

Avidin Is a Promising Tag for Fusion Proteins Produced in Baculovirus-Infected Insect Cells

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The baculovirus expression vector system (BEVS) has become one of the most versatile and powerful eukaryotic systems for recombinant protein expression. We have constructed a novel baculovirus transfer vector (pbacAVs+C) which allows for the efficient production, detection, and single-step purification of the desired molecule as a secretion-compatible avidin fusion protein in insect cells. It also enables fast construction of the baculoviruses by site-specific transposition in *Escherichia coli*. To demonstrate the power of this vector, we report here on the production of immunologically intact hevein, a major cysteine-rich latex allergen, as avidin fusion protein. Our results indicate that avidin is a stable and versatile tag in the BEVS. It retains its extraordinarily high biotin-binding activity and also enables independent folding of the fusion partner. The versatility with which avidin fusion proteins can be detected, purified, and immobilized is the basis for the use of our system as a useful alternative in eukaryotic fusion protein production. © 1999 Academic Press

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The baculovirus expression vector system (BEVS) provides an efficient way to produce large quantities of desired recombinant proteins (1). The insect cells which baculoviruses infect accomplish most eukaryotic posttranslational modifications (N- and O-linked glycosylation, phosphorylation, etc.) and are thus especially useful for production of proteins of eukaryotic origin. Recent improvements in constructing the recombinant baculoviruses have further increased the utility of BEVS (2).

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Chicken egg-white avidin is a glycoprotein (~63 kDa) consisting of four identical subunits (~15.7 kDa), each of which can bind one biotin molecule (3). Avidin is well known and a widely used tool for affinity-based separations, for diagnostic assays, and for a variety of other applications that have collectively become known as avidin–biotin technology (4). The ease of attaching biotin to most binders and probes without destroying their biological activity, the exceptional stability of the avidin–biotin complex ($K_d \sim 10^{-15}$ M), and the wide range of commercially available reagents provide the basis for the popularity of this system.

In order to combine the advantages of the versatile avidin–biotin system with production, detection, purification, and immobilization of recombinant proteins, we have reported previously successful production of C-terminal and N-terminal avidin fusion proteins in *Escherichia coli* (5). Although *E. coli* strains deficient in the biotin biosynthetic genes (6) can be used to avoid saturation of avidin by biotin during cell culture, biotin is essential for microbial growth. In contrast, the withdrawal of biotin does not seem to affect significantly the production rate of avidin in insect cells (7).

Hevein is a small (43 amino acids) cysteine-rich protein in the liquid latex of the rubber tree (*Hevea brasiliensis*; 8) and is reported to be a major allergen (Hev b6.02) in allergies to natural rubber latex products (9). We report here on the efficient production of secretion-compatible avidin–hevein (AvHev) in insect cells by the novel baculovirus transfer vector pbacAVs+C. The pbacAVs+C encodes avidin with its natural signal sequence and allows easy construction of avidin fusion proteins encoding baculoviruses (10). In addition to hevein, we have produced 10 mutated heveins (Karisola *et al.*, unpublished results) and some other proteins with this vector and the results indicate that pbacAVs+C may provide a general tool for facile production of avidin fusion proteins in BEVS. As a natu-

rally secreted and stable eukaryotic protein (3) avidin should also provide additional advantages over existing tags (11) for production, detection, purification, and immobilization of recombinant proteins which require transportation through intracellular compartments for correct folding and processing.

MATERIALS AND METHODS

Construction of Recombinant Baculovirus and Production of AvHev

The cDNA of preprohevein was synthesized from total latex RNA of the *H. brasiliensis* tree by polymerase chain reaction (PCR) and subcloned to get pIIProhev plasmid. The hevein coding fragment was further amplified from pIIProhev plasmid by PCR as previously described by Airenné *et al.* (7). Prohev6 (5'-TA TGT GGA TCC GAC GAC GAC GAC AAA GAG CAA TGT GGT CGG CAA GCA-3', *Bam*HI site underlined, enterokinase site in bold, hevein sequence in italics) was used as the forward primer and Prohev2 (5'-AAC ACA AGC TT **C TTA** GTC TTT GCA ATT GCT TTG GC-3', *Hind*III site underlined, stop codon in bold, hevein sequence in italics) as the reverse primer. The amplified fragment was subcloned into the pbacAVs+C-donor plasmid and recombinant viruses were then generated using the Bac-TO-Bac Baculovirus Expression System (GIBCO BRL, Gaithersburg, MD).

Production, Purification, and Characterization of AvHev

In order to produce AvHev, $\sim 5 \times 10^7$ Sf9 cells (ATCC CRL 1711) in SF-900 II SFM serum-free culture medium depleted of biotin and Pluronic F68 (GIBCO BRL; 041-94100A) were seeded to a final volume of 25 ml in a 250-ml Erlenmeyer flask. Recombinant viruses were added to give a multiplicity of infection of 2 pfu/cell. After 3 days of incubation at 27°C in a shaker at 125 rpm, the cells were pelleted by centrifugation (1000g, 22°C, 5 min) and the pellet was frozen under liquid nitrogen and stored at -70°C.

All the following steps were performed on ice unless otherwise stated. The cell pellet was resuspended in 12.5 ml of HILLO1 buffer (50 mM Tris-HCl, pH 8/1% Triton X-100/2 mM EDTA/150 mM NaCl). After 30 min of incubation the lysate was sonicated using a Vibra cell microtip sonicator (Sonics and Materials, Inc., Danbury, U.S.A.). The sonication was performed on ice in three 20-s bursts using a 50% duty cycle, power set at 3, and with a 1-min break between bursts. An aliquot of 100 μ l (referred to as the total cell protein sample, T) was taken, and the rest of the lysate was centrifuged (15,000g, 4°C, 10 min). After centrifugation an aliquot of 100 μ l was taken from the supernatant (referred to as the soluble fraction, S). The pellet

was resuspended in 6.25 ml of HILLO1 buffer and an aliquot of 100 μ l was taken (referred to as the insoluble fraction, I). The supernatant was mixed with 35 ml of binding buffer (50 mM Na₂CO₃, pH 11/1 M NaCl) and NaCl was added to a final concentration of 1 M. The crude recombinant AvHev mixture was then mixed with 2-iminobiotin-agarose, which had previously been equilibrated with the binding buffer. After slow mixing on a rotator at 4°C for 1 h, the resin was collected by centrifugation (500g, 4°C, 5 min), 20 ml of binding buffer was added to prewash the resin, and the centrifugation was repeated. Next, the resin was transferred to a column and washed three times with 10 ml of the binding buffer. An aliquot (100 μ l) was taken before (L, load) and after (F, flowthrough) affinity purification. The AvHev was eluted from the column with the elution buffer (50 mM ammonium acetate/0.1 M NaCl, pH 4) in eight 250- μ l fractions and stored at -20°C until further analyzed.

In order to purify AvHev from the medium, 12.5 ml of culture medium was mixed with 35 ml of binding buffer (50 mM Na₂CO₃, pH 11/1 M NaCl), and NaCl was added to make a final concentration of 1 M. The pH of the mixture was adjusted to 11 with NaOH and the sample was centrifuged (5000g, 4°C, 20 min). The AvHev was then purified from the clarified mixture as described above. A sample was taken before (L) and after (F) affinity purification.

Characterization of AvHev was basically carried out as previously described by Airenné *et al.* (7). A HiTrap desalting column (Pharmacia Biotech, Uppsala, Sweden) was used to exchange the AvHev-containing buffer for 50 mM Tris/50 mM NaCl (pH 8) before enterokinase cleavage. The cleavage was performed according to the instructions of the enzyme manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, about 0.6 μ g of affinity-purified AvHev in 12 μ l of 50 mM Tris/50 mM NaCl (pH 8) was treated with 0.6 μ g (in 2 μ l of H₂O) enterokinase, and an aliquot of 5 μ l was taken after 3 h or overnight incubation at 37°C. The immunoblotting was performed using 1:4000 diluted rabbit anti-avidin from Sigma (Sigma Aldrich Co., St. Louis, MO). The amount of eluted AvHev was determined by Bradford assay.

Interaction Analysis of AvHev by IASyS Biosensor

The IASyS Manual+ system (Affinity Sensor, Cambridge, UK) was used to evaluate the biotin-binding properties of AvHev. The association of avidin and AvHev (1×10^7 M) on the biotin surface of a cuvette was measured using PBS containing 1 M NaCl as a binding buffer. Dissociation was assayed with the same buffer in the absence of the proteins. The affinity of the proteins for the biotin surface and stability of the complexes were studied by 20 mM HCl and by an excess of free biotin.

Purification and Characterization of re-Hevein

Enterokinase-cleaved re-hevein (25 μ g in a volume of 700 μ l) was analyzed by reversed-phase chromatography on a PepRP 5/5 column (Pharmacia Biotech) using a linear gradient of acetonitrile (0–40% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min. Elution was monitored at 214 nm and the separated peptide was collected and further analyzed by N-terminal amino acid sequencing (using an ABI 494 A Procise sequencer; Perkin–Elmer Applied Biosystems, Foster City, CA) and mass spectrometry. Functional analysis was performed by IgE-ELISA and ELISA inhibition assays.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry was carried out using a Bruker Biflex II (Bruker-Franzen Analytik, Bremen, Germany). About 1 pmol (0.5 μ l) of the HPLC-purified peptide was applied to a thin-layer matrix preparation (0.5 μ l saturated α -cyano-4-hydroxycinnamic acid in acetone) and air-dried. External calibration was performed with insulin (human; Sigma).

For ELISA, microplates (Nunc, Roskilde, Denmark) were coated with the re-hevein and native hevein as previously described (9). Concentration of hevein during coating was 1.5 μ g/ml in 50 mM carbonate buffer, pH 9.6, for both preparations. Sera (diluted 1:10) from 16 latex-allergic patients (11 of them had previously been shown to have a specific IgE to hevein) and from 7 control subjects (with no latex allergy) were tested. In IgE-ELISA inhibition, the ability of the enterokinase-cleaved re-hevein to inhibit the binding of IgE to native hevein, immobilized on ELISA plates (1.5 μ g/ml), was assessed using patient sera with a high concentration of apparently monospecific IgE antibodies to hevein (9).

RESULTS

Construction of Recombinant Baculoviruses

As a first step to obtaining AvHev-producing baculoviruses, a PCR-amplified fragment, encoding a recognition site for enterokinase and the hevein cDNA sequence, was cloned between *Bam*HI and *Hind*III sites of pbacAVs+C vector (Fig. 1). The resultant plasmid was named pbAv+ent+hev. The pbacAVs+C vector was derived from pbAvt_r (7) by introducing a linker sequence C (5) encoding the hinge region of mouse IgG3 and a multiple cloning site (MCS) between the *Pst*I and the *Hind*III sites of pbAvt_r. The pbacAVs+C thus encodes for a secretion-compatible form of recombinant avidin (7) (i.e., the signal sequence and amino acids 1–123 of the mature avidin plus an extra Gln at the C-terminus) connected C-terminally to the IgG hinge region and MCS (5′-*Bam*HI, *Sma*I, *Sal*I, *Pst*I, *Hind*III-3′ in frame). All the above constructs were based on the pFastBAC1 donor plasmid and were designed for the construction of recombinant baculovirus

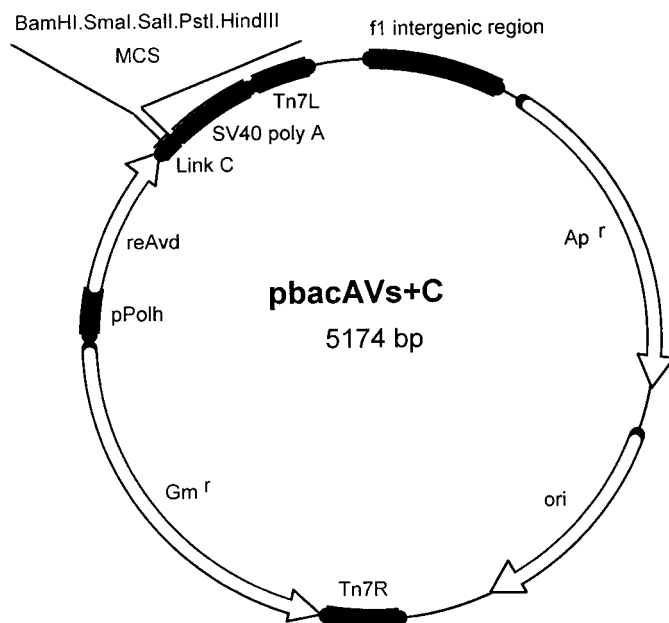


FIG. 1. Plasmid map of a baculovirus transfer vector pbacAVs+C. This transfer plasmid is a derivative of pFastBAC1 (Bac-TO-Bac system; GIBCO BRL), designed for the production and secretion of a desired avidin fusion protein in insect cells. The plasmid encodes for a secretion-compatible form of recombinant avidin (i.e., the signal sequence and amino acids 1–123 of the mature avidin plus an extra Gln at the C-terminus) connected C-terminally to linker C. Linker C contains the IgG hinge region and multiple cloning site (MCS) in frame (5′-*Bam*HI, *Sma*I, *Sal*I, *Pst*I, *Hind*III-3′).

genomes by site-specific transposition in *E. coli* (10) using the Bac-TO-Bac Baculovirus Expression System (GIBCO BRL). High-titer AvHev baculovirus stock was obtained by transfection of Sf9 cells with the constructed recombinant genome. Automated DNA dideoxynucleotide sequencing (ALF; Pharmacia Biotech) was used to confirm all the cloned sequences.

Production and Purification of AvHev

Three days after infection a protein near the computer-predicted size of glycosylated AvHev (~22 kDa; 20.2 kDa plus 10%, because avidin has one glycosylation site; 3) was detected by immunoblotting of samples derived from recombinant baculovirus-infected (pbAv+ent+hev) Sf9 cells (Fig. 2B). The discrepancy between the predicted and the observed migration (~28 kDa) of the band in the SDS-PAGE proved to be a gel artifact. According to the MALDI-TOF mass spectrometry the mass of purified AvHev was 21,738 $\pm$ 162, which is in good agreement with the predicted mass. The band was absent from cells infected with wild-type virus (Fig. 2B, lane WT). In addition to this major band, other forms of AvHev were also identified (Figs. 2B and 3, lane 4). The major band was detected by antibodies raised against avidin (Fig. 2B) and hevein (data not shown), which indicated that the fusion pro-

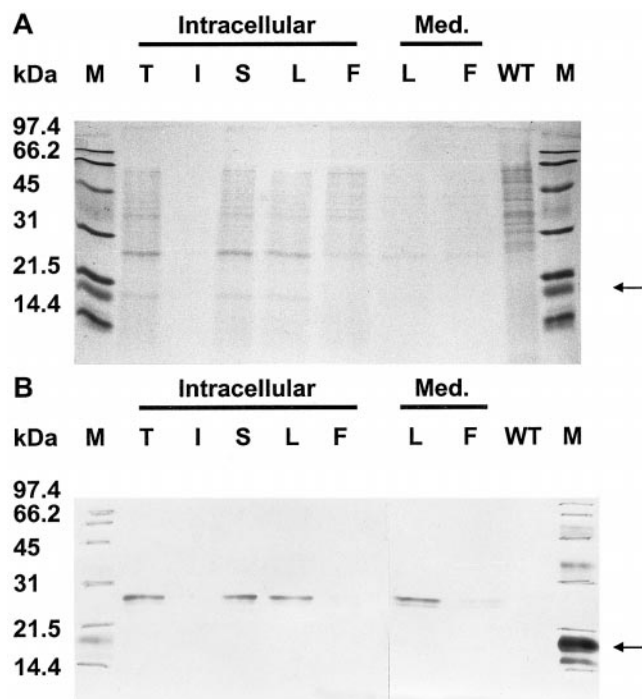


FIG. 2. Synthesis and purification of AvHev. (A) Coomassie-blue stained 15% SDS-PAGE gel showing samples from cell pellet and culture medium during different stages of AvHev purification. (B) Anti-avidin immunoblot from A. Each intracellular (cell pellet) and medium sample is from the same batch and normalized to correspond to 1×10^4 or 0.76×10^4 ($3.8 \mu\text{l}$ of culture medium) Sf9 cells, respectively. Lanes: M, molecular weight marker plus $1 \mu\text{g}$ of egg-white avidin ($\leftarrow$); T, total cell protein from recombinant virus-infected Sf9 cells; samples from insoluble (I) and soluble (S) fractions of the lysed recombinant virus-infected Sf9 cells; AvHev crude extract sample before (L, load) and after (F, flowthrough) application to a 2-aminobiotin-affinity purification; WT, total cell protein from wild-type (pFastBAC1-encoding virus) virus-infected Sf9 cells.

tein comprised antigenic determinants for both components. Like re-avidin (7), AvHev seemed to be secreted into the culture medium (Fig. 2B, Med.).

The AvHev was purified from the crude cell extract as well as from the culture medium by affinity chromatography on a 2-aminobiotin-agarose column (12). In order to enable efficient recovery of the AvHev, biotin and Pluronic F-68 were excluded from the culture medium. As a result, about 0.25 mg of highly purified fusion protein (Fig. 3, lane 4) was obtained in a single step from 25 ml culture containing $\sim 5 \times 10^7$ cells ($\sim 10 \text{ mg/L}$). Of this about 30% was from the culture medium and 70% from the cell pellet. An efficient yield was obtained from both the intracellular (cell pellet) and the extracellular (medium) source as can be seen by comparing lanes L and F in Fig. 2. The lanes show identical samples of AvHev crude extract before and after affinity purification by 2-aminobiotin-agarose, respectively. Most of the intracellular AvHev was soluble after disrupting the cells into lysis buffer (Fig. 2B, lane S).

Characterization of AvHev

Affinity-purified AvHev separated into two clearly distinct bands in SDS-PAGE after affinity purification (Fig. 3, lane 4), of which the upper band was near the calculated molecular weight of glycosylated AvHev ($\sim 22 \text{ kDa}$). The lower molecular weight band was close the calculated molecular weight ($\sim 15.7 \text{ kDa}$) of glycosylated egg-white avidin (Fig. 3, lane 1) and most probably reflects the avidin component as a stable degradation product of AvHev. In addition to the two distinct bands, several minor bands adjacent to these were detected (Figs. 2B and 3, lane 4). In order to further characterize these bands, the AvHev was deglycosylated with PNGase F glycosidase (Fig. 3, lane 6; the upper band represents the PNGase F). The PNGase F treatment affected the electrophoretic mobility of bands, which indicates that the majority of the purified AvHev and degradation product (avidin) was glycosylated. This is in a good agreement with our previous studies on re-avidin production by BEVS (7).

Egg-white avidin is a tetrameric protein composed of four identical subunits (3). In order to study whether avidin subunits connected to hevein are still capable of forming tetramers, the quaternary state of AvHev was studied by SDS-PAGE (13) and most of the AvHev seemed to be in the tetramer or oligotetramer state (Fig. 3, lane 5). The nature of the tetramers was not studied further, but they may well represent a mixture of avidin, AvHev, and their heterotetramers due to the fact that the hevein part of avidin seemed to be prone to proteolysis.

Properties of AvHev were also studied by real-time optical biosensor technology (IASyS; Affinity Sensor) (Fig. 4). The binding of purified AvHev to the biotin

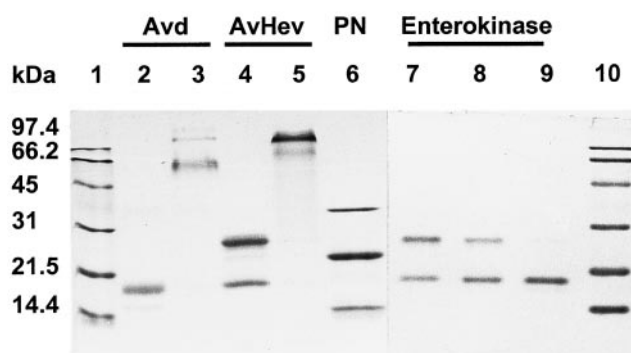


FIG. 3. Characterization of AvHev on Coomassie blue-stained 15% SDS-PAGE gel. The molecular weight markers are shown in lanes 1 and 10. Lanes 2 and 3 and lanes 4 and 5 show quaternary state of acetylated avidin (Avd) and AvHev after treatment for 5 min at 97°C or at room temperature, respectively. Lane 6 shows deglycosylated (PNGase-treated, PN) AvHev. Lane 7 shows AvHev control after overnight incubation in 50 mM Tris/ 50 mM NaCl (pH 8) at 37°C in the absence of enterokinase. Lanes 8 and 9 show AvHev after 3 h or overnight incubation in 50 mM Tris/ 50 mM NaCl (pH 8) at 37°C with enterokinase, respectively.

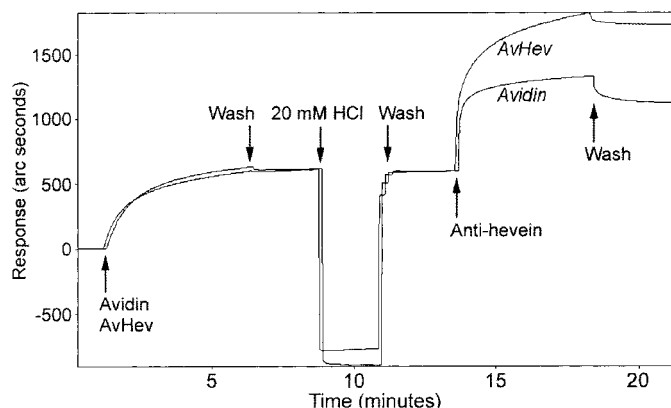


FIG. 4. Real-time binding assay of AvHev onto biotin surface. Association of avidin and AvHev at a concentration of 1×10^{-7} M in binding buffer (PBS, 1 M NaCl) onto a biotin surface was measured by an IASys Manual+ instrument. Dissociation was analyzed by the binding buffer or by treatment with 20 mM HCl. The presence of hevein on the surface was observed by specific binding of anti-hevein. An arrow shows the change in the solutions ($\downarrow$). The drop in response (arc seconds) after the addition of 20 mM HCl was due to the different reflection properties of HCl (compared to the binding buffer), and the equal response was recovered by the binding buffer.

surface of an assay cuvette was both rapid and irreversible at concentrations of 1×10^{-7} M, thus closely resembling commercial chicken egg-white avidin. We were unable to dissociate the proteins from the biotin surface by 20 mM HCl or by an excess of free biotin. The AvHev bound to the biotin surface was specifically recognized by anti-hevein after the HCl or biotin treatment. This strongly suggests that the biotin-binding property of avidin tag (in AvHev) is equivalent to that of native egg-white avidin.

In order to cleave hevein from the avidin moiety, a proteolytic cleavage site for Factor Xa, enterokinase, thrombin, and the tobacco etch virus (TEV) was constructed between the C-terminus of avidin and the N-terminus of hevein. AvHev was cleavable by enterokinase (Fig. 3, lanes 8 and 9) and by thrombin, but it seemed to be resistant to Factor Xa and TEV protease cleavage (results not shown).

Characterization of Enterokinase-Cleaved re-Hevein

In reversed-phase HPLC the enterokinase-cleaved hevein eluted after 20 min as a single narrow peak with base-line separation. N-terminal sequencing of the recombinant hevein revealed the sequence DDD-KEQCGRQAGGKLCPPNNLCCSQ. This indicated that the recombinant hevein contained four extra amino acids in front of the published N-terminus of hevein (14). This was also confirmed by MALDI-TOF mass spectrometry in which the purified recombinant hevein gave a molecular mass of 5191.8 Da. The determined molecular mass was equal to the mass of native hevein (4727.2 Da) with disulfide bridges plus the mass of the sequence DDDK.

In ELISA, the binding of IgE in sera from latex-allergic patients and control subjects both to the recombinant and to the native hevein correlated highly significantly ($r = 0.92$, Pearson's product moment correlation) (data not shown). The ability of the re-hevein (in solution) to inhibit the binding of hevein-specific IgE to native hevein (on solid phase) was assessed. Compared to native hevein, identical inhibition curves were observed (Fig. 5). This indicated that the main IgE-binding epitope(s) of native hevein was present also in the re-hevein and that in terms of their immunological properties, both preparations appeared to be identical.

DISCUSSION

Streptavidin (streptAv) is a bacterial relative of avidin (3) and the cytoplasmic production of luciferase-streptAv and green fluorescent protein-streptAv fusion proteins in BEVS has recently been described (15,16). However, streptAv seems not to be secretion-compatible in this system (not at least with its natural signal sequence, unpublished results). We have thus constructed a novel baculovirus transfer vector (pbacAVs+C) which allows for the efficient production, detection, and single-step purification of secretion-compatible avidin fusion proteins in insect cells. In order to facilitate the construction of desired baculovi-

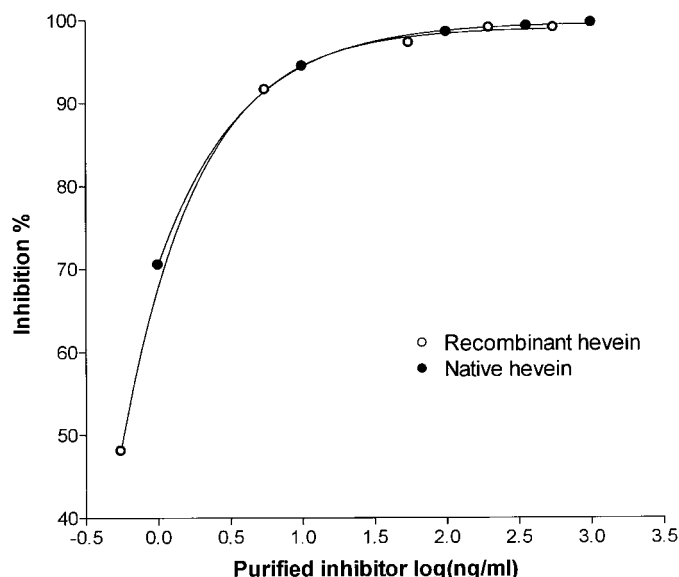


FIG. 5. ELISA analysis of inhibition of binding of anti-hevein IgE to native hevein. Microtiter wells were coated with native hevein (1.0 μ g/ml). Increasing concentrations of the inhibitors (native hevein and recombinant hevein) were incubated with equal volumes of a constant dilution (1:10) of serum from a patient with seemingly monospecific IgE to hevein. The mixtures were then applied to native hevein-coated wells and the binding of IgE was detected by biotinylated anti-human IgE and streptavidin-conjugated alkaline phosphatase. Enzyme activity was assayed using *p*-nitrophenyl phosphate.

ruses by site-specific transposition in bacterial cells, the pbacAVs+C vector was derived from the pFast-BAC1-vector. Moreover, to enable the removal of desired proteins from the avidin tag with their genuine N-termini, the construct was left without any built-in recognition site for site-specific protease, nor does it have built-in stop codons to ensure the native or desired C-terminal end of the fusion partner. Both the protease cleavage-site and the stop codon(s) can easily be attached to the fusion protein by adding the appropriate sequences into the PCR primers used for cloning. As clearly shown in this study by the production of AvHev, pbacAVs+C provides an efficient tool with which to combine the power of the baculovirus system with the multipurpose avidin tag.

Avidin meets most criteria for an ideal protein tag. It is relatively small, soluble in water, very stable, and secretion compatible. It can also be considered a versatile multidomain tag (17), since it can be used both as a specific label and as an affinity handle. In this study, avidin was successfully used as an antigenic domain and purification tag. AvHev could, however, also have been detected by the biotin-binding activity. A wide variety of biotinylated reagents, such as biotinylated alkaline phosphatase or biotinyl-FITC, are commercially available for this purpose. Similarly, the purification of AvHev could have been accomplished using a biotin column instead of 2-iminobiotin. This would not, however, have allowed the release of AvHev from the column, since avidin binds to biotin in an irreversible manner ($K_d \sim 10^{-15}$ M) (3). On the other hand, for some applications the irreversible nature of the binding offers a unique advantage by enabling the tight interaction of a desired fusion protein with different matrixes in an oriented and stable manner.

The general use of 2-iminobiotin for the purification of avidin fusion proteins might be restricted, since pH 9.5 (or higher) is needed for tight binding and relatively low pH 4 (or below) is required for the efficient elution of avidin from the column (12). The elution can, however, be performed at any pH value by using biotin as an eluent, but this will destroy the biotin-binding activity of the avidin tag. This is not a problem if biotin-binding activity is not needed after purification, since biotin does not affect the cleavage of the fusion partner from the avidin tag or antigenic properties of avidin. For this kind of application, biotin or biotin derivatives, such as S-S-biotin (18) or photocleavable biotin (19), might also offer an additional method of purification.

Our results demonstrated that AvHev formed tetramers, since its migration in SDS-PAGE, its association rate with the biotin surface (IASyS), and its stability properties were identical to those of the avidin tetramer. This indicates that the avidin tag folds correctly and acts as an independent N-terminal domain in the fusion protein. The observed difference of 8.8

mass units between the determined (5191.8 Da) and the calculated (5200.6 Da) molecular mass of re-hevein supports the idea that four disulfide bridges were formed in the re-hevein, thus also resulting in intact hevein.

The irreversible and oriented nature of AvHev binding to biotin surface should enable construction of biosensor cuvettes (IASyS) or ships (BIACore) as well as ELISA plates for analyses of patients with potential latex allergy. Indeed, one major advantage of using avidin as a fusion tag is that it can be used to form a stable binding between the desired fusion partner and different biotin surfaces in an oriented manner.

The small size of hevein (4.7 kDa) might partially explain the high efficiency of the avidin tag for purification seen in this study. Indeed, the size of the fusion partner is known to affect the purification efficiency of fusion tags (20). This may well also concern avidin.

The N-terminal sequencing of the purified hevein following cleavage by enterokinase revealed that it had four extra amino acids (DDDK) in the N-terminus. This was somewhat surprising since the enterokinase cleavage site (. . . GSSGSDDDDK ↓ EQCGR . . .) was used between avidin and hevein in order for the protease to cleave hevein in the native form from AvHev. The reason for unexpected cleavage is not clear. The cleavage is, however, clearly a consequence of enterokinase treatment, since incubation of AvHev in the absence of enterokinase did not cleave the protein. Interestingly only calf intestine enterokinase was able to cut AvHev. The antigenic properties of the DDDK-hevein were, however, indistinguishable from native hevein, which makes it (and its mutated forms) an interesting subject for detailed molecular studies of the immunological and allergic properties of hevein (9).

Despite the fact that hevein is considered a stable protein (9), it seemed to be prone to proteolysis in the insect cell system. Only small amounts of hevein were obtained when produced alone (results not shown). Although the avidin tag allowed for the efficient production of hevein, a part of the affinity-purified fraction continued to represent avidin as a stable degradation product of AvHev. Several reasons can tentatively be proposed to explain the instability of re-hevein. First, hevein is naturally synthesized as a preproprotein that is posttranslationally cleaved into amino-terminal hevein and the carboxy-terminal domain. This may protect hevein from "unnatural" proteolytic attack while it is being produced in the laticifer cells of the rubber tree (21). Second, the deleterious effect of baculovirus infection of insect cells may lead to proteolytic enzymes having access to the synthetic route of AvHev, an event which does not occur in healthy rubber tree cells. Third, it is well known that small proteins may be especially vulnerable to proteolytic attack when produced as recombinant forms, and their stability may be increased when produced as fusion proteins (22).

In addition to hevein, we have also produced 10 mutated heveins as avidin-tagged fusion proteins (Karisola *et al.*, unpublished results), and the results are in accordance with those reported in this paper. We have also gained good results from production and purification of glutathione *S*-transferase with our system. The purification of barley endoproteinase A (EPA) by avidin tag was not as successful, although AvEPA was easily detected in insect cells by avidin antibodies due to the avidin tag. For general usefulness of our system, a larger range of proteins should be produced. However, our results with the 13 avidin fusion proteins suggest that avidin has great potential as a versatile secretion-compatible tag for insect cells.

In conclusion, our results indicate that avidin works well in the baculovirus expression system as a multi-purpose secretion-compatible tag. The avidin tag is stable and retains its characteristically high biotin-binding activity. It is thus appropriate for use with the large range of commercially available avidin–biotin technology reagents. The ease and versatility with which avidin fusion proteins can be detected, purified, and immobilized provides the basis for the use of this system as a useful alternative in the production of eukaryotic fusion proteins.

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REFERENCES

- O'Reilly, D. R., Miller, L. K., and Luckov, V. A. (1994) "Baculovirus Expression Vectors—A Laboratory Manual," Oxford Univ. Press, New York.
- Davies, A. H. (1994) Current methods for manipulating baculoviruses. *Biotechnology* **12**, 47–50.
- Green, N. M. (1975). Avidin. *Adv. Protein Chem.* **29**, 85–133.
- Wilchek, M., and Bayer, E. A., Eds. (1990) Avidin–biotin technology. *Methods Enzymol.* **184**.
- Airenne, K. J., and Kulomaa, M. S. (1995) Rapid purification of recombinant proteins fused to chicken avidin. *Gene* **167**, 63–68.
- Lesley, S. A., Patten, P. A., and Schultz, P. G. (1993). A genetic approach to the generation of antibodies with enhanced catalytic activities. *Proc. Natl. Acad. Sci. USA* **90**, 1160–1165.
- Airenne, K. J., Oker-Blom, C., Marjomäki, V. S., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (1997) Production of biologically active recombinant avidin in baculovirus-infected insect cells. *Protein Expression Purif.* **9**, 100–108.
- Archer, B. L. (1960) The proteins of *Hevea brasiliensis* latex. *Biochem. J.* **75**, 236–240.
- Alenius, H., Kalkkinen, N., Reunala, T., Turjanmaa, K., and Palosuo, T. (1996) The main IgE-binding epitope of a major latex allergen, prohevein, is present in its N-terminal 43 amino acid fragment, hevein. *J. Immunol.* **156**, 1618–1625.
- Luckov, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* **67**, 4566–4579.
- Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M., and Nygren, P.-Å. (1997) Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expression Purif.* **11**, 1–16.
- Bayer, E. A., Ben-Hur, H., Gitlin, G., and Wilchek, M. (1986) An improved method for the single-step purification of streptavidin. *J. Biochem. Biophys. Methods* **13**, 103–112.
- Bayer, E. A., Ehrlich-Rogozinski, S., and Wilchek, M. (1996) Sodium dodecyl sulfate–polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin. *Electrophoresis* **17**, 1319–1324.
- Broekaert, W., Lee, H., Kush, A., Chua, N.-H., and Raikhel, N. (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc. Natl. Acad. Sci. USA* **87**, 7633–7637.
- Karp, M., Lindqvist, C., Nissinen, R., Wahlbeck, S., Åkerman, K., and Oker-Blom, C. (1996) Identification of biotinylated molecules using a baculovirus-expressed luciferase–streptavidin fusion protein. *BioTechniques* **20**, 452–459.
- Oker-Blom, C., Orellana, A., and Keinänen, K. (1996) Highly efficient production of GFP and its derivatives in insect cells for visual in vitro applications. *FEBS Lett.* **389**, 238–243.
- Nilsson, J., Larsson, M., Ståhl, S., Nygren, P.-Å., and Uhlén, M. (1996) Multiple affinity domains for the detection, purification and immobilization of recombinant proteins. *J. Mol. Recognit.* **9**, 585–594.
- Shimkus, M., Levy, J., and Herman, T. (1985) A chemically cleavable biotinylated nucleotide: Usefulness in the recovery of protein–DNA complexes from avidin affinity columns. *Proc. Natl. Acad. Sci. USA* **82**, 2593–2597.
- Olejnik, J., Sonar, S., Krzymańska-Olejnik, E., and Rothschild, K. J. (1995) Photocleavable biotin derivatives: A versatile approach for the isolation of biomolecules. *Proc. Natl. Acad. Sci. USA* **92**, 7590–7594.
- Frangioni, J. V., and Neel, B. G. (1993) Solubilization and purification of enzymatically active glutathione *S*-transferase (pGEX) fusion proteins. *Anal. Biochem.* **210**, 179–187.
- Lee, H. I., Broekaert, W. F., and Raikhel, N. V. (1991) Co- and post-translational processing of the hevein preproprotein of latex of the rubber tree (*Hevea brasiliensis*). *J. Biol. Chem.* **266**, 15944–15948.
- Marston, F. A. O. (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240**, 1–12.