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Elution of tightly bound solutes from concanavalin A Sepharose Factors affecting the desorption of cottonmouth venom glycoproteins

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Abstract

Some glycoproteins bind so tightly to concanavalin A Sepharose that common desorption techniques are ineffective, so a systematic exploration of factors affecting desorption of cottonmouth venom glycoproteins was undertaken. Glycoprotein desorption is greatly improved by introducing up to four pauses of 5-10 min duration into the elution step. Eluent concentrations above 250 mM methylglucoside or 500 mM methyl-mannoside reduced glycoprotein desorption. Eluent NaCl diminished glycoprotein desorption. Most venom glycoproteins desorb more readily as pH diminishes from 6.0 to 4.0, but phosphodiesterase shows the opposite pattern. Eluents recommended by the supplier for desorbing solutes or for column cleaning were ineffectual.

Keywords

Concanavalin A Sepharose; *Agkistrodon piscivorus leucostoma* venom; Phosphodiesterase; Affinity chromatography; Desorption pauses; Lectin; Glycoprotein; Methyl- α -D-glucopyranoside; Methyl- α -D-mannopyranoside; Ethylene glycol; Urea; Propanol; Ethanol; Triton X-100; NaCl; Ionic strength; pH

1. Introduction

Affinity chromatography of glycosylated compounds on concanavalin A Sepharose (Con A Sepharose) is a widely used chromatographic technique. More than 2500 publications employing immobilized Con A for biomolecule isolation are currently found in Medline.

The Con A Sepharose/agarose (Con A Sepharose) literature that addresses glycoprotein purification comprises a broad array of equilibration and desorption buffers, reflecting the tolerance of the technique to diverse conditions. A review of the literature reveals that most bound solutes are relatively easily desorbed from Con A Sepharose with modest concentrations of competitive eluents (usually 50-200 mM methyl- α -D-mannopyranoside, or methyl- α -D-glucopyranoside); however, some glycoproteins bind to Con A Sepharose so tightly that common elution techniques are ineffective [1]. Many authors report lower than expected yields [2-10] or solutes that are refractory to increasing sugar concentrations [11-13]. Undesorbed protein not only reduces the yield of the target protein, but also the column capacity [1]. Increased back pressure results in reduced flow rates and interrupted chromatography. In subsequent cycles the target protein begins to appear in the unbound peak.

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When Con A Sepharose was employed in this laboratory for the isolation of phosphodiesterase (E.C. 3.1.4.1) (PDE) from western cottonmouth (*Agkistrodon piscivorus leucostoma*) (Apl) venom, it was discovered that PDE has great affinity for the resin, as reported earlier for *Crotalus adamanteus* venom PDE [14]. Initial experiments in this laboratory failed to achieve quantitative elution of Apl PDE. Accordingly, a systematic exploration of factors affecting glycoprotein desorption was undertaken.

Adsorption and desorption of cottonmouth venom glycoproteins were monitored by 280 nm absorbance and Coomassie dye binding (Bradford assay), while elution of PDE activity was determined colorimetrically. The present paper examines the influence of various factors influencing the elution of cottonmouth venom glycoproteins from Con A Sepharose, and suggests a simple technique, applicable to any buffer system, to improve the desorption of stubbornly bound solutes.

2. Materials and methods

2.1. Venom and reagents

Venom of *Agkistrodon piscivorus leucostoma* was purchased from Kentucky Reptile Zoo (Slade, KY, USA). Na⁺ bis(*p*-nitrophenyl) phosphate (N3002), methyl- α -D-glucopyranoside (M9376) and methyl- α -D-mannopyranoside (M6882) were obtained from Sigma. Cottonmouth venom was dissolved at 25 mg/mL in equilibration buffer: 50 mM acetic acid/NaOH/500 mM NaCl/1 mM MnCl₂/1 mM CaCl₂/1 mM MgCl₂ (pH 6.0). This buffer must be titrated to pH 6.0 prior to addition of MnCl₂. Failure to do so results in a heavy brown precipitate [Mn(OH)₂]. The venom solution was aliquotted into 1.5 mL microcentrifuge tubes and centrifuged 5 min at 25,000 $\times$ g in an Eppendorf 5417C in order to pellet insoluble material. Aliquots were frozen until use.

2.2. Resin, instrumentation, and software

One milliliter of Con A Sepharose (17-0440-03, GE Healthcare, Piscataway, NJ, USA), packed in an HR column (5.0 cm $\times$ 0.5 cm) (GE Healthcare), was used for all experiments. According to product literature, this resin has a ligand density of 13 mg Con A/mL, attached with CNBr. Chromatography was performed on a BioPilot fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Piscataway, NJ, USA) equipped with 280 and 214 nm conductivity monitors. In each experiment, 5 mg of crude venom were injected in a volume of 200 μ L using a PTFE sample loop. Integration of peak areas and other manipulations of chromatographic data were performed using Unicorn 3.0 software (GE Healthcare).

2.3. Experimental design

The present study comprised >225 chromatographic experiments and a comparable number of column washes between different types of eluents. Each chromatographic method was run in triplicate from 1-4 times (3-12 individual chromatographic experiments). Following initial experiments to probe parameters of interest, an intersecting series of experiments were designed to gather data regarding the influences of various factors on the efficiency of glycoprotein desorption. Factors included the number of desorption pauses, pause length, the concentration of competitive eluents, NaCl concentration, and pH. All series included a common experiment employing two 10-min pauses and 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0) buffer. Methyl- α -D-mannopyranoside was compared with methyl- α -D-glucopyranoside in a parallel experiment. In addition, the influences of auxiliary eluents (20% ethylene glycol, 4 M urea, and 40% 2-propanol) and alternative eluents (50% ethylene glycol, 100 mM boric acid, 20% ethanol, and 0.1% Triton X-100) were also examined in lieu of pyranosides.

The equilibration buffer for all experiments was the same as that used to dissolve the venom [50 mM acetic acid/NaOH/500 mM NaCl/1 mM MnCl₂/1 mM CaCl₂/1 mM MgCl₂ (pH 6.0)]. Only desorption buffers varied between experiments. Elution was effected with a pulse of desorbent (square gradient) rather than with a linear gradient (Fig. 1). All buffers were filtered through 0.22 µm filters before use. This was particularly important with elution buffers because the pyranosides (especially glucopyranoside) were dirty. When 250 mL of a 1 M methyl- α -D-glucopyranoside buffer were passed through a 47 mm nylon filter, the filter was dark gray-brown and flow rate was negligible by the time filtration was complete. Column eluent was collected and pooled into two batches, comprising unbound proteins and desorbed, bound glycoproteins (Fig. 1). In all experiments, 20 column volumes (20 mL) of eluent were used.

2.3.1. Elution continuity—Desorption buffer was 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0). Elution was either continuous (no pauses), or was punctuated by one, two, three, or four 10-min pauses. These were effected at 4.0, 8.0, 12.0, and 16.0 column volumes after the initiation of desorption, respectively (Fig. 1).

2.3.2. Pause duration—Desorption buffer was 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0). Two pauses of either 5, 10, or 20 min were employed during the desorption. Glycoprotein desorption in these experiments was compared with that from the unpaused experiment above.

2.3.3. Concentration of competitive desorbents—Desorption was also accomplished using methyl- α -D-mannopyranoside instead of methyl- α -D-glucopyranoside. Both sugars were dissolved in 100 mM acetic acid/NaOH (pH 4.0) buffers at concentrations of 100, 250, 500, and 1 M.

2.3.4. pH of elution buffer—Desorption buffer was 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside. Elution buffers were adjusted to pH 3.0, 4.0, 5.0, or 6.0.

2.3.5. Influence of NaCl in elution buffer—Desorption buffer was 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0), containing either no NaCl, 0.5, 1, or 2 M NaCl. In two additional experiments, 500 mM NaCl was replaced with 500 mM ammonium sulfate or 500 mM sodium acetate (pH 6.0).

2.3.6. Auxiliary and alternative eluents—In a final series of experiments, auxiliary desorbents (20% ethylene glycol, 4 M urea, 40% 2-propanol) were added to 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0) to see if desorption were enhanced. Because GE Healthcare's product literature recommended that 50% ethylene glycol, 20% ethanol, 100 mM boric acid/NaOH (pH 6.50), or 0.1% Triton X-100 could be used to remove even the most stubbornly adsorbed solutes, these eluents were also tested. In these experiments, 50% ethylene glycol and 0.1% Triton X-100 were employed in lieu of methyl- α -D-glucopyranoside. Boric acid and ethanol were used instead of the acetic acid elution buffer.

2.3.7. PDE and protein assays—PDE activity was assayed by a modification of a previously published technique [15]. The assay was redesigned as a 1 mL reaction to be performed in microcentrifuge tubes, comprising 50 µL 200 mM MgCl₂, 700 µL 100 mM Tris/HCl (pH 9.0), 100 µL column eluent, and 100 µL 5 mM Na⁺ bis(*p*-nitrophenyl) phosphate. All reagents were warmed to 25 °C in a circulating water bath before use. Tubes were vortexed 1–2 s and then incubated 1 h at 37 °C in a dry block incubator. Reactions were stopped with 50 µL 1 M NaOH, vortexed again and centrifuged 5 min at 25,000 × *g* in an Eppendorf 5417C. Thereafter the absorbance of each tube was determined at 400 nm using an Ultrospec 2000

spectrophotometer (Pharmacia Biotech). Blanks contained elution buffer instead of column eluent. Positive controls contained 2 μL of venom solution plus 98 μL of elution buffer.

This assay is quite robust; however, positive controls are required to correct for the effects of salt, pH, and chaotropes on substrate binding and catalysis. Failure to centrifuge the tubes before absorbance determinations results in erroneous readings due to turbidity. Also, once the reactions are stopped with NaOH, absorbances should be read within a couple of hours, as gradual hydrolysis of uncleaved substrate occurs. Beyond these caveats, considerable latitude in incubation time and relative quantities of sample, substrate, and Tris buffer is possible.

In addition to direct absorbance measurements, protein was also quantified using Coomassie plus, a modified Bradford assay (Pierce, Rockford, IL, USA). This assay was also scaled down to be run in microcentrifuge tubes. One hundred μL of column eluent were mixed with 900 μL of Coomassie solution. Blanks employed 100 μL of elution buffer and positive controls comprised 95 μL of elution buffer plus 5 μL of venom solution (25 $\mu\text{g}/\mu\text{L}$). Tubes were allowed to stand 5 min at room temperature before absorbances were read at 595 nm.

A venom standard curve was used to convert Bradford absorbances to micrograms of venom protein. A venom standard curve based on 280 nm absorbance was also used to convert integrated peak areas from Unicorn 3.0 to micrograms of venom protein. The latter values had to be divided by five because the UV-1 detector of the BioPilot FPLC had a 2 mm flow cell, whereas the Ultrospec 2000 spectrophotometer employed cuvettes with a 10 mm path length.

2.3.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

—Samples were analyzed by SDS-PAGE on 4–12% bis-Tris 1 mm NuPAGE gels (Invitrogen, Carlsbad, CA, USA, NP0322BOX). Lyophilized sample was diluted with equal volumes of 20 mM DTT in water and 2 $\times$ NuPAGE sample buffer (as recommended by Invitrogen). Gels were run for 35 min at 190 V constant voltage. Gels were stained with Imperial protein stain (Pierce 24615) according to manufacturer instructions.

2.3.9. Statistical analysis and data transformation—Statistical analyses of total protein and enzymatic activity were performed using Prism 4.0c for the Macintosh and InStat 3.0 (GraphPad Software, San Diego, CA, USA). In most cases, desorbed, bound protein (AU mL at 280 nm) and enzymatic activity levels were analyzed with Kruskal-Wallis nonparametric analysis of variance (ANOVA), with Dunn's multiple comparisons post-test for pairwise comparisons. In selected cases, in which all groups passed a normality test and standard deviations did not differ significantly between groups, standard (parametric) ANOVA was run with Tukey-Kramer Multiple Comparisons (pairwise) Tests. The Mann-Whitney *U*-test was also employed for individual, pairwise, nonparametric tests. Nonparametric tests do not assume a Gaussian distribution of values, and are therefore appropriate with small or non-Gaussian samples. Because nonparametric tests make fewer assumptions about the distribution of the data, they are less powerful than parametric tests. As a result, *p*-values tend to be inflated, which means that real differences may be reported as statistically nonsignificant when they are actually significant. That is, non-parametric tests are prone to give false negatives rather than false positives. A result was considered nonsignificant if $p \geq 0.05$.

Despite the use of a sample loop, there was significant run-to-run variation in sample loading. To eliminate this variation, absorbance data were normalized by setting the unbound peak area to 0.75 AU mL. Because the equilibration buffer and sample buffer were identical for all experiments, the only variation should have been in the amount of bound glycoprotein successfully desorbed from the column, not in the amount of unbound protein, assuming that the column was not overloaded, and that column capacity had not been compromised by undesorbed glycoproteins, or by ligand deterioration or leaching. The factor used for

normalization of unbound protein was also applied to the bound glycoprotein peak, since both varied in tandem with sample load. Bradford assay values were likewise normalized by setting the 595 nm absorbance of the unbound peak to 0.575 AU/mL. PDE activity data were normalized with unbound peak A_{280} normalization values, as described above.

Eluent from triplicate chromatographic runs was assayed simultaneously. Bradford and PDE activity data were further normalized using positive control values to remove variation resulting from the influence of different elution buffers on the assays themselves. Positive controls, unbound protein, and bound glycoprotein were assayed in quintuplicate for each chromatographic run. This left only variation due to different amounts of protein or PDE present in the eluent, and random variation due to handling and pipetting errors.

3. Results and discussion

3.1. Paused elution

Glycoprotein desorption from Con A can be greatly improved by introducing one or more 10-min pauses into the elution step of the chromatography (Figs. 1 and 2). The largest benefit derives from introduction of the first pause; however, additional pauses continue to desorb additional material with a diminishing yield (Figs. 1 and 2). Glycoprotein desorption shows a sigmoid relationship with the number of pauses (0-4).

Integrated peak areas (AU mL) indicate that increasing numbers of elution pauses significantly increase the amount of glycoprotein desorbed from Con A Sepharose ($p < 0.0014$, ANOVA) (Fig. 2A). Pairwise comparisons of the amounts of glycoprotein eluted using no pause or 1 pause versus 3 and 4 pauses were highly significant ($p < 0.01$ or < 0.001). For Bradford data (Fig. 2B), the statistical significance of differences between groups was extremely high ($p < 0.0001$, ANOVA). When pairwise comparisons were performed using Dunn's multiple comparisons test, additional glycoprotein yields achieved with 3 versus 0 ($p < 0.01$) and 4 versus 0 ($p < 0.001$) pauses, were highly significant.

PDE data (Fig. 2C) are noisier, but still present a roughly sigmoid plot, indicating that PDE desorption also increases significantly with additional 10-min pauses ($p < 0.0001$, Kruskal-Wallis nonparametric ANOVA). Six of ten pairwise comparisons of the amount of glycoprotein eluted with different numbers of pauses were also significant, with p -values ranging from < 0.05 to < 0.001 (Dunn's multiple comparisons test).

3.2. Pause duration

When two pauses of 5, 10, or 20 min were employed and desorption efficiencies were compared with unpaused runs (Fig. 3), increased pause duration improved the glycoprotein yield (A_{280} : $p = 0.0242$, ANOVA; Bradford: $p = 0.015$, Kruskal-Wallis non-parametric ANOVA), although the curve shape differed between A_{280} and Bradford assay profiles (Fig. 3A and B). The biggest improvement was seen from 5 to 10-min pauses, while a smaller gain was achieved by increasing the pause length to 20 min (Fig. 3B); however, all pairwise comparisons were statistically nonsignificant ($p > 0.05$) except for the A_{280} no pause versus 20-min pause comparison ($p < 0.05$) and the Bradford 5-min pause versus 20-min pause ($p < 0.05$). PDE data are more definitive (Fig. 3C). Increasing pause length resulted in elution of significantly more PDE from Con A Sepharose ($p < 0.0001$, Kruskal-Wallis nonparametric ANOVA). Pauses of any length were more effective than no pauses ($p < 0.001$, Dunn's multiple comparisons test), but pairwise comparisons between 5, 10, and 20-min pauses were not statistically significant ($p > 0.05$, Dunn's multiple comparisons test).

From the data displayed in Figs. 1-3, it is clear that improvements in yield might be obtained with the addition of still more pauses, although the A_{280} and Bradford profiles give slightly

different impressions. It appears that in general, glycoprotein desorption from Con A Sepharose can be optimized by employing 4–6 pauses of between 5 and 10 min duration. Lengthy pauses, such as the 16 h pause employed by Dolapchiev et al. [16] would appear to be of little utility.

3.3. Eluent concentration

In this experiment, different concentrations of two competitive eluents were examined. Desorption buffers comprised 100 mM acetic acid/NaOH (pH 4.0), containing either methyl- α -D-glucopyranoside or methyl- α -D-mannopyranoside at concentrations of 100, 250, 500, or 1 M. The most efficacious eluent concentrations were 250 mM methyl- α -D-glucopyranoside and 500 mM methyl- α -D-mannopyranoside. Counterintuitively, higher pyranoside concentrations actually diminished the amount of venom glycoprotein desorbed from Con A Sepharose, regardless of which pyranoside was used (Fig. 4). For Bradford assay data, pairwise comparisons of both pyranosides indicated that 250 mM, 500 mM and 1 M concentrations were all more effective desorbents than 100 mM ($p < 0.01$ or 0.001 , Dunn's multiple comparisons test). Other pairwise comparisons were nonsignificant, although 250 mM methyl- α -D-glucopyranoside was nearly significant when compared with 1 M (two-tailed $p = 0.0768$, Mann-Whitney test).

PDE data, usually the noisiest of the three data sets, yielded the best fitted curves for eluent concentration (Fig. 4C). For methyl- α -D-glucopyranoside, 100 mM sugar was significantly less effective as a desorbent than either 250 or 500 mM ($p < 0.001$, Dunn's multiple comparisons test). Likewise, both 250 and 500 mM methyl- α -D-glucopyranoside were more effective desorbents than 1 M ($p < 0.001$, Dunn's multiple comparisons test). For methyl- α -D-mannopyranoside, by virtue of its flatter curve (Fig. 4C), most pairwise comparisons were nonsignificant. However, both 500 mM and 1 M methyl- α -D-mannopyranoside were more effective desorbents than 100 mM ($p < 0.001$, Dunn's multiple comparisons test). Because the curves for 280 nm absorbance, Bradford data and PDE activity are basically similar, we conclude that the most effective desorption of cottonmouth venom glycoproteins is achieved with 250 mM methyl- α -D-glucopyranoside or 500 mM methyl- α -D-mannopyranoside.

Despite the fact that 73% of authors¹ using Con A Sepharose as an affinity resin employ methyl- α -D-mannopyranoside as the desorbent, it was a less effective desorbent for Apl glycoproteins than methyl- α -D-glucopyranoside at a 250 mM concentration (two-tailed $p = 0.0032$, Mann-Whitney test) (Fig. 4). Given that methyl- α -D-mannopyranoside costs approximately 4 $\times$ as much as methyl- α -D-glucopyranoside, it would behoove researchers to test both desorbents before finalizing their protocols.

3.4. Influence of NaCl concentration in desorption buffer

Both the Bradford and PDE assays are negatively impacted by increasing sample NaCl concentrations (Fig. 5) ($p < 0.0001$, Kruskal-Wallis nonparametric ANOVA). Positive controls were used to correct for the NaCl inhibition of these assays (Fig. 6).

Addition of NaCl (0.5, 1.0, or 2.0 M) to the elution buffer also diminished the amount of Apl venom glycoprotein eluted from Con A Sepharose (280 nm absorbance, Bradford, and PDE, $p < 0.0001$, Kruskal-Wallis nonparametric ANOVA) (Fig. 6). Diminished desorption probably results in part from increased hydrophobic interaction between Con A (102 kDa) [17,18] and venom glycoproteins, induced by higher ionic strength of the elution buffer. A similar suppression of protein desorption was observed when the elution buffer included 500 mM

¹A search of Pubmed for ((Concanavalin A AND (agarose OR Sepharose)) yielded 2404 references. Subsearches yielded 96 references for (glucoside OR glucopyranoside) (3.9%) and 256 for (mannoside OR mannopyranoside) (10.6%). Thus 27% of the searchable references employed methyl- α -D-glucopyranoside.

ammonium sulfate or 500 mM acetic acid/NaOH (pH 6.0) instead of NaCl (results not shown). However, Stark and Sherry [19] have shown that in the presence of 1 M NaCl at pH 5.6-7.2 apo-Con A (with metal ions removed) binds sugars with the affinity of native Con A. Thus NaCl enhances sugar binding directly, in addition to increasing hydrophobic interactions between Con A and the peptidyl moieties of bound venom glycoproteins.

Some authors have reported that NaCl in the elution buffer promotes more complete desorption of the glycoprotein of interest [16-20]. Many have included NaCl in both equilibration and desorption buffers at concentrations as high as 1.0 M [10,13,21,22]. Baumstark [23] reported that Con A Sepharose chromatography using 1.0 M NaCl in the elution buffer results in earlier elution for most of the 14 serum proteins examined, particularly ceruleoplasmin and α_2 -macroglobulin. IgM appeared much later, while albumin, IgG, α_1 -acid glycoprotein and transferrin exhibited double peaks. While NaCl may be efficacious in eluting relatively hydrophilic glycoproteins, it is clearly counterproductive for more hydrophobic ones. In some cases, NaCl in the elution buffer may have contributed to the need for unusually high concentrations of competitive sugars (e.g. ≤ 1.5 M) to desorb the solute of interest [16,24], although in view of data presented in the previous section, the high sugar concentration employed in these studies may itself have been a hindrance.

3.5. pH of elution buffer

Across the range of mildly to strongly acidic pH employed in this study, sample pH had a negligible effect on the Bradford assay, in which acidic conditions are used to facilitate Coomassie dye binding (Fig. 7A). In a plot of positive controls versus sample pH, the slope of the curve deviates significantly from zero ($p < 0.0001$); however, the goodness of fit is low ($r^2 = 0.240$), so the slope is probably meaningless. In contrast, PDE, which has a pH optimum close to 9.0, was significantly inhibited by low sample pH (Fig. 7B) ($p < 0.0001$, ANOVA). The curve appears hyperbolic here because the pK_a of acetic acid is 4.75. At pH 6.0, acetic acid has little buffering capacity, so the sample buffer had a negligible effect on the PDE assay. Sample buffers at pH 5.0 and 6.0 did not differ significantly in their effect on the PDE assay, but pairwise comparisons with pH 4.0 and 3.0 buffers were significant ($p < 0.001$, Tukey-Kramer multiple comparisons test).

For cottonmouth venom glycoproteins, the response to pH is complex. As desorbent buffer pH drops from 6.0 to 4.0, protein desorption from the column increases (280 nm absorbance, $p < 0.01$; Bradford, $p < 0.001$, Dunn's multiple comparisons test) (Fig. 8A and B). Below pH 4.0, protein desorption appears to diminish, but this result was nonsignificant for both 280 nm absorbance and the Bradford assay ($p > 0.05$, Dunn's multiple comparisons test) (Fig. 8A and B). It should be noted that for acidic buffer species, higher pH buffers, which require much larger quantities of titrant, also have an ionic strength effect, similar to that of NaCl.

PDE elution behavior was almost the mirror image of the 280 nm absorbance profile for most venom glycoproteins (Fig. 8A and C). The least amount of PDE eluted at pH 4.0. A significant amount more eluted at pH 3.0 ($p < 0.01$), with much greater yields at pH 5.0 and 6.0 ($p < 0.001$, Dunn's multiple comparisons test).

With regard to *Crotalus adamanteus* PDE, Dolapchiev et al. [16] reported, "In order to elute exonuclease, a significant increase in the ionic strength, pH and concentration of mannoside were required." Dolapchiev et al. eluted the *Crotalus* PDE with 50 mM phosphoric acid/NaOH/1 M NaCl/300 mM methyl- α -D-mannopyranoside (pH 7.2). While we cannot confirm the efficacy of using pH 7.2, it seems entirely reasonable in light of our data. However, there is little doubt that the inclusion of NaCl in the elution buffer was counterproductive.

3.6. Auxiliary and alternative eluents

In these experiments, auxiliary eluents were added to the standard 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0) buffer. These included 20% ethylene glycol, 4 M urea, and 40% 2-propanol. Addition of 20% ethylene glycol to the standard buffer resulted in a sharp reduction in eluted glycoprotein (Fig. 9) (two-tailed $p < 0.0001$, Mann-Whitney test). Propanol also sharply reduced glycoprotein desorption from Con A Sepharose (Fig. 9) and this reduction was statistically significant (two-tailed $p < 0.0001$, Mann-Whitney test).

When 4 M urea was added to the standard buffer, the amount of desorbed glycoprotein was nearly twice that seen with the standard buffer system. Successive runs yielded progressively less glycoprotein until the fourth run was only about 20% greater than an average run under standard conditions; however, when all urea runs were averaged together, urea was still significantly more effective at desorbing protein than the standard conditions (two-tailed $p < 0.0001$, Mann-Whitney test).

One possible explanation for these observations was that 4 M urea dislodged glycoprotein that had accumulated gradually over the course of the study and that for each run, there was progressively less material to desorb. Presumably at some point the amount of glycoprotein would have stabilized at the amount of glycoprotein being applied to the column. However, Fountoulakis and Juranville [1] reported that high concentrations of chaotropes promote the release of Con A from the Sepharose matrix, significantly reducing the binding capacity of the column. They surmised that high concentrations of chaotropes may destroy the tetrameric structure of Con A. They employed 6 M urea at pH 7.0 and they also used guanidine-HCl, Triton X-100, and SDS in the course of their studies. They reported that SDS did not have the same effect as urea. Wan and Hasenstein [25] reported no complications when applying samples containing 4 M urea to Con A Sepharose.

A single chromatographic experiment using the standard buffer system (100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside, pH 4.0) was performed at the conclusion of the urea experiments. The unbound fraction comprised 89.3% of the venom protein applied to the column. Previously the unbound fraction mean of six experiments had been 82.2% (range 79.6-84.4%). The glycoprotein yield was only about 55% of that seen under standard conditions early in the study and was slightly less than that of the last urea run. Thus it appeared that column capacity had diminished significantly.

To resolve the question an extra experiment was performed. A virgin column of Con A Sepharose was packed and equilibrated as before, after changing both column filters. Then the standard gradient was run using 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside/4 M urea (pH 4.0). No sample was applied. Nonetheless, a large double peak eluted, reflecting the two-pause method used. When this experiment was repeated another double peak eluted, but much smaller than the first (Fig. 10). When eluent from the second run was examined by SDS PAGE, a very heavy band was seen at 25 kDa, which corresponds to the monomeric molecular weight of Con A. Lesser bands were also seen at 55 kDa (faint), 43 kDa (faint), 22 kDa (moderate), 14 kDa (heavy) and ~9 kDa (heavy). The 55 kDa band corresponds to the dimeric molecular weight, while the 14 and 9 kDa bands resemble those previously reported [18]. These results confirm the conclusion of Fountoulakis and Juranville [1] that Con A is removed from the resin by high concentrations of urea.

Influences of eluent concentration and pH proved more complex than indicated in GE Healthcare's Con A Sepharose instruction sheet [26], and the 0.5 M NaCl recommended for enhanced solute desorption was clearly a hindrance, rather than a help. Accordingly, additional eluents were investigated, including those suggested for regenerating the column, as well as for desorbing bound solutes. Absorbance of column eluent (280 nm) and the Bradford assay

provided similar pictures of glycoprotein desorption. For simplicity, only the Bradford data are reported.

According to the instruction sheet for Con A Sepharose, “Borate is known to form complexes with cis-diols on sugar residues and thus act as an competing eluent. For elution with borate, use a 0.1 M borate buffer, pH 6.5.” The recommended borate buffer desorbed only ~10% as much glycoprotein as the standard buffer system (two-tailed $p < 0.0001$, Mann-Whitney test) (Fig. 9). It was so ineffective that three, four-pause cleaning runs with 250 mM methyl- α -D-glucopyranoside were required to remove all of the accumulated glycoprotein before the column could be used in a subsequent set of experiments. Fountoulakis and Juranville [1] also found borate to be ineffective.

The supplier further recommended, “A 20% ethanol wash or a gradient wash with up to 50% ethylene glycol may be used to elute even the most strongly bound substances.” Elution with 20% ethanol was less effective than the standard buffer, although the difference was not statistically significant, owing to the variability between ethanol runs (two-tailed $p = 0.1261$, Mann-Whitney test) (Fig. 9). When 50% ethylene glycol replaced 1 M methyl- α -D-glucopyranoside in the standard buffer (100 mM acetic acid/NaOH/50% ethylene glycol, pH 4.0), significantly less glycoprotein was desorbed from the column (two-tailed $p < 0.0001$, Mann-Whitney test) (Fig. 9).

Nonionic detergents, such as Triton X-100 (0.1%), are also recommended by the manufacturer to regenerate the column. Ionic detergents are not recommended because of their potential to convert the affinity resin into an ion exchange resin. The Bradford assay indicated that no glycoprotein eluted from the column; however, the 0.1% Triton X-100 concentration used exceeded the interference limit (0.062%) recommended by Pierce for the assay. Absorbance (280 nm) indicated that the glycoprotein yield with Triton X-100 was lower than for all other eluents except borate and 20% ethanol (not shown).

For strongly bound glycoproteins, the elution and column cleaning procedures recommended by the manufacturer are largely ineffective. Having said this, it must be added that Con A Sepharose is a remarkably resilient resin. The column used in this study was employed in nearly 500 individual chromatographic experiments and showed no definite evidence of deterioration until subjected to 4 M urea buffers. Column care is simple. At the close of each day of experiments, ten column volumes of 20% ethanol were passed over the column. This was sufficient to maintain the column in good condition, even though it was often left for hours in equilibration buffer at pH 6.0.

The variable behavior of Con A Sepharose resins undoubtedly reflects the size and structural complexity of this ligand. Con A has a monomeric molecular weight of 25,500 D [17,27,28]. At physiological pH it exists primarily as a tetramer, but below pH 5.5 it is dimeric [17-29]. Each monomer possesses a transition metal binding site, a Ca_{2+} binding site, and a sugar binding site [30,31]. Some of the monomers occur in a naturally cleaved form [18,32,33].

Con A undergoes relatively slow, but significant structural shifts in response to different solvent conditions. The metal cofactors do not participate directly in carbohydrate binding, but stabilize a polypeptide loop that does [34,35]. This sugar binding conformation is known as the “locked” conformation. EDTA removes metal ions very slowly from the “locked” form of Con A at neutral pH, such that EDTA produces no detectable change in sugar binding over the space of several hours at room temperature [19]. Thus, under the conditions used by most researchers, desorption of glycoproteins via metal chelation is not a viable strategy.

4. Conclusions

The use of 4-6 pauses of 5-10 min duration during solute desorption greatly improves glycoprotein yields from Con A Sepharose without increasing the eluent volume or the concentration of desorbent used. In fact, with paused elution, it is possible to substantially reduce the amount of desorbent used. Given the cost of methylated pyranosides this is a significant benefit. With hydrophobic glycoproteins, NaCl and other salts enhance binding and should be avoided in elution buffers. Eluent concentrations above 250 mM methyl-glucoside or 500 mM methyl-mannoside reduce glycoprotein desorption, rather than enhancing it. pH effects vary from one protein to another and must be explored on a case-by-case basis; however, as general rule, pH 4.0 is most effective for the majority of cottonmouth venom glycoproteins. For strongly bound glycoproteins, auxiliary desorbents such as ethanol, propanol, or ethylene glycol and alternative eluents such as borate are ineffective. Urea elutes bound glycoproteins very effectively. Unfortunately, it also elutes Con A subunits very effectively, destroying the column and contaminating the sample. Urea should be avoided.

Subsequent to the submission of this manuscript, we employed *m*-aminophenyl boronic acid agarose (Sigma) as an alternative to Con A Sepharose. Hydrophobic interactions between the target glycoprotein and the phenyl group of the ligand are still problematic; however, the simpler nature of the ligand permits the use of any eluent that the target protein can tolerate. In the case of PDE, a desorbent containing 500 mM NaCl and 2 M urea seems to work well, at either acidic or mildly alkaline pH.

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Fig 1.

Glycoprotein elution profiles with 0-4 pauses introduced during desorption. The 1 mL Con A Sepharose column was equilibrated in 50 mM acetic acid/NaOH/500 mM NaCl/1 mM MnCl_2 /1 mM CaCl_2 /1 mM MgCl_2 (pH 6.0) and desorbed with 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0). A short, narrow depression preceding a peak indicates the point at which a 10-min pause was introduced (4, 8, 12, and 16 mL after desorption onset). In all experiments, 20 column volumes (20 mL) of eluent were used.

Fig 2.

Influence of pause number on glycoprotein and PDE desorption. Glycoprotein desorption increases significantly in sigmoidal fashion with the number of 10-min desorption pauses employed during elution ($p < 0.0001$, Kruskal-Wallis nonparametric ANOVA). The A_{280} curve and the results of Bradford and PDE assays suggest that additional pauses (>4) could still desorb additional material, a result also suggested by Fig. 1. In all experiments, 20 column volumes (20 mL) of eluent were used.

Fig 3.

Influence of pause length on glycoprotein and PDE desorption. Glycoprotein desorption is significantly enhanced by increasing pause length in a two-pause model ($p = 0.0242$, ANOVA). It is unclear whether the curve shape is sigmoidal or hyperbolic; however, pauses longer than 10 min yield diminishing amounts of bound glycoprotein, suggesting that there is little value in using pauses longer than 20 min. It is probably more effective to increase the number rather than the length of pauses. In all experiments, 20 column volumes (20 mL) of eluent were used.

Fig 4.

Influence of concentration and type of competitive desorbent. Counterintuitively, desorption of cottonmouth venom glycoproteins with methyl- α -D-glucopyranoside and methyl- α -D-mannopyranoside is generally maximal at 250 mM and 500 mM, respectively. Further increases in pyranoside concentration are counterproductive. PDE desorption is maximal at 500 mM methyl- α -D-glucopyranoside, a little later than for most other venom glycoproteins. Methyl- α -D-glucopyranoside is at least as effective a desorbent for cottonmouth venom glycoproteins as methyl- α -D-mannopyranoside.

Fig 5.

Influence of eluent NaCl concentration on the Bradford and PDE assays. Both assays are negatively impacted by increasing sample NaCl concentrations, as determined from positive controls ($p < 0.0001$, Kruskal-Wallis nonparametric ANOVA). Data in Fig. 6 were corrected for these effects.

Fig 6.

Influence of eluent NaCl concentration on glycoprotein and PDE desorption. Increasing eluent NaCl concentrations reduces glycoprotein desorption from Con A Sepharose even after the Bradford and PDE assays are corrected for direct effects on the assay [280 nm absorbance: $p < 0.0001$, ANOVA; Bradford, $p < 0.0001$, (Kruskal-Wallis nonparametric ANOVA); PDE: $p < 0.0001$, (Kruskal-Wallis nonparametric ANOVA)].

Fig 7.

Influence of eluent pH on the Bradford and PDE assays. Across the range of mildly to strongly acidic pH employed in this study, there was no discernible effect of sample pH on the Bradford assay, which employs acidic conditions to facilitate Coomassie dye binding. The slope of the curve deviates significantly from zero ($p < 0.0001$); however, the goodness of fit is low ($r^2 = 0.240$). PDE, which has a pH optimum close to 9.0, was inhibited by low sample buffer pH. The curve appears hyperbolic here because the pK_a of acetic acid in the sample buffer is 4.75. At pH 6.0 acetic acid has little buffering capacity, so above pH 5.0 the sample buffer has a negligible effect on the PDE assay.

Fig 8.

Influence of pH on venom glycoprotein and PDE desorbed from Con A Sepharose. Eluents consisted of 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside titrated with NaOH to pH 3.0, 4.0, 5.0, or 6.0. Bradford data and PDE activities have been corrected for the effects of pH and ionic strength so that assay data shown here represent only the quantities of glycoprotein and PDE, respectively, eluted from the column (see Fig. 7). The 280 nm absorbance suggests that in general, more glycoprotein is desorbed at lower pH, with no improvement in yield below pH 4.0 ($p = 0.0011$, Kruskal-Wallis nonparametric ANOVA). This pattern is consistent with supplier literature. The Bradford assay provides a slightly different picture at lower pH, but also suggests that significantly less glycoprotein is desorbed

at more neutral pH ($p = 0.0001$, Kruskal-Wallis nonparametric ANOVA). In contrast, venom PDE desorbs more completely at higher pH, its elution curve being almost a mirror image of the 280 nm curve for all venom glycoproteins ($p = 0.0001$, Kruskal-Wallis nonparametric ANOVA).

Fig 9.

Influence of auxiliary and alternative eluents on glycoprotein and PDE desorption from Con A Sepharose. Auxiliary eluents were added to the standard 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside (pH 4.0) buffer. These included 20% ethylene glycol, 4 M urea, and 40% 2-propanol. Alternative eluents included 100 mM acetic acid/NaOH/50% ethylene glycol (pH 4.0), 100 mM boric acid/NaOH (pH 6.50), 20% ethanol in water and 0.1% Triton X-100. Absorbance (280 nm) and the Bradford assay provide generally similar pictures of glycoprotein desorption, so only the Bradford data are shown here. With the exception of 4 M urea, none of the auxiliary or alternative eluents appeared to be as effective as the standard conditions; however only 40% Propanol and borate were significantly less effective (Bradford assay, $p < 0.01$, $p < 0.001$, Kruskal-Wallis nonparametric ANOVA, respectively). The amount of glycoprotein desorbed with 4 M urea was significantly greater than that desorbed with all other eluents except for the standard buffer (Bradford assay, $p < 0.001$, Kruskal-Wallis nonparametric ANOVA); however, 4 M urea also desorbed Con A itself (Fig. 10).

Fig 10.

Effects of 4 M urea on Con A Sepharose. A virgin Con A Sepharose column (column filters replaced) was subjected to a standard chromatographic experiment with 100 mM acetic acid/NaOH/1 M methyl- α -D-glucopyranoside/4 M urea (pH 4.0) as the eluent. Sample was never injected onto this column. Con A subunits were released from the resin. Note the reduction in Con A released by a second experiment. SDS PAGE of the released material showed major bands identical to those previously reported for Con A and its naturally occurring fragments [1,18].