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Cloning, expression and purification of three Chaperonin 60 homologues

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Abstract

The Chaperonin 60 (Cpn60) proteins have, in addition to their well-known functions of protein folding and protection, a range of intercellular signalling activities. As part of a study to investigate the biological activity of the Cpn60 proteins, particularly from pathogenic organisms, we have cloned and expressed three Cpn60 proteins from *Homo sapiens*, *Helicobacter pylori* and *Chlamydia pneumoniae*. The Cpn60 proteins were purified to apparent homogeneity using a combination of nickel column affinity chromatography and Reactive Red dye affinity columns. Insoluble protein was solubilised using 8 *M* urea and then re-folded on the nickel column by stepwise removal of the urea. The immunostimulant LPS was removed by addition of the antibiotic polymyxin B as part of the purification process.
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1. Introduction

The molecular chaperones are proteins which, assist other proteins to fold correctly and reach their native state [1]. They are divided into families on the basis of sequence homology and each protein family is highly conserved across species. Of all the molecular chaperones much work has focussed on the chaperonin (Cpn60) family. The Cpn60 proteins are ubiquitous proteins; they are essential for cell viability [2], facilitate the correct folding of a subset of proteins under normal conditions [3] and prevent aggregation of proteins following cellular stress [4]. The *E. coli* Cpn60 homologue, GroEL has been the most widely studied to date and its structure and mechanism of action with its co-chaperone Cpn10 largely elucidated [5–11]. However, the Cpn60s have roles beyond those of protein folding and protection, recently, evidence has emerged about their other perhaps surprising properties.

Cpn60s have other varied roles. Rather than being solely intracellular proteins, as was initially thought, a number of bacteria have Cpn60 proteins present on the outer surface [12–15]. It was postulated that the Cpn60s could mediate bacterial invasion of the host cells, leading to the suggestion that the Cpn60s may be virulence factors [16,17]. Furthermore, the human homologue of Cpn60 has been detected on the surface of a variety of cells particularly leukocytes and vascular endothelial cells [18,19]. *Mycobacterium tuberculosis* Cpn60.2 (mtCpn60.2), a potent immunogen, was the first Cpn60 protein demon-

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strated to have cell-signalling properties. Following stimulation with *M. tuberculosis* Cpn60.2, monocytes secreted pro-inflammatory cytokines [20], other studies then confirmed this result [21]. Later Cpn60 homologues from a wide range of bacteria were shown to increase the levels of pro-inflammatory cytokines in a number of monocytic cells and human cell lines [22–28]. Mycobacterial, chlamydial, human and *E. coli* Cpn60 homologues stimulated the expression of adhesion molecules; ICAM-1, VCAM-1 and E-selectin on cultured human vascular endothelial cells (HUVECs) in a cytokine-independent manner [26,29,30]. These findings indicate a role for the Cpn60s in leukocyte recruitment, since monocytes and endothelial modulate the inflammatory process.

The Cpn60s have also been implicated in disease states, particularly autoimmune diseases and are thought to be pathogenic factors in atherosclerosis. One hypothesis states that antibodies to prokaryotic Cpn60 present in the circulation following a bacterial infection, cross-react with human Cpn60 expressed on stressed endothelial cells leading to the cells being destroyed and initiating the atherogenic process [31,32]. Other hypotheses link chronic infection of pathogenic bacteria such as *M. tuberculosis, C. pneumoniae* and *H. pylori* with the atherogenic process. The release of the Cpn60 proteins by these bacteria may accelerate the inflammatory process, aiding in leukocyte trafficking, thus playing a role in atheroma formation.

Although the Cpn60s share a high degree of homology [33], they have differences in their respective sequences which could indicate different biological activity. There is evidence that Cpn60s from different organisms have different effects and mechanisms of action [24,28,34].

Our aim is to compare the potency and efficacy of Cpn60s from different organisms in various biological assays and determine if they are pathogenic factors in coronary heart disease. To this end as part of a wider study involving six Cpn60 homologues, we have purified three Cpn60 proteins from *H. pylori, C. pneumoniae* and *Homo sapiens*. Criticisms of the early work, which identified the Cpn60s as signalling molecules ascribed the results to either contaminating proteins or the presence of lipopolysaccharide (LPS) a major component of the Gram-negative bacterial cells wall and an extremely potent immunostimulant. We have developed a purification strategy, which, not only allows purification of protein from inclusion bodies and subsequent solubilisation, but also rids the Cpn60 protein of contaminating polypeptides and results in negligible levels of LPS contamination.

2. Experimental

2.1. Chemicals, solutions and buffers

Imidazole, glycine, polymyxin B, lysozyme, urea, 2-mercaptoethanol, Reactive Red dye affinity resin, ATP, Luria broth, isopropyl-β-D-thiogalactopyranoside (IPTG), arabinose, ampicillin, KCl, $MgCl_2$, Tris, NaCl, sodium acetate, methanol, Tween20, phosphate buffered saline (PBS), protease inhibitor cocktail (suitable for use with His-tagged proteins) (Sigma–Aldrich, Poole, Dorset, UK). HCl, acetic acid, hypersolv water (BDH, Poole, Dorset, UK). *Limulus* amebocyte lysate (LAL) chromogenic endpoint assay kit (Associates of Cape Cod, Liverpool, Merseyside, UK). Sample reducing buffer (Pierce, Tattenhall, Cheshire, UK).

All buffers and solutions were prepared using hypersolv water, HPLC grade from BDH, where appropriate the pH of each solution was adjusted with either HCl or NaOH. The solutions were freshly prepared before purification.

B1: 0.1 *M* $NiSO_4$
B2: 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 10 m*M* imidazole
B3: 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 10 m*M* imidazole, 2.5 mg/ml polymyxin B
B4: 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 250 m*M* imidazole
B5:20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 1 m*M* 2-mercaptoethanol
B6: 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 1 m*M* 2-mercaptoethanol, 2.5 mg/ml polymyxin B
B7: 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 1 m*M* 2-mercaptoethanol, 250 m*M* imidazole
B8: 0.1 *M* Tris–HCl pH 8.0, 0.5 *M* NaCl

B9: 0.1 *M* sodium acetate pH 4.5, 0.5 *M* NaCl.
B10: 20 m*M* Tris–HCl pH 7.5, 1 m*M* 2-mercaptoethanol, 2 m*M* $MgCl_2$, 2 m*M* KCl
B11: 20 m*M* Tris–HCl pH 7.5, 1 m*M* 2-mercaptoethanol, 2 m*M* $MgCl_2$
B12: 20 m*M* Tris–HCl pH 7.5, 1 m*M* 2-mercaptoethanol, 2 m*M* $MgCl_2$, 2.5 mg/ml polymyxin B
B13: 20 m*M* Tris–HCl pH 7.5, 1 m*M* 2-mercaptoethanol, 2 m*M* $MgCl_2$, 1 *M* KCl
B14: 20 m*M* Tris–HCl pH 7.5
B15: 39 m*M* glycine, 48 m*M* Tris base, 20% methanol
B16: PBS, 0.05% Tween20, 10% dried skimmed milk
B17: PBS, 0.05% Tween20

2.2. Cloning and expression of the genes for recombinant proteins

Genomic DNA was purified from *H. pylori* (a kind gift from J. Halton, University College London) using the Promega genomic DNA purification kit and the *cpn60* gene amplified by PCR using the primers shown in Table 1 using the Qiagen Hotstar PCR kit. The cycling protocol was 95 °C for 5 min (1 cycle), then 30 cycles of: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, followed by one cycle of 72 °C for 15 min. The gene was inserted into the PCR4-TOPO vector (Invitrogen) and then excised and inserted into the multiple cloning site of the pBADA vector following digestion with *Xho*I and *Eco*RI (Invitrogen) for subsequent expression. The expression vector containing *H. pylori cpn60* was named pMM16. The recombinant *H. pylori* Cpn60 protein contains 585 amino acids, has a calculated molecular mass of $62.673 \cdot 10^3$ and a theoretical p*I* of 5.49.

The cloning of *C. pneumoniae cpn60* gene was accomplished in two stages using nested PCR. The genomic DNA was a kind gift from Yuri Boroskin (St. Georges Hospital Medical School). A 1840 bp fragment of the *Cpn60* operon (including the *cpn60* gene) was PCR amplified using primers CPHISFE and CPHISRB (Table 1) using Ready-to-Go PCR beads (Amersham Biosciences, Little Chalfont, Buckinghamshire) with the following PCR protocol, 95 °C for 5 min (1 cycle), then 30 cycles of: 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1 min, followed by a 15-min extension at 72 °C. The gene was inserted into PCR4-TOPO vector, which was then used as a template in a subsequent PCR reaction to amplify the 1634 bp *cpn60* gene using primers CPHISFZ and CPHISRZ. The Amersham Biosciences Ready-to-Go PCR beads and a cycling protocol of 95 °C for 1 min (1 cycle), then 30 cycles of: 95 °C for 0.5 min, 45 °C for 0.5 min, 72 °C for 1 min and a final extension of 15 min at 72 °C was used to amplify this gene. The *C. pneumoniae cpn60* gene was inserted into PCR4-TOPO and then excised from the PCR4-TOPO vector and inserted into the pBADA vector following digestion with *Xho*I and *Eco*RI, creating the plasmid pMM18. The recombinant *C. pneumoniae* Cpn60 protein is made up of 582 amino acids, has a calculated molecular mass of $62.559 \cdot 10^3$ and a theoretical p*I* of 5.42.

The human Cpn60 gene cloned into pQE60 (plasmid PQE60HH) was a kind gift from Mark Lillicrap. The recombinant protein contains 514 amino acids and has a calculated molecular mass of $56.09 \cdot 10^3$, it has a theoretical p*I* of 5.75. The plasmids pMM16 and pMM18 were transformed into the *E. coli* TOP10 strain and pQE60HH transformed into *E.coli* strain XL-2 blue for protein expression.

Table 1
The primers used in the PCR reaction to amplify *cpn60* genes from *H. pylori* and *C. pneumoniae*

Primer name	Primer sequence	Restriction site
HPBDF	5′ CTC GAG ATG GCA AAA GAA ATC	*Xho*I
HPBDR	5′ GAA TTC TTA CAT CAT GCC GCC CAT GCC	*Eco*RI
CPHISFE	5′ CCT TTC GAA GTT CAA GTT GGC G	None
CPHISRB	5′CTA GTA GTC CAT TCC TGC GCT TGG	None
CPHISFZ	5′ CTC GAG GCA GCG AAA AAT ATT AAA TAT AAT	*Xho*I
CPHISRZ	5′ GAA TTC CTA GTA GTC CAT TCC TGC GCT TGG	*Eco*RI

The restriction enzyme sites within the primers are highlighted.

2.2.1. Protein expression

The *cpn60* genes in pMM16 and pMM18 were under the control of the *BAD* promoter and therefore expression of the recombinant protein was induced by adding arabinose. The human *cpn60* was under the control of the *lac* promoter and therefore the protein was induced by adding IPTG. The cultures were grown overnight in an incubator (with agitation) at 37 °C in Luria Broth (LB) medium, supplemented with 100 μg/ml ampicillin (final concentration). The cultures were diluted into 400 ml of LB medium to an optical density at 600 nm (OD_{600}) of 0.1 and grown until an OD_{600} of 0.4 was reached then either 0.002%, 0.02% arabinose or 1 m*M* of IPTG (final concentration) was added to cultures containing plasmids pMM16, pMM18 or pQE60HH respectively. The cells were incubated for a further four h and then harvested by centrifugation at 4 °C at 6084*g* in a Sorval RCB5 plus centrifuge for 20 min. The supernatant was discarded and the cell pellets stored at −70 °C.

2.3. Extraction of soluble protein (H. pylori Cpn60)

The cell paste was resuspended in 10 ml buffer B2 supplemented with 10 mg/ml lysozyme, 100 μl protease inhibitor cocktail and incubated at 4 °C on a rotary mixer for 30 min. The cells were disrupted by sonication on ice using a IKA Laborteknik sonicator (amplitude 100%, cycle 1) using 3×30 s sonication with a 30-s rest on ice between each sonication. The lysate was centrifuged for 30 min at 4 °C at 11 235 *g* in a Sorvall RCB 5plus centrifuge and the supernatant applied to a nickel-charged HiTrap chelating column (Amersham Biosciences).

2.3.1. Extraction and solubilisation of C. pneumoniae and Human Cpn60

For the insoluble proteins, (*C. pneumoniae* and Human Cpn60), the cell paste was resuspended in 10 ml buffer B5 supplemented with 8 *M* urea and 100 μl protease inhibitor cocktail. The insoluble proteins were therefore rendered soluble but denatured by the addition of 8 *M* urea. Lysozyme (10 mg/ml) was added and the cells were subsequently treated as detailed in Section 2.3.

2.4. Affinity chromatography

2.4.1. Preparation of the HiTrap chelating column

The 1-ml column was prepared by washing (using a syringe) with 5 ml hypersolv water, then loading 0.5 ml of 0.1 *M* $NiSO_4$ solution on to the column. The column was then washed with 5 ml hypersolv water. The column was equilibrated by adding binding buffer, (either B2 in the case of soluble protein, *H. pylori* Cpn60, or B5 supplemented with 8 *M* urea for the insoluble *C. pneumoniae* and human Cpn60).

2.4.2. Purification of H. pylori Cpn60

The purification was performed at room temperature (RT), the buffers were left on ice prior to use. The supernatant (following centrifugation of the cell lysate, described in Section 2.3) was applied to the nickel-charged HiTrap chelating column and the column was washed twice with 5 column volumes (CVs) of buffer B2. Five CVs of buffer B3 were run through the column, followed by two washes of five CVs of buffer B2. The protein was eluted from the column with 2.5 CVs of buffer B4. The protein was then applied to an Amersham Biosciences PD-10 column (described below) to remove the imidazole and the eluate supplemented with 2 m*M* ATP, 5 m*M* $MgCl_2$, 5 m*M* KCl and 1 m*M* 2-mercaptoethanol in a final volume of 10 ml and left overnight at 4 °C.

2.4.3. Purification of C. pneumoniae and Human Cpn60

The purification was performed at RT, the buffers were left on ice prior to use. The same methodology was used for both proteins, which were originally in the insoluble cellular fraction, but which were made soluble by the addition of 8 *M* urea as described in Section 2.3.1. The supernatant (from centrifugation of the cellular lysate, described in Section 2.3) was applied to the nickel-charged HiTrap chelating column and the column washed with five CVs of buffer B5 supplemented with 8 *M* urea. The denatured protein was allowed to re-fold on the column by passing a urea gradient down the column from 8 *M* urea to 0 at 2-*M* intervals. The column was washed twice with five CVs of buffer B5 and once with five CVs of buffer B6 followed by another two washes with five CVs of buffer B5. The soluble and re-

folded protein was eluted with 2.5 CVs of buffer B7. The protein was then applied to an Amersham Biosciences PD-10 column (described below) to remove the imidazole and the eluate supplemented with 2 m*M* ATP, 5 m*M* $MgCl_2$, 5 m*M* KCl and 1 m*M* 2-mercaptoethanol in a final volume of 10 ml and left overnight at 4 °C.

2.4.4. Reactive Red column

The purification was performed at RT using ice-cold buffers. One millilitre of Reactive Red slurry was applied to a 1-ml Qiagen (Crawley, West Sussex, UK) polypropylene column. The column was prepared by washing with 10 CVs of buffer B8, then 10 CVs of buffer B9 and finally 10 CVs of buffer B10. The protein was applied to the column and left for 30 min. An ATP gradient was then applied to the column from 2 m*M* ATP to 0 at 0.5 m*M* intervals in buffer B10. The column was washed with four CVs of buffer B11, four CVs of buffer B12 and two washes of four CVs B11. The Cpn60 protein was eluted in 2.5 ml of buffer B13 and then applied to an Amersham Biosciences PD-10 column.

2.4.5. Gel filtration

The PD-10 column was prepared by washing with 25 ml of buffer B14. The protein was applied to the column and then eluted with 3.5 ml of buffer B14.

2.5. Analytical techniques

2.5.1. Electrophoresis

Samples of the protein taken during each stage of the purification process were analysed by SDS–PAGE gel electrophoresis. The Bio-Rad Protean 3 system (Bio-Rad, Hemel Hamptstead, Hertfordshire) was used and the samples were run on a 10% Tris–HCl precast gel (Bio-Rad) according to the manufacturers instructions. The protein samples were diluted 1:5 in reducing sample buffer (Pierce) and boiled for 10 min prior to loading on the gel. A pre-stained broad-range protein marker (Bio-Rad) was also loaded on the gel. Electrophoresis was performed at 100 V for 45 min. The gel was fixed in a solution of 15% acetic acid, 40% methanol for 1 h and stained using Brilliant blue colloidal concentrate according to the manufacturers instructions (Sigma–Aldrich).

2.5.2. Western blot analysis

The proteins were electroblotted on to a Hybond ECL nitrocellulose membrane (Amersham Biosciences) in buffer B15 at 350 mA for 60 min. The membrane was then incubated in buffer B16 overnight at 4 °C supplemented with a 1:3000 dilution of mouse anti-polyHistidine IgG2. The membrane was washed at RT three times, for 15 min each wash with buffer B17, then incubated for 60 min at RT in buffer B16 supplemented with 1:5000 dilution of goat anti-mouse IgG horse radish peroxidase (HRP) conjugated antibody. The membrane was washed as before with buffer B17, the proteins were detected using the Amersham Biosciences ECL western blotting reagent kit.

2.5.3. Quantitation

The concentration of the purified protein was determined by using an Ultraspec 2000 spectrophotometer (Amersham Biosciences) and measuring the absorbance at 280 nm. The concentration (mg/ml) was determined using the extinction co-efficient at A_{280} and calculated molecular mass. The Bio-Rad protein assay kit II (based on the Bradford assay) was used to determine the concentration of the protein in the crude extracts.

2.5.4. Measurement of the levels of LPS

The levels of LPS contamination was determined using the LAL assay (Associates of Cape Cod) according to the manufacturers instructions. All standards and protein samples were prepared in pyrogen-free water in pyrogen-free tubes using pyrogen-free pipette tips. A concentration of 0.05 ng/ml (or 0.5 EU/ml) LPS was used as top standard and six further two-fold serial dilution were prepared to give a standard curve. The purified recombinant proteins (or crude extracts) were diluted to 200 μg/ml and six further 10-fold serial dilutions were prepared. Ten microlitres of each standard or protein was mixed with 10 μl of *Limulus* lysate and incubated at 37 °C for 60 min. Two microlitres of dye (0.2% methylene blue in 70% ethanol) was added to each reaction and the level of LPS determined by dye exclusion.

3. Results and discussion

3.1. Extraction and solubilisation of recombinant proteins

The *H. pylori* Cpn60 is soluble, therefore in order to extract this protein, the cells had to be lysed and disrupted. Different methods for disrupting the cells to release the proteins were tested both individually and in combination: B-Per solution (Pierce), lysozyme, soniciation and Bug-buster (Invitrogen). The combination of using lysozyme and sonication to disrupt the cells was found to be necessary in order to extract the proteins. This method was also applied to *C. pneumoniae* and Human Cpn60 following their solubilisation.

In the case of the proteins found in the insoluble cellular fraction (*C. pneumoniae* and Human Cpn60), supplementing buffer B5 with 8 *M* urea enabled the concomitant resuspension of the cellular pellet and solubilisation of the protein in one step. Urea was chosen as it is a powerful denaturing agent and relatively easy to remove during the purification process by the application of a urea gradient whilst the proteins were immobilised on the nickel column.

3.2. Nickel affinity chromatography

The use of the poly-Histidine tag for initial capture of the protein was an integral part of the purification strategy. Other metals such as cobalt were tried but we found that nickel had the highest affinity of binding for the Cpn60s purified in this work. Immobilisation of the proteins had two advantages in this purification procedure. Firstly, it allowed the denatured proteins to re-fold on the column, by passing a urea gradient down the column. Secondly, we were able to pass the LPS-binding antibiotic, polymyxin B down the column to remove contaminating LPS. An imidazole titration was used to determine the optimum concentration for elution of the Cpn60 protein and most Cpn60 protein eluted at a concentration of 250 m*M*. Figs. 1–3 show the level of contaminating proteins still present following capture and purification with this column. Tables 2–4 shows the calculated yields and levels of LPS contamination.

The eluate from the nickel column was passed down an Amersham Biosciences PD-10 column to remove any contaminating imidazole and had the advantage of also exchanging the buffer B4 to 20 m*M* Tris–HCl, thus allowing the addition of 2 m*M* ATP, 5 m*M* KCL, 5 m*M* $MgCl_2$ and 1 m*M* 2-mercaptoethanol without the need for time-consuming dialysis stages.

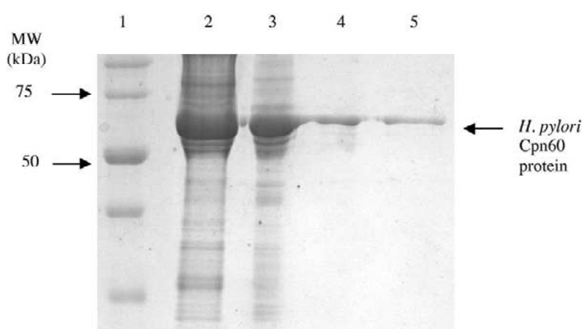

Fig. 1. Lane 1 Bio-Rad broad range marker, lane 2 elution from the nickel affinity column (250 m*M* Imidazole fraction), lane 3 elution from PD-10 column, lane 4 elution from Reactive Red column (1 *M* KCl fraction), lane 5 purified *H. pylori* Cpn60 protein eluted from a final PD-10 column.

3.3. Reactive red dye affinity column

The Reactive Red dye affinity column was used as a powerful clean-up stage to remove the remaining contaminating proteins. Due to the nature of Cpn60

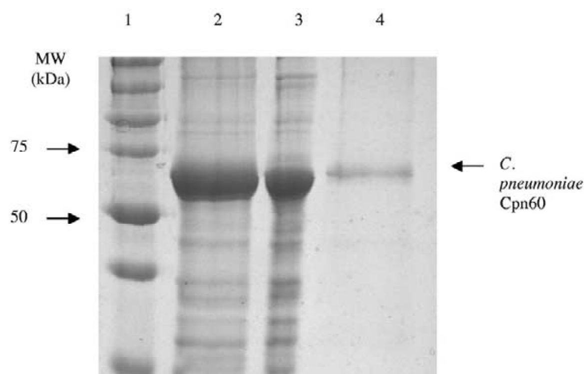

Fig. 2. Lane 1 Bio-Rad broad range marker, lane 2 elution from the nickel affinity column (250 m*M* Imidazole fraction), lane 3 elution from PD-10 column, lane 4 purified *C. pneumoniae* Cpn60 after Reactive Red column and PD-10 column.

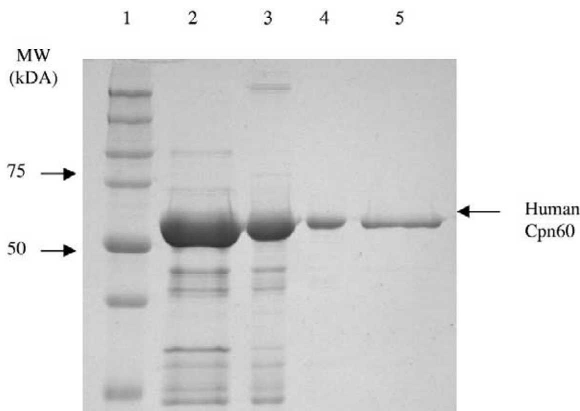

Fig. 3. Lane 1 Bio-Rad broad range marker, lane 2 elution from the nickel affinity column (250 m*M* Imidazole fraction), lane 3 elution from PD-10 column, lane 4 elution from Reactive Red column (1 *M* KCl fraction), lane 5 purified human Cpn60 protein eluted from a final PD-10 column.

protein and its ability to bind proteins, not only the contaminating proteins in solution, but also any peptides or proteins, which are bound to the Cpn60 have to be removed. Supplementing buffer B10 with 2 m*M* ATP, not only primes the release of bound proteins from Cpn60 but also facilitates Cpn60 binding to the Reactive Red column. Again the immobilisation of the Cpn60 to the Reactive Red column also allowed the further use of polymyxin B to remove more contaminating LPS resulting in extremely low levels of contamination at the end of the purification process (Tables 2–4). The Cpn60 proteins were eluted from the column with 1 *M* KCl. This was the optimal concentration for elution of the protein, following a titration experiment. The final, purified protein was passed through a PD-10 column and this removed the contaminating KCl resulting in pure, relatively LPS-free protein (Figs. 1–3).

3.4. SDS–PAGE and western blot analysis of the cpn60 proteins

Western blot analysis (Fig. 4) confirmed the presence of the His-tagged proteins following purification.

4. Conclusion

We report here, the purification of three Cpn60 proteins in order to compare their biological activities as intercellular signalling molecules. For this purpose, the Cpn60s not only have to be soluble, free

Table 2
Purification of the recombinant *H. pylori* Cpn60 protein

Sample	Calculated total protein (%)	Purity of protein (%)	LPS level (EU/ml)
Cell pellet	100	<1	>0.5
250 m*M* imidazole fraction from nickel column passed through a PD-10 column	25	40	0.25
1 *M* KCl fraction from Reactive red column passed through a PD-10 column	0.3	>95	0.015

Table 3
Purification of recombinant *C. pneumoniae* Cpn60 protein

Sample	Calculated total protein (%)	Purity of protein (%)	LPS level (EU/ml)
Cell pellet	100	<1	>0.5
250 m*M* imidazole fraction from nickel column passed through a PD-10 column	30	45	0.125
1 *M* KCl fraction fraction from Reactive Red affinity column passed through a PD-10 column	0.2	>95	0.03

Table 4
Purification of human Cpn60

Sample	Calculated total protein (%)	Purity of protein	LPS level (EU/ml)
Cell pellet	100	<1	>0.5
250 m*M* imidazole fraction from nickel column passed through a PD-10 column	15	55	0.125
1 *M* KCl fraction from Reactive Red affinity column passed through a PD-10 column	0.3	>95	0.03

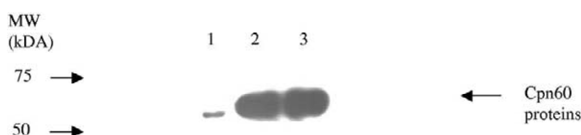

Fig. 4. Lane 1 human Cpn60, lane 2 *H. pylori* Cpn60, lane 3 *C. pneumoniae* Cpn60.

from contaminating proteins, but also free from the immunstimulant LPS. Two of the Cpn60s (those from *C. pneumoniae* and *Homo sapiens*) were insoluble, therefore, a key part of the purification strategy was to solubilise the proteins using 8 *M* urea. Designing the recombinant proteins to have a poly-Histidine tag enabled immobilisation on to an Ni-NTA column, which facilitated on-column refolding of the *C. pneumoniae* and Human Cpn60s. The use of the Reactive Red dye affinity column enabled the removal of contaminating proteins. The addition of Polymyxin B to both columns lowered the LPS to extremely low levels. The procedure detailed above is a fast, reproducible method for the purification of pure, soluble Cpn60 protein.

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