Purification, Refolding, and Angiogenic Characterisation of Isthmin Produced as Inclusion Bodies in Escherichia coli.

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Abstract

Isthmin, a 60 kDa novel secreted protein, is predicted to be an anti-angiogenic protein based on its TSR domain. To study the function of isthmin, recombinant proteins containing full-length, N- and C-terminal truncations, as well as the TSR domain alone, were generated from Escherichia coli. However, all these proteins were present in insoluble inclusion bodies. Previous attempts to make the protein soluble through urea denaturation followed by dialysis refolding lead to significant loss of the protein through precipitation. In this work, a working method was developed and optimized to produce soluble, pure recombinant isthmin with biological activity by employing an Immobilized Metal Affinity Chromatography (IMAC) on-column refolding procedure. Recombinant proteins were obtained as refolded single species monomers that were >95% pure and with an average yield of 14 mg per 1L bacterial culture. Proteins purified in this manner also had a low endotoxin level (<0.047 EU/ml) and low endothelial cell cytotoxicity. The purified Isthmin was biologically active and exhibited dose-dependant inhibition of the early stage of endothelial cell tube formation and endothelial cell proliferation in Human Umbelical Vein Endothelial Cells (HUVECs). Our study provides a feasible approach to produce soluble and biologically active Isthmin proteins with high purity and permits subsequent studies into the structure, function, and mechanisms of action of Isthmin.

Introduction

Isthmin is a novel protein that is predicted to have anti-angiogenic activity based as it has a thrombospordin repeat (TSR) domain that is highly identical to the TSR domain in thrombospordin-1 (TSP-1) a highly active anti-angiogenic protein that has been shown to be able to reduce tumor angiogenesis (Chen et al., 2000). TSP-1’s TSR domain has been implicated in antiangiogenic functions (Dawson, 1997). To study the hypothesized anti-angiogenic functions of isthmin, recombinant isthmin, the TSR domain, as well as truncated N- and C-terminal peptides were synthesized. However, peptides were expressed in Escherichia coli as insoluble inclusion bodies. As such, isthmin and its peptide fragments have to be expressed in E. coli, purified, and properly refolded by immobilized metal-affinity chromatography (IMAC) refolding before they can be studied for anti-angiogenic functions and thus proven to be biologically active.

Materials and Methods

All four peptides were cloned with an appended 6x His tag into pET-M plasmids and used to transform E. coli BL21 cells. 1L bacteria culture of these transformed cells were induced with IPTG and harvested.

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1 Student
2 Principal Investigator, Assistant Professor
Inclusion bodies were isolated by centrifugation and washed repeatedly to remove contaminating proteins and endotoxins. Purified inclusion bodies were solublized with 6 M guanidine hydrochloride and applied to a nickel nitroacetic acid (Ni-NTA) column where the peptides were refolded by applying a continually decreasing concentration of denaturant. Refolded proteins were eluted and dialyzed. Purity and refolding was determined by SDS PAGE and native gel electrophoresis.

Limulus Amoebocyte Lyaste (LAL) assay and EZ4U assay were carried out to determine endotoxin content and cytotoxicity in the purified protein.

Cell proliferation assays using BrdU and tube formation assays on Matrigel were carried out on Human Umbelical Vein Endothelial Cells (HUVECs) to test for anti-angiogenic biological activity.

**Results**

**Purification of isthmin and truncated peptides**

Isthmin and its truncated peptides were isolated as pure, properly refolded proteins.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>After Solublization</th>
<th>After Elution</th>
<th>After Dialysis</th>
<th>Final Purity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISM-FL</td>
<td>~15.23 mg</td>
<td>11.88 mg</td>
<td>11.22 mg</td>
<td>&gt;95%</td>
<td>73.7%</td>
</tr>
<tr>
<td>ISM-N</td>
<td>~16.31 mg</td>
<td>11.64 mg</td>
<td>10.74 mg</td>
<td>&gt;95%</td>
<td>65.9%</td>
</tr>
<tr>
<td>ISM-C</td>
<td>~18.12 mg</td>
<td>14.98 mg</td>
<td>14.23 mg</td>
<td>&gt;95%</td>
<td>78.5%</td>
</tr>
<tr>
<td>TSR</td>
<td>~19.89 mg</td>
<td>15.71 mg</td>
<td>14.68 mg</td>
<td>&gt;95%</td>
<td>73.8%</td>
</tr>
</tbody>
</table>

*Table 1: Summarized purification table for the various isthmin peptides.*

**Endotoxin contents**

All proteins were isolated with low endotoxin contents below the detection limit of the LAL assay. All had < 0.047 EU/ml, well below the safe limit for cell and pharmacological use of 0.5EU/ml (FDA, 1987).

**Protein cytotoxicity**

All proteins were not found to be cytotoxic up to 1 mM concentrations.

**EC cytotoxicity**

*Table 1: Recombinant Isthmin and Isthmin fragments produced do not exhibit detectable cytotoxicity in Human Umbelical Vein Endothelial Cells up to 1uM concentrations*
Concentration dependant tube formation assay

Both isthmin and its C-terminal truncate was able to inhibit endothelial tube formation on Matrigel though full-length isthmin was much more potent. The N-terminal and TSR domain had no effect by themselves.

Tube formation time course assay

Isthmin is only able to disrupt tube formation if applied early to the endothelial cells. After 4 hours, isthmin has minimal effect on tube formation.

Figure 1: 1 μM of isthmin applied to endothelial cell cultures on Matrigel at various time points. Full length isthmin significantly disrupts endothelial cell tube formation if applied at 0 hour and 2 hours. Inhibition at 4 hours is minimal.

Endothelial cell proliferation assay

Isthmin inhibits VEGF-stimulated endothelial cell proliferation in a dose dependant manner and reduces proliferation levels to baseline (no VEGF).

Figure 2: Various concentrations of full-length isthmin applied to VEGF induced proliferating endothelial cells. Cell proliferation decreases to the baseline, non-VEGF induced control level in a dose-dependant manner.

Discussion

Angiogenesis is critical in tumor progression and metastasis. Without angiogenesis, tumors are unable to grow beyond 1-2mm ((Folkman, 1972) and are less
likely to metastasize as blood vessels form a primary path of escape of tumor cells that can form metastatic colonies. Hence, anti-angiogenic molecules can be important anti-cancer drugs.

Isthmin and the synthesized peptide fragments expressed as inclusion bodies can be refolded and purified with a high purity and yield. The refolded polypeptides are then shown to be biologically active. The proteins were refolded as their His tags bind to Ni-NTA beads which function as molecular chaperones by isolating protein molecules and minimizing aggregation of unfolded protein or folding intermediates. In addition, a redox couple of oxidized and reduced glutathione allows disulphide bond shuffling to occur, where disulphide bonds continually form, break, and reform as the polypeptide chain refolds to its lowest energy state thus preventing misformed disulphide bonds from stabilizing folding intermediates, and allowing only correct disulphide bonds form in the refolded protein.

Inclusion body washing with Tx-114 was shown to be effective in removing endotoxins from bacterial cell wall. Furthermore, proteins exhibited low cytotoxicity. Therefore, cell assay results are unlikely to be artifacts of cell cytotoxicity of endotoxin mediated cell reactions.

Isthmin was found to inhibit endothelial cell proliferation and tube formation, two critical steps in angiogenesis, the formation of new blood vessels from pre-existing ones. Isthmin inhibits endothelial cell proliferation in the early stages by preventing cell attachment to the basement membrane (Matrigel). The AMOP domain on the C-terminal is associated with cell adhesion in other proteins. Here, it possibly prevents endothelial cell adhesion and hence tube formation.

References

