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HETEROLOGOUS EXPRESSION AND PURIFICATION OF RECOMBINANT CROTOXIN B, THE PHOSPHOLIPASE A2 SUBUNIT OF CROTOXIN

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ABSTRACT. Crotoxin is a heterodimeric β-neurotoxin isolated from the venom of *Crotalus durissus terrificus*, consisting of two, non-covalently bound subunits, crotapotin (CA) and crotoxin B (CB). Both subunits present four different isoforms, consequently there are up to 16 different crotoxin complexes, each with different activity levels. The biological activities usually associated with crotoxin include neurotoxicity, myotoxicity and cardiotoxicity, however several other important biochemical and pharmacological effects of crotoxin have been observed, such as the antibacterial, antiviral, anti-inflammatory, antitumoral and analgesic activity. In this study we present the production of recombinant crotoxin B (isoform C). Expression of the protein was carried out using *E. coli Rosetta^{TM} (DE3)pLysS* strain, and the obtained protein was separated and purified using Ni^{2+}-affinity chromatography. To the best of our knowledge the developed method represents the first reported method for obtaining the crotoxin B (isoform C) through heterologous expression using an efficient and cost-effective procedure.

Keywords: *Crotoxin, neurotoxin, phospholipase A2, heterologous expression*

INTRODUCTION

Crotoxin (CTX) is a highly potent β-neurotoxin representing the main toxic component of *Crotalus durissus terrificus* (South American rattlesnake) venom. Crotoxin was the first protein isolated and crystallized from animal venom [1,2].

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Crotoxin is a heterodimeric protein that consists of a non-toxic, non-enzymatic, acidic subunit (crotapotin, crotoxin A, CA) non-covalently associated with a weakly toxic, basic subunit (crotoxin B, CB) that expresses phospholipase A_2 activity. The CA subunit acts as a chaperone protein, potentiating the toxicity of the CB subunit [1,3]. Four different isoforms have been identified for each subunit, consequently the venom of *Crotalus durissus terrificus* contains up to 16 different crotoxin complexes, each presenting different activity levels [4,5].

The main biological activities associated with CTX include neurotoxicity, myotoxicity and cardiotoxicity. Along with these well-known activities, several other biochemical and pharmacological effects have been reported, some of them highlighting the potential pharmaceutical use of this protein. These effects include the antibacterial, antiviral, anti-inflammatory, antitumoral, and analgesic activities of CTX and its subunits (CA and CB) [1,2].

The neurotoxic effect of CTX is exerted mainly at the presynaptic level of the neuromuscular junctions, and is caused by inhibition of acetylcholine release [6]. Furthermore, a postsynaptic effect exerted on acetylcholine receptors which are stabilised in a desensitized form by CTX has also been observed [1,7]. The myotoxicity of CTX can be characterized as a systematic and generally irreversible damage caused to skeletal muscles. The systemic myotoxicity caused by CTX is accompanied by an increase in plasma creatine-kinase and myoglobinuria, which often leads to acute renal failure [8–10]. Studies regarding the cardiotoxic effect of CTX showed that it induces a significant decrease in the contractile force of the heart, and causes an increase in creatine-kinase activity [11]. Later studies demonstrated that CTX potentiates L-type Ca^{2+} currents in cardiomyocytes, and also alters significantly the ultrastructure of the cardiac autonomic nervous system [12,13].

The antibacterial effect of CTX and its CB subunit has been demonstrated against several bacterial strains, such as *Burkholderia pseudomallei, Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Xanthomonas axonopodis pv. passiflorae* [14–16]. The chemical modification of the CB subunit did not influence its antibacterial activity on *E. coli*, suggesting that this effect is not linked with the enzymatic activity of the protein, as other molecular mechanisms might be involved in the bacterial membrane disruption [17]. In a different study, CTX and the CB subunit showed antiviral activity against dengue and yellow fever viruses, possibly by disrupting the virus lipid bilayer envelope [18,19].

Another study has shown that the CTX is able to influence the immune and inflammatory responses by inhibiting macrophage spreading and phagocytic activities [20], by reducing the number of lymphocytes in blood and lymph and promoting leukocyte adherence to endothelial cells,

possibly through the involvement of lipoxygenase-derived mediators [21]. Similar effects have been observed with the CB subunit, but not with the CA subunit [22].

The antitumoral activity of CTX has been demonstrated in both *in vitro* and *in vivo* studies. CTX was found to be effective *in vitro* against mammary ductal carcinoma, glioblastoma and lung adenocarcinoma cells [23], but also showed promising results when used *in vivo* against different forms of advanced cancer [24,25]. A more recent study suggests that autophagy and apoptotic mechanisms might be involved in the cytotoxic effects of crotoxin [26].

Several studies have been conducted to demonstrate the analgesic effect of CTX. The results of these studies showed that CTX induces analgesia by a direct action, presumably by activating the central muscarinic receptors, along with central serotonergic and noradrenergic receptors. Eicosanoids derived from the lipoxygenase pathway have also been observed to mediate the analgesic effect of CTX, which indicates that the PLA_2 activity of the protein is also important in the exertion of this effect [27,28].

Considering the various pharmacological effects and possible therapeutic applications of CTX and its CB subunit, our aim was the development of a simple and cost-effective method for obtaining the pure CB subunit. The first step consisted of the heterologous expression of the recombinant CB (isoform C), followed by the separation and purification of the obtained protein.

RESULTS AND DISCUSSION

Assembly of the CB_pET-30b(+) vector

The expression vector was obtained by inserting the coding sequence of CB (isoform C) into the pET-30b(+) vector using the NotI and HindIII restriction sites resulting in the restriction map of the designed vector (Figure 1).

The CB_pET-30b(+) vector was designed and assembled to be compatible with the chosen prokaryotic host cells (*E. coli Rosetta™ (DE3)pLysS*) and the methods used for protein separation. The selection of host cells was based on experiments in which these cells proved to be adequate for synthesis of proteins of vegetal [29], animal [30] or human origin [31]. The planned separation method for the recombinant protein was based on Ni^{2+}-affinity, thus a 6xHis-tag was added to the coding sequence of CB (isoform C).

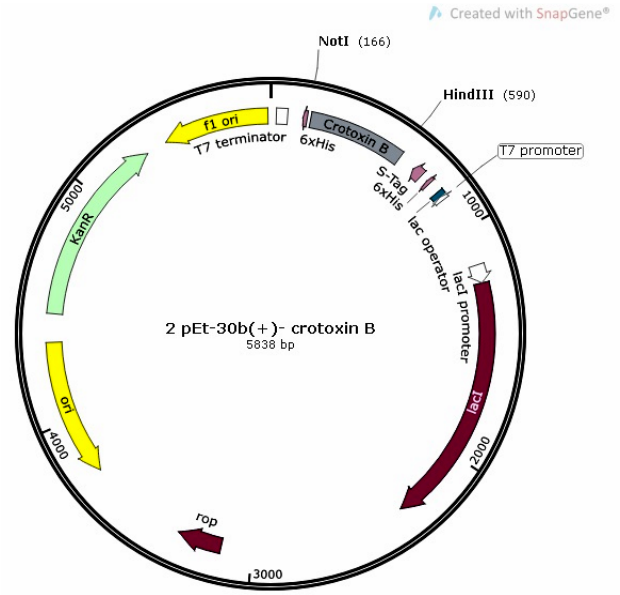

Figure 1. Restriction map of CB_pET- 30b(+) expression plasmid, outlining the significant elements of the vector (Map created in SnapGene® 1.1.3.)

Transformation of competent cells and colony PCR

The CB_pET-30b(+) vector was transformed into chemically competent *Rosetta™ (DE3)pLysS* cells, plated on Luria-Bertani (LB) agar plates containing 50 µg/ml kanamycin and incubated overnight at 37°C. After colony formation, the presence of the plasmid and plasmid insert in the selected bacterial colony was confirmed by colony PCR (Figure 2).

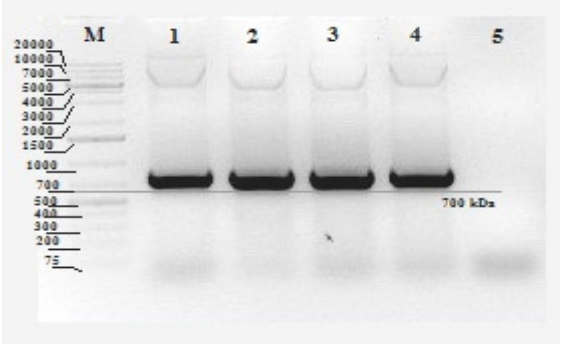

Figure 2. Agarose gel showing the DNA fragments obtained after colony PCR of samples taken from four individual bacterial colony. (M) – 1 kb Plus DNA Ladder (Thermo Scientific); (1-4) – PCR product of transformed *E. coli Rosetta*™ bacterial colonies; (5) – Reaction mixture without added sample (negative control)

Method optimisation for protein expression

The effect of incubation temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration on protein expression has been determined using transformed bacterial strains inoculated in LB broth containing kanamycin at a concentration of 50 μg/ml. The protein expression has been observed at 30 °C, respectively at 37 °C. For induction we have used 0.01 mM, 0.5 mM or 1.0 mM IPTG.

Biomass levels have been determined during incubation, by measuring the optical density at 600 nm (OD_{600}). The bacterial growth curves for each condition used are presented in Figure 3.

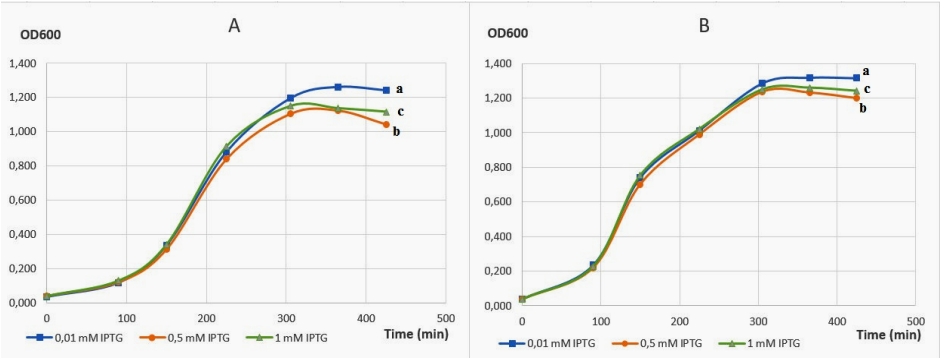

Figure 3. Bacterial growth curves obtained during method optimisation. (A) - Samples incubated at 30 °C, (B) – Samples incubated at 37 °C. (a) – 0.01 mM IPTG, (b) – 0.5 mM IPTG, (c) – 1.0 mM IPTG used for induction.

Protein expression has been assessed by SDS-PAGE analysis of samples taken hourly after induction, for each experimental condition (Figure 4 and Figure 5). The presence of CB (isoform C, 15.9 kDa) was confirmed by visible bands at approximately 16 kDa. Based on the visual examination of these bands, it was concluded that neither the temperature, nor the IPTG concentration have a significant influence on the yield of the protein expression. However, the distortion of the bands corresponding to CB (isoform C) at 3 hours post-induction, suggested that an additional conformational change might take place in the structure of the protein after a prolonged induction.

Based on the obtained results, it was concluded that an optimal biomass level can be obtained at 37°C when the protein expression was induced with 0.5 mM IPTG, and induction time was limited to two hours to prevent possible modifications in the protein structure. Using these parameters, an optimised expression process was conducted, and the presence of the protein confirmed through SDS-PAGE analysis (Figure 6). The obtained cell culture was subsequently used for the optimization of the purification method.

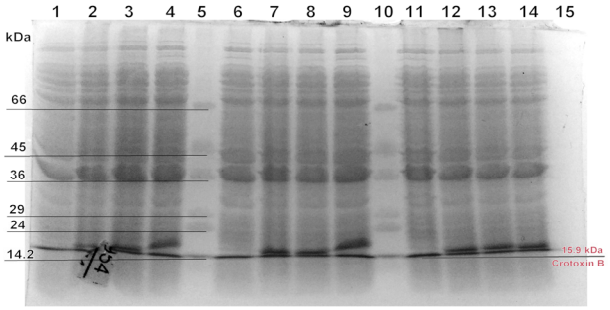

Figure 4. SDS-PAGE gel analysis of samples taken from cell cultures incubated at 30 °C, before and after induction with different concentrations of IPTG. (1) – Before induction, (2-4) – 60, 120, 180 min after induction with 0.01 mM IPTG; (6) – Before induction, (7-9) – 60, 120, 180 min after induction with 0.5 mM IPTG; (11) – Before induction, (12-14) – 60, 120, 180 min after induction with 1.0 mM IPTG; (5),(10) – Molecular Weight Marker 14-66 kDa (Sigma-Aldrich).

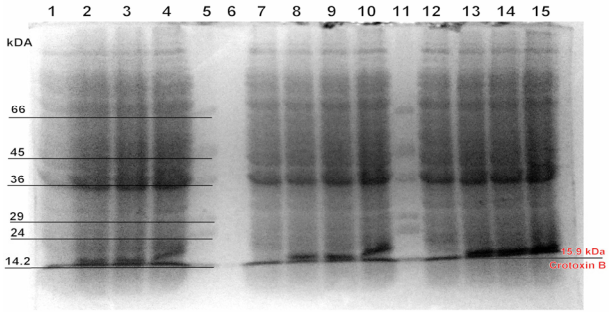

Figure 5. SDS-PAGE gel analysis of samples taken from cell cultures incubated at 37 °C, before and after induction with different concentrations of IPTG. (1) – Before induction, (2-4) – 60, 120, 180 min after induction with 0.01 mM IPTG; (7) – Before induction, (8-10) – 60, 120, 180 min after induction with 0.5 mM IPTG; (12) – Before induction, (13-15) – 60, 120, 180 min after induction with 1.0 mM IPTG; (5), (10) – Molecular Weight Marker 14-66 kDa (Sigma-Aldrich).

Batch purification of recombinant Crotoxin B

To obtain a purified CB (isoform C) solution a single-step separation approach was used, using Ni-affinity chromatography. Separation of the protein was accomplished using two different buffer systems (tris(hydroxymethyl)aminomethane (Tris) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)). Theoretical isoelectric point (pI) of the protein was computed using *Expasy ProtParam* and determined to be 8.74. The purification was carried out at pH=7.6 to allow at least one pH unit difference between the pI of CB and the pH of the solutions. After the Ni-NTA chromatographic step, the protein purity has been verified by SDS-PAGE analysis (Figure 6 and Figure 7).

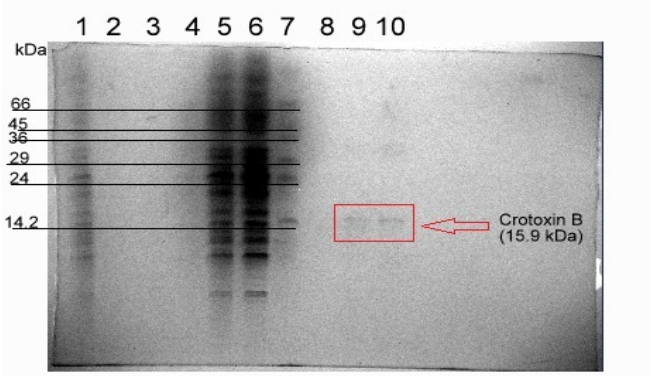

Figure 6. SDS-PAGE gel analysis of samples taken from cell cultures incubated using the optimal conditions (37°C, 0.5 mM IPTG concentration) and the affinity chromatography purification of the recombinant protein using Tris-HCl buffer (pH=7.6). (1) – 120 min after induction; (2) – 60 min after induction; (3) – before induction; (4) - Molecular Weight Marker 14-66 kDa (Sigma-Aldrich), (5) – elution fraction 1, (6) – elution fraction 2, (7) – elution fraction 3.

Using Tris-HCl the desired protein was not selectively separated on Ni-NTA resin column (it was bonded on the stationary phase along with other proteins). On the other hand, changing the Tris-HCl with HEPES buffer a better selectivity for the separation of the protein was achieved. However, we have noticed that the CB (isoform C) was eluted in two separated fractions (2 and 3) increasing the total volume and decreasing the concentration of the final solution.

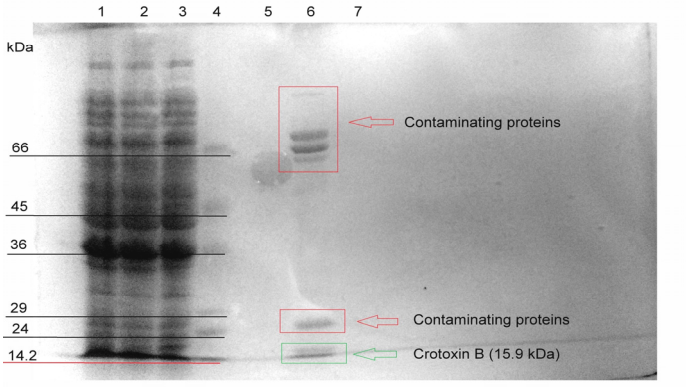

Figure 7. Tricine-SDS-PAGE gel analysis of affinity chromatography purification of the recombinant protein using HEPES buffer (pH=7.6). (1-4) – washing (I) fraction 1-4; (5-6) – washing (II) fraction 1-2; (7) - Molecular Weight Marker 14-66 kDa (Sigma-Aldrich); (8) – elution fraction 1; (9) – elution fraction 2; (10) – elution fraction 3.

The separation method was further optimized by using a HEPES buffer at pH=8.0, purification results are shown in Figure 8. As our results confirm, the target protein was specifically bound by the Ni-NTA resin. Furthermore, the complete amount of CB was eluted in a single fraction, thus slightly increasing the concentration of the solution.

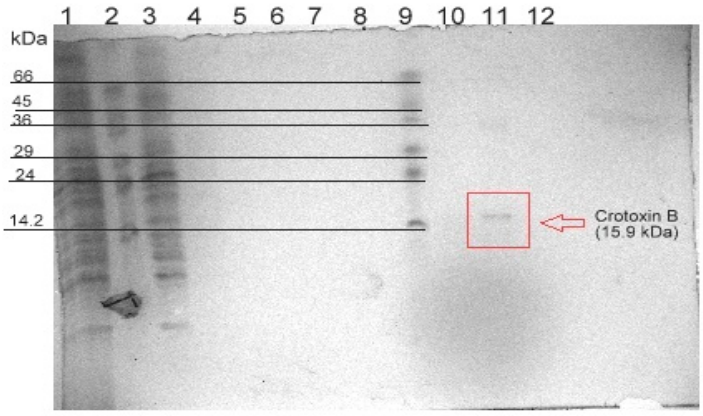

Figure 8. Tricine-SDS-PAGE gel analysis of affinity chromatography purification of the recombinant protein using HEPES buffer (pH=8.0). (1) – flow-through; (2)(9) – Molecular Weight Marker 14-66 kDa (Sigma-Aldrich); (3-6) – washing (I) fraction 1-4; (7)(8) -empty lanes; (10) – elution fraction 1; (11) – elution fraction 2; (12) – elution fraction 3.

CONCLUSIONS

Based on the available coding sequence of CB (isoform C), we have successfully managed to design an experimental protocol for expressing, separation and purification of Crotoxin B using CB_pET-30b(+) vector. The recombinant vector was used to transform the *Rosetta™ (DE3)pLysS* expression cells, and the recombinant protein was successfully obtained in the following optimized conditions: incubation at 37 °C and 250 rpm until optimal optical density of the cell culture was reached, and induction with 0.5 mM IPTG concentration, followed by two hours of effective protein expression.

Using different buffer systems and optimized pH conditions, the purification of the target recombinant protein was achieved through a single-step Ni^{2+}-affinity chromatographic method.

To the best of our knowledge this is the first literature reported method for heterologous expression of the crotoxin B. Furthermore, the protein can be used as positive control for further pharmacological studies being an elegant method for expressing large-scale quantities of crotoxin B.

EXPERIMENTAL SECTION

Plasmids and bacterial strains

The coding sequence of CB (isoform C) obtained from the National Center for Biotechnology Information (NCBI) database (GenBank: X12603.1) was used as a reference. The cloning and expression of the recombinant CB (isoform C) was carried out using a pET-30b(+) vector, which contains and operates using the bacteriophage T7 promoter. The cloning of the coding sequence and the subcloning of the sequence in the pET-30b(+) vector was performed by Genscript USA Inc, using the NotI-HindIII restriction sites.

Protein expression was conducted using *Rosetta™ (DE3)pLysS* cells, genotype F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE (Cam^R), which work as a protease deficient expression host.

Transformation of competent cells

The lyophilised CB_pET-30b(+) plasmid was dissolved in nuclease-free water to obtain a solution with a final concentration of 100 ng/µl. 5 µl of the plasmid solution was added to 100 µl *Rosetta™ (DE3)pLysS* competent cells. The resulting suspension was placed on ice for 15 minutes, heat shocked for two minutes at 42 °C, then put again on ice for 5 minutes. LB broth was added to the suspension up to a final volume of 1000 µl, followed by incubation at 37°C and 250 rpm for one hour (Biosan TS-100 Thermo Shaker). The obtained cell cultures were plated on LB agar plates containing 50 µg/ml kanamycin and incubated overnight at 37 °C.

A single colony was selected and inoculated in 3 ml LB broth containing kanamycin 50 µg/ml and incubated at 37 °C and 250 rpm (Biosan Orbital Shaker-Incubator ES-20), until OD_{600} reached a value of 0.5-0.6, which indicated the logarithmic growth phase of the cells. Glycerol stock was prepared from the cell culture and stored at -80 °C for subsequent use.

Colony PCR

The composition of the PCR reaction mixture used for colony PCR is presented in Table 1. To each cold PCR tube containing the reaction mixture a small amount of the selected bacterial colony was added.

Amplification of the DNA was accomplished using a SureCycler 8800 PCR (Agilent Technologies) and the PCR program shown in Table 2. The obtained DNA fragments were separated using a 1 % agarose gel, and the bands were visualised using a Bio-Rad Gel Doc XR System under UV light (λ = 312 nm).

Table 1. Composition of the PCR reaction mixture

Component	Volume
Sterile distilled water	28 µl
10X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH=9,0), 1.0 % Triton X-100)	5 µl
$MgCl_2$ (25 mM)	3 µl
dNTPs (10 mM each dATP, dTTP, dGTP, dCTP)	3 µl
Forward primer (T7 promoter) (20 µM)	5 µl
Reverse primer (T7 terminator) (20 µM)	5 µl
Taq polymerase	1 µl
Total volume	50 µl

Table 2. Program used for colony PCR

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	1 min	35
Primer annealing	54 °C	1 min 30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

Method optimisation

A cell culture with an approximate OD_{600} value of 0.04 was prepared and equally divided in flasks. The flasks, each containing 100 ml cell culture, were incubated at 30 °C or 37 °C and 250 rpm. OD_{600} value was measured (Zuzi spectrophotometer 4201/50) from samples taken from each flask until the target value of 0.8-1.0 was reached.

Protein expression was induced using 0.01 mM, 0.5 mM or 1.0 mM IPTG solution in both temperature ranges (Table 3). The cultures were incubated for three more hours at 30 °C or 37 °C and 250 rpm. Samples for measuring OD_{600} and electrophoretic analysis were taken before induction and hourly after induction from each flask. Protein expression was halted three hours after induction by centrifuging the cell culture at 4500 rpm for 10 minutes (Hettich Mikro 22R Centrifuge).

Table 3. Distribution of samples based on conditions used for method optimisation

Samples*	IPTG concentration	Temperature
I	0.01 mM	30 °C
II	0.5 mM	
III	1.0 mM	
IV	0.01 mM	37 °C
V	0.5 mM	
VI	1.0 mM	

* All samples have been prepared in duplicate

Expression of recombinant Crotoxin B

100 µl of transformed cell culture containing the CB_pET-30b(+) vector was added to 10 ml LB broth containing 50 µg/ml kanamycin and incubated for four hours at 37 °C and 250 rpm. The obtained cell culture was then added to 1000 ml LB broth solution containing kanamycin 50 µg/ml and incubated at 37 °C and 250 rpm (Sartorius Certomat BS-T). OD_{600} was measured hourly, and the protein expression inducted with 0.5 mM IPTG when OD_{600} value was in the range of 0.9-1.1.

For measuring OD_{600} and SDS-PAGE analysis samples were taken before induction at 1 h and 2 h after induction. Two hours after the induction with IPTG, the protein expression was stopped, separating the cell culture by centrifugation at 4500 rpm and 4 °C for 10 minutes. The obtained pellets were stored at -80 °C until further processing.

Cell lysis was performed by thawing the pellets and resuspending the cells in 25 ml lysis buffer (Table 4). In order to disrupt the cell structures and obtain a homogeneous solution the cell suspensions were sonicated (Dr. Hielscher UP200H sonicator) at 70 % amplitude for 12 minutes, in three-minute cycles with one-minute pause between the cycles, keeping the suspension on ice in order to prevent overheating and denaturation of proteins. The resulting cell extracts were centrifuged (Beckman Coulter Allegra 64R Centrifuge) at 19000 rpm and 4 °C for 50 minutes.

Table 4. Composition of lysis buffers used

Component	Tris buffer	HEPES buffer	
Tris	50 mM	-	
HEPES	-	50 mM	
NaCl	300 mM	300 mM	
DTT	2.0 mM	2.0 mM	
PMSF	1.0 mM	1.0 mM	
Triton X-100	1.0 %	1.0 %	
Imidazole	20 mM	20 mM	
Protease inhibitor	1 tablet	1 tablet	
pH	7.6	7.6	8.0

Batch purification of recombinant CB protein

Based on the 6xHis tag added to the protein, the purification was performed manually using Ni^{2+}-affinity chromatography at 4 °C. Separation and purification was carried out using Tris-HCl buffer (pH=7.6) and HEPES buffer (pH=7.6 and pH=8.0). The composition of buffer solutions is presented in Table 5.

Table 5. Composition of the washing and elution buffers used

Component	Tris-HCl buffer			HEPES buffer		
	Washing buffer (I)	Washing buffer (II)	Elution buffer	Washing buffer (I)	Washing buffer (II)	Elution buffer
Tris	-	50 mM	50 mM	-	-	-
HEPES	-	-	-	-	50 mM	50 mM
Na_2HPO_4	50 mM	-	-	50 mM	-	-
NaCl	500 mM	1 M	500 mM	500 mM	1 M	500 mM
DTT	2 mM	2 mM	2 mM	2 mM	2 mM	2 mM
Imidazole	40 mM	20 mM	400 mM	40 mM	20 mM	400 mM
Glycerol	-	-	5 %	-	-	5 %
pH	7.6			7.6 / 8.0		

The stationary phase (Ni-NTA resin) was equilibrated with washing buffer (I). Supernatant and 2 ml of HisPur Ni-NTA resin (Thermo Scientific) were mixed (Premiere Roll Mixer) for 60 minutes to allow the binding of the His-tag to the resin.

The suspension was added to an empty column, and the flow-through collected. The Ni^{2+}-NTA resin was washed with 4 x 2 ml washing buffer (I), 4 x 2 ml washing buffer (II) then eluted with 6 x 1.5 ml elution buffer. Each fraction from the washing and elution process was collected separately and analysed using SDS-PAGE for protein content.

SDS-PAGE analysis

Samples for SDS-PAGE analysis was prepared according to Laemmli [32]. Proteins were separated on either 15 % SDS-PAGE gels or 10 % tricine SDS-PAGE gels. SDS-PAGE gels were run using 90 V voltage for 15 minutes, followed by 120 V for 90 minutes (Bio-Rad PowerPac Basic). Tricine SDS-PAGE gels were run using 120 V for 90 minutes, on ice. The separated proteins were stained using Coomassie Brilliant Blue.

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