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Optimalisation of expression conditions for production of round-leaf sundew chitinase (*Drosera rotundifolia* L.) in three *E. coli* expression strains

Optimalizácia podmienok expresie droserovej chitinázy (*Drosera rotundifolia* L.) v troch expresných kmeňoch *E. coli*

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Abstract

Round-leaf sundew (*Drosera rotundifolia* L.*),* family *Droseraceae,* genus *Drosera*, is one of a few plant species with a strong antifungal potential. Chitinases of carnivorous plants play an important role in decomposition of chitin-containing cell structures of insect prey. The cell wall of many phytopathogenic fungi also contains chitin, which can be utilized by chitinases, thus round-leaf sundew represents an interesting gene source for plant biotechnology. The purpose of this study was to compare the suitability of 3 different *E. coli* expression strains (*E. coli* BL21-CodonPlus® (DE3)-RIPL, *E. coli* ArcticExpress (DE3)RIL and *E. coli* SHuffle® T7) for production and isolation of heterologous round-leaf sundew chitinase (DrChit). Results showed that the recombinant protein was successfully expressed in all three strains, but occurred in insoluble protein fraction. To get the DrChit protein into soluble protein fraction some modifications concerning to induction temperatures and concentration of the IPTG inductor were tested. In addition, composition of lysis buffer has been modified with supplementation of strong non-ionic detergents, Triton® X100 and Tween® 20, respectively. As these modifications didn't increase the amount of the DrChit protein in soluble fraction, therefore, its isolation under denaturing conditions and subsequent refolding for activity assays is recommended.

Keywords: *Drosera rotundifolia* L., *Escherichia coli* expression, protein solubility

Abstrakt

Rosička okrúhlolistá (*Drosera rotundifolia* L.), rodina *Droseraceae*, rod *Drosera*, je jednou z mála rastlinných druhov so silným antifungálnym potenciálom. Chitinázy mäsožravých rastlín zohrávajú dôležitú úlohu pri dekompozícií bunkových štruktúr obsahujúcih chitín z tela chyteného hmyzu. Bunkové steny viacerých fytopatogénnych húb obsahujú chitín, ktorý môže byť využívaný chitinázami, čo robí z rosičky okrúhlolistej zaujímavý genetický zdroj pre rastlinné biotechnológie. Cieľom práce bolo porovnanie vhodnosti použitia 3 rozdielnych expresných kmeňov *E. coli* (*E. coli* BL21-CodonPlus® (DE3)-RIPL, *E. coli* ArcticExpress (DE3)RIL a *E. coli* SHuffle® T7) pre produkciu a izoláciu heterológnej chitinázy rosičky okrúhlolistej (DrChit). Výsledky ukázali, že rekombinantný proteín bol úspešne exprimovaný vo všetkých 3 kmeňoch, ale vyskytoval sa v nerozpustnej proteínovej frakcii. Aby sa DrChit proteín dostal rozpustnej proteínovej frakcie, boli testované modifikácie teploty indukcie a koncentrácie induktora IPTG. Ďalej bolo modifikované zloženie lyzačného pufru pridaním silných neiónových detergentov, Triton® X100 a Tween® 20. Pretože tieto modifikácie nezvýšili množstvo získaného DrChit proteínu v rozpustnej frakcii, je odporúčaná jeho izolácia za denaturačných podmienok a následná obnova štruktúry proteínu.

Kľúčové slová: *Drosera rotundifolia* L., expresia v *Escherichia. coli,* rozpustnosť proteínov

Introduction

Chitin, homopolymer of the β-(1,4) -N-α-acetyl-D-glucosamin, is the second most abundant biopolymer occurring in living organisms. Crustacean shells, insect exoskeletons and fungal cell walls contain a substantial quantity of chitin. Cleavage of the chitin polymer chains is accomplished by hydrolytic enzymes, chitinases. Endochitinases (EC 3.2.1.14) cleave the β-1,4-glycoside bonds of the chitin randomly, alongside the whole polymer chain, releasing a mixture of oligomers with various chain lengths. Exochitinases (EC 3.2.1.29) cleave the β-1,4-glycoside bonds of the chitin from the non-reducing end and release dimers of the β-N-acetylglucosamine. Further cleavage to the β-N-acetylglucosamine monomers is accomplished by chitobiases (EC 3.2.1.52) (Cletus et al., 2013).

Plants do not contain an immune system, and thus they evolved a system of defence responses to pathogen attacks. One of them involves the expression of PR (pathogenesis-related) proteins, including chitinases. The increase of chitinases expression in plant tissues after an infection by various types of pathogens suggests their important role in plant defence response. As a major cell wall component of many pathogenic fungi is chitin, its digestion by plant chitinases during an infection contributes to pathogen growth inhibition (Ebrahim et al., 2011).

Current research in the agricultural production aims at increasing the resistance of crops to biotic and abiotic stress factors. One of the most widely used strategy involves overexpression of the genes for PR proteins. In some cases, the increased

resistance to fungal pathogens was achieved in transgenic plants overexpressing specific chitinase genes (Dana et al., 2006; Amian et al., 2011; Girhepuje and Shinde, 2011; Prasad et al., 2012).

Various chitinase genes from diverse source organisms show different enzyme and antifungal activity. Round-leaf sundew (*Drosera rotundifolia* L.) belongs among plant species with a strong chitinolytic and an antifungal activity (Ďurechová et al., 2013). Results of study of Matušíková et al. (2005) suggest that the round-leaf sundew chitinase is likely to be involved in the decomposition of the insect prey. Therefore, the isolation of chitinase gene involved in carnivory processes of round-leaf sundew, and characterisation of enzymatic and antifungal properties of chitinase protein, seems to be justified in its next application in plant biotechnology.

Choosing a suitable expression system for candidate genes is often a crucial step in protein engineering. There are many factors that determine the suitability of the expression system for individual experiments, such as a gene source organism, production capacity of a recombinant protein, its solubility and localisation, stability, structural flexibility or forming of inclusion bodies. *Escherichia coli* is one of the organisms of choice for production of various recombinant proteins (Rosano and Ceccarelli, 2014). *E. coli* expression system continues to dominate among the bacterial expression systems and remains to be the preferred system for laboratory investigations and initial research of recombinant proteins (Chen, 2012).

When the active purified recombinant protein is needed, it is important to find the conditions for its high expression as well as maximum possible solubility before its purification from cellular mass. The expression of the polypeptide (fusion partner) or short sequence of amino acids (fusion/affinity tag) alongside with the recombinant protein can help to achieve these goals (Rosano and Ceccarelli, 2014). Glutathione S-transferase (GST), thioredoxin A (TrxA) and small ubiquitin related modifier (SUMO) are the most commonly used fusion proteins which provide potential advantages in the protein expression and solubility of a recombinant protein. Fusion or affinity tags are highly efficient tools for protein detection, characterization and affinity purification. C-myc, the hemaglutinin antigen (HA), FLAG epitope, 1D4 epitope, polyArg and polyHis tags are among the most commonly used affinity tags (Young et al., 2012).

Heterologous protein overexpression in *Escherichia coli* often leads to an accumulation of a target protein in dense insoluble aggregates known as inclusion bodies (Hwang et al., 2014). Inclusion bodies are formed when the overexpression of heterologous genes leads to the forming of protein products that fail to attain in a soluble, bioactive conformation (de Groot et al., 2008). This is the reason why they are considered as a major bottleneck in the recombinant protein production. Recent studies of bacterial inclusion bodies revealed, that protein deposition in recombinant cells is fully reversible and a relevant fraction of inclusion body's proteins is actually functional (García-Fruitós et al., 2012). There are two strategies of obtaining bioactive recombinant proteins from inclusion bodies. The first strategy is used for refolding of inclusion body proteins into bioactive forms and generally involves two steps: solubilization of inclusion body *via* denaturation by high concentration of denaturants like urea, SDS or guanidin hydrochloride, which is followed by the second step - refolding of a solubilized protein with slow removal of a denaturant.

The second strategy focuses on modification of cultivation conditions for production of recombinant protein in a soluble fraction (Clark, 1998).

The aim of this work is to analyse and compare the suitability of 3 various *E. coli* strains for the expression of the recombinant DrChit protein and to consider the possibility of its isolation from soluble cell protein fraction.

Materials and methods

Bacterial strains and plasmid construction

Bacterial *E. coli* strains used for the expression of the target recombinant chitinase protein:

Escherichia coli BL21-CodonPlus® (DE3)-RIPL strain (Agilent Technologies, USA), genotype B F^- ompT hsdS(r_B^- m_B^-) dcm^+ Tet^r gal λ(DE3) endA Hte [argU proL Cam^r] [argU ileY leuW Strep/$Spec^r$]

Escherichia coli ArcticExpress (DE3)RIL strain (Agilent Technologies, USA), genotype B F^- ompT hsdS(r_B^- m_B^-) dcm^+ Tet^r gal endA Hte [cpn10 cpn60 $Gent^r$] [argU ileY leuW Str^r]

Escherichia coli SHuffle® T7 strain (New England Biolabs, England), genotype fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbC ($Spec^R$, $lacI^q$) ΔtrxB sulA11 R(mcr-73::miniTn10-Tet^S)2 [dcm] R(zgb-210::Tn10 – Tet^S) endA1 Δgor Δ(mcrC-mn)114::IS10

Plasmid construction, isolation, and verification of cloning procedure were carried out as described by Sambrook and Russell (2001). Plasmid transformation was performed according to the manufacturer's recommendation (Agilent Technologies, New England Biolabs).

Induction of target protein expression

Induction of the target protein expression in *E. coli* BL21-CodonPlus® (DE3)-RIPL strain was performed according to the manufacturer's recommendations (Agilent Technologies, USA). One ml aliquot of Luria–Bertani (LB) broth [1% (w/v) pepton; 0.5% (w/v) yeast extract; 1.5% (w/v) NaCl] supplemented with 50 $\mu g.ml^{-1}$ chloramphenicol and 100 $\mu g.ml^{-1}$ ampicillin was inoculated with 30 µl of the bacterial glycerol stock and incubated with shaking overnight at 220 rpm and 37°C. Then 50 µl of the culture was pipetted into fresh 1 ml aliquots of LB medium without selection antibiotics and incubated with shaking for 2 hours at 220 rpm and 37°C until the OD_{600} reached approx. 0.4 – 0.6. Next, 100 µl of the culture was pipetted into a fresh microcentrifuge tube and placed on ice until needed for SDS-PAGE (non-induced control sample). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added up to a final concentration of 1 mM in the bacterial cells. The culture was incubated with shaking for 2 hours at 220 rpm and 37°C; and subsequently placed on ice until needed for SDS-PAGE (induced sample).

Induction of the target protein expression in *E. coli* Arctic Express (DE3) RIL strain was performed according to the manufacturer's recommendations (Agilent Technologies, USA). One ml aliquot of LB broth containing 20 $\mu g.ml^{-1}$ gentamycin and 100 $\mu g.ml^{-1}$ ampicillin was inoculated with 30 µl of the bacterial glycerol stock and incubated with shaking overnight at 220 rpm and 37°C. Next, 60 µl of the overnight culture was pipetted into a 50 ml tube with 3 ml of LB broth without selection antibiotics. The culture was cultivated with shaking for 3 hours at 220 rpm and 30°C, until the OD_{600} reached approx. 0.4 – 0.6. After 3 hours, 100 µl of the culture was pipetted into a fresh microcentrifuge tube and placed on ice until needed for SDS-PAGE (non-induced control sample). Next, the culture tube was incubated with shaking for approx. 10 minutes at 220 rpm and 12°C. After equlibration of the bacterial culture to 12°C, IPTG up to a final concentration 1 mM was added, and bacteria were incubated with shaking for 24 hours, at 220 rpm. After the incubation period, the culture was placed on ice until needed for SDS-PAGE (induced sample).

Induction of the target protein expression in *E. coli* SHuffle® T7 strain was performed according to the manufacturer's recommendations (New England Biolabs, England). One ml aliquot of LB broth containing 100 $\mu g.ml^{-1}$ ampicillin was inoculated with 30 µl of the bacterial glycerol stock and incubated with shaking at 220 rpm and 30°C until the OD_{600} reached 0.4 – 0.6 (incubation period approx 2 hours). After the incubation period, 100 µl of the culture was pipetted into a microcentrifuge tube and placed on ice until needed for SDS-PAGE (non-induced control sample). Then the bacterial culture was supplemented with IPTG up to a final concentration of 1 mM and incubated with shaking for 3 hours at 220 rpm and 37°C. After the end of the incubation period, culture was placed on ice until needed for SDS-PAGE (induced sample).

Lysis of bacterial cells

Induced cells were harvested by centrifugation at 8,000 rpm for 10 min and the pellet was resuspended in lysis buffer [50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole, pH adjusted to 8.0). Lysozyme to a final concentration of 1 $mg.ml^{-1}$ was added to the mixture and the incubation continued on ice for 1 hour. Then the lysate was centrifuged at 10,000 rpm and 4°C for 30 minutes. Next, the supernatant was decanted (soluble protein fraction) and pellet (insoluble protein fraction) was resuspended in lysis buffer and placed on ice until the SDS-PAGE analysis. For protein solubility experiments, modification of lysis buffer with 1% (w/w) Triton® X100 (PanReac AppliChem, Germany) or 1% (w/w) Tween® 20 (PanReac AppliChem, Germany) was performed.

SDS – PAGE electrophoresis

Expression of the recombinant chitinase was detected by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook and Russell (2001). The analysed samples of total proteins of the non-induced control (10 µl) and of the induced sample (10 µl) were loaded onto the gel. The gels were run at 8°C, at constant voltage of 120V for 2 hours, followed by staining overnight in a solution containing 0.05% Coomassie Brilliant Blue R-250, 40% (v/v)

methanol and 10% (v/v) acetic acid. Next the gel was destained with 20% (v/v) methanol and 7% (v/v) acetic acid for about 5 – 6 hours, visualised and photographed on the Quantum ST4 1000/26MX UV-transluminator (Vilber Lourmat, Germany).

Results and discussion

Induction of the high-level expression of the recombinant protein in *E. coli* can result in the production of large amounts of incorrectly folded proteins, which generate aggregates of inactive protein known as inclusion bodies (Song et al., 2012). Obtaining active protein from inclusion bodies often requires protein-specific and labour-intensive *in vitro* refolding steps with no guarantee of obtaining biologically active product (García-Fruitós et al., 2012).

The aim of the work was to compare the suitability of 3 different *E. coli* expression strains for the production of the heterologous round-leaf sundew chitinase in soluble fraction. Various cultivation temperatures and concentrations of IPTG were used for the recombinant protein expression. After protein detection *via* SDS-PAGE, 3 different compositions of the lysis buffer were used to obtain heterologous DrChit protein in soluble form.

E. coli BL21-CodonPlus® (DE3) - RIPL expression

E. coli BL21-CodonPlus® (DE3)-RIPL strain (Agilent Technologies, USA) enables efficient high-level expression of heterologous proteins. BL21-CodonPlus strain is engineered to contain extra copies of the *argU*, *ileU*, *leuW,* and *proL* tRNA genes, which encode tRNAs that recognize arginin codons (AGA, AGG), the isoleucin codon (AUA), the leucin codon (CUA) and the proline codon (CCC). These tRNA most frequently restrict the translation of heterologous proteins from organisms with AT- or GC-rich genomes (Wu et al., 2004).

E. coli BL21-CodonPlus® (DE3)-RIPL cells harboring the plasmid pET32aDrChit were grown in the LB broth as described in “Material and Methods“. Expression of the heterologous round-leaf sundew chitinase was carried out under different cultivation conditions as described in Table 1, while induction period lasted for 2 hours. In the first experiment, *E. coli* BL21-CodonPlus® (DE3)-RIPL cells with pET32aDrChit plasmid were cultivated and induced according to the manufacturer's recommendations (temperature 37°C, 1 mM concentration of IPTG). These conditions enabled significant recombinant protein production, as shown in Figure 1. However, when cultivation temperature was lowered to 30°C and the induction time period was unchanged, the recombinant protein production wasn’t detected. Concentration of IPTG (1 mM and 0.5 mM respectively), didn’t positively affect the production of recombinant chitinase at 30°C as well. Further lowering of temperature to 25°C with 0.5 mM or 0.25 mM IPTG concentration showed similar results as cultivation at 30°C, namely no detectable recombinant protein production (Figure 1). Induction of *E. coli* BL21-CodonPlus® (DE3)-RIPL cells at 25°C with 0.5 mM IPTG was successfully performed by Assadi-Porter et al. (2008), while production of the eucaryotic plant brazzein protein was detected in the soluble protein fraction. Canella et al. (2010) used E. coli BL21-CodonPlus® (DE3)-RIPL expression strain for

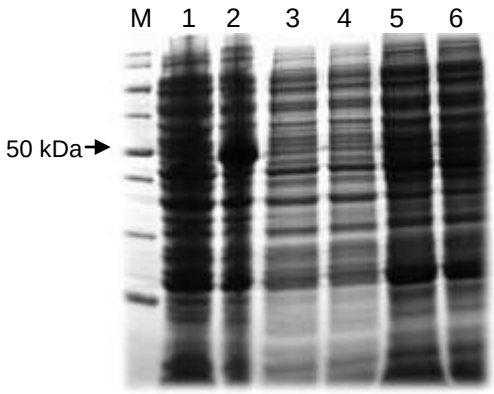
production of heterologous protein from Arabidopsis. Transcription factor CBF1 was fused with MBP tag and with 6xHis tag. The expression was successfully induced with 1 mM IPTG. MBP- and His-tagged CBF1 protein was purified onto amylase and nickel column, respectively. Batch production of heterologous protein in E. coli BL21-CodonPlus® (DE3)-RIPL was performed by Collins et al. (2013). Maximise production of the silk-elastin-like protein involved optimalisation of media, induction time and temperature. Optimal production conditions were comparable to ones used in this experiment (induction temperature 37°C, 1 mM IPTG concentration).

Table 1. Different conditions for induction of round-leaf sundew chitinase expression in *E. coli* BL21-CodonPlus® (DE3)-RIPL expression strain

Table 1. Rozdielne podmienky indukcie expresie droserovej chitinázy v expresnom kmeni *E. coli* BL21-CodonPlus® (DE3)-RIPL

Sample	Temperature (°C)	Concentration of IPTG (mM)	Detectable production of DrChit *via* SDS-PAGE
Non-induced sample	37	--	-
Induced sample	37	1	+
Induced sample	30	1	-
Induced sample	30	0.5	-
Induced sample	25	0.5	-
Induced sample	25	0.25	-

IPTG - Isopropyl β-D-1-thiogalactopyranoside, DrChit – recombinant round-leaf sundew chitinase, SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis, "+" - protein was detected after SDS-PAGE, "-" - protein was not detected after SDS-PAGE



M – PageRuler™ Unstained Broad Range Protein Ladder (ThermoFisher Scientific), 1 – crude protein extract of non-induced control sample, 2 – crude protein extract of induced sample (37°C, 1 mM IPTG), 3 – crude protein extract of induced sample (30°C, 1 mM IPTG), 4 – crude protein extract of induced sample (30°C, 0.5 mM IPTG), 5 – crude protein extract of induced sample (25°C, 0.5 mM IPTG), 6 – crude protein extract of induced sample (25°C, 0.25 mM IPTG)

Figure 1. SDS-PAGE analysis of different induction conditions of round-leaf sundew chitinase expression in *E. coli* BL21-CodonPlus® (DE3)-RIPL expression strain

Figure 1. SDS-PAGE analýza rozdielnych podmienok indukcie expresie droserovej chitinázy v expresnom kmeni *E. coli* BL21-CodonPlus® (DE3)-RIPL

E. coli ArcticExpress (DE3)RIL expression

E. coli ArcticExpress (DE3)RIL strain (Agilent Technologies, USA) is engineered to solve two common bacterial gene expression problems: protein insolubility and codon bias. Suitability of *E. coli* strains for expression of a wide range of heterologous proteins in soluble form can be enhanced by low temperature cultivation. Problem with low temperature cultivation is, that *E. coli* chaperonins, proteins which facilitate proper protein folding, lose their activity at reduced temperatures. *E. coli* ArcticExpress strain enables improved protein processing at low temperatures, because of co-expression of cold-adapted chaperonins Cpn10 and Cpn60 from the psychrophylic bacteria *Oleispira antarctica.* Cpn10 and Cpn60 chaperonins show 74% and 53% amino acid identity with homologues from *E. coli*. The chaperonins display high refolding activities at temperatures of 4–12°C (Ferrer et al., 2004;). *E. coli* ArcticExpress (DE3)RIL strain also contains extra copies of the *argU*, *ileU*, and *leuW* tDNA genes, which enhance expression of heterologous proteins from organisms that have AT-rich genomes (Rosano and Ceccarelli, 2014).

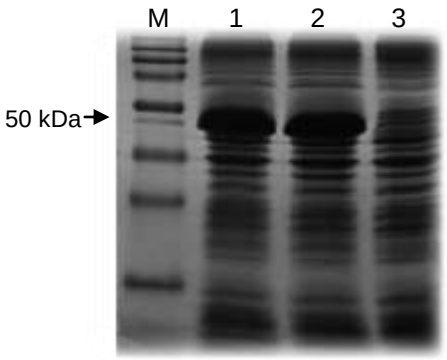
E. coli ArcticExpress (DE3)RIL cells harboring the plasmid pET32aDrChit were grown in the LB broth as described in “Material and Methods“. Expression of the heterologous round-leaf sundew chitinase was carried out under various concentrations of inductor – IPTG, as described in Table 2, while induction period lasted for 24 hours. The conditions - temperature 13°C, 1 mM concentration of IPTG and induction 24 hours - were recommended by the manufacturer and enabled significant production of recombinant DrChit protein (Figure 2). When the concentration of IPTG was lowered to 0.5 mM, heterologous protein expression was detected as well. Quantity of produced recombinant DrChit protein was almost the same in both cases (1 and 0.5 mM IPTG concentration). It implies that lowering concentration of IPTG to 0.5 mM didn’t affect the amount of produced heterologous protein significantly in case of *E. coli* ArcticExpress (DE3)RIL expression strain (Figure 2). Song et al. (2012) tested 3 different *E. coli* strains, Rosetta(DE3), ArcticExpress(DE3)RIL, and Rosetta-gami2(DE3), for production of soluble eucaryotic proteins. The crude lysates were separated into soluble and insoluble fractions by centrifugation and both fractions were analyzed by SDS–PAGE. ArcticExpress(DE3)RIL strain produced eucaryotic proteins in the insoluble fraction and partially in soluble fraction. However, the recombinant proteins in the soluble fraction didn’t show any enzymatic activity. Pacheco et al. (2012) compared suitability of various *Escherichia coli* expression strains, including *E. coli* ArcticExpress(DE3)RIL, for heterologous protein expression. Set of 71 expressed heterologous proteins was tested and *E. coli* ArcticExpress(DE3)RIL showed 37% success rate with their expression in soluble form.

Table 2. Different conditions for induction of round-leaf sundew chitinase expression in *E. coli* ArcticExpress (DE3)RIL strain

Table 2. Rozdielne podmienky indukcie expresie droserovej chitinázy v expresnom kmeni *E. coli* ArcticExpress (DE3)RIL

Sample	Temperature (°C)	Concentration of IPTG (mM)	Detectable production of DrChit *via* SDS-PAGE
Non-induced sample	--	--	-
Induced sample	13	1	+
Induced sample	13	0.5	+

IPTG - Isopropyl β-D-1-thiogalactopyranoside, DrChit - recombinant round-leaf sundew chitinase, SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis, "+" - protein was detected after SDS-PAGE, "-" - protein was not detected after SDS-PAGE



M – Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific), 1 – crude protein extract of induced sample (13°C, 1 mM IPTG), 2 – crude protein extract of induced sample (13°C, 0.5 mM IPTG), 3 – crude protein extract of non-induced control sample

Figure 2. SDS-PAGE analysis of different induction conditions of round-leaf sundew chitinase expression in *E. coli* ArcticExpress (DE3)RIL expression strain

Figure 2. SDS-PAGE analýza rozdielnych podmienok indukcie expresie droserovej chitinázy v expresnom kmeni *E. coli* ArcticExpress (DE3)RIL

E. coli SHuffle® T7 expression

E. coli SHuffle® T7 strain (New England Biolabs, England) is engineered to promote disulfide bond formation in the heterologous protein in the cytoplasm of the host cell. *E. coli* SHuffle® T7 strain is based on the *E. coli* trxB gor suppressor strain SMG96, which allows the formation of disulfide bonds in the cytoplasm *via* diminishing of the bacterial cytoplasmic reductive pathways. *E. coli* SHuffle® T7 strain constitutively expresses a chromosomal copy of the disulfide bond isomerase DsbC, which promotes the correction of mis-oxidized proteins into their correct form (Lobstein et al., 2012).

E. coli SHuffle® T7 cells harboring the plasmid pET32aDrChit were grown in the LB broth as described in “Material and Methods“. Expression of the heterologous round-leaf sundew chitinase was carried with various concentrations of inductor – IPTG, as

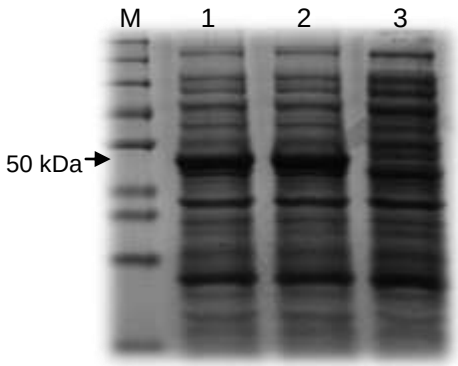
described in Table 3, while induction period was constant - 3 hours. Conditions for induction of recombinant protein expression - temperature 37°C, 1 mM concentration of IPTG - were recommended by manufacturer of *E. coli* SHuffle® T7 strain and enabled obvious DrChit expression. Lobstein et. al. (2012) suggested that the effect of optimal concentration of inducer is protein-specific and recommended values of IPTG concentration range from 0.01 mM to 1 mM. Lowering the concentration of IPTG to 0.5 mM resulted in comparable expression of DrChit protein (Figure 3). Hofzumahaus and Schallmey (2013) used *E. coli* SHuffle® T7 expression strain for initial cytosolic expression tests of β-cinnamomin from *Phytophthora cinnamomi.* Expression of the heterologous protein led exclusively to formation of large amounts of inclusion bodies probably due to wrongly formed disulfide bonds.

Table 3. Different conditions for induction of round-leaf sundew chitinase expression in *E. coli* SHuffle® T7 expression strain

Table 3. Rozdielne podmienky indukcie expresie droserovej chitinázy v expresnom kmeni *E. coli* SHuffle® T7

Sample	Temperature (°C)	Concentration of IPTG (mM)	Detectable production of DrChit *via* SDS-PAGE
Non-induced sample	--	--	-
Induced sample	37	1	+
Induced sample	37	0.5	+

IPTG - Isopropyl β-D-1-thiogalactopyranoside, DrChit - recombinant round-leaf sundew chitinase, SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis, "+" - protein was detected after SDS-PAGE, "-" - protein was not detected after SDS-PAGE



M – Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific), 1 – crude protein extract of induced sample (37°C, 1 mM IPTG), 2 – crude protein extract of induced sample (37°C, 0.5 mM IPTG), 3 – crude protein extract of non-induced control sample

Figure 3. SDS-PAGE analysis of different induction conditions of round-leaf sundew chitinase in *E. coli* SHuffle® T7 expression strain

Figure 3. SDS-PAGE analýza rozdielnych podmienok indukcie droserovej chitinázy v expresnom kmeni *E. coli* SHuffle® T7

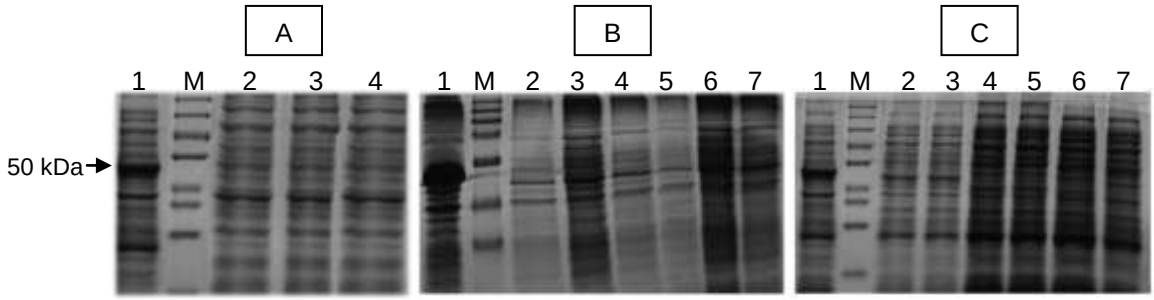
Bacterial cell lysis and isolation of round-leaf sundew chitinase protein from soluble cell fraction

After the successful expression of the heterologous DrChit protein, induced *E. coli* cells were centrifuged and lysed in various lysis buffers for isolation of proteins under native conditions. *E. coli* BL21-CodonPlus® (DE3)-RIPL strain showed production of heterologous chitinase only when induction with 1 mM IPTG at 37°C was used. After lysis of induced cells in manufacturer's recommended lysis buffer (as described in "Material and Methods") there wasn't detected heterologous chitinase protein in the soluble fraction *via* SDS-PAGE. Modification of the lysis buffer with addition of 1% Triton® X100 and 1% Tween® 20, respectively, didn't increase the amount of DrChit protein in the soluble fraction either (Figure 4). Assadi-Porter et al. (2008) used *E. coli* BL21-CodonPlus (DE3)RIPL strain for production of the eucaryotic brazzein protein in soluble form, which was purified to greater than 90% purity by single-step Ni–NTA column chromatography.

E. coli ArcticExpress (DE3)RIL cells with recombinant DrChit protein production (induction temperature 13°C, 1 and 0.5 mM concentration of IPTG) were lysed in manufacturer's recommended lysis buffer; in lysis buffer with the addition of 1% Triton® X100 and 1% Tween® 20, respectively. Recombinant protein wasn't detected in soluble protein fraction in none of three used lysis buffers (Figure 4). Miernikiewicz et al. (2012) used standard expression strains (without cold-adapted chaperones), for expression of gp23 and gpsoc phage proteins that were produced as inclusion bodies and were presumably misfolded. Folding and solubility were substantially improved when these proteins were co-expressed with Cpn10 and Cpn60 derived from *Oleispira antarctica*, that allowed slow expression at 10°C. Co-expression with chaperones had little effect on the solubility of gphoc protein, though.

E. coli SHuffle® T7 cells producing heterologous round-leaf sundew chitinase (induction temperature 37°C, 1 and 0.5 mM concentration of IPTG), were lysed in three previously used buffers (lysis buffer, lysis buffer with the addition of the 1% Triton® X100 and lysis buffer with the addition of the 1% Tween® 20). Unfortunately, the recombinant DrChit protein wasn't detected in the soluble fraction in any of used lysis buffer systems (Figure 4).

Results showed that use of 3 different E. coli expression strains (E. coli BL21 CodonPlus® (DE3)-RIPL, E. coli ArcticExpress (DE3)RIL, E. coli SHuffle® T7) didn't affect production of the round-leaf chitinase in the soluble form significantly. Enrichment of lysis buffer with strong nonionic detergent (Triton® X100 or Tween® 20), didn't increase the amount of the recombinant DrChit protein in the soluble protein fraction either. As shown in Figure 4 recombinant DrChit protein was detected only in the insoluble fraction.



A - *E. coli* BL21-CodonPlus® (DE3)-RIPL strain (1 – crude protein extract of induced sample – recombinant chitinase ~50kDa, M – Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific), 2 – soluble protein fraction after lysis in lysis buffer, 3 - soluble protein fraction after lysis in lysis buffer + 1% Triton® X100, 4 - soluble protein fraction after lysis in lysis buffer + 1% Tween® 20); B - *E. coli* ArcticExpress (DE3)RIL strain (1 - crude protein extract of induced sample – recombinant chitinase ~50kDa, M – Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific), 2 – soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer, 3 - soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer + 1% Triton® X100, 4 - soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer + 1% Tween® 20, 5 - soluble protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer, 6 - soluble protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer + 1% Triton® X100, 7 - protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer + 1% Tween® 20); C - *E. coli* SHuffle® T7 strain (1 - crude protein extract of induced sample – recombinant chitinase ~50kDa, M – Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific), 2 – soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer, 3 - soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer + 1% Triton® X100, 4 - soluble protein fraction of cells induced with 1 mM IPTG after lysis in lysis buffer + 1% Tween® 20, 5 - soluble protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer, 6 - soluble protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer + 1% Triton® X100, 7 - protein fraction of cells induced with 0.5 mM IPTG after lysis in lysis buffer + 1% Tween® 20)

Figure 4. *E. coli* induced cell lysis and detection of round-leaf sundew chitinase protein in soluble protein fraction

Figure 4. Lýza indukovaných buniek *E. coli* a detekcia droserovej chitinázy v rozpustnej frakcii proteínov

Conclusion

The suitability of 3 different *E. coli* expression strains was tested for production of heterologous round-leaf sundew chitinase. When manufacturer's recommended induction conditions were used DrChit protein was successfully expressed in all three strains. However, the recombinant protein was produced in insoluble protein fraction, regardless of tested expression strain. Modifications of induction temperatures and concentrations of IPTG were tested for optimalisation of expression procedure and for increase of amount of DrChit protein in soluble protein fraction. Modification of lysis buffer with strong non-ionic detergents, Triton® X100 or Tween® 20, didn't increase the recombinant protein expression level in soluble protein fraction as well. Results showed, that the lysis buffer containing denaturant is probably necessarry for recombinant DrChit protein isolation followed by protein refolding step.

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