4. *Струговщикова Л.П., Шавловський Г.М., Федорович І.П., Кучерас Р.В.* Очищення та деякі властивості лужної фосфатази І дріжджів *Pichia guilliermondi* // Укр. біохім. журн. – 1973. – Т. 45, № 3. – С. 312-317.
5. *Шавловский Г.М., Жарова В.П., Щелокова И.Ф., Трач В.М., Сибирный А.А., Кшеминская Г.П.* Флавиногенная активность природных штаммов дрожжей *Pichia guilliermondii* // Прикл. биохимия и микробиология. – 1978. – Т.14. – С. 184-189.
6. *Bessey O.A., Lowry J.Y., Love R.H.J.* The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues // J. Biol. Chem. – 1949. – Vol. 180, N 2. – P. 378-381.
7. *Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J.* Protein measurement with the Folin phenol reagent // J. Biol. Chem. – 1951. – Vol. 193. – P. 265-275.

Резюме

Досліджено флавіногенну активність рекомбінантних штамів дріжджів *Candida famata*, що містять ген РФ-кінази *FMN1* під контролем сильного конститутивного промотора *TEF1*. Оптимізовано умови нагромадження ФМН у культуральній рідині.

Изучена флавиногенная активность рекомбинантных штаммов дрожжей *Candida famata*, содержащих ген РФ-киназы *FMN1* под контролем сильного конститутивного промотора *TEF1*. Оптимизированы условия накопления ФМН в культуральной жидкости.

The flavinogenic activity of recombinant strains of the yeast *Candida famata* that express the *FMN1* gene encoding riboflavin kinase under control of the strong constitutive *TEF1* promoter was studied. Conditions for flavinmononucleotide production were optimized.

LYPOVA N.M., SINDAROVSKA Y.R, GERASYMENKO I.M., SHELUDKO Y.V., BANNIKOVA M.A., KUCHUK N.V.
Institute of Cell Biology and Genetic Engineering NAS of Ukraine,
Zabolotnogo str. 148, Kiev 03680, Ukraine, e-mail: ysheludko@ukr.net

COMPARISON OF DIFFERENT SYSTEMS FOR PURIFICATION OF RECOMBINANT PROTEINS PRODUCED BY TRANSIENT EXPRESSION IN PLANTS

Plants as source of recombinant proteins have important advantages over microbial or animal cell systems. Plant cells, unlike bacteria, are able to produce proteins with post-translational modifications, as well as correctly folded and assembled multimeric proteins, e. g. antibodies [1]. In contrast to animal cells, plants are free from human pathogens like viruses and prions, so the recombinant proteins of plant origin are considered to be safer [2]. The main obstacle on the way of using transgenic plants for high-scale production of recombinant proteins is the low level of foreign gene expression in case of stable integration into plant nuclear genome (usually about 0.1-0.5 % TSP) [3]. Transient gene expression may allow for rapid accumulation of considerably larger amount of recombinant proteins (in the range 0.5-10 % TSP or sometimes more) [4], but even in this case an efficient purification system can substantially improve the yield of pure target product.

Here we describe comparative analysis of different approaches for purification of transiently expressed recombinant proteins from plants using green fluorescent protein (GFP) as a reporter. The purification scheme including ammonium sulfate precipitation and anion-exchange chromatography was compared with two tag-based protocols applying metal affinity chromatography with a 6xHis tag (Qiagen) and intein mediated purification with a chitin-binding affinity tag (New England Biolab).

Materials and methods

For transient expression we used a 35S expression system carrying the GFP gene driven by the CaMV 35S promoter (pICH5290) and a viral-based expression system (pICH10881, pICH10570 and pICH7410) carrying the same reporter gene described in details in the recent publications [5-7]). In all experiment p19 protein of *Tomato bushy stunt virus*, a suppressor of post-transcriptional gene silencing, was co-expressed (pICH6692) [8]. For tag-based purification, either the sequence coding for six histidine residues or the fragment encoding intein and chitin-binding domain (derived from the pTYB1 plasmid, New England Biolabs) was fused to the GFP gene immediately before the stop-codon.

Infiltration of *Nicotiana excelsior* plants with *Agrobacterium tumefaciens* strain GV3101 and transient expression was performed as it was described [7]. Leaf tissue was extracted with 100 mM KPi buffer, pH 7.8, containing 5 mM EDTA and 10 mM β-mercaptoethanol. The protein fraction precipitated between 50 % and 70 % of ammonium sulfate saturation was separated by anion-exchange chromatography on Q-sepharose with a linear NaCl gradient (0-1 M NaCl) [9]. For purification of the His-tagged GFP, the leaf extracts (prepared in 50 mM Tris/HCl buffer, pH 8.0, containing 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, and 2.5 % PVP) were applied onto Ni-NTA sorbent, the column was washed with extraction buffer and the target protein was eluted with the same buffer containing 250 mM imidazole. For intein-mediated purification, the leaf extracts (prepared in 100 mM KPi buffer, pH 7.8, containing 5 mM EDTA, 10 mM β-mercaptoethanol, and 2.5 % PVP) were applied onto chitin sorbent, the column was washed with extraction buffer and the target protein was eluted after intein cleavage induced by 50 mM dithiothreitol (DTT).

The content of GFP was calculated by measurements of fluorescence intensity in dilutions of leaf extracts using fluorescence spectrophotometer Hitachi 4000 (Hitachi, Tokyo, Japan) (excitation at 395 nm, emission at 509 nm) on the basis of standard values. The background fluorescence of control extracts (from leaves infiltrated with bacteria carrying pICH6692 only) was subtracted from values of GFP containing extracts. The identity of GFP in the extracts to the standard was proved by recording their fluorescence spectra. The concentration of total soluble protein was determined by the method of Bradford. The protein mixtures were analyzed by SDS-PAGE with Coomassie staining.

Results and discussion

N. excelsior was used in our experiments because it was shown to be a promising host for production of recombinant proteins by means of *Agrobacterium*-mediated transient expression [7]. The GFP gene was expressed under control of CaMV 35S promotor or as a part of a module viral-based system which allows considerable increase of the reporter protein accumulation [5, 6]. However, in order to confirm that the effectiveness of the purification scheme does not depend on high initial recombinant protein level we performed transient expression under conditions which were not optimal for the maximal GFP accumulation (plant developmental stage and cultivation temperature [9]). GFP content in the crude protein extract amounted to 3.3 % TSP. The crude protein extract from infiltrated leaf tissue was subjected to ammonium sulfate precipitation followed by Q-sepharose anion-exchange chromatography. The concentration of GFP in fractions during purification procedure was estimated by measurements of fluorescence intensity. The developed scheme of enrichment resulted in 26-fold purification of the recombinant GFP. GFP was recovered in high yield (77 %) with about 85 % purity. The described purification procedure was performed in gentle environment conditions and, although some optimization may be required, we consider this scheme may be regarded as a benchmark for purifying of GFP-fusion proteins as well as GFP.

After cloning from the jellyfish *Aequorea victoria* the GFP gene has undergone substantial modifications that resulted in high expression rate, increased fluorescence, stability and low toxicity for wide range of hosts, including plant cells [10]. Such modifications are not always desirable for pharmaceutically valuable proteins, thus their level

of accumulation in plants is often considerably lower than that of GFP. It renders classical purification schemes ineffective. Adding of affinity tags to the protein of interest simplifies the purification procedure and improves the yield of the refined recombinant protein. Proteins tagged with 6 consecutive histidine residues can be efficiently purified using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrix (Qiagen).

The GFP gene with additional sequence coding for six histidine residues was successfully transiently expressed in *N. excelsior* under control of CaMV 35S promoter. The level of His-tagged GFP reached 5.5 % TSP, which is normal mean for such kind of expression system [7]. One-stage purification procedure resulted in obtaining of homogenous target protein (Fig. 1), although the yield of GFP was about 40 %. The effectiveness of His-tag-mediated purification was shown for many recombinant proteins in different host systems, but for production of pharmaceutically valuable proteins in plants this method has some drawbacks. The protease cleavage of His-tag, which is necessary for obtaining of native product, is an expensive procedure. In addition, some plant proteins containing histidine residues can interact with Ni-NTA matrix and pollute the target product. To overcome these problems, other affinity tag-based systems may be used.

IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag, New England Biolabs) is a protein purification system which utilizes the inducible self-cleavage activity of a protein splicing element (intein) to separate the target protein from the affinity tag (chitin-binding domain). It allows purifying in a single chromagraphic step without further protease cleavage a native recombinant protein. The drawback of this system is the lower level of fused protein expression, presumably due to the big size of the tag. The GFP gene with additional sequence coding for intein and chitin-binding domain was transiently expressed in *N. excelsior* under control of CaMV 35S promoter. Although starting with comparatively low accumulation level (about 1 % TSP), we were able to obtain homogenous native GFP after one-step purification procedure (data not shown). Although further studies are necessary for optimization of the expression protocol, we can conclude that IMPACT system may be useful for purification of recombinant pharmaceutically valuable proteins transiently expressed in plants in low amounts.

Conclusions

All three tested purification systems can be applied for obtaining of refined recombinant GFP from plant tissues. After optimization they can be considered for purification of other recombinant proteins produced in plants by means of *Agrobacterium*-mediated transient expression.

References

1. *Stoger E., Sack M., Fischer R., Christou P.* Plantibodies: applications, advantages and

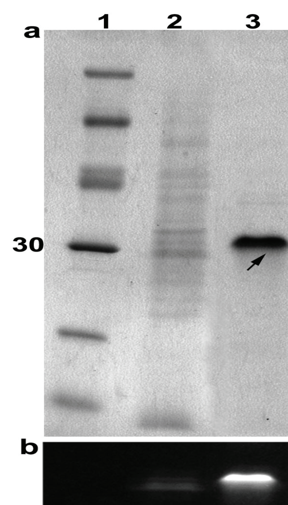

Figure 1. GFP-6xHis SDS-PAGE analyses: **a**) Coomassie staining of proteins (all samples except of marker without prior heat denaturation); 1 - molecular weight marker proteins, 2 – crude protein extract of *N. excelsior* infiltrated with GFP-6xHis; 3 – Ni-NTA purified protein; GFP is indicated with arrow **b**) GFP fluorescence in protein extracts without prior heat denaturation (under UV-light).

bottlenecks. // Curr. Opin. Biotechnol. – 2002. – Vol. 13. – P. 161-166.
2. *Larrick J.W., Thomas D.W.* Producing proteins in transgenic plants and animals. // Curr. Opin. Biotechnol. – 2001. – Vol. 12. – P. 411-418.
3. *Daniell H., Streatfield S.J., Wycoff K.* Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. // Trends in Plant Sci. – 2001. – Vol. 6. – P. 219–226.
4. *Gleba Y., Klimyuk V. Marillonnet S.* Magnifection – a new platform for expressing recombinant vaccines in plants. // Vaccine. – 2005. – Vol. 23. – P. 2042–2048.
5. *Marillonnet S., Giritch A., Gils M., Kanzia R., Klimyuk V., Gleba Y. In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium.* // PNAS. – 2004. – №101. – P. 6852–6857.
6. *Marillonnet S., Thoeringer C., Kandzia R., Klimyuk V., Gleba Y.* Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. // Nat. Biotechnol. – 2005. – Vol. 23. – P. 718–723.
7. *Sheludko Y. V., Sindarovska Y. R., Gerasymenko I. M., Bannikova M. A., Kuchuk N. V.* Comparison of several *Nicotiana* species as hosts for high-scale *Agrobacterium*-mediated transient expression. // Biotechnol. Bioeng. – 2007. – Vol. 96. – P. 608-614.
8. *Voinnet O., Rivas S., Mestre P., Baulcombe D.* An enhanced transient expression system in plants based on suppression of gene silecing by the p19 protein of tomato bushy stunt virus. // Plant J. – 2003. – Vol. 33. – P. 949–956.
9. *Sindarovska Y. R., Sheludko Y. V., Gerasymenko I. M., Bannikova M. A., Kuchuk N. V.* Purifikation of recombinant GFP produced by *Agrobacterium*-mediated transient expression in *Nicotiana excelsior.* // Cytology and Genetics. – Vol. 42 (N2). – (in press).
10. *Chiu W., Niwa Y., Zeng W., Hirano T., Kobayashi H., Sheen J.* Engineered GFP as a vital reporter in plants. // Curr. Biol. – 1996. – Vol. 6. – P. 325–330.

Summary

Three different approaches for purification of transiently expressed recombinant proteins from plants using GFP as a reporter have been successfully applied. The purification scheme including ammonium sulfate precipitation and anion-exchange chromatography was compared with two tag-based protocols applying metal affinity chromatography with a 6xHis tag and intein mediated purification with a chitin-binding affinity tag.

Три различных подхода для очистки рекомбинантных белков, полученных методом транзиентной экспрессии в растениях, были успешно применены с использованием GFP как репортера. Схему очистки, включающую преципитацию сульфатом аммония и анионообменную хроматографию, сравнивали с протоколами теговой очистки с использованием 6xHis-тега и хитин-интеин-опосредованной очистки.

Три різних підходи для очищення рекомбінантних білків, отриманих методом транзієнтної експресії в рослинах, було успішно застосовано з використанням GFP як репортера. Схему очищення, яка включала преципітацію сульфатом амонію і аніонообмінну хроматографію, порівнювали з протоколами тегового очищення з використанням 6xHis-тега та хітин-інтеїн-опосередкованого очищення.