acid–water (10:10:80) [rather than methanol–acetic acid–water (5:7:88)]. Comparison of "reducing" *versus* "non-reducing" conditions indicated optimal resolution under "reducing" conditions with detection of up to 30 polypeptide bands. "Non-reducing" conditions revealed a greater proportion of high-molecular-mass proteins (presumably oligomers) but the low-molecular-mass proteins were less well defined with a tendency to smear (Fig. 1B). The patterns obtained for individual venoms were distinctly different irrespective of the sample preparation (Fig. 1).

Silver staining enhanced detection sensitivity 100- and 200-fold ["reducing" (Fig. 2A, B) and "non-reducing" (Fig. 2C, D), respectively]. The detection limit for CBB staining was approximately 1 μg of snake venom (Fig. 2A, C), whilst the respective values for silver staining were 10 ng ("reducing" conditions, Fig. 2B) and 5 ng ("non-reducing" conditions, Fig. 2D).

Fig. 3 demonstrates 2-DE of snake venom and compares the detection sensitivity of Coomassie brilliant blue and silver staining. Fixation of the gels in 20% TCA prior to Coomassie brilliant blue staining resulted in a background "shadow" of ampholyte staining in the low-molecular-mass cathodal region of the gel. Fixation in 20% TCA prior to silver staining impaired detection sensitivity. Whilst fixation in methanol–acetic acid–water (50:10:40) eliminated these staining problems the spots were less sharp suggesting an increased tendency for the protein to diffuse. In contrast to the 30 polypeptide bands detected by SDS-PAGE (Fig. 1), 2-DE revealed up to 400 polypeptide spots in microgram amounts of snake venom (particularly when combined with silver staining). The higher-molecular-mass protein bands ($M_r > 30\,000$, Fig. 1A) were separated into strings of up to 10 spots reflecting microheterogeneity whilst the lower-molecular-mass bands ($M_r < 30\,000$, Fig. 1A) were shown to consist of apparently unrelated spots.

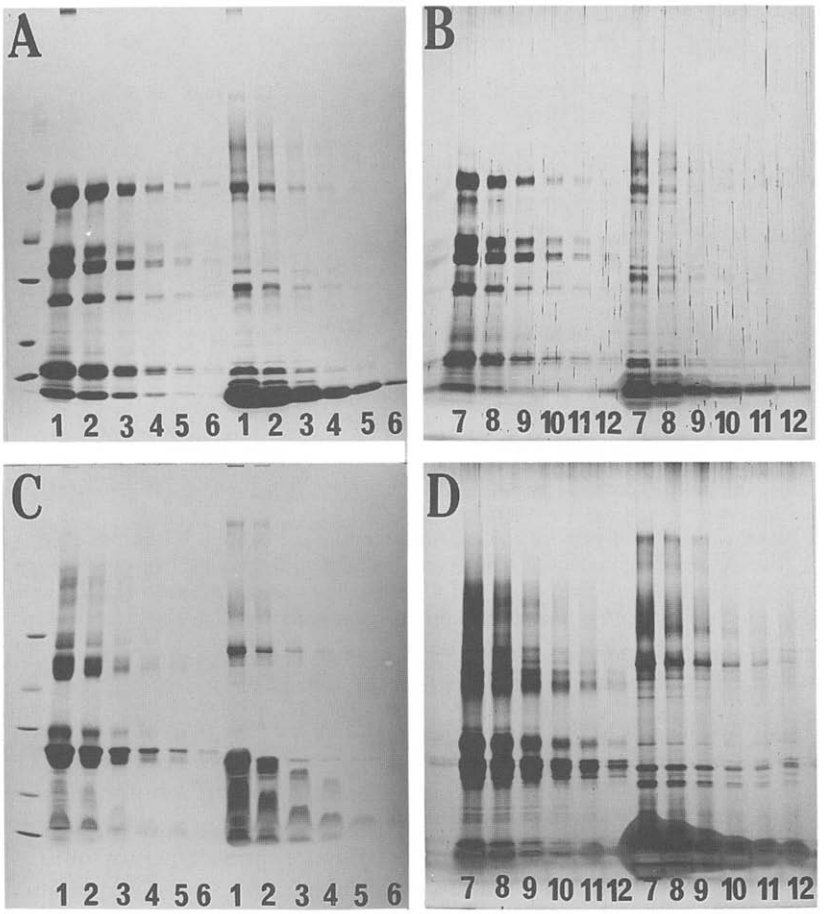

Fig. 2. Comparison of Coomassie brilliant blue (A, C) and silver (B, D) staining following SDS-PAGE of decreasing amounts of two venoms (*Crotalus horridus horridus* and *Naja haje*) after sample preparation in the presence (A, B) or absence (C, D) of 2-mercaptoethanol ("reducing" and "non-reducing" conditions, respectively). Venom amounts: 1 = 50 μg; 2 = 25 μg; 3 = 10 μg; 4 = 5 μg; 5 = 2.5 μg; 6 = 1 μg; 7 = 0.5 μg; 8 = 0.25 μg; 9 = 0.1 μg; 10 = 0.05 μg; 11 = 0.025 μg; 12 = 0.01 μg.

DISCUSSION

The present study suggests that one-dimensional SDS-PAGE may be of limited value for inter-species comparison of the polypeptide constituents of snake venoms. 2-DE clearly indicates that individual SDS-PAGE bands consist of multiple strings of related spots and/or numerous unrelated spots. This overlap is a common problem when applying one-dimensional electrophoretic methods to complex proteins mixtures. In this respect, 2-DE demonstrates a previously unknown degree of complexity in the polypeptide constituents of snake venom. This complexity may be partly explained by microheterogeneity whereby minor differences in the charge or molecular size of a polypeptide manifests itself as delicate trails of closely packed spots. Thus, apparent similarities in the SDS-PAGE banding patterns of different snake venoms need to be confirmed by techniques of higher resolution, including 2-DE. Such comparisons should also include 2-DE using non-equilibrium pH gradient electrophoresis [15] to account for additional proteins of a more basic nature.

Mendoza *et al.* [10] recently suggested that "certain proteins from native venoms and polypeptides derived from SDS-treated venoms do

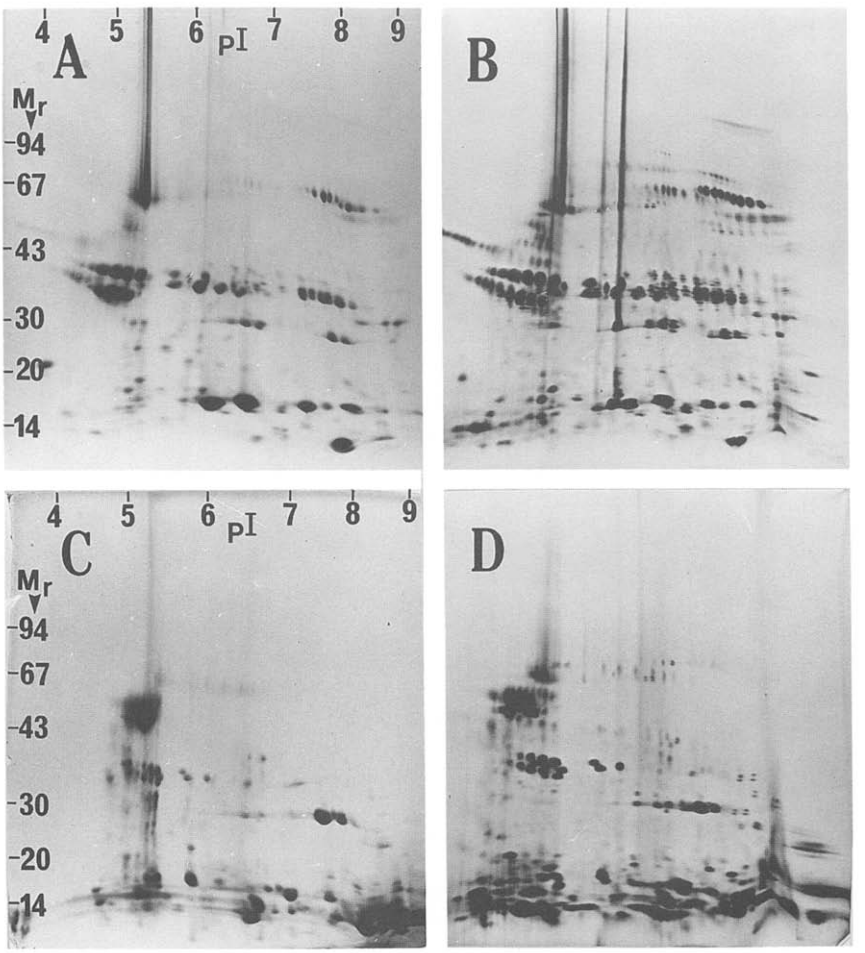

Fig. 3. 2-DE of snake venom from *Crotalus horridus horridus* (A, B) and *Crotalus ruber* (C, D) as detected by Coomassie brilliant blue staining (A, C; 200 μg venom) or silver staining (B, D; 10 μg venom). Co-electrophoresis of human serum and a serum/venom mix were used to calibrate p*I* and M_r.

not react strongly with the silver nitrate stain and are not readily detectable. In contrast, they produce an intense reaction with the Coomassie brilliant blue stain and are readily detectable". We have found no evidence to support this claim. On the contrary, our silver staining method consistently improved the detection sensitivity of Coomassie brilliant blue staining at least 100-fold. This discrepancy may arise from the choice of silver stain as different methods vary widely in their response and commercially available stains often sacrifice sensitivity for improved reproducibility.

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