Production of Heat Shock Proteins by Expression Cloning and Subsequent Cytokine Induction in Dendritic Cells

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ABSTRACT

Three heat shock protein genes were cloned, namely groEL from E. coli M15[pREP4] strain, and hsp70 and hsp60 from B. pseudomallei KHW strain. Gene expression was subsequently induced, and the Hsps were isolated and purified. B. pseudomallei Hsp70 and Hsp60 are novel Hsps which have not been characterized and reported in literature to-date. It was then verified that the recombinant HSPs were able to induce TNF-α production in DCs in vitro. Future work would include more functional assays to examine the immunomodulating activity of Hsps, which would bring us closer to the eventual use of Hsps as adjuvants in the production of vaccines against various diseases.

INTRODUCTION

Heat shock proteins (Hsps) are able to elicit both humoral and cellular immunity against associated peptides, stimulate cytokine production, and bring about DC maturation quickly. These immunological properties make Hsps powerful adjuvants. B. pseudomallei is the causative agent of melioidosis, and to date there is neither effective treatment nor vaccine for the disease. With the production of B. pseudomallei Hsps, it is hoped that this project would contribute to the ongoing investigation of Hsps as potential adjuvants against diseases like melioidosis.

MATERIALS AND METHODS

Cloning of Hsp genes

Full length E. coli groEL (1648 bp), B. pseudomallei hsp70 (1958 bp) and hsp60 (1641 bp) were amplified by polymerase chain reaction. Amplified genes were then ligated into TA vector pGEM-T Easy (Promega). Vector inserts were then transformed into competent E. coli JM109 cells. Plasmids of successfully transformed bacteria were then isolated for DNA sequencing. The plasmids were then cut with with restriction enzymes Hind III and XmaI for groEL and Hind III and Kpn I for hsp60 and hsp70. The expression vector pQE-30 (QIAGEN) were also treated with appropriate restriction enzymes to produce compatible sticky ends for ligation. Ligation and transformation into competent E. coli M15[pREP4] cells were then performed. Correct insert orientations were then verified by further restriction digests.

Protein induction and purification

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Successfully transformed *E. coli* M15[pREP4] were grown in 1 L LB cultures, and Hsp production was with Isopropyl-beta-D-thiogalactopyranoside (IPTG). After 3 hrs of induction, the cells were harvested by centrifugation. Harvested cells were lysed and sonicated, and cell debris was removed by further centrifugation. Hsps contained within the supernatant were purified by affinity purification with Ni-NTA resins (CLONTECH Labs Inc.). Affinity purification was made possible with the 6 x Histidine tag at the N-terminal of proteins expressed through pQE-30. The column was washed with sodium deoxycholate to remove lipopolysaccharides (LPS) before eluting the Hsps.

**Stimulation of cytokine production in vitro**

**Table 1. Hsps used in for in vitro stimulation of JAWSII**

<table>
<thead>
<tr>
<th>Well number</th>
<th>Stimulation with</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No stimulation</td>
</tr>
<tr>
<td>2</td>
<td>200 µg/ml Hsp65 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>3</td>
<td>1 mg/ml Hsp65 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>4</td>
<td>200 µg/ml Hsp60</td>
</tr>
<tr>
<td>5</td>
<td>200 µg/ml Hsp60 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>6</td>
<td>1 mg/ml Hsp60</td>
</tr>
<tr>
<td>7</td>
<td>1 mg/ml Hsp60 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>8</td>
<td>200 µg/ml Hsp70</td>
</tr>
<tr>
<td>9</td>
<td>200 µg/ml Hsp70 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>10</td>
<td>1 mg/ml Hsp70</td>
</tr>
<tr>
<td>11</td>
<td>1 mg/ml Hsp70 + 20 µg/ml polymyxin B</td>
</tr>
<tr>
<td>12</td>
<td>1 mg/ml lactoferrin + 20 µg/ml polymyxin B</td>
</tr>
</tbody>
</table>

The cell-line used for in vitro stimulation was JAWSII, an immortalized bone marrow-derived immature DC line from C57BL/6 mouse (MacKay and Moore, 1997). JAWSII cells were plated at a density of 5 x 10⁴ cells in twelve separate wells on a 96-well plate. *B. pseudomallei* Hsp60, Hsp70, *M. bovis* Hsp65, lactoferrin and polymyxin B were added to respective wells to final concentrations as indicated in table 1. GM-CSF was also added to a final concentration of 20 ng/ml for each well. The total volume for each well was 200µl. The cell samples were then left in a 37°C incubator for 8 hrs to allow TNF-α production to be stimulated. TNF-α levels in the supernatant were then measured by enzyme-linked immunosorbent assay (ELISA) with a TNF-α ELISA kit (BD PharMingen) according to the manufacturer’s instructions.

**RESULTS**

PCR products that were subjected to gel electrophoresis were viewed under UV light. This showed correct sized DNA bands of 1.6 kb for *groEL* and 2.0 kb for *hsp70* (Data for *hsp60* not shown). Amplified Hsp genes were eventually cloned into the expression vector pQE-30. DNA sequencing revealed that there were missense mutations in *groEL* and *hsp70*. Work with *groEL* was discontinued due to time constraints.
Affinity purification of Hsps

During affinity purification, sodium deoxycholate was added in the wash buffer to solubilize and remove lipopolysaccharides (LPS). LPS removal was essential as they are known to have stimulatory effects on immune cells, and this may mask similar effects that were actually elicited by Hsps. The Hsps were eluted with elution buffer after washing in seven fractions of 1.5 ml each. 2 µl from each of the 1.5 ml seven fractions were then analysed by SDS-PAGE (Fig 2 and 3). Hsp70 had an approximate size of 70 kDa and Hsp60 had a size of approximately 66 kDa.

![Fig 3. SDS-PAGE for Hsp60](image)

![Fig 4. In vitro induction of TNF-α production by Hsps](image)

TNF-α induced by Hsps in vitro

Once purified recombinant Hsps were obtained, the ability of the Hsps to stimulate cytokine production in vitro was investigated. Earlier experiments in the lab showed that Hsp65 was able to stimulate TNF-α production in JAWSII, an immature dendritic cell-line, therefore it acts as a positive control. The sample incubated with medium only (no stimulation) serves as a negative control. The sample incubated with lactoferrin was a specificity control to show that other transporter proteins did not induce TNF-α production. 20 µl polymyxin B were added to duplicates of the samples incubated with Hsps. The addition of polymyxin B inhibits the action of any residual LPS that was not removed by sodium deoxycholate during affinity purification, hence further validating that TNF-α production is not due to effect of LPS.

From the data (fig 4), it can be seen that all of the samples incubated with Hsps induced high levels of TNF-α. In fact, JAWSII incubation with Hsp60 and Hsp70 exhibited greater levels of TNF-α than the Hsp65 positive control. Furthermore, when the concentration of Hsp used was increased from 200 µg/ml to 1 mg/ml, TNF-α production also increased. However, the response to Hsp70 peaked at 200 µg/ml. Another important point to note is that many of the samples had concentrations of TNF-α greater than the 2000 pg/ml standard maximum. Hence, the result of the functional assay is not accurately quantitative. The experiment has to be repeated with at least three times dilution to the supernatant before the results could be ascertained. Another observation was that TNF-α levels decreased when polymyxin B was added to the samples. This suggests that residual LPS might have been incubated with the samples and contributed to cytokine induction. In fact, many researchers had raised concern over the similarities between Hsp and LPS induced responses. However, the addition of polymyxin B should be sufficient to
prove that the responses were due to Hsp and not LPS. One way to avoid the issue of LPS contamination altogether is perhaps to avoid the use of bacteria for recombinant protein expression. A good alternative would be yeast cells or insect cells.

DISCUSSION

This project had achieved two main objectives. First was the cloning of Hsp genes into an expression vector and the subsequent production of recombinant Hsps. The cloning and production of \textit{B. pseudomallei} Hsps is novel because there has been no published report on the proteins from this bacterium before. Second was the verification that the recombinant Hsps were able to bring about cytokine production in DCs \textit{in vitro}. However, one major setback was that most of the cloned genes contained mutations.

All of the cloned genes sequenced so far were found to harbour mutations. Mutations in groEL and hsp70 were missense whereas mutations for hsp60 were silent, resulting in no change in amino acid composition. Taking into account that the Hsp gene templates are GC rich, the mutations were most likely introduced during PCR amplification. However, it is interesting to note that although hsp70 had two missense mutations, it possesses the ability to induce TNF-\(\alpha\) production in JAWSII cells. For future work, it is necessary to compare the immunogenicity of the mutated and non-mutated Hsp70, and verify if the mutations had actually enhanced or decreased its immunogenicity. In conclusion, it was found that recombinant \textit{B. pseudomallei} Hsp60 and Hsp70 could both stimulate the production of TNF-\(\alpha\) production from DCs, similar to \textit{M. bovis} Hsp65.

Even though Hsp70 did not show significant sequence homology to the rest of the Hsps (Hsp70 and Hsp60 are classified into different families), they are known to exhibit many common immunological effects, including the ability to induce TNF-\(\alpha\) as demonstrated in this project. This suggests that although the Hsps do not share significant amino sequence homology, it is still possible that the Hsp60 and Hsp70 families bind to a common or similar pattern recognition receptor on DCs, and work through a common signalling pathway. Another hypothesis is that they bind to different receptors that mediate similar downstream signals.

Future studies could be directed to exploring the immunological properties of the recombinant Hsps. For example, the ability of the recombinant Hsps to cross-prime antigens and hence activate T cell responses should be investigated. Likewise, activation of the humoral response against peptides carried by Hsps could also be tested. Antibodies produced against recombinant \textit{B. pseudomallei} Hsps should also be considered for two main reasons. First, Hsps are known to play critical roles especially when cells are subjected to stress. Hence antibodies against Hsps may decrease the survivability and hence virulence of \textit{B. pseudomallei}. Another reason is that it was recently found that Hsps itself could act as virulence factors. (Dobbin \textit{et al}, 2002). In this case, antibodies against Hsps pertain to removing bacterial virulence altogether.

REFERENCES
