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PURIFICATION OF RECOMBINANT GFP PRODUCED BY *AGROBACTERUM*-MEDIATED TRANSIENT EXPRESSION IN *NICOTIANA EXCELSIOR*

Green fluorescent protein (GFP) is commonly used as a reporter protein in a wide range of biological experiments. The efficient protocol of Agrobacterium-mediated transient expression in Nicotiana excelsior was applied for quick preparative production of recombinant GFP. The protein purification scheme has been developed and included ammonium sulfate precipitation and Q-sepharose anion-exchange chromatography. It results in obtaining of a fraction with about 85 % GFP homogeneity and the protein yield of about 75 %.

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Introduction. During the recent decade green fluorescent protein (GFP) has become one of the most popular *in vivo* marker molecule used in great variety of biological and medical experiments, e.g. researches on recombinant protein expression in different cell systems, studies on promoter activities, protein targeting, localization, kinetics and functional analysis of cytoskeleton and cytoskeleton-associated proteins etc [1, 2]. At the present time GFP gene can be regarded as a substitution of β-glucuronidase gene, which is widely used as a reporter (gene) in transformed plants. In contrast to GUS gene product, GFP can be directly monitored or quantified in living cells without destructive tests.

GFP is derived from jellyfish *Aequorea victoria* and contains a chromophore which does not require any substrates or cofactors for fluorescence. After cloning GFP has undergone substantial modifications that resulted in high expression rate, increased fluorescence, stability and low toxicity for a wide range of hosts, including plant cells [3–5]. The present GFP-based protocols include qualitative and quantitative analyses of GFP expression by detection of its fluorescence from the cellular level to the whole plant level [1, 2]. For correct quantitation considerable amount of purified GFP is necessary for building of calibration curve.

Transient expression of foreign genes is a recently developed method allowing production of large amount of recombinant proteins within a very short (days) time [6]. It occurs without stable integration of foreign DNA into the host genome. Our recent work describes optimization of protocol for rapid and high-scale production of recombinant GFP using transient expression in *Nicotiana* species [7].

Although numerous publications exist concerning recombinant GFP production and purification, most of them describe purification of GFP from bacterial source. It includes tag-based protocols [8, 9], which not always allow to separate improperly folded or cyclized GFP from correctly folded form of this protein [10], as well as size exclusion chromatography [11], chromatofocusing [12], organic extraction [11] etc. On the other hand, GFP production and purification from plant source under physiological conditions using ion-exchange chromatography may be regarded as an alternative method (including GFP purification from constitutively transformed plant tissue). In the present work we describe a scheme including

two stages of protein purification: ammonium sulfate precipitation and Q-sepharose anion-exchange chromatography. It results in obtaining of fraction with about 85 % GFP purity and the yield of the initial fluorescent protein of about 75 %.

Materials and methods. *Plant materials.* Seeds of *Nicotiana excelsior* J.M. Black were obtained from the National Germplasm Bank of World Flora of the Institute of Cell Biology and Genetic Engineering (Kyiv, Ukraine). Plants were grown in greenhouse at 20–25 °C and 14 h light period (3000–4000 lux).

Bacterial strains and genetic constructs. Constructs pICH10881, pICH10570 and pICH7410 represent a viral-based module system carrying GFP gene which is described in details in the recent publications [7, 13, 14]. The plasmid pICH6692 [15] contained the gene of the p19 protein of tomato bushy stunt virus, a suppressor of post-transcriptional gene silencing [16]. All the mentioned plasmids were generously donated by Icon Genetics GmbH (Halle/Saale, Germany).

Agrobacterium tumefaciens strain GV3101 transformed with individual constructs was grown overnight in LB medium supplemented with 50 mg/L rifampicin and 50 mg/L carbenicilin or kanamicyn, and 100 μM acetosyringone.

Transient expression assay. Plant infiltration was performed as it was described by Schob et al. [17] with several modifications [7, 13]: *A. tumefaciens* cells of overnight culture were centrifuged (5 min, 5000 g) and resuspended in the infiltration buffer (10 mM MES, pH 5.5; 10 mM $MgSO_4$; 100 μM acetosyringone). The *Agrobacterium* suspensions harboring different plasmid vectors were mixed in the equal volumes so that the final optical density (OD 600) of each suspension in the infiltration buffer amounted 0.5 for viral-based cassette (pICH10881, pICH10570, pICH7410) coexpressed with the suppressor of silencing (pICH6692). For typical assay the leaves of greenhouse grown *N. excelsior* plants were filled with *Agrobacterium* mixture by using a syringe without a needle. After infiltration, the plants were further grown under greenhouse conditions.

Protein extraction and precipitation. Accumulation of GFP in the infiltrated leaves was monitored at the 18th day after infiltration with a hand-held black ray lamp (UVP, Upland, USA) and the fluorescent areas were cut out. For typical assay leaf tissue (app. 15 g) was grounded in blender for 1–2 min with 75 mL of buffer A (100 mM KPi, pH 7.8; 5 mM EDTA; 10 mM β-mercaptoethanol). After filtration through 3 layers of nylon, extract was centrifuged at 10000 g for 15 min at +4 °C. The supernatant was collected and used for ammonium sulfate precipitation.

For protein precipitation, ammonium sulfate was added to the clarified extract (19.47 g $(NH_4)_2SO_4$ per 66 mL of solution) up to 50 % of saturation and incubated in ice for 1 h. After centrifugation (15 min, 10000 g, +4 °C) ammonium sulfate was added to the supernatant (9.4 g $(NH_4)_2SO_4$ per 74 mL of solution) to a final concentration of 70 % saturation. After 1 h incubation the solution was centrifuged again, and the precipitated proteins were dissolved in 5 mL of buffer B (20 mM KPi, pH 7.8; 2 mM EDTA; 10 mM β-mercaptoethanol). The protein extract was dialyzed two times against 2 L of buffer B.

Q-Sepharose chromatography. The dialyzed proteins were filtered through 0.44 μm membrane and applied onto a column filled with Q-Sepharose FF (Amersham Biosciences, Piscataway, USA; volume 11 mL) equilibrated with buffer B. After washing the column with 4 column volumes of buffer B, the proteins were eluted with a linear NaCl gradient (18 column volumes, 0–1 M NaCl) prepared from buffer B and C (20 mM KPi, pH 7.8; 2 mM EDTA; 10 mM β-mercaptoethanol, 1 M NaCl) at a flow rate of 3 mL/min collecting 5 mL fractions.

GFP analysis. GFP content was calculated by measurements of fluorescence intensity in dilutions of protein extracts using fluorescence spectrophotometer Hitachi 850 (Hitachi, Japan) (excitation at 395 nm, emission at 509 nm) on the basis of standard values (GFP standard was generously granted by Icon Genetics GmbH (Halle/Saale, Germany)). The background fluorescence of control extracts (from leaves infiltrated with bacteria carrying pICH6692 only) was subtracted from values of GFP containing crude protein 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 [18].

The SDS-PAGE analyses of protein extracts with Coomassie staining were carried out according to Blakesly and Boezi [19]. Crude protein extracts were mixed with 3 X loading buffer, boiled during 5 min and applied on 12 % gel.

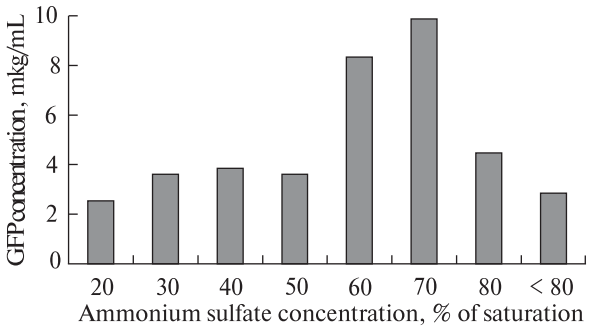

Fig. 1. GFP precipitation with ammonium sulfate

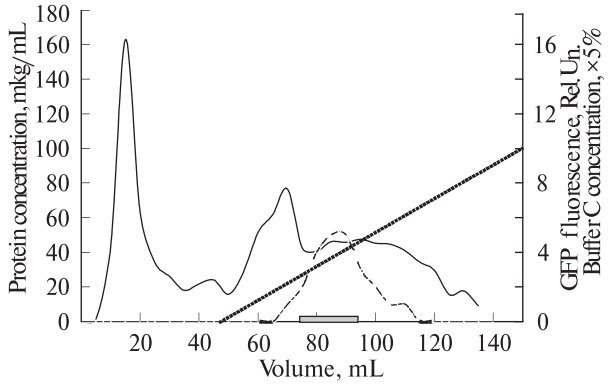

Fig. 2. Protein fractionation by Q-Sepharose chromatography. Solid line – protein concentration, μg/mL; dashed line – GFP fluorescence (Relative Units); dotted line – buffer C concentration, ×5 %; gray rectangle indicates collected fractions

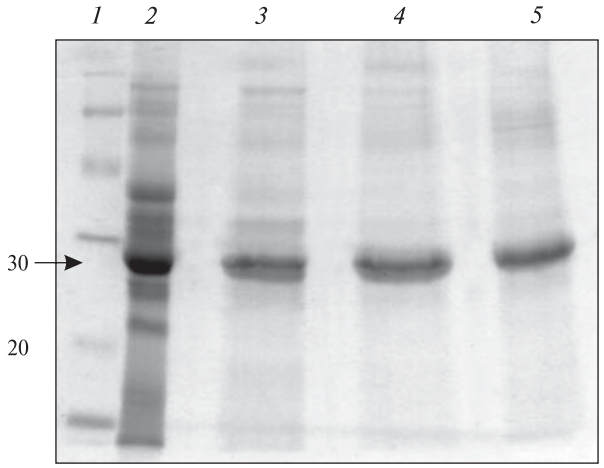

Fig. 3. SDS-PAGE analysis of protein fractions (Coomassie staining): *1* – molecular weight marker proteins, kDa; *2* – fractions after ammonium sulfate precipitation; *3–5* – fractions collected after Q-Sepharose chromatography. GFP position is indicated with arrow

For determination of relative abundance of GFP band on Coomassie stained SDS gel we used Gel-Pro Analyzer (v. 3.1.00.00, Media Cybernetics). Background correction: Join valleys with maximal slope 20.

Results and discussion. *Transient expression of GFP in N. excelsior.* In our recent publication we showed that as a host for *Agrobacterium*-mediated transient expression *N. excelsior* displayed the best characteristics in regard to biomass yield as well as GFP accumulation level for different types of the expression cassettes. For preparative production of recombinant GFP 2nd–4th upper leaves of greenhouse grown plants of *N. excelsior* were filled with *Agrobacterium* suspensions harboring different plasmid vectors mixed in equal volumes (viral-based module system which was described in details in the recent publications [7, 13, 14]). The p19 protein of tomato bushy stunt virus, a suppressor of post-translational gene silencing, was co-expressed with the GFP gene to increase the reporter protein accumulation [18].

In order to confirm that the effectiveness of the purification scheme does not depend on the 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 [7]). Leaf tissue of the infiltrated plants was harvested and extracted at the 18th day after infiltration. GFP content in the crude protein extract amounted to 3.3 % of the total soluble protein.

Protein purification scheme. For extraction of GFP from leaf tissue we used a buffer without additional protectors against proteases because GFP proteolysis resistance was described recently [10]. As the first purification stage we selected precipitation with ammonium sulfate. Ammonium sulfate precipitates cellular debris, ribosomes, and membrane fragments and stabilizes most of the proteins in solution. This stage has been used in several GFP purification schemes [10, 11, 20]. In our experiments we initially performed stepwise ammonium sulfate precipitation of GFP from crude protein extract in order to determine the optimal concentration range. We observed the highest GFP concentration in the fractions between 50 % (app. 2.3 M) and 70 % (app. 3 M) of ammonium sulfate saturation (Fig. 1). These data are in agreement with other protocols which have

Summary of GFP purification

Purification step	Total protein, mg	GFP content, % TSP	Yield, %	Purification factor (-fold)
Crude extract	30.19	3.26	100	1
Ammonium sulfate precipitation	5.4	16.38	89.94	5.03
Q-Sepharose chromatography	0.89	84.8	76.58	26.03

used salting-out conditions to fractionate GFP. For example, range 40–70 % of ammonium sulfate saturation was applied as an initial GFP purification step from *E. coli* protein extract [10].

At the next step we used anion-exchange chromatography. The dialyzed protein extract was applied onto a Q-Sepharose FF column. After washing the column, the proteins were eluted with a linear NaCl gradient and the highest GFP concentrations were observed at the fractions collected from 75 to 95 mL after start of chromatography (35–55 mL after start of elution that corresponds to app. 0.14–0.25 M NaCl concentration in the buffer, respectively) (Fig. 2).

The concentration of GFP in fractions during purification procedure was estimated by measurement of fluorescence intensity and comparison with standard values. The background fluorescence of the initial crude protein extract (from leaves infiltrated with bacteria carrying pICH6692 only) was subtracted from values of initial GFP containing extract.

The developed scheme of enrichment resulted in 26-fold purification of the recombinant GFP (Table). GFP was recovered in high (77 %) yield with about 85 % purity as it was calculated by comparison of GFP containing fraction fluorescence with the standard fluorescence means.

The collected GFP containing fractions were additionally tested with SDS PAGE (Fig. 3) and relative abundance of GFP band on Coomassie stained gel was estimated by densitometry analysis using Gel Pro Analyzer software algorithm. The obtained results are in good agreement with those calculated by fluorescence measurements: the average GFP homogeneity in the collected fractions was 85.1 % reaching in several fractions more then 90 %.

In conclusion, we developed the efficient protocol for production and purification of recombinant GFP from plant source using *Agrobacterium*-mediated transient expression.

The developed protein purification scheme included ammonium sulfate precipitation and Q-sepharose anion-exchange chromatography and integrates effective purification (85 % homogenity) with high protein yield (about 75 % of initial GFP content).

The described purification procedure was performed in gentle environment conditions (in comparison with several other protocols including organic solvent extraction [10] or high temperature precipitation [20]) 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.

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РЕЗЮМЕ. Зелений флуоресцентний білок (GFP) часто використовують як репортерний білок у різних галузях біологічних досліджень. Ефективний протокол *Agrobacterium*-опосередкованої транзієнтної експресії в *Nicotiana excelsior* було використано для швидкого одержання препаративної кількості рекомбінантного GFP. Розроблено схему очищення рекомбінантного білка, яка включає стадію осадження сульфатом амонію та іонообмінну хроматографію на сорбенті Q-sepharose. В результаті очищення було отримано фракцію білків з вмістом GFP близько 85 %. В ході очищення було отримано близько 75 % від початкової кількості рекомбінантного GFP.

РЕЗЮМЕ. Зеленый флюоресцентный белок (GFP) часто используется в качестве маркерного белка в разных областях биологических исследований. Эффективный протокол *Agrobacterium*-опосредованной транзиентной экспрессии в *Nicotiana excelsior* был использован для быстрого получения препаративных количеств рекомбинантного GFP. Разработана схема очистки рекомбинантного белка, включающая стадию осаждения сульфатом аммония и ионообменную хроматографию на сорбенте Q-sepharose. В результате очистки была получена фракция белков с содержанием GFP около 85 %. В ходе очистки было выделено около 75 % от начального количества рекомбинантного GFP.

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