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Efficient isolation, purification, and characterization of the *Helicoverpa zea* VHDL receptor

Deryck R. Persaud,* Vandad Yousefi, and Norbert Haunerland

Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada V5A 1S6

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Abstract

The study of fat body receptors (e.g., VHDL receptor) in Lepidoptera has been irksome due to the fact that isolation and purification of these proteins are difficult and resulted in extremely low yields. A rapid and efficient method is presented for the purification of *Helicoverpa zea* VHDL receptor by the use of VHDL–biotin ligand complexed to streptavidin coated magnetic beads. The technique can be easily applied to other ligands and allows for the purification of membrane proteins with higher yields compared to previously used methods involving immunopurification. Although the purified protein can be characterized by Western and non-radioactive ligand blots using enhanced chemiluminescence (ECL), a non-radioactive ligand blot method using VHDL–FITC is presented, which allows for the quick analysis of the receptor directly from the blot under standard UV light. Sufficient receptor protein has been derived for amino acid analysis, receptor-ligand and xenobiotic binding studies.

The VHDL[1]-receptor is important for the uptake of storage proteins (e.g., VHDL) into the fat bodies of the larvae of the moth *Helicoverpa zea* [1]. Storage proteins are later used by the insect in the pupal stages as amino acid reserves for the development of new tissues, which results in the formation of the adult. *H. zea*, also notoriously known as the corn earworm, causes significant damage to corn as well as tomatoes and strawberries. A good knowledge of the nature of this receptor is important in understanding the development of this insect and also its eventual control. Although a number of mammalian lipoprotein receptors have been identified and well characterized, e.g., the LDL receptor [2–4], there have been relatively few examples of insect lipoprotein receptors in the literature [1,5,6]. With respect to the VHDL receptor, there has not been much advance in the research due to the difficulty in obtaining enough protein for biochemical and molecular analysis. The isolation of the *H. zea* VHDL receptor, as stated in the literature, was an elaborate procedure, which was time intensive and produced low yields [1]. A number of methods [7,8] were later tried to improve the purification and protein yields of the VHDL receptor but these were unsuccessful.

With the advent of new protein isolation procedures involving the use of magnetic beads, the purification of the receptor protein, using a functional approach, could be easily carried out. Paramagnetic Dynabeads (Dynal, Lake Success New York, USA) have been used for the removal of receptors and other proteins as well as whole cells (*Dynabeads Biomagnetic Application in Cellular Immunology*, Dynal, Lake Success New York, USA). Dynabeads are uniform, supermagnetic, monodispersed polystyrene microspheres. For the isolation of the VHDL-receptor, the ligand (VHDL protein, $K = 7.8 \times 10^{-8}$ M [9,10]) was first biotinylated and then later bound to streptavidin coated Dynabeads. The VHDL was then bound to the Dynabeads via the biotin–streptavidin link. This VHDL complex was used to bind the VHDL receptor and isolate it from the mixture of crude protein. Biotin is a water-soluble vitamin

* Corresponding author. Founder and CSO, Infogenetica Bioinformatics, Coquitlam, BC, Canada V3B 6E2. Fax: +6044644365.
E-mail address: infogenetica@telus.net (D.R. Persaud).

[1] *Abbreviations used*: VHDL, very high density lipoprotein; ECL, enhanced chemiluminescence; *H. zea*, *Helicoverpa zea*; FITC, fluorescein isothiocynate; PMSF, phenylmethylsulfonyl fluoride; LDL, low density lipoprotein; PBS, phosphate buffered saline; TBS, Tris buffered saline; TBST, Tris buffered saline with Tween; PVDF, polyvinylidine difluoride; HRP, horseradish peroxidase.

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belonging to the B-complex group of vitamins. This vitamin displays one of the highest affinities amongst biomolecules; for example, it binds to avidin ($K = 10^{-15}$ M) and streptavidin ($K = 10^{-14}$ M). The nature of the strong and specific binding of streptavidin to biotin also proved useful for isolation of the VHDL receptor whose identity was confirmed by Western and ligand blots.

Methods

Insect rearing

Helicoverpa zea colonies were reared in a controlled environment. The temperature was maintained at 26 °C. Eggs (AgriPest, Zebulon, NC) were hatched on paper towels enclosed in plastic bags. Once hatched, the larvae were immediately placed into plastic containers containing an artificial diet (Corn Earworm Diet, Southland Products, Lake Village, AR). After the larvae had reached the 3rd instar, they were placed into individual 2 oz cups filled with 2 mL of diet. The cups were sealed with a perforated lid (Dixie PL2) to allow for the exchange of gases. After approximately a week, the larvae turned into pupae; adult ecdysis occurred between 10 and 14 days afterwards.

Crude preparation of the VHDL receptor

Perivisceral fat body tissues were isolated from 5 to 8-days-old 5th instars. Hemolymph was first removed by bleeding the insect through an excised proleg. The larvae were dissected and the remaining hemolymph was washed away with protein extraction buffer. The fat bodies from 10 larvae (≈450 mg, wet weight) were excised out and placed immediately in 6 mL cold protein extraction buffer at 4 °C, (50 mM Tris–base, 150 mM NaCl, 1% Nonidet P-40, and 1 mM PMSF, and pH 7.8). The fat body tissues were completely homogenized using a polytron homogenizer set at maximum speed. The homogenized tissues were first centrifuged at 800*g* for 1 h at 4 °C. The supernatant (Fraction B) was poured out into a clean protease-free tube. The subnatant (Fraction A) was stored at −20 °C and later used for analysis. A 1 mL aliquot of Fraction B was stored at −20 °C for use in Western and ligand blots. The rest of the isolate (Fraction B) was centrifuged at 30,000*g* for 1 h at 4 °C. The supernatant (Fraction D) was carefully poured into a clean tube, stored at −20 °C, and later used for analysis. A 1 mL aliquot of the subnatant (Fraction C) was removed and stored at −20 °C for Western and ligand blots. The remainder of Fraction C was washed twice with protein extraction buffer and then placed in 200 μL of 2% solution of Triton X-100. The detergent mixture was then gently vortexed and left overnight at 4 °C with gentle rocking. The overnight mixture was then centrifuged at 100,000*g* for 1 h at 4 °C. The supernatant (Fraction F) was carefully pipetted out and placed into a clean Eppendorf tube. The subnatant (Fraction E) was resuspended in 100 μL protein extraction buffer. Both supernatant and subnatant were stored at −20 °C until required for analysis.

Purification of VHDL receptor protein on Dynabeads

Protein biotinylation was done using the ECL protein biotinylation system (Amersham–Pharmacia Biotech, Piscataway, NJ). Protein stock solutions of VHDL were prepared to a concentration of 1 mg/mL in bicarbonate buffer. One hundred microliters of biotinylation reagent (*N*-hydroxysuccinimide-biotin ester) was added to a 2.5 mL aliquot of the protein stock solution and the mixture was incubated for 1 h under constant agitation. The labeled protein was then loaded onto a Sephadex G25 column (1 cm × 10 cm), later eluted with 5 mL PBS, and stored at 4 °C. Streptavidin coated M-280 Dynabeads (Dynal, Lake Success New York, USA) were prepared and conditioned for use according to manufacturer's protocols. However, it was found appropriate to replace the traditional washing buffer with special VHDL ligand binding buffer (20 mM Tris–HCl, 0.15 M NaCl, 4 mM $CaCl_2$, and 0.1% Triton X-100, and pH 7.0) during all washing steps. Lyophilized VHDL–biotin was dissolved in 100 μL ligand binding buffer to make a final concentration of 1 μg/μL. One hundred microliters streptavidin coated Dynabeads was incubated with 100 μL of biotinylated VHDL for 90 min at r.t. with gentle mixing using a Dynal Sample Mixer (Dynal, Lake Success New York, USA). The beads were washed twice with ligand binding buffer. Washing steps were carried out using a Dynal MPC magnetic holder. The VHDL–biotin–streptavidin–Dynabeads complex was stored at 4 °C. For receptor isolation, it was found useful to utilize the Fraction D and Fraction F (from the crude preparation as stated earlier) as these showed the greater presence of the receptor in Western blots. The crude receptor isolate (1 μg/μL) was first desalted (Spectra/Por 1, 8000 Da cutoff; Spectrum, Houston, Texas, USA) and lyophilized before being redissolved in 100 μL ligand binding buffer. The redissolved crude isolate was then incubated with the VHDL–biotin–streptavidin–Dynabeads complex for 60 min at room temperature under gentle mixing. The Dynabeads were then washed three times with ligand binding buffer, each washing being for 5 min duration. To remove the wastes, the Eppendorf containing the beads was then placed into a magnetic holder for 2 min, which caused the beads to align themselves to the sides of the Eppendorf. All waste solutions (e.g., VF1, VF2, and VF3 fractions) were then pipetted out and kept for analysis to monitor the VHDL and receptor binding efficiency. After the washing steps,

the beads were incubated with elution buffer (20 mM Tris–HCl, 0.15 M NaCl, 0.17 mM PMSF, 0.1% Triton X-100, and pH 9.5) for 5 min. The elution buffer had a higher pH and contained no calcium ions (compared to the ligand binding buffer), which are not optimum binding conditions for the VHDL ligand and its receptor [10]. The mixture was then placed a magnetic holder and the receptor eluent was pipetted out. The eluted VHDL receptor (VE1 fraction) was then stored at −20 °C. For analytical purposes, 20 μL of all washes and of the eluted receptor protein was used in SDS–PAGE, Western and ligand blots. The used beads can be stored at 4 °C in ligand binding buffer containing sodium azide (0.01%) for 6 months.

Protein assay

Protein quantification was carried out using a commercial kit (Bio-Rad Protein Assay, BioRad laboratories, California), which was based on the method of Bradford [11]. The assay was performed according to manufacturer's protocol. Absorbance readings were taken at 595 nm.

Protein separation and transfer to PVDF membrane

Separation of proteins was achieved using an SDS–PAGE gel (stacking gel 4% [from a 40% acrylamide/bisacrylamide mixture, 37.5:1; Bio-Rad Laboratories, California] and a 12% resolving gel [40% acrylamide/bisacrylamide]) [12]. Before loading, 20 μL of protein loading dye was added to the 20 μL of protein samples and the entire mixture was boiled for 10 min. Forty microliters of the prepared samples was loaded into each well. The separation was carried out either on a Miniprotein II electrophoresis Cell (BioRad Laboratories) or on a Mighty Small SDS–PAGE apparatus (Hoefer Scientific Instruments, San Francisco, CA). The settings for the BioRad instrument were 30 mA for 1 h and 50 mA for 3 h using the Mighty Small equipment. A polyvinylidine difluoride (PVDF) membrane was conditioned by soaking it in 100% methanol for 5 min, after which, it was rinsed with transfer buffer and then left wet in transfer buffer. Whatman 3M filter paper was cut to the size of the resolving gel and soaked in transfer buffer. The gel was placed onto the PVDF membrane and then sandwiched between the layers of wet filter papers. The transfer of protein onto the membrane was carried out for 7 h at a constant voltage of 200 mV using a semi-dry ElectroBlotting apparatus (Pharmacia LKB, Sweden).

Western and non-radioactive ligand blotting

The blotted PVDF membrane was removed from the blotting apparatus and rinsed with transfer buffer. The membrane was immediately placed into a filtered solution of 5% milk powder (Carnation milk powder) and incubated for 1–2 h under gentle agitation. Membranes were washed three times with TBST, each wash being of 15 min duration. Primary rabbit anti-VHDL-receptor antibodies (1:5000) were added to the membrane and incubated for 1 h under gentle rocking [13]. The membrane was washed three times for 15 min duration as described above. Secondary antibody (goat anti-rabbit–HRP conjugate, BioRad Laboratories) was added at a concentration of 1:10,000 and the mixture was left for 1 h at room temperature. The membrane was washed and then drained to remove excess liquid. Detection of the protein of interest was obtained by chemiluminescence detection using the ECL kit from Amersham–Pharmacia Biotech. For detection of each reaction, 3 mL of ECL reagent 1 and 3 mL of ECL reagent 2 were mixed together and quickly poured over the moist membrane. The membrane was enclosed in a piece of saran wrap and exposed to X-ray film using an X-ray film cassette. Detection of positive signals on the X-ray film occurred in as little as 5 s. The exposed X-ray films were developed using an automated photo image developer system.

Dot blots and normal SDS–PAGE blots were prepared for Western blot of the crude fractions of the VHDL receptor. For dot blots, equal amounts of protein (pure VHDL, pure VHDL-receptor stock, 30,000 supernatant {Fraction D}, and bovine serum albumin, and VE1) were blotted onto a pre-conditioned PVDF membrane by using a dot-blotter (Bio-Dot Microfiltration Apparatus, BioRad Laboratories). For normal SDS–PAGE blots, 20 μL of equal amounts of protein (VE1, pure VHDL-receptor, and, BSA) was resolved on a 12% SDS–PAGE gel. The concentrations of primary (rabbit anti-VHDL) and secondary antibodies (goat anti-rabbit–HRP conjugate) were 1:3000 and 1:5000, respectively.

For Non-radioactive ligand blots, the VHDL ligand was conjugated to FITC. This VHDL–FITC (FITC, Fluorescein Isothiocyanate) complex was prepared by using the FluoroTag FITC Conjugation Kit (Sigma, St. Louis MO, USA). Lyophilized and dialyzed VHDL was dissolved in 0.1 M carbonate buffer, pH 9.0, to make a 1.0 mg/mL protein solution. The conjugation of FITC to 1 mg of VHDL was carried out according to manufacturer's protocol using one of the following molar ratios in the reaction mixture: 5:1, 10:1, and 20:1 of FITC (MW 389) to VHDL (MW 150,000). Each of the vials containing the reaction mixtures was sealed in aluminium foil and incubated for 2 h at room temperature. Three Sephadex G-25M columns were labelled 5:1, 10:1, and 20:1. The protein/FITC mixture was then added to each of the columns. To obtain the purified VHDL–FITC complex, the columns were eluted with 2.5 mL PBS. As the samples were eluting, 0.25 mL fractions were collected and absorbance readings (at 280 and 495 nm) were taken. Protein fractions with an

absorbance ratio (A280/A495 = 1.0) were used in ligand blot experiments. Molar ratios of FITC–VHDL (10:1) gave the best results for ligand blot experiments. For normal SDS–PAGE blots, 20 μL of equal amounts of protein (VE1, pure VHDL-receptor, and BSA) were resolved on a 12% SDS–PAGE gel. The primary incubation step was with VHDL–FITC (1:5000) and the secondary incubation step was with anti-FITC–HRP conjugate (1:5000). The VHDL–FITC could also be used to identify the receptor directly on the blot under UV light using the 1:5000 dilution.

Results and discussion

The VHDL-receptor was not found in the hemolymph of 5–8-days-old 5th larval instar. However, it was found most commonly in the 800*g* precipitate (Fraction A) and the 30,000*g* supernatant (Fraction D) fraction of the fat body isolate (Fig. 1). The 100,000*g* subnatant and supernatant (Fractions E and F) also showed the presence of the VHDL-receptor. A possible explanation for the presence of the positive signals in the 30,000*g* supernatant (Fraction D) could be due to the presence of proteins with similar epitopes that are recognized by the VHDL-receptor antibodies. This is a reasonable concern because many insect larval hemolymph proteins belong to the hemocyanin superfamily. Since positive signals were not detected in hemolymph but only in fat body tissues, it implies that in the final few days of the last instar, the VHDL-receptor is found only in fat body tissues. The presence of the receptor in the 30,000*g* supernatant would suggest that the receptor is most likely a peripheral protein or a weakly attached integral membrane protein that is easily separated from the cell membrane without the use of non-ionic detergents. The purification of the VHDL receptor using Dynabeads was effective and led to protein of about 98% purity and a yield of 100 μg (0.022%). On a 12% SDS–PAGE gel, a single protein band (≈80 kDa) was detected from the VE1 eluted fraction (Fig. 2). This fraction is believed to contain the VHDL-receptor. The final washing step (VF3) showed no presence of proteins and hence suggests that the elution step was effective in dissociating the bond between the VHDL–biotin–streptavidin–Dynabeads and the VHDL receptor. Dot blots initially helped in optimizing the conditions for obtaining a strong positive signal on normal Western and ligand blots. The SDS–PAGE Western blot showed that the protein band was of the correct molecular weight (Fig. 3A). With ligand blots, the identity of the VE1 isolate was also confirmed (Fig. 3B).

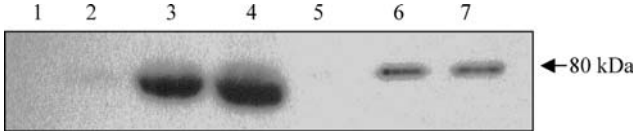

Fig. 1. Western blot of the various fractions obtained during the isolation of VHDL receptor. For Lanes 1–5, 1 μg of protein was loaded on the SDS–PAGE gels. In lanes 6 and 7, 0.2 μg of protein was loaded on the SDS–PAGE gels. The receptor was identified by Western blot using anti-VHDL receptor primary antibodies (1:10,000) and goat-anti-rabbit-Ig HRP conjugate (1:3,000). Lane 1, protein markers. Lane 2, *Helicoverpa zea* hemolymph. Lane 3, 800*g* pellet (Fraction A). Lane 4, 30,000*g* supernatant (Fraction D). Lane 5, 100,000*g* pellet (Fraction E). Lane 6, 100,000*g* supernatant (Fraction F). Lane 7, pure VHDL receptor [13].

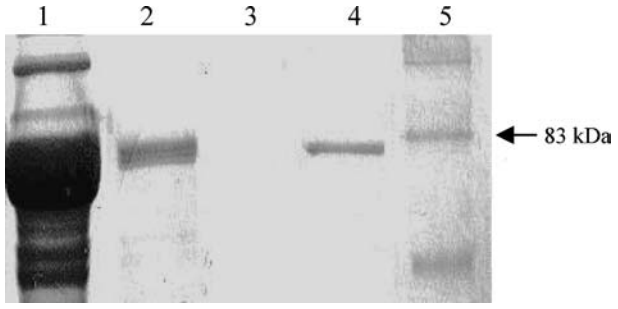

Fig. 2. SDS–PAGE gel of various fractions from the purification of the VHDL-receptor by Dynabeads. Lane 1, first wash step (VF1). Lane 2, second wash step (VF2). Lane 3, third wash step (VF3). Lane 4, purified VHDL-receptor isolate (VE1). Lane 5, broad range protein markers (New England Biolabs, Beverly MA, USA).

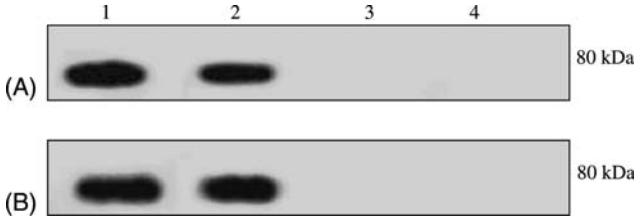

Fig. 3. (A) SDS–PAGE Western blot. Lane 1, VE1 isolate. Lane 2, pure VHDL-receptor (control). Lane 3, protein markers. Lane 4, bovine serum albumin. (B) Normal SDS–PAGE ligand blot (VHDL–FITC). Lane 1, VE1 isolate. Lane 2, pure VHDL-receptor (control). Lane 3, protein markers. Lane 4, bovine serum albumin.

This rapid purification scheme stated here in this paper allows for sufficient amount of VHDL-receptor to be isolated to be used in amino acid analysis and for ligand binding studies. Current work being carried out involves the development of a suitable non-radioactive ligand binding assay for in-depth receptor-ligand binding studies and the determination of the amino acid sequence of this protein, molecular cloning, and recombinant expression. Future work will incorporate the determination of other ligands (e.g., chemical compounds) which will bind to the receptor and inhibit or stimulate its function. Such lead compounds can lead to further understanding of storage protein uptake and hence insect development. In addition, potential inhibitors of receptor activity can be used to design novel pesticides.

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