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The concept of two-dimensional electrophoresis-guided purification proven by isolation of rhodocetin from *Calloselasma rhodostoma* (Malayan pit viper)

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Abstract: Two-dimensional gel electrophoresis (2DE) is an important tool for investigating the complexity of snake venom proteomes. Apart from applications based on whole proteome analysis, we suggest that 2DE can be used as an assay to guide the progress of protein purification. The aim of this study was to prove the feasibility of this concept by using it to purify rhodocetin from *Calloselasma rhodostoma* venom. Rhodocetin (α subunit) spot on the 2DE profile of *C. rhodostoma* venom was first identified and confirmed by mass spectrometry, with a molecular mass of 16 kDa and calculated pI of 5.16. Rhodocetin was subsequently purified by successive anion-exchange and gel filtration chromatography. Every peak from both chromatography profiles was collected and tested on 2DE. The presence of rhodocetin (α subunit) spot in the 2DE profile of the peak DP2 indicated the presence of the protein. The purified compound was used to spike the crude venom. A spiked spot with a 1.6-fold increase in intensity was observed and its position matched to that of rhodocetin (α subunit) on the 2DE profile. Together, these spots confirmed the identity of the purified compound as rhodocetin. Hence, our results have demonstrated the effectiveness of the concept we now term 2DE-guided purification.

Key words: protein fingerprinting, liquid chromatography, proteomics, venom.

INTRODUCTION

Since its first description thirty years ago, two-dimensional gel electrophoresis (2DE) has been the primary tool for detecting proteins in an organism and complex biological extracts (1, 2). By separating proteins based on two independent parameters – pI values by isoelectric focusing (IEF) in one dimension and molecular mass by SDS-PAGE in the other – samples can be resolved to a few thousand protein spots (3). This power of 2DE has given it the ability to provide a representative view of complex proteomes. Complemented by the development of mass spectrometry techniques which help identify these protein spots, 2DE reference maps of numerous samples have been built and published over the years, providing standardized libraries of the proteins in these samples to researchers worldwide (3).

The revitalization of 2DE has also pushed for a more global approach in the study of the snake venom proteome (4). Two-dimensional gel electrophoresis profiles give good representative views of venom complexity – an important factor that helps to improve proteome coverage (4). Protein profiling efforts and reference map publications have been of great value in the study of snake venom. Protein identification on 2DE profiles and between-gel comparisons allow investigation into snake venom variations that have applications in basic venom research and envenomation management (4).

Two-dimensional gel electrophoresis profiles and their analyses have also emerged as new tools for taxonomic studies by allowing investigation

into interspecies and intraspecies variations (5, 6). It can also be employed in selecting donor snakes for antivenom production, as well as in quality control by detecting degradation of venom components in the preparation of venom samples for immunization (4, 7). Apart from these applications which are mainly based upon complex whole proteome analysis, we suggest that the concept of protein identification and between-gel comparison can be used for another purpose that has previously never been described – as an assay to guide the process of protein purification. This concept was first hypothesized during the mapping of five snake venoms found in Malaysia and was then appropriately termed "2DE-guided purification" (8).

In recent years, natural product drug discovery has received a renewed interest and snake venom has also been investigated for pharmacologically important components (9, 10). The major bottleneck that continues to affect natural product drug discovery is the isolation and purification of active components from an exceptionally complex matrix (9). Once an active source has been identified, bioassay-guided isolation is typically used to isolate the active component. While the general paradigm of this process can be relatively straightforward in the academic laboratory setting, the design of a suitable bioassay, however, can often present a major limitation to the purification process (9). There are some requirements that have to be taken into consideration when designing a bioassay – sensitivity, validity, lack of ambiguity, accuracy, reproducibility, and reasonable cost (10, 11). On top of these criteria, several other aspects of bioassay, which can pose major challenges to progression of the purification process, have to be taken into account. To begin with, turnaround time for a bioassay can be lengthy, prolonging the time needed for a single round of fractionation (9). Moreover, certain bioassays require large amounts of the active component, in addition to the materials needed for further fractionation and structural characterization (12).

When a protein spot has been identified on the 2DE profile, the specificity of its spot location can be an indication of its presence in a particular sample. Expanding on this principle of 2DE, our study describes the purification of rhodocetin from crude *Calloselasma rhodostoma* (Malayan pit viper) venom, employing 2DE as an alternative to bioassay. Although the purification of rhodocetin has already been described, our paper uses rhodocetin purification to prove the feasibility of this novel concept known as 2DE-guided purification (8, 13, 14).

MATERIALS AND METHODS

Materials

Crude *C. rhodostoma* (CR) venom was obtained from Bukit Bintang Enterprise Sdn. Bhd., Sungai Batu Pahat, Perlis, Malaysia. The venom was lyophilized and stored at –20°C. All chromatography materials, including the Mono Q 5/50 GL® (1 mL) column, HiTrap Desalting® (5 mL) column, and 2DE materials, were all products from GE-Healthcare (Sweden).

Protein Content Determination

CR venom protein content was estimated using the dye binding technique as per Bradford (15) with BSA at 2.0 mg/mL (Thermo Scientific).

Purification of Rhodocetin from *C. rhodostoma*

Rhodocetin was purified from CR venom using a two-step protocol. Lyophilized CR venom (5 mg) was dissolved in 250 µL of 20 mM Tris-HCl, pH 8.5 and injected into the Mono Q 5/50 GL® anion-exchanger (1 mL) column pre-equilibrated with equilibration buffer (20 mM Tris-HCl, pH 8.5) on AKTAprime® (GE-Healthcare, Sweden). Five milliliters of equilibration buffer was used to wash the unbound proteins and a linear salt gradient was subsequently achieved with 20 mL of elution buffer (0.4 M NaCl in 20 mM Tris-HCl, pH 8.5) at a flow rate of 1.0 mL/minute. Peak P2 fraction was collected and directly injected into the G25 HiTrap Desalting® (5 mL) column which was pre-equilibrated with distilled water on AKTAprime®. The proteins were eluted using distilled water at a flow rate of 1.0 mL/minute. Fractions from each peak were pooled and lyophilized. The remaining peaks obtained from the Mono Q separation were all pooled, desalted, and lyophilized. All peaks from both purification stages were tested for the presence of rhodocetin by 2DE.

Two-Dimensional Gel Electrophoresis

IPG strips (7 cm) were rehydrated overnight with 125 µL of rehydration buffer. Protein

samples were dissolved in 100 µL of rehydration solution containing 8 M urea, 2% (w/v) CHAPS, 20 mM dithiothreitol (DTT), 0.5% (v/v) IPG buffer, and 0.002% (w/v) Bromophenol blue. The dissolved protein samples were introduced into the rehydrated IPG strip via sample cup loading.

IEF was performed using the following parameters modified from manufacturer's instructions: 200 V step and hold for one hour, 1000 V gradient for one hour, 5000 V gradient for four hours, 5000 V step and hold for one hour. When salt content was too high in the sample, the IEF was prolonged to an overnight run by introducing an additional first step of 12 hours at 50 V step and hold, followed by the described four-step protocol. Before proceeding to the second dimension, the isofocused strips were equilibrated with two steps: reduction buffer with 50 mM Tris/HCL, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of Bromophenol blue and 1% (w/v) DTT on a rocking table for 10 minutes; alkylation buffer with 50 mM Tris/HCL, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of Bromophenol blue and 2.5% (w/v) iodoacetamide for additional ten minutes. The equilibrated strips were then directly applied onto 15% polyacrylamide Laemmli gels (10 cm x 10.5 cm) and run using the SE260 system with a two-step electrophoresis program – an initial step of 10 mA/gel for 15 minutes followed by 20 mA/gel until the dye front reached the bottom of the gel. The gels were stained with Coomasie brilliant blue to visualize the separated proteins.

Mass Spectrometry

The protein spot for rhodocetin (α subunit) was identified on the 2DE profile of crude CR venom by comparing it with our previous work profiling crude CR venom on larger format 18 cm 2DE gel (unpublished data). The identified rhodocetin spot was also cleaved from the smaller gel (7 cm IPG strip) and sent for confirmation by MALDI-TOF/TOF analysis to Proteomics International, Australia. Peptide mass fingerprinting combined with tandem mass spectrometry (PMF + MS/MS) was used to identify the protein.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (16) with resolving solution containing 15% acrylamide and stacking solution containing 6% acrylamide. The gels were stained with Coomasie brilliant blue and silver to visualize the separated bands.

Spiking

Spiking was based on the method described by Vejayan *et al* (17). Crude CR venom (60 µg of protein) was introduced into an IPG strip via sample loading cup. On another IPG strip, two sample loading cups were placed at separate ends. A 60-µg sample of crude CR venom was loaded into the sample loading cup at the anodic end and purified rhodocetin was loaded into the sample loading cup at the cathodic end. All other parameters used in spiking were the same as those mentioned above under 2DE.

RESULTS

Crude Venom and Mass Spectrometry

Figure 1 shows the 2DE profile of crude CR venom (60 µg of protein). The rhodocetin (α subunit) spot was identified on the profile and cleaved for mass spectrometry analysis. Peptide mass fingerprinting confirmed the cleaved protein as rhodocetin α subunit from CR. The hit matched to rhodocetin subunit α (accession number: P81397) of Tax_Id = 8717 (*Agkistrodon rhodostoma*); using Ludwig NR database search with five peptide masses matched; score of 246; nominal subunit mass: 15951 Da; calculated pI value: 5.16 and sequence coverage of 36%.

2DE-Guided Purification

Crude CR venom fractionated on a Mono Q 5/50 GL® (1 mL) column gave six peaks: U, P1, P2, P3, P4, and P5 (Figure 2 – A). The fractions from each peak were pooled for desalting. Injection of P2 fraction into the G25 HiTrap Desalting® (5 mL) column revealed two distinct peaks (Figure 2 – B). As such, the G25 column was also used as the second fractionation stage. Eluted peaks DP1 and DP2 were pooled and lyophilized. The remaining peaks obtained from Mono Q (U, P1, P3, P4, and P5) were also desalted and lyophilized. All peaks from both stages were tested for the presence of rhodocetin using 2DE (Figure 2 – C and D). The 2DE profiles of all peaks were then compared with the crude CR venom profile. Rhodocetin was found prominently present in the DP2 peak (Figure 2 – D).

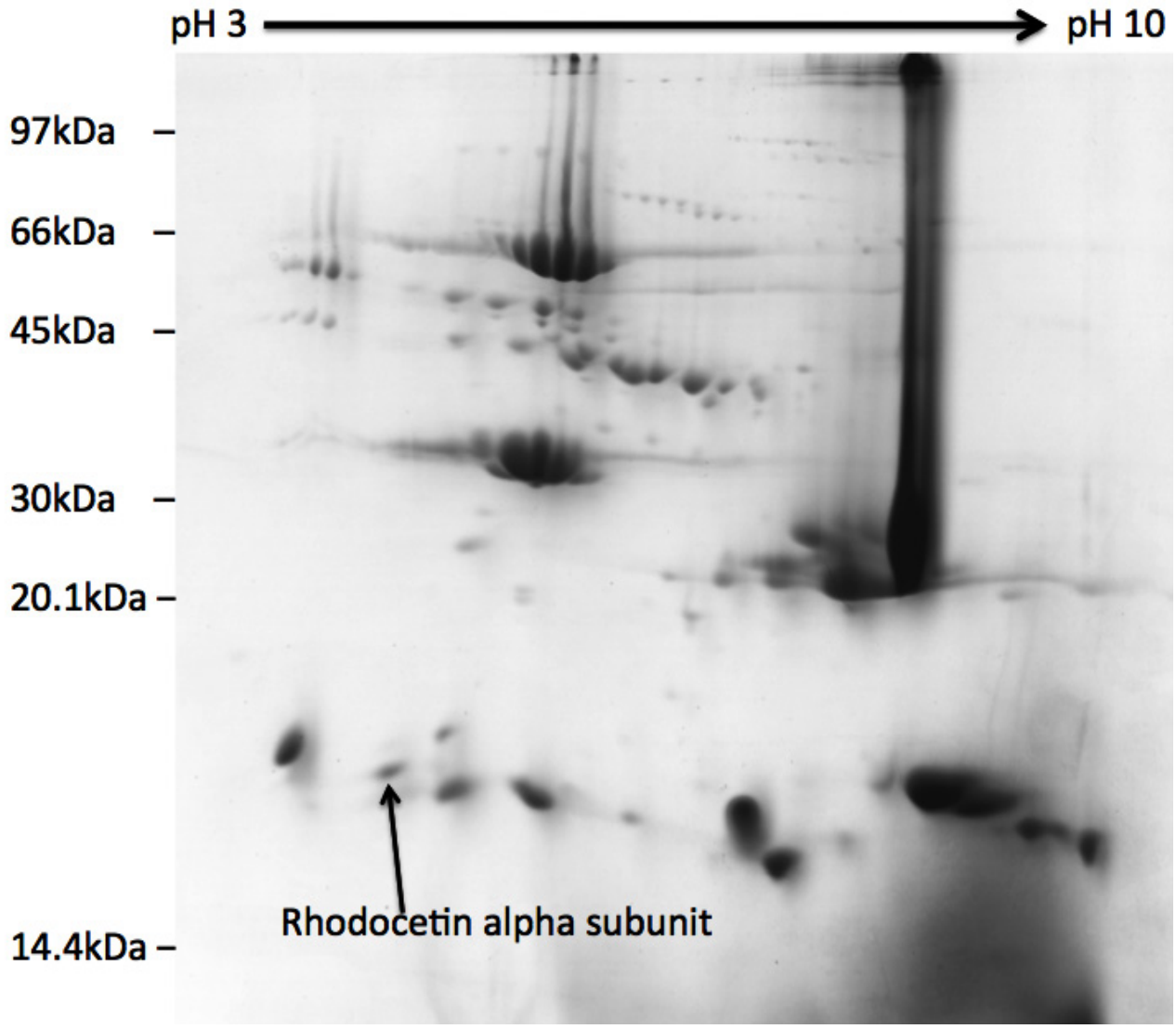

Figure 1. Two-dimensional gel electrophoresis profile of CR (60 µg of protein) highlighting the rhodocetin (αsubunit) spot. The profile was obtained by IEF on a 7-cm IPG strip (pH 3-10) and the proteins subsequently separated in the second dimension by 15% SDS-PAGE. The separated proteins were visualized by Coomasie brilliant blue staining.

SDS-PAGE

Homogeneity of DP2 was assessed using 15% SDS-PAGE. The two rhodocetin subunits were shown as two distinct bands at approximately 15 kDa with Coomasie brilliant blue and silver staining (Figure 3 – A and B). No other bands, even faint ones, were visualized in lane 6 or 9 of the very sensitive silver stained gel.

Spiking

Spiking was performed using the pure compound obtained from DP2 to further confirm its identity as rhodocetin. Comparison was made between 2DE profiles for crude venom (Figure 4 – A) and venom spiked with pure rhodocetin (Figure 4 – B). The latter revealed a spot of increased intensity at the position matching rhodocetin on the crude venom 2DE profile (Figure 4 – A). With the aid of Image Master 2D® Platinum 7.0 (GE Healthcare, USA), spots on both profiles were detected and matched. The spiked rhodocetin spot was quantified as 1.6 times the intensity of the matching spot on the unspiked venom profile. Figure 4 (C and D) clearly show the increased intensity spot in 3D view. Together, these all confirm the identity of the purified compound as rhodocetin.

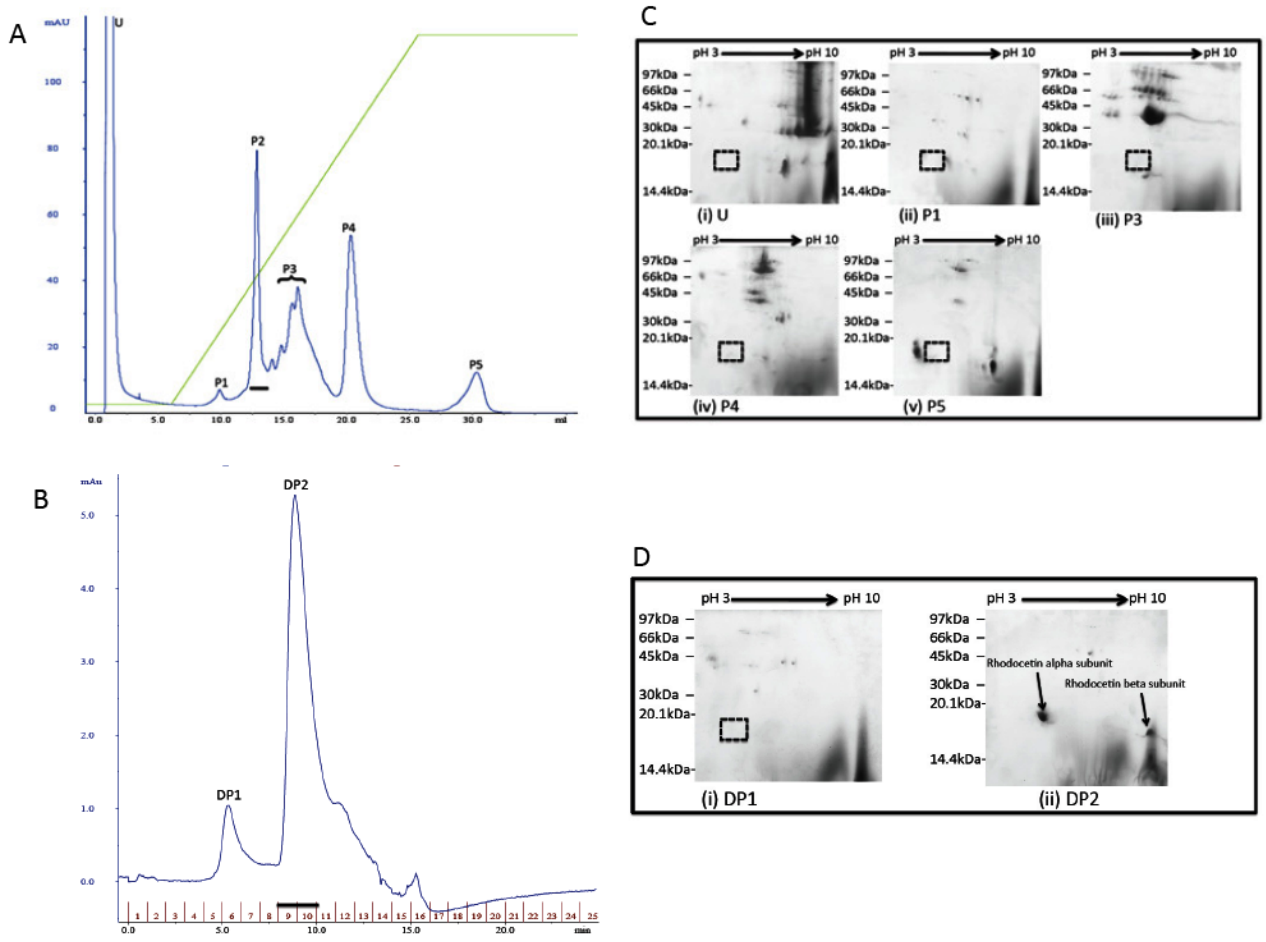

Figure 2. Purification profiles of rhodocetin from CR and the 2DE profiles for each peak obtained from the two-stage purification protocol. The horizontal bars indicate the collected fractions containing rhodocetin. The black grids represent our area of interest in which the rhodocetin (α subunit) spot should have been present. (**A**) Five milligram of crude CR venom dissolved in 250 µL of 20 mM Tris-HCl, pH 8.5 and loaded into a Mono Q 5/50 GL® (1 mL) column, equilibrated with 20 mM Tris-HCl, pH 8.5. Six peaks were obtained. (**B**) The P2 fraction collected from the Mono Q column was directly injected into a G25 HiTrap Desalting® column, equilibrated with distilled water. Two peaks, DP1 and DP2, were obtained. (**C**) Two-dimensional gel electrophoresis was performed on five peaks obtained from the anion-exchange separation and the profiles clearly displayed. 2DE assay was not performed on peak P2 as it was directly injected into the G25 HiTrap Desalting column without prior lyophilisation. (**D**) Two-dimensional gel electrophoresis profile of DP2 clearly showing the presence of rhodocetin α and β subunits.

DISCUSSION

Conventional 2DE has primarily been used as a technique for whole proteome analysis. In recent years its popularity has reduced due to its tediousness and the advent of non-gel based proteomics. However, with the introduction of newer 2DE applications it could possibly see a revival. The results from our study have proven its feasibility as an assay to guide progression in the purification process. Rhodocetin appeared as two spots on the 2DE profile of CR and purified rhodocetin showed two separate bands in SDS-PAGE, owing to its unique characteristic of being a heterodimer with α and β subunits held together by non-covalent bonds (13, 18). When comparing 2DE profiles between crude venom and all the peaks from two-stage purification, we used the spot for the rhodocetin α subunit as it is far more distinct than the β subunit. The appearance of the α subunit spot helped us determine which peak in the chromatography spectrum contained rhodocetin. Based on these results, 2DE in place of an assay is sufficiently selective and specific to determine which peaks contain the protein of interest, thus allowing a researcher to decide which

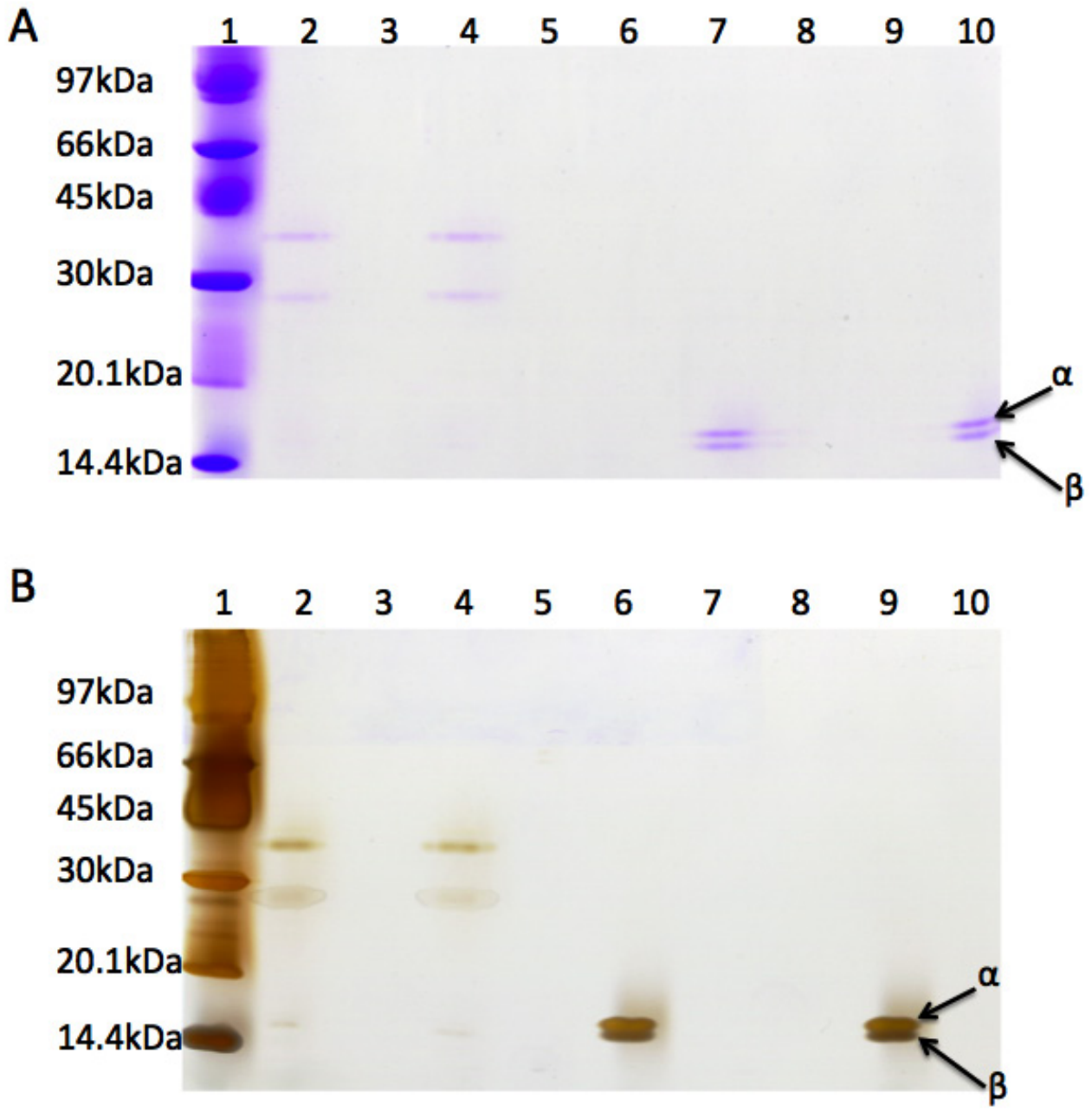

Figure 3. Homogeneity of purified rhodocetin from DP2 assessed using 15% SDS-PAGE. The purified rhodocetin showed two distinct bands due to separation of the heterodimer into its α and β subunits by SDS denaturation. The separated bands were visualized with both (**A**) Coomasie brilliant blue and (**B**) silver staining. (**A**) Lane 1: GE Healthcare low molecular weight (LMW) markers; lane 2: DP1; lane 3: blank; lane 4: DP1; lanes 5 and 6: blank; lane 7: DP2; lanes 8 and 9: blank; lane 10: DP2. (**B**) Lane 1: GE Healthcare LMW markers; lane 2: DP1; lane 3: blank; lane 4: DP1; lane 5: blank; lane 6: DP2; lanes 7 and 8: blank; lane 9: DP2; lane 10: blank. The blank wells were intentionally skipped to prevent any effect from inter-well spillage.

peak should be selected for further fractionation.

We intentionally selected 2DE over SDS-PAGE to guide progression in the purification process. One-dimensional SDS-PAGE has limited differentiation efficiency for crude venom proteins, owing to the overlapping of protein bands with similar molecular weights (19). Protein spots on the 2DE profile are more specific and more definite indications of the presence of proteins in a particular sample.

We have only described the use of this method with snake venom; 2DE is a versatile technique that can be applied to any sample, as long as it contains protein (1, 3). The technique has undergone many developments and efforts to standardize it over the years. These efforts have helped improve 2DE into a

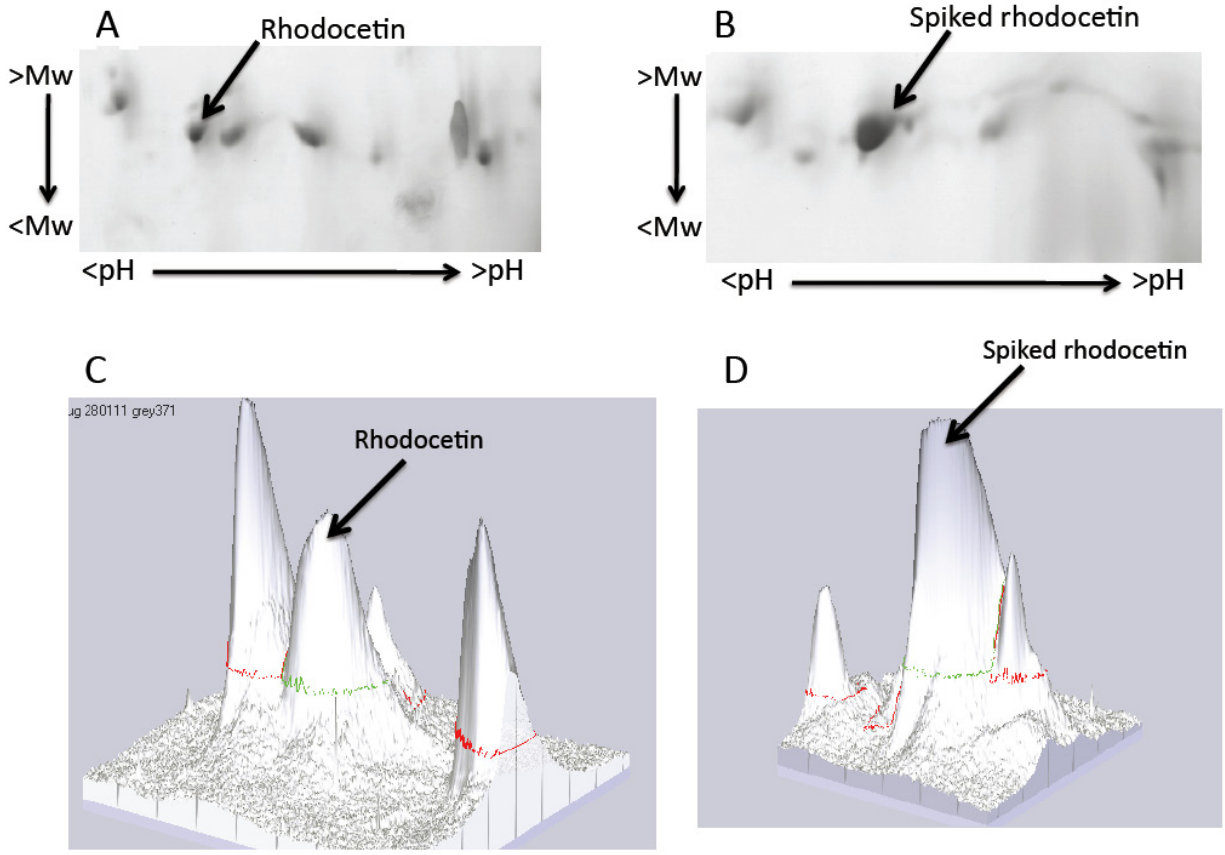

Figure 4. (**A**) Area of interest on the 2DE profile of crude CR venom with the rhodocetin (α subunit) spot labeled. (**B**) The same area showing the spot of spiked rhodocetin with an observed increased intensity. (**C**) Three dimension representation of the rhodocetin (α subunit) spot on the crude venom alone and (**D**) of the spiked rhodocetin (α subunit) spot, the latter having a quantified 1.6 fold increase in intensity.

method with a standardized protocol that requires little optimization and is often reproducible.

One of the major limitations of 2DE has always been the time required to perform a single run. The time needed to complete a general large format 2DE gel is often estimated as 3 to 5 days (3, 20). However, we have selected mini-gels for use in our 2DE-guided purification. This has decreased the overall time required allowing several simultaneous runs to be completed in a single day (20). In our study context, the use of mini-gels was also adequate for identifying the rhodocetin spot by comparing the crude CR profile on mini-gel with that previously performed on a larger 18-cm format 2DE gel (unpublished data).This is in line with a study that also demonstrated that data transfer between large format gel and mini-gel was compatible (20). Also with the recent advent of 2DE innovations such as the ZOOM® IPGRunner (Invitrogen, USA) bench top proteomics system that allows rapid first and second dimension protein separation in 2DE, any laboratory can achieve faster, simpler, and easier high-resolution 2DE (21).

Spot detection in 2DE critically relies on staining method and our use of Coomasie brilliant blue was sufficiently sensitive for our progression. The two common staining methods, silver staining and Coomasie brilliant blue, stain between 0.04 to 2 ng/mm^2 and 10 to 200 ng/mm^2 respectively (22). Several recent modifications to the Coomasie brilliant blue staining protocol have also greatly increased its sensitivity (23, 24). As such, 2DE is a sensitive assay requiring relatively low amounts of sample compared to certain bioassays. In addition, the sensitivity of this technique is expected to improve with the development of fluorescent staining (25). This is especially important, since progression into a further fractionation cycle only results in reducing the quantity of available sample while bioassay-guided purification of

venom neurotoxins using animal assays require fairly large amounts of the sample material (12). Although microinjection has been described as addressing this issue, it can be labor intensive and time consuming (12).

As liquid chromatography frequently employs salt gradient and non-volatile buffer (such as Tris-HCl), salt can still be present even after desalting and lyophilization of the peaks. This was evident by our inability to increase the voltage during IEF resulting in under focusing of the protein spots. Subsequently, whenever this problem appeared, we prolonged the IEF protocol to an overnight run by introducing an additional first step of 50 V at step and hold for 12 hours. This was found to improve IEF and voltage could be increased up to 5000 V. This is in line with the electrophoretic desalting concept described by Gorg *et al.* (26), in which samples with high salt concentration were directly desalted in IPG strip using a low voltage for the first few hours of IEF. Davidsson *et al.* (27) also reported that prolonging IEF run could improve the problem of incomplete focusing due to the presence of ampholytes in cerebrospinal fluid samples.

The biggest limitation of 2DE-guided purification is its dependence on protein profiling efforts and the publication of 2DE reference maps. In our study, without the prior profiling of rhodocetin into the 2DE reference map for CR, the rhodocetin spot would not have been located and, consequently, it would have been impossible to determine the presence of rhodocetin in chromatography peaks by 2DE testing. However, the prospect is for this limitation to disappear if the rise in protein profiling over recent years continues. In the field of snake venoms, the revitalization of 2DE has prompted a more global approach to studies on the snake venom, and the venom proteome of 55 snake genera has been solved using a variety of approaches (4). In other words, the reliance of 2DE-guided purification on prior protein profiling means that it can only be used for proteins already known in proteomic databases.

CONCLUSION

We have proven that 2DE-guided purification is a feasible concept. Two-dimensional gel electrophoresis can be used as an alternative assay, given that it fulfills the requisite criteria of a purification assay: selectivity, sensitivity, and specificity. This is evident by its efficiency in identifying the protein of interest from chromatography peaks in the fractionation process. While we do not deny that there are limitations to this newly-developed technique and its robustness may not be compatible with the many automated, miniaturized high-throughput screening (HTS) assays that are being developed, for small academic laboratories that do not have access to such complicated HTS assays, 2DE-guided purification could be an important tool that will potentially give them an equal opportunity to purify important bioactive materials from the complex matrix of natural products.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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