Biochemical isolation and identification of DnaK and GroEL from urease-positive thermophilic campylobacters

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Urease-positive thermophilic campylobacter (UPTC), a microaerophilic and Gram-negative bacterium, has only relatively recently been isolated from the natural environments and identified in England.[1] Following the original description of UPTC, isolates were reported in France,[2,3] Northern Ireland,[4,5] The Netherlands[6] and Japan.[7,8]

Heat shock proteins (HSPs) are produced by cells in response to a variety of physical and chemical stresses.[9] DnaK (HSP70) and GroEL (HSP60) are the most highly conserved proteins known, and are two major bacterial HSPs.[10-14] Reports on HSPs of thermophilic *Campylobacter jejuni* have been published;[15-18] however, there have been no reports of the analysis of HSPs from thermophilic *C. lari*, including UPTC. Therefore, the present study aims to isolate biochemically and identify DnaK and GroEL from a Japanese UPTC strain.

Cells from the Japanese strain (CF89-12)[7] were first precultured on blood agar base No. 2 (Oxoid, Hampshire, UK) that contained defibrinated horse blood (Nippon Bio-Test Tokyo, Japan), supplemented with Butzler campylobacter-selective medium (Nissui, Tokyo, Japan), under microaerophilic conditions at 37˚C for 48 h.

In order to isolate DnaK, cells were further cultured on Mueller-Hinton agar (30 Petri dishes) under the same microaerophilic conditions, washed (x2) in phosphate buffered saline (PBS) and four volumes were homogenised. A crude cell extract was prepared by centrifugation (20,000 x**g** at 4˚C for 15 min) and then ultracentrifugation (100,000 x**g** at 4˚C for 90 min). The resultant supernatant fluid was fractionated using 50% and then 70% ammonium sulphate. The resulting precipitate was dissolved into four volumes of PBS and the buffer was then changed to ADP buffer (20 mmol/L Tris-acetate, 20 mmol/L NaCl, 15 mmol/L mercaptoethanol, 3 mmol/L $MgCl_2$, 0.1 mmol/L EDTA [pH 7.5]) using a PD-10 column (Sephadex G25; Amersham Pharmacia Biotech, Tokyo, Japan). This was followed by ADP agarose affinity column chromatography using stepwise elution from 0.1 to 3.0 mmol/L ADP.[19] Coomassie protein assay reagent (Pierce Chemical Company, Ill, USA) was employed to determine protein concentration.

For the fractionation of the protein components, sodium dodecyl sulphate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) was used, and the protein bands were visualised by Coomassie brilliant blue straining. Protein *N*-terminal sequencing was performed on a Beckman LF3000 (Beckman Coulter, CA, USA) following SDS-PAGE and subsequent membrane blotting of components.

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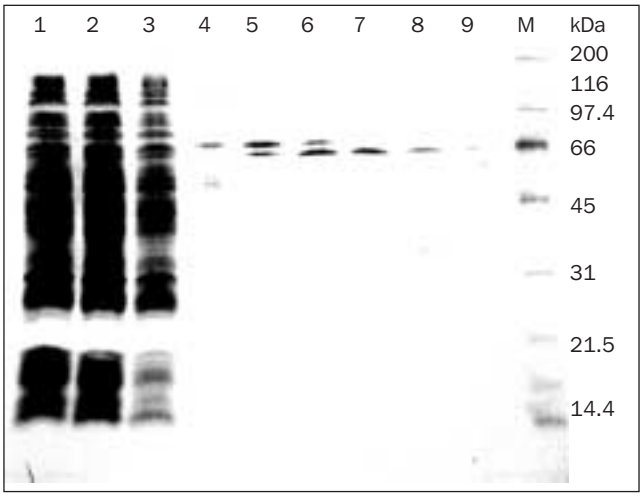

Fig. 1. SDS-PAGE of the fractions eluted on ADP affinity column chromatography. Lane 1: flow-through fraction from the ADP column; lane 2: the first wash of the column; lane 3: the second wash from the column; lane 4: the eluate from the column with 1.0 mmol/L ADP; lane 5: 0.5 mmol/L; lane 6: 1.0 mmol/L; lane 7: 2.0 mmol/L; lane 8: the first eluate from the column with 3.0 mmol/L ADP; lane 9: the second eluate from the column with 3.0 mmol/L ADP; lane M: protein molecular weight standard markers.

As shown in Figure 1, two protein components were found at the 70 kDa and 64 kDa regions by SDS-PAGE of the fractions eluted by 0.5 mmol/L (lane 5) and 1.0 mmol/L ADP (lane 6) using ADP affinity column chromatography. In addition, a component of approximately 64 kDa was detected in the 2.0 and 3.0 mmol/L ADP eluates (lanes 7 to 9).

In order to identify the two protein components of UPTC eluted from the ADP affinity column, an amino (*N*-) terminal sequencing procedure was employed. In Figure 2, N-terminal sequences of approximately 70 kDa (A) and approximately 64 kDa (B) protein components of UPTC are shown, as are the sequences of the corresponding regions from other organisms such as *C. jejuni*, *Escherichia coli* and *Salmonella typhimurium*.

Figure 2A shows that the 11 *N*-terminal amino acid sequence of the 70 kDa UPTC protein was identical (100% sequence homology) to the sequence of the corresponding region of DnaK from *C. jejuni*, except for the two terminal amino acid residues of the DnaK protein (M and S) of *C. jejuni*. This suggested that the 70 KDa UPTC protein was DnaK or a DnaK-like protein.

Similarly, the amino acid sequence of the 19 *N*-terminal residues of the 64 kDa UPTC protein identified it as UPTC GroEL or a GroEL-like protein. Because the sequences of 16 of the 19 *N*- terminal amino acids were identical, 84% amino acid sequence homology between UPTC and *C. jejuni* was demonstrated. Thus, the results strongly suggested that the 64 kDa UPTC protein was GroEL or a GroEL-like protein.

In the present study, one methionine residue present in the amino acid sequences of DnaK and GroEL at the *N*-terminal end could not be detected in the corresponding UPTC regions. This may have been due to the fact that the cited sequences were deduced from the determined DnaK and GroEL DNA sequences. Here, we analysed the amino

A)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
UPTC 70 kDa		*	K	V	I	G	I	D	L	G	T	T	N		
Campylobacter jejuni DnaK	M	S	K	V	I	G	I	D	L	G	T	T	N	S	C
Salmonella typhimurium DnaK	M	G	K	I	I	G	I	D	L	G	T	T	N	S	C
Escherichia coli DnaK	M	G	K	I	I	G	I	D	L	G	T	T	N	S	C
Human HSP70.1	M	A	K	A	A	A	V	G	I	D	L	G	T	T	Y

B)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
UPTC 64 kDa		A	-	K	E	I	F	F	S	D	E	A	Q	N	K	L	S	E	G	V
Campylobacter jejuni GroEL	M	A	-	K	E	I	I	F	S	D	E	A	R	N	K	L	Y	E	G	V
Escherichia coli GroEL	M	A	A	K	D	V	K	F	G	N	D	A	R	V	K	M	L	R	G	V
Human HSP60	M	L	R	L	P	T	V	F	R	Q	M	R	P	V	S	R	V	L	A	P

** no amino acid residue analysed.*

Fig. 2. Amino (*N*-) terminal amino acid sequences of 70 kDa (A) and 64 kDa (B) protein components of the Japanese UPTC strain (CF89-12). *N*-terminal amino acid sequences of DnaK and GroEL from the other organisms are shown for comparison. The sequences cited for DnaK from *C. jejuni* (accession number Y17165), *E. coli* (D10765), *S. typhimurium* (U58360) and a human source (M11717) and for GroEL from *C. jejuni* (Y13334), *E. coli* (AE000487) and a human source (M34664) were from EMBL/GenBank. Amino acids are designated by a single-letter code.

acid sequences of the two protein components but not the base sequences of DnaK and GroEL, and the methionine residue at the *N*-terminal end of the two protein components may have been processed after translation.

Furthermore, the *N*-terminal amino acid sequence of the *C. jejuni* 64 kDa protein, which is stress-inducible and a homologue of *E. coli* GroEL, lacks a methionine at the *N*-terminal end of the sequence.[15] In the present study, one amino acid residue in the DnaK sequences shown in Figure 2A (at the second N-terminal end of the 70 kDa UPTC protein component) could not be analysed.

Peng and colleagues first reported the substitution of ATP affinity chromatography by ADP affinity chromatography for the isolation of HSP70 molecules from cells and mouse liver tissue.[19] Here, we used ADP affinity column chromatography after ammonium sulphate fractionation and the results strongly suggest that it can be very useful for simultaneously obtaining both DnaK and GroEL preparations from UPTC strains. □

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