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Production of Hev b5 as a fluorescent biotin-binding tripartite fusion protein in insect cells

Henri R. Nordlund [a,*,1], Olli H. Laitinen [b], Sanna T.H. Uotila [a], Minna Kulmala [c], Nisse Kalkkinen [c], Markku S. Kulomaa [a,1]

[a] *Department of Biological and Environmental Science, NanoScience Center, P.O. Box 35, FIN-40014 University of Jyväskylä, Finland*
[b] *AI Virtanen Institute, Department of Molecular Medicine, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland*
[c] *Institute of Biotechnology, University of Helsinki, P.O. Box 56 (Viikinkaari 9), FIN-00014 Helsinki, Finland*

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Abstract

The presented green fluorescent protein and streptavidin core-based tripartite fusion system provides a simple and efficient way for the production of proteins fused to it in insect cells. This fusion protein forms a unique tag, which serves as a multipurpose device enabling easy optimization of production, one-step purification via streptavidin–biotin interaction, and visualization of the fusion protein during downstream processing and in applications. In the present study, we demonstrate the successful production, purification, and detection of a natural rubber latex allergen Hev b5 with this system. We also describe the production of another NRL allergen with the system, Hev b1, which formed large aggregates and gave small yields in purification. The aggregates were detected at early steps by microscopical inspection of the infected insect cells producing this protein. Therefore, this fusion system can also be utilized as a fast indicator of the solubility of the expressed fusion proteins and may therefore be extremely useful in high-throughput expression approaches.
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The fusion protein concept was introduced in the late 1970s when the expression of small peptide hormones as fusion constructs was described [1,2]. Two of the most important reasons to construct variable fusion proteins are: (1) a need to easily purify desired proteins from heterologous expression systems and (2) a demand to label protein components for detection particularly inside living cells and in intact organisms. Therefore, numerous different affinity purification and other fusion tag systems have been constructed for protein purification and visualization [3,4].

Green fluorescent protein (GFP) from *Aequorea victoria* is known for its ability to emit green light (peak at 509 nm wavelength) upon excitation with 395 nm wavelength. It is a considerably stable protein and its 3D structure is known. A GFP monomer consists of eleven β-strands and one α-helix, which is located in the middle of the β-strand barrel. Wild-type GFP can form a dimer composed of two identical monomers, each having a molecular mass of 25 kDa. Interaction between the two monomers is relatively weak and oligomerization does not affect the fluorescence properties of the GFP monomers [5]. In addition to the wild-type protein and its analogues from different species, different genetically modified GFP spectra variants are available [6–8]. GFP is widely used in applications and methods for visualization and localization of fusion proteins containing it [9–11].

Streptavidin from *Streptomyces avidinii* displays an extremely high affinity and specificity towards water-soluble vitamin-H (d-biotin). The dissociation constant of streptavidin–biotin complex has been estimated to be $\sim 10^{-14}$ M, which is among the strongest known non-covalent

* Corresponding author. Fax: +358 3 2157710.
E-mail address: henri.nordlund@uta.fi (H.R. Nordlund).
[1] Present address: Institute of Medical Technology, FIN-33014 University of Tampere, Finland.

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protein–ligand interactions in the nature [12]. Due to this tight affinity, streptavidin is a widely used protein tool in biotechnological applications generally known as the (strept)avidin–biotin technology [13]. Many applications of this methodology rely on biotinylated probes and detection molecules, such as enzymes and fluorochromes, conjugated either chemically or via biotin to the tetravalent (strept)avidin. However, these (strept)avidin conjugates usually show some disadvantageous structural heterogeneity and reduced biotin-binding activity. Moreover, the stoichiometry of streptavidin, specific probe, and detection molecule cannot be adjusted at a molecular level, leading to a diverse population of different combinations. Therefore, it is beneficial to fuse the partners to (strept)avidin by recombinant DNA technology and produce the desired fusion proteins in heterologous expression systems [14–21]. Apart from its use as a labeling partner, (strept)avidin fusion moiety provides an efficient way to purify the fusion protein in a single step by 2-iminobiotin affinity chromatography [19].

An ideal fusion tag would contain properties that allow easy purification of the construct as well as its fast detection without laborious labeling steps. In one approach, Wu et al. [22] described a His-GFP-tag that they fused to organophosphorous hydrolase. This construct was easy to visualize due to its GFP part and also the purification was straightforward because of the His-tag. However, the IMAC matrixes used for the purification of His-tag fusion proteins also bind naturally occurring histidine-rich proteins, and when purification is done from crude cell lysates these proteins contaminate the sample. In addition, His-tag can have detrimental effects on the fusion partner leading to a non-functional protein product [23,24]. Oker-Blom et al. [25] have described a GFP–streptavidin fusion protein that was produced in baculovirus-insect cell expression system with high efficiency. They were able to detect the fusion product through the GFP part, and they also demonstrated that it was possible to attach a probe molecule to the fusion protein via streptavidin–biotin linkage. In the present study, we have gone one step further and fused a third partner to the GFP–streptavidin core fusion complex and included protease cleavage sites to the construct as well (Fig. 1). Since the N- and C-termini in each streptavidin monomer are spatially close to one other at the closed end of the eight-stranded antiparallel β-barrel [26], both N- and C-terminal fusion partners are far from the biotin-binding site of streptavidin. The developed tripartite fusion system enables easy purification through the streptavidin–biotin linkage, visualization alternatively through GFP or streptavidin, and probing through the selected third fusion partner, for example, in analytical applications.

As model proteins we used Hev b1 and Hev b5, the two potent allergens present in the natural rubber latex of *Hevea brasiliensis*. Hev b5 is commonly known as a problematic protein to detect by conventional biochemical methods, i.e., SDS–PAGE and Western blotting [27]. The simple affinity chromatography purification via 2-iminobiotin–streptavidin interaction and easy visualization through the GFP part of the fusion constructs during the purification steps as well as the straightforward partitioning of the fusion protein by cleaving it within the engineered protease recognition sites are shown. Furthermore, we demonstrate that the successful production of Hev b5 was rapidly distinguished from the unsuccessful Hev b1 production by monitoring the GFP fusion protein distribution in insect cells at the very early stage of protein expression.

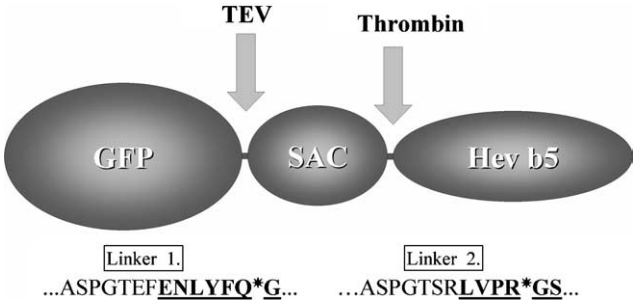

Fig. 1. Schematic representation of the tripartite fusion protein GFP–SAC–Hev b5. GFP and SAC are joined with the linker 1, which contains the recognition site (underlined and bold) for TEV protease. SAC and Hev b5 are fused to each other with the linker 2, which bears the recognition site for thrombin protease (underlined and bold). In both linkers, the asterisk (*) indicates the protease cleavage site.

Materials and methods

Construction of recombinant baculoviruses. The recombinant baculoviruses were generated according to the instruction of the manufacturer of the Bac-To-Bac baculovirus expression system (Gibco-BRL). The GFP coding region was PCR-amplified from a recombinant baculovirus genome having the GFP cDNA under polyhedrin promoter [25] with primers (forward) 5′-GGTGTGGATCCTATAAATATGAGTAAAGGAG-3′ and (reverse) 5′-ACACCGAATTCAGTTCCTGGGGAAGCCTTGTAG AGCTCATCCATGC-3′ (*Bam*HI and *Eco*RI sites underlined, respectively). The PCR product was digested with *Bam*HI and *Eco*RI, and ligated into a similarly treated pFASTBAC1 vector (serves as en expression cassette donor in the Bac-To-Bac system), which was designated as pFB_GFP. Coding region of streptavidin core (nucleotides 161–538, EMBL:X03591) was PCR-amplified from the pFASTBAC1-derivative containing the streptavidin gene with primers (forward) 5′-GGTGTGAATTCGAGAACCTGTACTTCCAGGGAGAGGCCGGCA TCACCGGCAC-3′ and (reverse) 5′-ACACCTCTAGAAGTTCCT GGGGAAGCGGAGGCGG CGGACGGCTTCA-3′ (*Eco*RI and *Xba*I sites underlined, respectively). The PCR product was digested with *Eco*RI and *Xba*I, and ligated into a similarly treated pFB_GFP vector and the resultant vector was designated as pFB_GFP–SAC. The coding regions of Hev b1 and Hev b5 were PCR-amplified using their cDNAs as templates. For Hev b1, we used primers (forward) 5′-GGTGTTCTAGACTG GTGCCAAGAGGCTCCGCTGAAGACGAAGACAACCA-3′ and (reverse) 5′-CAAATTAAGCTTTAATTCTCTCCATAAAACACC-3′ (*Xba*I and *Hin*dIII sites underlined, respectively) and for Hev b5 primers (forward) 5′-GGTGTTCTAGACTGGTGCCAAGAGGCTCCGCCAG TGTTGAGGTTGAATC-3′and (reverse) 5′-AATAGAAAGCTTTATT CCTCTGTTTTTTCCA CCG-3′ (*Xba*I and *Hin*dIII sites underlined, respectively). The PCR products were digested with *Xba*I and *Hin*dIII, and ligated into a similarly treated pFB_GFP–SAC vector. The final donor vectors were designated as pFB_GFP–SAC–Hev b1 and

pFB_GFP–SAC–Hev b5. The expression cassettes were transferred from the donor vectors to baculovirus genomes by site-directed transposition and the resultant recombinant genomes were used to transfect Sf-9 insect cells according to the instructions of the Bac-To-Bac system.

Production of proteins. The Sf-9 insect cells (ATCC CRL 1711) were maintained in SF-900 SFM (Gibco-BRL) in a 2 L Erlenmayer flask at 27 °C in an orbital shaker (125 rpm). Change of medium to biotin-free derivative of SF-900 preceded introduction of the recombinant baculoviruses. Cells were collected 72 h post-infection by centrifugation (1000*g*, RT, 10 min) and the cell pellet was stored at −70 °C freezer.

Protein purification. The frozen cell pellets were melted (RT, 20 min) and the cells from a 400 ml culture (2×10^6 cells/ml) were resuspended into 100 ml HILLO1 buffer (50 mM Tris–HCl, pH 8/1% Triton X-100/2 mM EDTA/150 mM NaCl). Incubation (30 min) and all the following steps were performed on ice unless otherwise stated. After that, the lysate was sonicated with Vibra cell microtip sonicator (Sonics and Materials) twice for 1 min with the following settings: 50% duty cycle, power set 4. The sonicated lysate was centrifuged (10,000*g*, 20 min, 4 °C) and the pH of the resultant supernatant was adjusted to 11 with 1 M NaOH and the NaCl concentration was brought to 1 M. In the next step, the supernatant was mixed with 2-iminobiotin agarose beads, which were previously balanced with pH 11 buffer (50 mM Na_2CO_3, pH 11/1 M NaCl) and incubated in a rotator (4 °C, 1 h). The resin was collected by centrifugation (500*g*, 5 min, 4 °C) and washed two times with 45 ml pH 11 buffer. After that, the resin was transferred to a column and washed further with 20 ml pH 11 buffer. Bound fusion proteins were eluted with 8 ml pH 4 buffer (50 mM Na-acetate, pH 4). Centricon YM-3 centrifugal filter devices (Millipore) were used to change the buffers of the purified fusion proteins. Cleavages with TEV and thrombin proteases were carried out in 50 mM Tris–HCl buffer (pH 8). The thrombin cleaved GFP–SAC–Hev b5 was subjected to cation exchange chromatography to get Hev b5 separated from GFP–SAC and thrombin. The sample was diluted with buffer A (20 mM citrate, pH 3.8) and applied to cation exchange column. Bound Hev b5 was eluted with buffer B (20 mM citrate, pH 3.8/1 M NaCl).

Characterization of purified proteins. Thrombin cleaved protein samples were analyzed with SDS–PAGE. The gel was silver stained according to the manufacturer's instructions (Bio-Rad). The band corresponding to Hev b5 was extracted from the gel and analyzed with N-terminal sequencing as described elsewhere [28]. Accessibility of the fusion partners for antibodies was studied in an ELISA. Protein A (50 μl, 10 μg/ml)-coated Nunc Maxisorb 96-well plate was blocked with 200 μl of 1% BSA in PBS. Polyclonal anti-GFP (Invitrogen), anti-streptavidin [29], anti-avidin [30], monoclonal anti-Hev b5, anti-Hev b6.02 or commercial anti-GFP (Invitrogen) was allowed to bind to protein A. In the subsequent step, the fusion protein GFP–SAC–Hev b5 (50 μl, 20 μg/ml) was applied into the wells after which biotinylated alkaline phosphatase (Sigma) was allowed to bind. After each step, the wells were washed three times with PBS–Tween and finally the substrate, PNPP in DEA buffer (Sigma), for alkaline phosphatase was added and the absorbance at 405 nm was measured after a 15 min incubation.

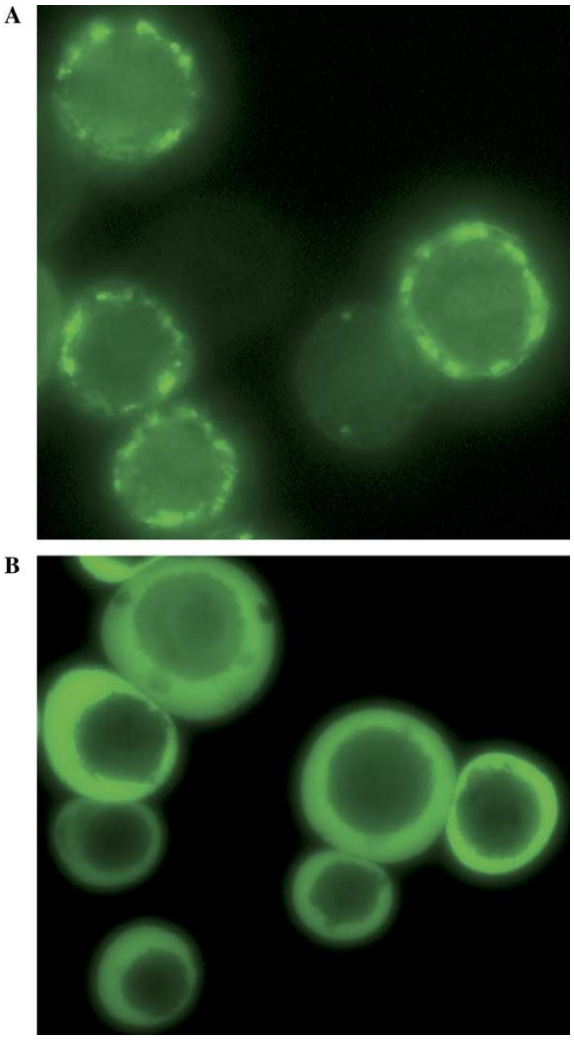

Fig. 2. Inspection of the insect cells expressing the fusion constructs with fluorescence microscope. GFP–SAC–Hev b1 expressing cells (A) contained large and bright aggregates, which indicated poor solubility of the construct. The cells expressing GFP–SAC–Hev b5 (B) showed evenly dispersed green color in cytoplasm and the protein was found to be soluble and purification by 2-iminobiotin affinity chromatography was straightforward and efficient in this case. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Results

Protein production

In order to produce viruses bearing GFP–SAC–Hev b1 or GFP–SAC–Hev b5 (Fig. 1) expression cassettes under polyhedrin promoter, the corresponding recombinant bacmids were transfected to Sf-9 cells by Cellfectin (Invitrogen). We were able to monitor the efficacy of transfection and the spread of infection when the recombinant baculoviruses were prepared. Expression of the tripartite fusion proteins could be detected easily at 48 h post-infection by fluorescence microscopy. Roughly 10% of the cells expressed the fusion proteins at detectable level after 72 h transfection culturing period (not shown). In successive larger-scale productions insect cells that expressed the constructs in suspension cultures showed a bright green color when exposed to UV light. In addition, the study of cells with a microscope showed that the GFP distribution in the cells expressing GFP–SAC–Hev b1 differed radically from the cells expressing GFP–SAC–Hev b5 (Fig. 2).

Affinity chromatography

The one-step affinity purification of GFP–SAC–Hev b5 was successful. It was completely soluble and the cell lysis supernatant contained all the detectable GFP-fusion

whereas no green color was detected in the insoluble fraction (Fig. 3A). The soluble fraction was subjected to purification on 2-iminobiotin affinity chromatography column as described in Materials and methods. Elution of the fusion construct by lowering the pH was easily tracked (Fig. 3B). After 2-iminobiotin resin purification, the fusion protein was subjected to a change of buffer using a Centricon YM-3 filter (Figs. 3C and D). In contrast to Hev b5 fusion, the Hev b1 fusion construct was found to be almost completely in the non-soluble fraction and therefore its purification failed (not shown).

Protease treatments

Functionality of the thrombin cleavage site in GFP–SAC–Hev b5 fusion protein was confirmed (Fig. 4). Cleavage was successful and bands of expected size were present on SDS–PAGE. Furthermore, the band corresponding to Hev b5 was sequenced N-terminally and the amino acid sequence confirmed that the digested band was composed of Hev b5 and that the cleavage occurred at the correct place leaving glycine and serine residues to the N-terminus of Hev b5 as expected according to the design of the construct (Fig. 1). Cleavage with TEV protease was successful as well (Fig. 4). Thrombin cleavage of the GFP–SAC–Hev b5 fusion was scaled up to liberate hundreds of micrograms of Hev b5. After that, the cleaved Hev b5 was recovered from the sample by cation exchange chromatography. Mass

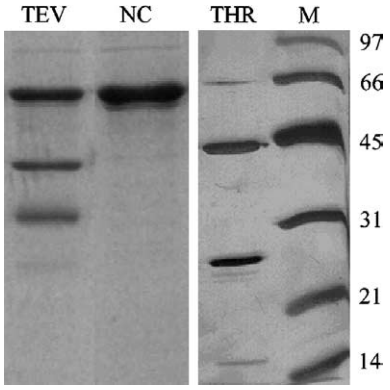

Fig. 4. GFP–SAC–Hev b5 treated with site-specific proteases. From left to right: a sample from TEV protease cleavage after 16 h. The lowest MW band in TEV sample is GFP (~30 kDa), the middle band is SAC–Hev b5, and part of the protein remained intact, despite the treatment. In the second lane is a negative control sample incubated without protease (NC). This gel was stained with Coomassie brilliant blue. On the right panel, a sample after 16 h thrombin cleavage in a silver-stained gel is indicated with THR. The ~25 kDa band was confirmed by mass-spectrometer to be Hev b5 and the ~43 kDa band is GFP–SAC.

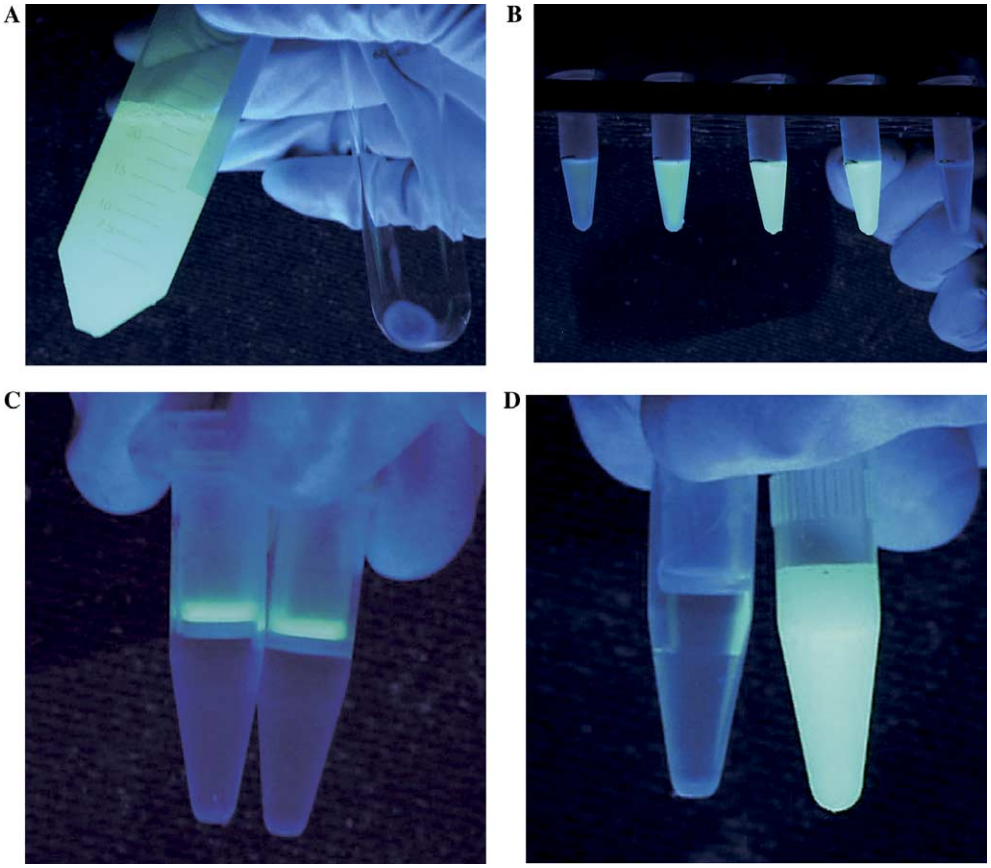

Fig. 3. Visualization of GFP–SAC–Hev b5 purification using UV-illuminator. (A) After cell lysis and centrifugation of the lysate, GFP–SAC–Hev b5 was found only in the supernatant transferred to a 50 ml Nunc tube, whereas the pellet in the Corex tube was not fluorescent. (B) Appearance of the fusion protein in the five eluted fractions was also easily detected. (C) Upon concentration, the fusion protein was again detected on the filter and not in the effluent below the filter. (D) Efficiency of the resuspension to a new buffer was also easily monitored.

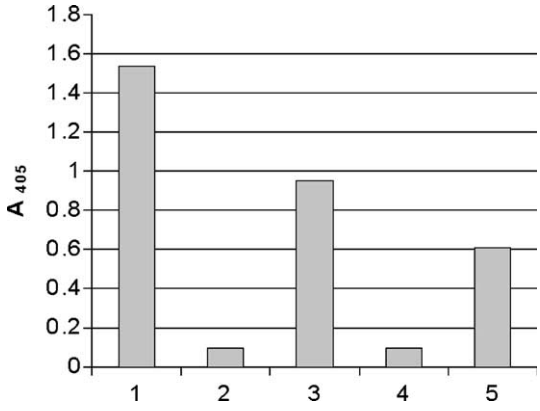

Fig. 5. ELISA of GFP–SAC–Hev b5. Antibodies for GFP (1), SAC (3) and Hev b5 (5) recognized their targets specifically. Samples 2 and 4 were negative controls and contained antibodies for Hev b6.02 (2) and chicken avidin (4). The signals obtained from these controls were essentially equal to that of a reagent control, which has not been subtracted from the data shown.

spectrometry gave a value of 16112.95 Da for the cleaved and cation exchanged Hev b5, which is in good agreement with its theoretical mass (16104.1 Da).

ELISA

Recognition of the purified fusion protein by antibodies was evaluated with an ELISA. All of the fusion partners (i.e., GFP, streptavidin core, and Hev b5) were found to be accessible for protein A immobilized antibodies, and the antibodies recognized their targets specifically (Fig. 5).

Discussion

Different fusion protein strategies have facilitated research in multiple fields of biological sciences. For example, the use of GFP fusion proteins has enabled the labeling of extracellular and intracellular structures and the tracking of particles, like viruses [31–33], inside living cells. On the other hand, the introduction of numerous fusion affinity handles has enabled the purification of hard-to-handle proteins, and their use has helped scientists gather such proteins that have previously been practically impossible to produce and purify [3]. The two most often used affinity handles are His-tag and GST [34], but (strept)avidin has also been utilized successfully as an affinity tag in numerous cases [18–21,35,36]. In addition, avidin-related proteins [30] and the recently characterized bradavidin [37] are alternative biotin-binding tags, displaying differences in the properties of stability, ligand binding, and immunological cross-reactivity as compared to (strept)avidin.

A usual problem in the production of recombinant proteins in heterologous expression systems is that although a strong expression can be achieved the produced proteins may not be biologically active. There may be several reasons behind this phenomenon like inappropriate post-translational modifications or proteolytic degradation. One of the most common problems is, however, the aggregation of the produced proteins [38,39], which may occur simply because the protein folding guidance systems of the host cannot handle such an enormous expression pressure which the strong promoter-driven recombinant protein production creates. Alternatively, lack of appropriate chaperones, altogether in the used expression host, may be the reason for protein aggregation. The solubility and functionality of fusion proteins have been scanned in GFP fusion protein systems by the intensity of green light that fusions were capable emitting [40,41]. We were also able to distinguish a successful and an unsuccessful case, but merely as a difference in GPF distribution in insect cells, instead of the intensity differences of the emitted light between functional and non-functional products containing cells.

Our successful product, Hev b5, can be regarded as a difficult protein to work with. The reasons for that are its considerably low p*I*, high proline composition, and the fact that it does not contain any aromatic amino acids [27]. Therefore, its detection in SDS–PAGE or Western blots is challenging. The deduced molecular mass and the mass measured by mass spectrometer for Hev b5 are ~16 kDa, although its apparent molecular mass in SDS–PAGE is around 25 kDa (Fig. 4). This difference is explained by its unusual proline-rich amino acid composition and its incomplete denaturation during sample preparation [27]. The fusion strategy described in the present study overcomes these problems and provides visibility due to the GFP-tag, and various possibilities for purification and detection via the streptavidin handle. In practice, the presence and localization of the Hev b5 fusion was easily verified during the different steps of the purification procedure (Fig. 3) without any special instruments (normal UV-light table found in every laboratory was used).

Since natural GFP can form dimers [42] and streptavidin is tetrameric [26], one could imagine that a fusion protein containing both of them would aggregate. In contrast, GFP–SAC–Hev b5 did not form aggregates, and it was efficiently purified by 2-iminobiotin agarose affinity chromatography. It is possible that steric hindrances, in this kind of complex fusion protein, would break the tetrameric structure of the streptavidin part. However, the affinity of a non-tetrameric streptavidin towards 2-iminobiotin would be so low that it would not be possible to purify such fusion efficiently by affinity chromatography using 2-iminobiotin agarose. Based on our results with monomeric avidin [43] and less tightly biotin-binding AVR forms [30], biotin agarose can be used to purify such low affinity forms. Therefore, we suggest that in this complex streptavidin forms the natural tetramer, which leaves two options for the GFP part. It either manages to form a dimer without disturbing the tetramerization and biotin-binding activity of the fusion complex or then the streptavidin part, Hev b5 part or the overall assembly of the fusion protein would block the GFP dimerization due to physical obstruction.

Both possibilities, however, can be functional because GFP dimers are not very stable and monomers themselves are also fluorescent. In contrast, GFP–SAC–Hev b1 fusion formed aggregates, which occurred probably because Hev b1 has a strong intrinsic property to form tetramers [44]. If monomeric forms of streptavidin [45,46] and a monomeric fluorescent protein tag [6] had been used instead of the wild-type forms, the third fusion partner could probably have been oligomeric without causing disadvantageous aggregations. Another option would be to replace streptavidin in this construct by stoichiometrically different, but high affinity biotin-binding avidin, forms, i.e., dual chain avidin [47] and single chain avidin (EMBL:AJ966780), which are topologically engineered avidin scaffolds.

In successful cases, our GFP–streptavidin visualization and affinity handle overtake a comparable system based on GFP and His-tag [22]. First of all, direct detection or quantification of the fusion protein through the GFP fluorescence could be possible in many applications with an ordinary fluorometer. The interaction between streptavidin and biotin is also markedly tighter and more specific than that between the His-tag and metal ions. Therefore, purification is more efficient when the streptavidin–biotin system is used. As a tight and stable affinity handle, streptavidin could also be utilized in the detection, quantification, and labeling instead of GFP also in challenging conditions. The signal could easily be amplified with branched biotin molecules [48] and with a second layer of (strept)avidin followed by biotinylated enzyme. Therefore, if the GFP moiety causes disturbance in any applications it is possible to cleave it off and leave the streptavidin part intact. One interesting application could also be immunomodulation, where suitable biotinylated adjuvant molecules, such as oligonucleotides, would be incorporated into SAC–allergen fusion and the complex would be administered to a patient.

In conclusion, we have shown that it is possible to fuse a third protein C-terminally to GFP–streptavidin core fusion construct and that the resultant complex can be produced in soluble and functional form in insect cells. We were able to produce the natural rubber latex allergen Hev b5 as a tripartite fusion protein. The purification of the fusion was performed in single step by utilizing the streptavidin–biotin linkage and the fusion was easily visualized during downstream procedures via the GFP part. On the other hand, the unsuccessful product, Hev b1, was easy to distinguish because it formed insoluble aggregates seen inside the insect cells. Therefore, this novel tripartite fusion protein system may be a valuable tool, for example, in high-throughput expression and purification approaches like library screenings.

Acknowledgments

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