Acta Biochimica Polonica

Vol. 50 No. 2/2003

455–459

QUARTERLY

Communication

Overproduction and purification of the CcpA protein from *Lactococcus lactis*★✪

Magdalena Kowalczyk and Jacek Bardowski✉

Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics PAS, Warszawa, Poland

Received: 12 May, 2003; accepted: 21 May, 2003

Key words: CcpA, His-tag, cross links, dimers

In this work we present cloning and overexpression of lactococcal CcpA protein in *Escherichia coli* Xl1blue strain as a fusion with 6×His tag. A high yield of the CcpA protein was obtained when the cells were cultured in liquid medium LB with 100 μg/ml ampicillin at 37°C and subsequently for 4 h after induction by IPTG. The procedure let us obtain 5 mg of homogenous CcpA protein. Glutaraldehyde crosslinking analysis indicated the formation of dimer or tetramer forms of the CcpA protein.

CcpA, a catabolite control protein A, belongs to the LacI/GalR family of regulators and acts as a global transcriptional regulator. It is a well conserved protein, responsible for catabolic repression in Gram-positive bacteria (Guédon *et al.*, 2002). CcpA activity is regulated by the HPr protein phosphorylated in position Ser-46 (Deutscher *et al.*, 1994). Such a phosphorylation is stimulated by fructose-1,6-bisphosphate and occurs in the presence of easily metabolizable carbon sources. CcpA protein has a HTH (helix-turn-helix) motif responsible for its binding to DNA. CcpA binds to conserved 14 nucleotide palindromic sequence *cre* (catabolite responsive element). The consensus sequence of *cre*, TGNNAN-

★Presented at the 4th Summer School on Lactic Acid Bacteria, May 2003, Spala near Tomaszow Mazowiecki, Poland.

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) grant No. 6 P06G 055 20.

✉Corresponding author: Jacek Bardowski, Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics PAS, A. Pawińskiego 5a, 02-106 Warszawa, Poland; phone: (48 22) 659 7072 ext. 1223; fax: (48 22) 658 4636; e-mail: jacek@ibb.waw.pl

Abbreviations: Amp^r, resistance to ampicillin; CcpA, catabolite control protein A; *cre*, catabolite responsive element; DTT, dithiothreitol; IPTG, isopropylthio-β-D-galactoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

CGNTNNCA results from research on catabolic repression in *Bacillus subtilis* (Weickert & Chambliss, 1990). Depending on the position of the *cre* sequence, CcpA can act as an activator or repressor (Titgemeyer & Hillen, 2002). Interaction with the *cre* sequence located within the promoter hampers the initiation of transcription and its location within the coding region leads to the block of transcription. Binding of CcpA upstream to the promoter region is characteristic of transcriptional activation and probably leads to stabilization of the RNA polymerase–promoter complex. The CcpA protein in *Lactococcus lactis* subsp. *lactis* IL1403 is encoded by the *ccpA* gene (GenBank accession number AF106673) containing 999 bp (Aleksandrzak *et al.,* 2000). The homology of this protein to the CcpA from *Bacillus subtilis* is 48%, while to that from *Lactococcus lactis* subsp *cremoris* MG1363 reaches 96% (GenBank accession number Z97202). The molecular mass of the CcpA protein calculated from the amino-acid sequence is approximately 37 kDa.

Up to the present, most studies on CcpA have involved the physiological and DNA/RNA analysis of the $ccpA^-$ mutants and $ccpA^+$ strains. Studies on purified CcpA protein were done in *Bacillus subtilis* (Miwa *et al.*, 1994), *Bacillus megaterium* (Deutscher *et al.* 1995; Gosseringer *et al.*, 1997; Tebbe *et al.*, 2000), *Lactobacillus delbrueckii* subsp. *lactis* (Schick *et al.*, 1999) and *Lactobacillus casei* (Mahr *et al.*, 2002).

The aim of this work was to overproduce and purify the CcpA protein from *Lactococcus lactis*. Purified CcpA protein is necessary for studies of the interactions of CcpA with potential *cre* sequences in selected regions of *Lactococcus lactis* IL1403 genome.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Lactococcus lactis* IL1403 strain was used as a donor of the *ccpA* gene. PCR products were first cloned in *Escherichia coli* TG1 strain (Gibson, 1984). In the cloning procedure the following vectors were used: the pGEM-T Easy plasmid (Amp^r) (Promega U.S.A.) which improves cloning of PCR products in *E. coli* and the pQE30 plasmid (Amp^r) (Qiagen). The pQE30 vector allows the sequence coding for 6×His to be placed at the 5′-end of a *ccpA* gene resulting in CcpA protein extended in frame at its N-terminus by 6 His residues.

Recombinant DNA methods. General recombinant DNA methods were as described (Sambrook *et al.*, 1989). The *ccpA* gene from *Lactococcus lactis* IL1403 was amplified by the PCR method, with TaKaRa Ex Taq DNA polymerase (TaKaRa) and specific primers containing *Bam*HI or *Kpn*I restriction sites that enable efficient cloning of the *ccpA* gene in the pQE30 vector. The following primers, CcpA*Bam*HIfor 5′- CGG GAT CCA TGG TAG AAT CAA CAA CA - 3′ and CcpA*Kpn*Irev 5′- CGG GGT ACC GCG ACT TTT CTC TCA AAT GTC - 3′, were used in order to join in frame the N-terminus of the *ccpA* gene with the 6-His affinity tag. The PCR reaction was carried out for 30 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min). The PCR product and pGEM-T Easy were ligated and used for electroporation of *E. coli* TG1 cells. Transformants growing as white colonies on the LB solid medium with X-gal, IPTG and 100 μg/ml ampicillin were tested for the presence of the recombinant plasmid. Digestion of their plasmid DNA with *Kpn*I and *Bam*HI restriction enzymes generated the PCR insert, which was subsequently sequenced (Sanger *et al.*, 1977) to eliminate the possibility of mutation. The *Kpn*I/*Bam*HI fragment was subsequently cloned into the pQE30 vector in the *E. coli* Xl1blue strain. Transformants were selected as above and then analyzed by colony PCR method for the presence of the recombinant plasmid. The plasmid DNA was isolated from the correct clone and used as a template DNA for sequencing of the *ccpA* gene and the promoter region from pQE30 expression vector, using primers pQE30for 5′- CCC GAA AAG

TGC CAC CTG -3′ and CcpA*Kpn*Irev 5′- CGG GGT ACC GCG ACT TTT CTC TCA AAT GTC -3′.

Overproduction of the CcpA protein. The *Escherichia coli* Xl1blue strain containing pQE30 vector with *ccpA* was cultured in liquid medium LB with 100 μg/ml ampicillin at 37°C with agitation. At A_{660} about 0.6 the IPTG was added to the final concentration of 1 mM and incubation was continued for 4 h. The cells were harvested and kept frozen at –20°C.

Preliminary control of CcpA production. Cell pellets from 2 ml portions of culture with or without IPTG induction were resuspended in 400 μl of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8) and incubated with lysozyme for 30 min on ice. The efficiency of CcpA production was checked by SDS/PAGE (Sambrook *et al.*, 1989).

Purification of the CcpA protein. The CcpA protein was purified using a modification of the standard protocol (The QIAexpressionist 03/2001). The cell pellet from 100 ml of IPTG induced culture of the *E. coli* Xl1blue strain containing the pQE30 vector with *ccpA* was resuspended in 3 ml of the lysis buffer in the presence of lysozyme. The cell extract was obtained by disruption of cells with glass beads (3 one-minute pulse in MBB-8 apparatus, Biospec U.S.A.). Protein purification was performed by the Ni-NTA (nickel-nitrilotriacetic acid) metal-affinity chromatography and the Qiagen purification kit. Proteins were separated on 1 ml batches of 50% Ni-NTA resins. The washing solution (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8) was used for elimination of proteins nonspecifically bound to the matrix and the CcpA protein was eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8. The protein concentration was determined spectrophotometrically by the method of Bradford (1976), with Bio-Rad reagents and bovine serum albumin as a standard.

Glutaraldehyde crosslinking. 10 μl portions of crosslinking buffer (0.5 M *N,N*-bis (2-hydroxyethylo)glycine-NaOH, pH 8.5, 400 mM NaCl, 100 mM DTT) were mixed with 2 μg of purified protein and glutaraldehyde (final concentration 0.01–0.1%) and incubated for 20 min at room temperature. The reactions were stopped by adding 1.25 μl of 1.4 M ethanolamine, pH 8, and analyzed by SDS/PAGE.

Protein electrophoresis. Protein electrophoresis was performed in 12% polyacrylamide (acrylamide and bis-acrylamide 37.5:1) with 0.1% SDS in appropriate buffer (Sambrook *et al.*, 1989). Gels were stained with Bio-Safe Coomassie solution from Bio-Rad. Before loading onto the gel all samples were mixed with 3×Laemmli loading buffer, boiled for 5 min. and centrifuged. The Premix Protein Molecular Weight marker from Boehringer (Fig. 1) and the Wide Range SigmaMarker from Sigma (Figs. 2 and 3) were used.

RESULTS AND DISCUSSION

The CcpA protein was overproduced in *E. coli* by the method described in Materials and Methods. The *E. coli* Xl1blue strain with pQE30 vector, producing no CcpA, was used as a negative control.

In Xl1 blue cells carrying the pQE30 vector alone (Fig. 1, lanes 1 and 2) no protein band at 37 kDa (corresponding to the calculated CcpA mass) that would be inducible by IPTG was observed. This demonstrated that in the absence of the *ccpA* gene no protein synthesized in *E. coli* can mimic the CcpA protein. In the culture lysate of induced Xl1 blue with pQE30 vector fused with *ccpA* (Fig. 1, lanes 3 and 5), a much stronger protein band appears at 37 kDa, than in the noninduced strain (Fig. 1, lanes 4 and 6). This result shows that under the conditions applied, the Xl1 blue strain with the pQE30 *ccpA* fusion is able to overproduce the CcpA protein.

The CcpA protein had been purified by Ni-NTA affinity chromatography. Since studies of protein : DNA interaction require a biologically active protein and CcpA is soluble

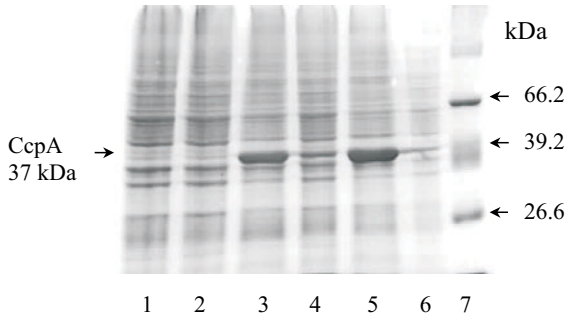

Figure 1. Overproduction of CcpA protein in *E. coli* Xl1 blue.

Samples were analyzed by 12% SDS/PAGE and visualized by Coomassie staining. Lane 1, negative control, Xl1 blue with pQE30 vector (induced culture); lane 2, negative control, Xl1 blue with pQE30 vector (noninduced culture); lanes 3 and 5, Xl1 blue with pQE30 vector fused with *ccpA* (induced culture); lanes 4 and 6, Xl1 blue with pQE30 vector fused with *ccpA* (noninduced culture); lane 7, molecular mass standard (Boehringer).

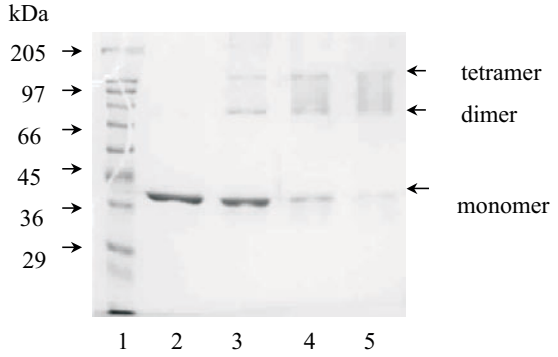

Figure 3. Oligomeric forms of CcpA protein (glutaraldehyde crosslinking).

The crosslinking was performed at increasing concentrations of glutaraldehyde. Lane 1, molecular mass standard (Sigma); lane 2, CcpA protein without glutaraldehyde; lanes 3–5, CcpA protein at 0.01%, 0.05% and 0.1% glutaraldehyde, respectively.

(not shown), the purification was performed under native conditions in the presence of imidazole.

The purity and molecular mass of eluted fractions tested by SDS/PAGE confirmed the presence of a protein of 37 kDa which corresponds to CcpA (Fig. 2). The extra protein bands migrating at 74 kDa and 148 kDa could be either contaminants or correspond to oligomeric forms of CcpA. The ability of CcpA to form multimers was checked by glutaraldehyde crosslinking (Fig. 3). These results confirmed that CcpA can form dimers and tetramers and that the CcpA protein was purified to homogeneity.

The expression system employed and the described procedure allowed us to obtain 5 mg of pure CcpA protein, stable at –20°C in 10% glycerol.

The CcpA protein from *Lactococcus lactis* IL1403 was overexpressed and efficiently purified to homogeneity. The quality as well as quantity of the purified protein is sufficient for *in vitro* analysis of protein : DNA interac-

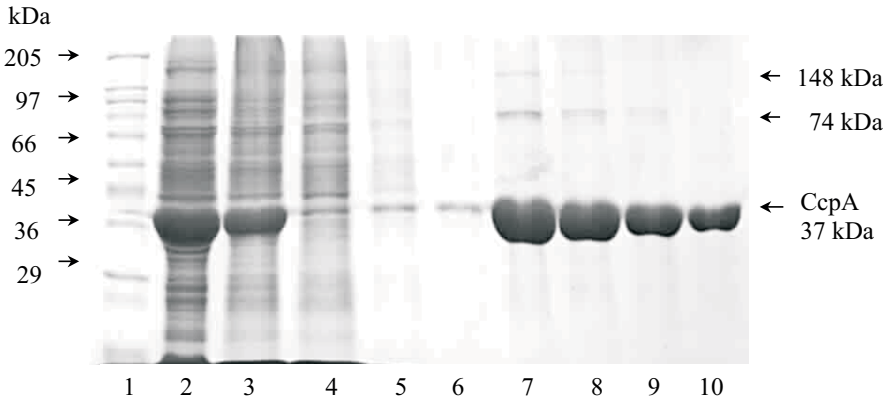

Figure 2. Purification of CcpA protein under native conditions.

Expression of 6×His-CcpA protein was performed at 37°C, at inducing conditions. Analysis as in Fig. l. Lane 1, molecular mass standard (Sigma); lane 2, pellet of cells Xl1 blue with pQE30 vector fused with *ccpA* (induced culture); lane 3, cell extract from Xl1 blue with pQE30 vector fused with *ccpA* (induced culture); lane 4, proteins not bound to Ni-NTA agarose (flow-through); lanes 5 and 6, wash; lanes 7–10, proteins eluted with 250 mM imidazole.

tions. According to our knowledge this is the first report describing the overexpression in *Escherichia coli* and efficient purification of lactococcal CcpA protein.

REFERENCES

Aleksandrzak T, Kowalczyk M, Kok J, Bardowski J. (2000) Regulation of carbon catabolism in *Lactococcus lactis*. In *Food Biotechnology*; Bielecki S, Tramper J, Polak J. eds, **17**: 61–6. Elsevier Science B.V.

Bradford MM. (1976) *Anal Biochem.*; **72**: 248–54.

Deutscher J, Reizer J, Fischer C, Galinier A, Saier MH, Jr, Steinmetz M. (1994) *J Bacteriol.*; **176**: 3336–44.

Deutscher J, Kuster E, Bergstedt U, Charrier V, Hillen W. (1995) *Mol Microbiol.*; **15**:1049–53.

Gibson TJ. (1984) *Studies on the Epstein-Barr virus genome.* Ph.D. Thesis.; Cambridge University Press.

Gosseringer R, Kuster E, Galinier A, Deutscher J, Hillen W. (1997) *J Mol Biol.*; **7**: **266**: 665–76.

Guédon E, Jamet E, Renault P. (2002) *Antonie Van Leeuwenhoek.*; **82**: 93–112.

Mahr K, Esteban CD, Hillen W, Titgemeyer F, Perez-Martinez G. (2002) *J Mol Microbiol Biotechnol.*, **4**: 489–94.

Miwa Y, Saikawa M, Fujita Y. (1994) *Microbiology.*; **140**: 2567–75.

Sambrook J, Fritsch EF, Maniatis T. (1989) *Molecular cloning a laboratory manual.* Cold Spring Harbor Laboratory Press.

Sanger F, Nicklen S, Coulson AR. (1977) *Proc Natl Acad Sci U S A.*; **74**: 5463–7.

Schick J, Weber B, Klein JR, Henrich B. (1999) *Microbiology.*; **145**: 3147–54.

Tebbe J, Orth P, Kuster-Schock EK, Hillen W, Saenger W, Hinrichs W. (2000) *Acta Crystallogr D Biol Crystallogr.*; **56**: 67–9.

Titgemeyer F, Hillen W. (2002) *Antonie Van Leeuwenhoek.*; **82**: 59–71.

Weickert MJ, Chambliss GJ. (1990) *Proc Natl Acad Sci U S A.*; **87**: 6238–42.