Removal of endotoxin by reverse phase HPLC abolishes anti-endothelial cell activity of bacterially expressed plasminogen kringle 5

Andrew Dudley, William McKinstry, David Thomas, James Best, and Alicia Jenkins
The University of Melbourne, Melbourne, Australia


INTRODUCTION

The ability to clone human genes in bacteria and express and purify the recombinant proteins enables the production of significant quantities of material for use in in vitro or in vivo activity assays, structural studies, and even in the clinical setting. For example, in the field of angiogenesis research, following the discovery of proteins such as angiostatin (plasminogen kringle 1–3), which specifically target endothelial cell growth, bacterial and other expression systems have been utilized to express and purify recombinant proteins and then assay for anti-angiogenesis activity (1–3). However, bacterial expression systems are prone to contamination with endotoxin, a ubiquitous lipid-rich carbohydrate moiety found in the cell wall of Gram-negative bacteria. As endothelial cells are highly sensitive to endotoxin compared to other cell types, care must be taken to ensure that negligible concentrations of endotoxin are present in any purified recombinant proteins that will be assayed in a downstream in vitro endothelial cell system (4). In this study, we describe the expression, purification, and characterization of plasminogen kringle 5, a protein, like other plasminogen kringle, previously reported to inhibit angiogenesis (5–13).

MATERIALS AND METHODS

Preparation of Constructs

Human liver RNA was reverse transcribed to cDNA using reverse transcription PCR (RT-PCR). The regions spanning plasminogen kringle 5 or kringle 4 were then amplified from cDNA using the primers 5′-TGTGAATTCGCGACATGAGAGTCTCTCTTC-3′ and 5′-GGAAAGCTTGGCCACACTGGAGACATCACAG-3′ for kringle 4. The amplified products were gel-purified and ligated into the expression vector pET22b+ (Novagen, Darmstadt, Germany) in frame with an N-terminal periplasmic localization sequence and a C-terminal hexa-histidine (HIS)-tag. The resulting constructs were transformed into the Escherichia coli strain BL21 (DE3).

Expression and Purification

For expression, a 50-mL starter culture was grown in LB plus 50 µg/mL ampicillin with shaking for 2 to 3 h at 37°C. This was then used to inoculate 1 L of LB containing 50 µg/mL ampicillin. The culture was grown with shaking for an additional 1 to 2 h until the A280nm reached 0.5 units. Isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) was added, and the culture was left for 4 h at 37°C with shaking. Following the 4-h incubation, cells were harvested by centrifugation (10,000× g for 20 min), and the supernatant was discarded. The cell pellet was resuspended in 20 mM Tris, 20% sucrose, 1 mM EDTA, pH 8.0, and stirred gently at 25°C for 10 min. The cells were again centrifuged as before and then resuspended in ice-cold 5 mM MgSO4. Periplasmic proteins were released into the supernatant by stirring on ice for 10 min. Following a final centrifugation (10,000× g for 20 min at 4°C), the supernatant was collected and adjusted to contain 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole (all final concentrations), and the pH was titrated to 8.0. Periplasmic proteins were bound to a 4-mL column of Ni2+-NTA affinity agarose (Qiagen, Victoria, Australia) (previously equilibrated with five column volumes wash buffer containing 50 mM NaH2PO4, 35 mM imidazole, pH 8.0) before eluting with washing buffer containing 250 mM imidazole. The eluted proteins were then dialyzed at 4°C against sterile phosphate-buffered saline (PBS), with two buffer changes over 48 h. Finally, purified proteins were filter-sterilized using a 0.22-µm filter (Millipore, Bedford, MA, USA), and the protein concentration was determined using the Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA).
Anion Exchange Purification

Following initial purification on Ni⁺-NTA agarose, both kringle 5 and kringle 4 were dialyzed against 20 mM Tris buffer, pH 7.5, before loading onto a Q HyperD™ column (4.6 × 100 mm; Beckman Coulter, Fullerton, CA, USA). Bound proteins were eluted with NaCl (0–1 M gradient), desalted on Sephadex™ G-25 (Amersham Biosciences, Uppsala, Sweden), and then combined and lyophilized. Lyophilized proteins were resuspended in pyrogen-free water and then filter-sterilized using a 0.22-µm filter before assaying in bovine aortic endothelial cells (BAEC).

Reverse Phase High-Performance Liquid Chromatography Purification

Initial purification on Ni⁺-NTA agarose was carried out as before. Proteins eluted from the Ni⁺-NTA column were loaded directly onto a Brownlee™ Aquapore C4 column (4.6 × 220 mm; Applied Biosystems, Foster City, CA, USA) and eluted with a 0%–60% acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA). Eluted proteins were speed-vacuumed until dry and then washed twice with pyrogen-free water. Finally, proteins were resuspended in pyrogen-free water and then filter-sterilized using a 0.22-µm filter before assaying in BAEC.

Kringle 5 and Kringle 4 Activity in Bovine Aortic Endothelial Cells

The activity of kringle 5 and kringle 4 were assayed in BAEC as described previously (6). Cell numbers were determined by dispersing cells in trypsin at the end of the 72-h incubation period and counting by a model Z1 Coulter counter (Beckman Coulter).

Figure 1. Purification and activity of plasminogen kringle 5 and kringle 4. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of 3 µg purified protein. Samples were run on a 15% gel under reducing conditions and stained (left) or blotted (right) with a monoclonal HIS-tag antibody. The molecular weight marker sizes are indicated in kDa. (B) Dose response assessment of both kringle 5 and kringle 4 in bovine aortic endothelial cells (BAEC) after 72 h in culture. The assay was done in triplicate wells and repeated three times. The error bars are the standard error of the mean (SEM). (C, top panel) Five micrograms of kringle 5 were digested with the indicated proteases for 2 h and analyzed by SDS-PAGE. (C, bottom panel) Digested kringle 5 was assayed in BAEC in triplicate. Error bars are the SEM. No effect on cell growth was seen in control wells, which were treated with the equivalent concentration of enzyme only. K5, kringle 5; K4, kringle 4.

Endotoxin Testing

Endotoxin testing was performed following the manufacturer’s instructions, using a colorimetric quantitative limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA).

RESULTS AND DISCUSSION

Expression and Purification of Recombinant Plasminogen Kringle 5 and Kringle 4

Figure 1A shows a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a Coomassie™ Brilliant Blue stain analysis of purified recombinant kringle 5 and kringle 4 (left). Both proteins migrated at about 18 kDa, which is somewhat shifted from the expected 12 kDa for each protein. About 3 µg of each protein were then blotted and probed with a monoclonal HIS-tag antibody, confirming the presence of the HIS-tag (right). Finally, a chloroform extraction was performed with each sample prior to electrospray ionization double quadrupole time-of-flight (ESI-Q-Q-TOF) mass spectroscopy on an API-QSTAR® Pulsar 1 MS (Applied Biosystems). The results showed that the majority of the sample had a molecular mass of 12.82743 kDa, which is in good agreement with the expected molecular mass of 12.827 kDa. Similarly, the majority of the kringle 4 sample had a mass of 11.834 kDa, which also agrees well with the theoretical monoisotopic mass of 11.832 kDa. Taken together, these results confirm the purity and fidelity of both our recombinant kringle 5 and kringle 4.

Activity of Kringle 5 and Kringle 4 in Bovine Aortic Endothelial Cells

The activity of each purified protein was then assayed in BAEC following the method of Cao et al. (6). The results show that the dose response curves for both kringle 5 and kringle 4 are identical with a median effective dose (ED50) of about 50 nM, which is in good agreement with the results of Cao et al. for kringle 5 (6). However, contrary to previously published data,
kringle 4 also inhibited BAEC growth, leading us to speculate that a contaminant might be present (14) (Figure 1B). To test this hypothesis, kringle 5 was subjected to proteolysis with various proteases at an enzyme to protein ratio of 1:100 for 2 h at 37°C or heat denaturing at 100°C for 10 min. The digested material was then analyzed by SDS-PAGE, and 1 µM was assayed for activity in BAEC cultures (Figure 1C). The results show that proteolysis or heat denaturing (data not shown) did not abrogate kringle 5’s activity even when kringle 5 was digested with proteinase K, an enzyme that will theoretically cleave kringle 5 at 35 sites, thus generating peptides no larger than approximately 7 amino acids. These results suggested the presence of a non-protein contaminant in recombinant kringle 5 purified by Ni⁺-NTA agarose that was not sensitive to heat denaturing or proteolysis.

**Additional Purification by Anion Exchange Chromatography**

Figure 2A is the elution profile and SDS-PAGE analysis of kringle 5 showing a broad peak eluting between 100–200 mM NaCl. Kringle 4 demonstrated similar behavior to kringle 5 on anion exchange purification and also eluted as a broad peak between 100–200 mM NaCl. As before, each protein was then assayed in BAEC. The results show that even after ion exchange chromatography, both proteins inhibited BAEC growth, albeit at higher concentrations (ED50 of about 1 µM for each), compared to proteins purified by Ni⁺-NTA agarose alone (Figure 2B). Again, when anion exchange-purified kringle 5 was subjected to heat denaturing or proteolysis with either chymotrypsin or proteinase K, no loss in activity was observed compared to the undigested control (data not shown).

![Figure 2. Anion exchange chromatography and reverse phase high-performance liquid chromatography (HPLC) purification of kringle 5 and kringle 4.](image-url)
A standard curve was prepared using known amounts of the mean (kringle 5 were plotted. The experiment was repeated three times, and the error bars are the standard error counted. The dose response curves for pure endotoxin and the endotoxin concentration calculated for being added into previous cell growth assays. The cells in triplicate wells were dispersed in trypsin and with known amounts of endotoxin based on the endotoxin units (EU)/mL in each sample and the amount performance liquid chromatography agarose alone was compared to the endotoxin concentration in samples purified by reverse phase HPLC. Compared to the other purification methods, reverse phase HPLC removed nearly all of the endotoxin present by 100- to 500-fold.

Finally, we calculated the concentration of endotoxin that was being added into our previous BAEC growth assays based on the endotoxin concentration for each sample. For example, it was found that a 5 µM concentration of kringle 5 equaled approximately 3.75 EU/mL. We then added comparable concentrations of endotoxin to BAEC cultures to determine if this might account for the observed inhibition of cell growth. The dose response curves for pure endotoxin and the calculated EU/mL for kringle 5 were fit to a second-order polynomial. The results show that the dose response curves are nearly identical. It was noted that even at the lowest pure endotoxin concentration of 0.625 EU/mL (equivalent to 0.625 µM or 0.468 EU/mL kringle 5), >50% growth inhibition was observed despite very little evidence of cell toxicity (reflected by no changes in cell morphology, such as cell rounding) (Figure 3D). Therefore, at doses of kringle 5 that are calculated to achieve a similar concentration of pure endotoxin (as seen in Figure 3D), the magnitude of growth inhibition is comparable. Taken together, these results strongly indicate that recombinant kringle 5’s anti-endothelial cell activity is related to its contamination with endotoxin.

CONCLUSIONS

This study highlights the potential pitfalls of utilizing bacterially expressed recombinant proteins in angiogenesis-type assays in which endothelial cells are the specific target. Indeed, many previous studies have been confounded by endotoxin contamination, which can be tightly bound to a protein of interest and resistant to removal even with harsh detergents (15–17). We found that an endotoxin removal col-
REFERENCES


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Address correspondence to Andrew Dudley, The Department of Medicine, St. Vincent’s Hospital, The University of Melbourne, Corner of Princes and Regent Streets, Fitzroy, Melbourne, VIC, 3065, Australia. e-mail: drew@medsv.unimelb.edu.au