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Expression of recombinant GFP-actin fusion protein in the methylotrophic yeast *Pichia pastoris*

Vladislav V. Verkhusha [a,1], Mikhail M. Shavlovsky [b], Olga V. Nevzglyadova [c], Andrey A. Gaivoronsky [c], Aleksey V. Artemov [c], Olga V. Stepanenko [c], Irina M. Kuznetsova [c], Konstantin K. Turoverov [c,*]

[a] *Center for Molecular Medicine, Building B, Moscow State University, Moscow 119899, Russia*
[b] *Institute for Experimenal Medicine, Russian Academy of Medical Science, St. Petersburg 197376, Russia*
[c] *Institute of Cytology, Russian Academy of Science, Tikhoretsky Ave., 4, St. Petersburg 194064, Russia*

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Abstract

The integrative vector pPIC3 for the yeast *Pichia pastoris* and a cDNA fragment encoding a fusion protein consisting of green fluorescent protein (GFP) and actin 5C of the fruit fly *Drosophila melanogaster* were used to construct a pPIC3-GFP-actin 5C expression plasmid. The *P. pastoris* host strain GS115 was transformed with the pPIC3-GFP-actin 5C carrying *HIS4* as a selective marker. The transformants were selected on a histidine-deficient medium, and were shown to contain the gene of GFP-actin 5C fusion protein. Expression was induced by cultivation of the transformant cells in a methanol-containing medium. Production of the fusion protein in the yeast was detected by the bright green fluorescence of the GFP tag. The pattern of yeast cytoskeleton labeling by the fusion indicated proper folding and functioning of GFP-actin 5C in a heterologous system in vivo. After cell destruction, purification of GFP-actin 5C was performed by DNase I-Sepharose. Efficient binding of the chimera to the DNase I indicated nativity of the actin 5C fusion in vitro. SDS electrophoresis and further Western blot confirmed the purified protein to exhibit the expected molecular mass of about 70 kDa. The recombinant GFP-actin 5C was used to produce polyclonal antibodies, which had not been reported so far but are extremely needed for immuno-labeling and isolation of wild-type and mutant forms of actin 5C.

Keywords: Recombinant actin; GFP-actin fusion; *Pichia pastoris*; Cytoskeleton labeling

1. Introduction

Actin is one of the major proteins of muscle tissues and the cytoskeleton of non-muscle eukaryotic cells [1–3]. At low ionic strength actin exists as a monomer (G-actin), but in the presence of neutral salts it is polymerized into a double-stranded polymer (so-called fibrous form of actin, F-actin). F-actin forms the backbone of thin filaments in muscle fibers. In the non-muscle eukaryotic cell it can exist both in fibrous and in monomeric form, though in vitro under physiological conditions it polymerizes. The polymerization–depolymerization processes are determined by its interaction with actin-binding proteins [3]. Actin is a remarkably conserved protein: the homology between α-actin from skeletal muscle and cytoplasmic β-actin from higher eukaryotes is more than 90% [4].

Due to the essential role of actin in generating motility of both muscle and non-muscle cells, the interaction of actin with regulator proteins is being extensively studied [1–3]. Great attention is also paid to studying the actin structure, its dynamics, structural changes and problems of its folding [5–12].

A powerful method for such kind of research could be the study of the mutant recombinant forms of the protein generated by directed mutagenesis [10–13]. Therefore, it has become an important task to establish a genetic sys-

* Corresponding author. Fax: +7 (812) 247 0341.
E-mail address: kkt@mail.cytspb.rssi.ru (K.K. Turoverov).

[1] Present address: Department of Pharmacology, University of Colorado Health Science Center, Denver, CO 80262, USA.

Abbreviations: GFP, green fluorescent protein; DNase I, deoxyribonuclease I (EC 3.1.21.1); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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tem for high-level production of recombinant actin. A cytoplasmic actin 5C from *Drosophila melanogaster*, which is more than 90% homologous to β-actin of higher eukaryotes, was chosen as a target protein. Its cDNA has been cloned and hence is available for recombinant manipulation [14–15]. An easily detectable tag for the actin 5C, namely a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, was utilized. In recent years GFP has been widely used to visualize protein expression and localization in tissues and living cells, as well as to monitor behavior and isolation of fusion proteins in biochemistry and biotechnology [16–18].

This work is aimed at verifying whether biosynthesis of functionally active heterologous actin 5C fused with GFP is possible in the methylotrophic yeast *Pichia pastoris*, to determine the optimal conditions for expression of the recombinant actin, to produce specific antibodies against it (not reported previously) and, finally, to establish a procedure for the purification of recombinant actin 5C in the native state.

2. Materials and methods

2.1. Construction of the integrative plasmid for expression in *P. pastoris*

For preparation of the GFP-actin 5C fusion construct, the pRmHa-3 plasmid [19] was digested with *Eco*RI and *Kpn*I, and PCR-amplified cDNA of GFP (Quantum Biotechnologies, Carlsbad, CA, USA) lacking a stop codon was introduced, resulting in pRmHa-3-GFP. A *Kpn*I-*Bam*HI fragment encoding the actin 5C with an additional 15 nucleotides in the upstream region was amplified by PCR as above, and was cloned downstream of the GFP sequence in the pRmHa-3-GFP, resulting in cDNA encoding a fusion protein, GFP-Gly-Thr-Ala-Ser-Ala-actin 5C. The PCR-amplified 1.9-kb *Bam*HI-*Eco*RI fragment of the GFP-actin 5C construct was introduced into pPIC3 vector (Invitrogen, Carlsbad, CA, USA), resulting in the pPIC3-GFP-actin 5C plasmid.

2.2. Transformation, selection and cultivation procedures

The *P. pastoris* GS115 host strain (His^-) was transformed by the pPIC3-GFP-actin 5C plasmid linearized at the *HIS4* region by a *Stu*I restriction enzyme (10–20 μg for probe). Transformation was performed by a modified lithium chloride procedure described for *Saccharomyces cerevisiae* [20]. Denatured fragmented salmon sperm DNA (25 μl for probe) in a concentration of 2 mg ml^{-1} was used as helper DNA. Transformants (His^+) were selected on histidine-deficient medium. pPIC3-GFP-actin 5C recombinant clones were studied by detection of the GFP fluorescence in the cells. Several selected colonies of transformants were cultured till the exponential-phase stage in buffered glycerol complex medium and after harvesting were resuspended in buffered methanol complex medium to induce the protein production. The content of the media was according to the '*pichia* expression kit' manual (Invitrogen, Carlsbad, CA, USA). To induce the expression, methanol was added till the final concentration of 0.5% every 24 h during 3 d of culturing. The level of protein synthesis was evaluated by yeast cell fluorescence and by immunochemical analysis of GFP. The samples for microscopic and immunochemical analysis were taken at different times of culture growth.

2.3. Fluorescence microscopy and image processing

P. pastoris cultures expressing GFP-actin 5C were analyzed by an Axioskop fluorescent upright microscope using the standard FITC filter set (Zeiss). Images of freshly cultured cells grown in the methanol-containing medium were acquired by a digital CCD camera VarioCam (PCO). Images were quantitatively analyzed by Quantum Image and assembled in Adobe Photoshop software.

2.4. Isolation and purification of fusion protein

After 72 h of methanol induction yeast cells were lysed mechanically by vigorous vortexing in the presence of glass beads (425–600 μm, Sigma) in buffer A (Tris–HCl 2 mM, ATP 0.2 mM, 2-mercaptoethanol 0.5 mM, $CaCl_2$ 0.2 mM, sodium azide 0.005%, pH 8.5) supplied with 1 mM phenylmethylsulfonyl fluoride (PMSF), a cocktail of protease inhibitors (in concentrations recommended by the supplier, Boehringer Mannheim), and acetone powder of rabbit actin muscles (0.03 g of powder per 1 g of cells). Then the material was centrifuged in the cold for 10 min at 10 000*g*. The supernatant containing soluble fusion protein was separated from host proteins by ion-exchange chromatography on DEAE–Sepharose. The column was washed 3 times with buffer A, and bound proteins were eluted by a 0.3-M linear gradient of NaCl. The GFP-actin 5C content of the fractions was determined by the green fluorescence as indicated below. The fractions containing the GFP were pooled and additionally purified by DEAE–Sepharose under the same conditions. Final purification was performed by large-scale native polyacrylamide gel electrophoresis (PAGE). Recombinant EGFP used as a control was expressed in *Escherichia coli* and purified exactly as described [21]

2.4.1. Preparation of DNase I-Sepharose

Sepharose 2B was sequentially washed with water, 30%, and 60% solution of acetone in water. An equal volume of 60% acetone was added, and the mixture was cooled down till −20°C by ethanol. Then 0.2 ml of triethanolamine in 60% acetone (150 mg ml^{-1}) and 0.2 ml of cyanogen bromide in acetone (100 mg ml^{-1}) were supplied per 1 ml to activate the Sepharose mixture. After 15 min of activation

the mixture was washed by 60% and 30% solution of acetone, followed by cold (0°C) acidic water (pH 2.0–3.0). Immediately after the activation an equal volume of deoxyribonuclease I (EC 3.1.21.1; DNase I; 5 mg ml^{-1}) was added, and the pH was adjusted to 8.5 by crystalline sodium bicarbonate. The coupling of DNase I with activated Sepharose progressed during 16 h at 4°C, and unbound protein was further washed out by buffer A with 0.14 M NaCl.

2.4.2. Binding of the fusion protein to DNase

After the yeast lysis and centrifugation described above, the supernatant was applied for affinity chromatography on DNase I-Sepharose. Following protein binding, the DNase I-Sepharose was sequentially washed by pure buffer A and buffer A containing 10% glycerol and 10% formamide, respectively, and the fusion protein was finally eluted with buffer A containing 10% glycerol and 40% formamide. To avoid actin denaturation due to prolonged incubation in 40% formamide, the protein was diluted 5 times with buffer A immediately after elution.

2.4.3. Co-polymerization of the fusion protein with actin from rabbit muscles

The ability of GFP-actin 5C to polymerize with native skeletal muscle actin was used to verify the nativity of the eluted fusion protein. The G-actin from rabbit muscles purified according to [22] was dissolved in the lysis supernatant of yeast cells in the ratio of 2 mg of muscle actin to 10 ml of cleared lysate, followed by the addition of KCl, $MgCl_2$ and ATP to the final concentrations of 50 mM, 2 mM and 1 mM, respectively. After incubation for 1 h at 0°C, the solution was centrifuged at 100 000*g*, and the precipitate was homogenized in buffer A. After the depolymerization of the actin mixture, the level of GFP fluorescence from GFP-actin 5C fusion was evaluated.

2.5. Characterization of the recombinant protein

The degree of GFP-actin 5C purification was determined by PAGE without or in the presence of sodium dodecyl sulfate (SDS) by the Laemmli method [23]. Western blotting was performed as described [24]. Rabbit polyclonal antibodies produced as described below were used as primary antibodies, and horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham, Little Chalfont, UK) as secondary antibodies. The fusion protein was also revealed by immuno-electrophoresis and by double immunodiffusion according to Ouchterlony [25].

The intrinsic tryptophan and green fluorescence of GFP-actin 5C were used to characterize the homogeneity, degree of purification, and correct folding of each fusion partner. Spectroscopic experiments were carried out using a spectrofluorimeter with steady-state excitation [26]. The intrinsic fluorescence of the actin 5C partner or the GFP fluorescence was recorded with excitation at 297 nm or 480 nm, respectively, and the emission spectra were further corrected for instrument sensitivity.

2.6. Production of polyclonal antibodies

The material purified by native PAGE was used for direct immunizations without extraction from the gel. The high molecular bands of 60–80 kDa with the green fluorescence were cut out, homogenized in phosphate-buffered saline, followed by addition of Freund's adjuvant. Immunization of rabbits was done by three injections with intervals of 2 weeks. The complete adjuvant was used only for the first injection. Blood was collected 10 d after the last immunization, incubated for 2 h at 37°C, and then centrifuged to obtain the serum. Immunoglobulins were precipitated by 1.75 M ammonium sulfate and, after elimination of the salt, lyophilized.

3. Results and discussion

It has been shown that native recombinant actin could not be obtained in a bacterial system [27]. Our own attempts to produce recombinant *Drosophila* actin 5C and a GFP-actin 5C fusion protein in an *E. coli* system under inducible T7 promoter had also resulted in their expression in the insoluble fraction and accumulation in the inclusion bodies (not shown). Therefore, in this paper we have attempted to verify an eukaryotic system for production of a GFP-actin 5C fusion protein in order to further use it for expression of actin 5C mutant forms alone.

It has been shown that chicken β-actin can be successfully produced in *S. cerevisiae* [28] at the level of 40 μg l^{-1}. Later, expression of the recombinant actin has been enhanced 40-fold, mainly due to the introduction of continuous cultivation, more efficient cell disintegration and probably by the increase of the volume of the DNase I-Sepharose column [29]. We decided to use the advantages of another lower eukaryotic organism, namely, the yeast *P. pastoris*. The features of protein processing, folding, and posttranslational modification (e.g., glycosylation) in *P. pastoris* are similar to those of higher eukaryotes. *P. pastoris* is a methylotrophic yeast, which can efficiently utilize methanol as the only carbon source, using two highly homologous genes for alcoholoxidases, *AOX1* and *AOX2*. The forced synthesis of alcoholoxidases occurs due to positive regulation by methanol, i.e. methanol activates promoters of *AOX* genes. In the pPIC3-GFP-actin 5C plasmid we have constructed the fusion gene of GFP-actin 5C is located downstream of the *AOX1* promoter, which allows the fusion gene to become activated in the presence of methanol as effectively as the intrinsic *AOX1*. The GS115 strain of *P. pastoris*, mutated for histidinol dehydrogenase (*HIS4*), was transformed by the pPIC3-GFP-actin 5C plasmid, which besides the target fusion had the intact gene of *HIS4*. Thus, selection of His^+ clones

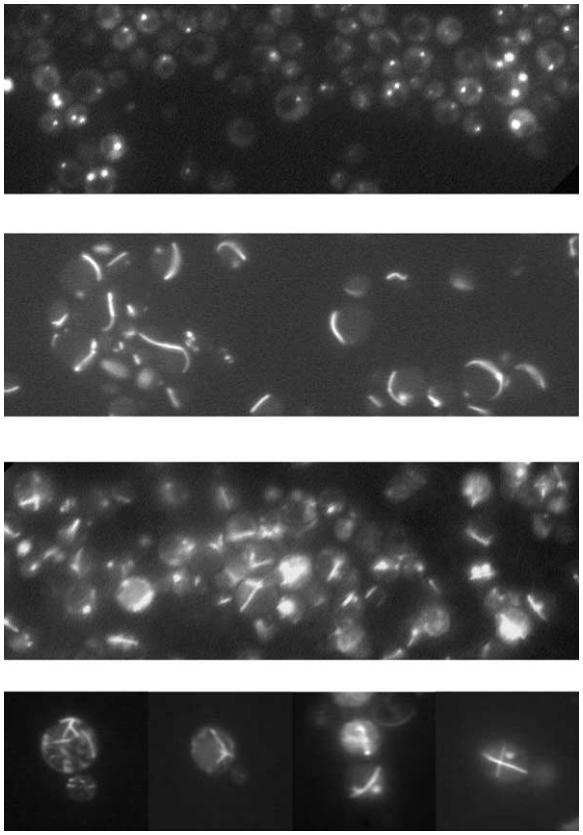

Fig. 1. Expression of GFP-actin 5C fusion protein in living cells of *P. pastoris*. Panels A, B, and C represent labeling of endogenous actin structures by the GFP-tagged *Drosophila* actin 5C after one, two and three days of growth in methanol, respectively. Panel D emphasizes incorporation of exogenous GFP-actin 5C into actin patches and cytoplasmic cables of yeast.

allowed the isolation of transformants carrying the integrated gene of the GFP-actin 5C fusion. Cultivation of the transformants in the presence of methanol as the only carbon source stimulated expression of the fusion protein. The bright green fluorescence of GFP allowed to visualize actin fusion synthesis and to control its induction and localization inside cells (Fig. 1).

It is known that in non-muscle cells the actin-based microfilament system forms structures, which re-organize in response to intra- or extracellular signals. Monomeric actin can reversibly polymerize into filaments forming a loose network or tight bundles. The filamentous actin of the yeast cytoskeleton has been found to localize into two morphologically distinct pools, patches and cables, which are similar to the pools of filamentous actin of other eukaryotic cells. Genetic studies have revealed the necessity of actin for both secretion and endocytosis, without an indication of particular roles for patches or cables.

Formation and enlargement of buds occur by polarization of the cytoskeleton and transport processes toward the selected bud sites. Polarized orientation of actin cables and patches is critical for the establishment of cell polarity [30–32]. Actin patches are labile structures, and their localization during establishment of cell polarity occurs by their assembly at the sites of polarized cell surface growth. Actin cables are bundles of actin that align along the axis of polarization. Patches and cables appear to depend on each other for assembly and function [33].

There are no viable yeast mutants that have no patches, but in a recent work one case of absence of cables was detected in a *tmpl* mutant [34], which suggests that cables might not be essential for viability. Moreover, because there was no evidence that the cables existed and remained stable without patches, one could also suggest that the assembly of cables might require patches. In our experiments we observed that both structures were efficiently labeled by GFP-actin 5C. Mobile actin patches observed at the exponential stage of growth were complemented later by labelled actin cables that were probably formed due to GFP-actin 5C co-polymerization with endogenous microfilaments. During the first day after methanol induction of GFP-actin 5C expression, the level of fusion protein was low. Approximately 20 h after the induction some cells and, particularly, buds did not manifest any fluorescence. At this stage of the culture, intensive proliferation resulted in uneven distribution of GFP-actin 5C; most of it was remaining in the older mother cells (Fig. 1A). However, 24–30 h after induction the number of cells exhibiting a high level of fusion protein had increased considerably, which resulted in clearly observed GFP-actin 5C polymerization (Fig. 1B and C). After 48–72 h of growth significant amounts of cross-shaped and branched structures were detected (Fig. 1D).

The fact that GFP fluorescence revealed the structures typical for endogenous cytoskeleton proved that the heterologous actin 5C was able to co-polymerize with yeast actin in vivo, showing the *Drosophila* actin 5C, as a part of

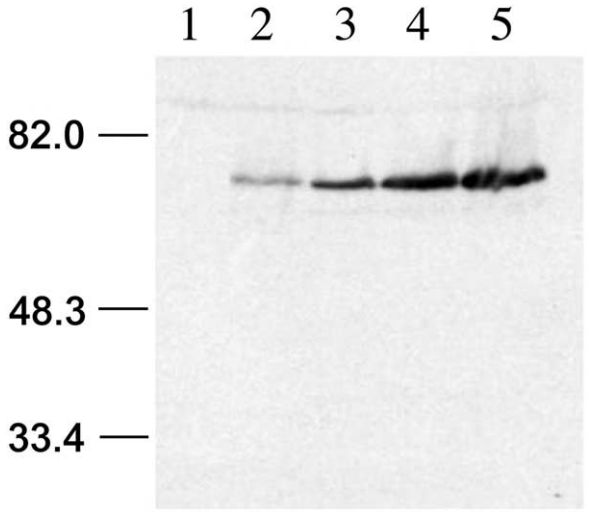

Fig. 2. Western blot of GFP-actin 5C fusion protein from *P. pastoris*. Yeast cells bearing pPIC3-GFP-actin 5C before (lane 1), or 12 (2), 24 (3), 48 (4), and 72 (5) h after induction of the protein expression, respectively, were analyzed. The optimal time for induction of the protein expression was estimated to be 48–72 h.

the fusion protein, to be functionally active. In transformants of *S. cerevisiae* carrying fusion protein actin-GFP, fluorescent patches but not visible cables have been found [35]. Observation of single living cells of *Dictostelium discoideum* expressing GFP-actin fusion has revealed that filaments containing up to 30% of GFP-actin exhibit almost normal sliding velocity and motility behavior [36]. On the other hand, filaments containing more than 30% of GFP-actin have been found to exhibit deficiencies in sliding motion in vitro, probably because the increased contents of GFP-tagged actin sterically disturbed actin-myosin interactions. Moreover, it is interesting to note that when GFP was fused to the C-terminus of the actin partner, only very weak expression was obtained, without distinct localization of the fusion protein to any specific structures within the cells [36]. It suggests that our N-terminally GFP-tagged construct of actin 5C fusion resulted in quite correct folding and rather complete functionality of the actin partner allowing it to incorporate into the yeast cytoskeleton.

Western blot with polyclonal rabbit antibodies showed that the amount of fusion protein with molecular mass of about 70 kDa was increasing with the time after induction (Fig. 2). The maximum was obtained 48–72 h after the first addition of methanol. It correlated with the results of microscopic observations. Immunological estimation proved the protein yield to be 3–4 mg l^{-1}.

The recombinant actin expressed in *P. pastoris* was further characterized by biochemical assays. The fusion protein GFP-actin 5C was extracted from disintegrated yeast cells by buffers usually used for the isolation of rabbit muscle globular actin. For extraction of the fusion protein we used DEAE–Sepharose chromatography (Fig. 3) that efficiently separated the immunoreactive and fluorescent material from the whole protein contents. Repeating of this chromatography step resulted in significant enrichment of the fluorescent fraction. Fig. 4 presents the SDS–PAGE of GFP-actin 5C obtained after DEAE–Sepharose in comparison with purified EGFP and muscle actin (lanes b, a, and c, respectively). The major band probably corresponds to the fusion protein, although material with lower molecular mass was also detected. Furthermore, some chromatographic fractions contained immunoreactive material with molecular mass equal to that of the EGFP control. It indicated that part of the fusion protein was proteolytically destroyed, which resulted in separation of actin 5C from the GFP tag (not shown). This suggested that although several inhibitors were added during the isolation procedures the degradation of the fusion protein was not prevented completely. Therefore, fast purification by large-scale native PAGE was utilized, which yielded the intact fluorescent fusion protein with about 95% purity. This recombinant protein was successfully used in immunizations for production of polyclonal antibodies.

To verify proper folding and nativity of the actin 5C partner as well as to attempt purification of the fusion protein in large quantities, an affinity chromatography

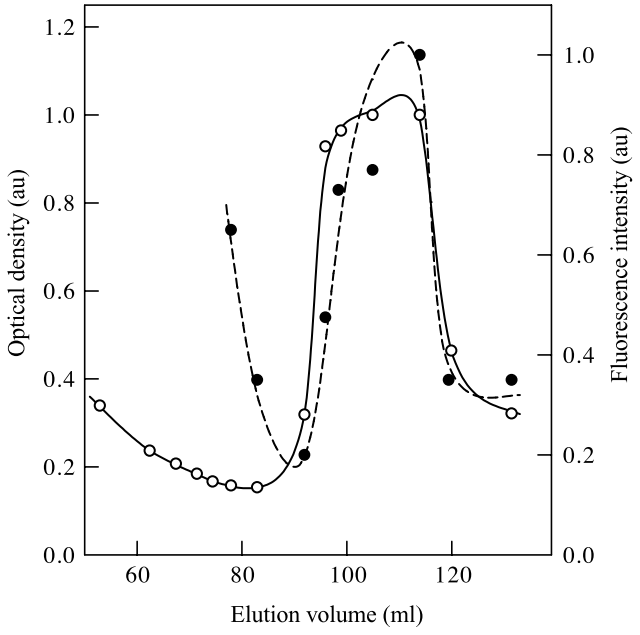

Fig. 3. Elution profile of cell lysate of GFP-actin 5C expressing *P. pastoris* after DEAE–Sepharose chromatography. Solid line is optical density at 280 nm; dashed line is fluorescence intensity at 512 nm (λ_{ex} = 365 nm).

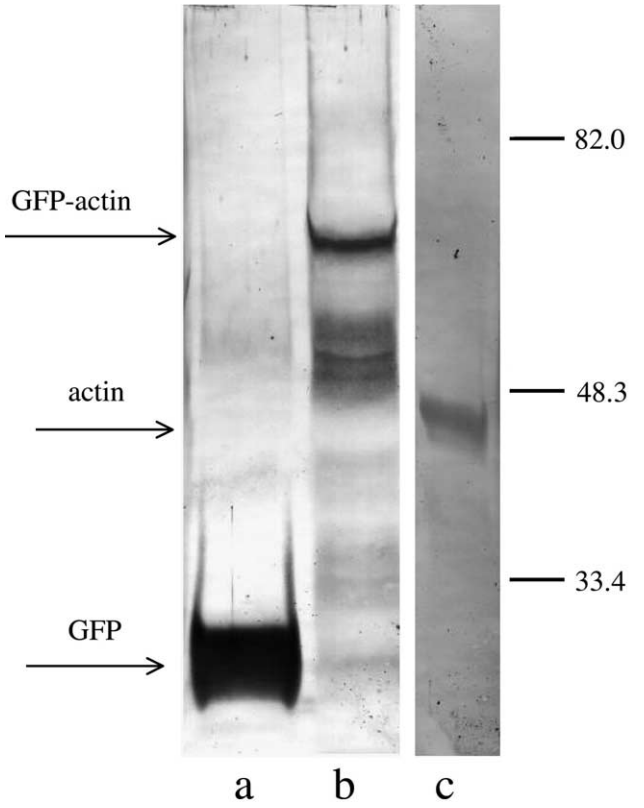

Fig. 4. SDS–PAGE of control EGFP (lane a), GFP-actin 5C fusion from *P. pastoris* (lane b), and rabbit muscle actin (lane c). The estimated size of the GFP-actin 5C band was consistent with the expected molecular mass of 70 kDa for the fusion protein.

on DNase I-Sepharose has been tried. The purification was based on the well-known phenomenon that native actin effectively binds pancreas DNase I. The procedure was performed according to the approach described in [10]. Indeed, when a yeast cell extract was filtrated through the DNase I-Sepharose, the protein fraction with green fluorescence bound to the sorbent. The protein was eluted by 40% formamide followed by immediate dilution to a formamide concentration below 10%. According to the fluorescence and immunofluorescence analysis, the eluted protein contained the GFP tag. PAGE and Western blot with polyclonal antibodies produced against the native GFP-actin 5C revealed that this was the fusion protein with molecular mass of about 70 kDa (not shown). However, spectroscopic analysis of the purified fraction detected a shift of the intrinsic fluorescence of the actin 5C partner to red wavelengths that was indicative for protein inactivation [11]. Control experiments with material from rabbit muscles have also shown that treatment by DNase I-Sepharose and formamide caused inactivation of muscle actin.

The results of this work demonstrate that *P. pastoris* expressed a significant amount of GFP-actin 5C fusion protein with a molecular mass equal to the sum of those of the components. *Drosophila* actin 5C fused with GFP had the native structure. This was confirmed by actin 5C binding to DNase I, by co-polymerization with the endogenous yeast actin in vivo, and with the rabbit muscle actin in vitro. Thus, *P. pastoris* can be successfully utilized as the producer of native recombinant actins and their mutants for biochemical and biotechnological applications. Furthermore, the produced polyclonal rabbit antibodies against the fusion protein will be used for labeling endogenous actin structures in *Drosophila* embryo and larva, as well as for detection of mutant forms of actin 5C in our future studies.

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