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Research paper

Selection and expression of recombinant single domain antibodies from a hyper-immunized library against the hapten azoxystrobin

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ABSTRACT

Three V_HHs against the model hapten, azoxystrobin (MW 403), were isolated from a hyper-immunized phage-displayed V_HH library. This library was constructed by isolating the V_HH-coding genes from the lymphocytes collected from a *Llama glama* that was immunized with azoxystrobin conjugated to bovine serum albumin (BSA). Six rounds of panning were performed against azoxystrobin conjugated to either ovalbumin (OVA) or rabbit serum albumin (RSA) to enrich clones containing V_HHs specific to the hapten. After screening 95 clones, three V_HHs (A27, A72, and A85) with different amino acid sequences were identified, expressed in soluble format in *Escherichia coli* HB2151, and purified using nickel-immobilized metal affinity chromatography. Competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) showed that A27 and A85 were specific to azoxystrobin while A72 was not. The IC_{50} values of A27 and A85 V_HHs were 7.2 and 2.0 μM, respectively. To our knowledge A85 is one of the highest affinity V_HHs that has yet been isolated against a hydrophobic hapten such as azoxystrobin.

1. Introduction

In addition to conventional IgG, camelid species, e.g., camels and llamas, produce a unique class of IgG known as HcIgG, which are devoid of light chains and the first heavy chain constant (C_{H1}) domain (Hamers-Casterman et al., 1993). Despite the fact that these IgGs lack light chains, their variable heavy chains are able to bind to protein and peptide antigens efficiently (Riechmann and Muyldermans, 1999; Spinelli et al., 2000). The antigen binding fragments of HcIgGs are known as the *v*ariable domain of the *h*eavy chain of *h*eavy chain antibodies (V_HH). V_HHs are classified into four different subgroups based on the similarities of the amino acid sequences of both the *f*ramework *r*egions (FRs) and *c*omplementarity *d*etermining *r*egions (CDRs) (Harmsen et al., 2000). Generally, V_HHs have a longer CDR3, with an average of 13–18 amino acids, in comparison to V_Hs in mouse and human, which generally contain 9 and 12 amino acids, respectively (Vu et al., 1997; Arbabi-Ghahroudi et al., 2005). The extended CDR3 loop makes V_HHs more suitable for recognition of concave epitopes and cryptic sites inside proteins (Dumoulin et al., 2003).

V_HHs have a number of advantages when compared to conventional antibody fragments such as single chain variable fragments (scFvs) and fragment antigen binding (Fabs). For example, V_HHs have a higher solubility than scFvs and Fabs; this property is due to the substitution of four hydrophobic residues, Val37, Gly44, Leu45 and Trp47, in V_Hs to the less hydrophobic residues Phe/Tyr37, Glu44, Arg45 and Phe/Leu/Gly47 in V_HHs (Kabat numbering system) (Muyldermans, 2001). V_HHs are also thermostable and can remain active after heat treatment at temperatures as high as 90 °C (van der Linden et al., 1999; Dumoulin et al., 2002).

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Furthermore, the small size of V_HH fragments (~14 kDa) offers an advantage in terms of genetic manipulation compared to scFvs and Fabs. V_HHs also have advantages in therapeutic applications due to their low immunogenicity, which is a result of their homology to human IGHV3 subgroup genes (Conrath et al., 2003).

Despite the many advantages of using V_HHs, there is an uncertainty associated with isolation of these fragments against low molecular weight haptens (<1000 Da), especially molecules with low water solubility. There are a number of studies on anti-hapten V_HHs that suggest that factors such as the size and chemical structure of haptens play important roles in successful isolation of V_HHs against target haptens. For instance, Spinelli et al. (2000) isolated an anti-Reactive Red 6 (RR6) V_HH with a K_d of 22 nM from an immune library. The crystal structure of RR6-V_HH showed that the V_HH provided an efficient binding site for RR6 by forming a deep cleft. It was further shown that CDR1 had a strong interaction with the copper atoms in RR6, which is probably the main reason for the high affinity of this V_HH to RR6. The water solubility of target haptens is another important characteristic for successfully isolating V_HHs. Alvarez-Rueda et al. (2007) suggested that camelid single-domain antibodies are capable of binding with high affinity to hydrophilic haptens of low molecular weight. However, compared to other antibody fragments, the isolation of V_HHs against hydrophobic haptens is more challenging. This is due to the fact that during the conjugation reaction hydrophobic haptens react with hydrophobic domains of the carrier protein, consequently, the hapten may "hide" within the protein structure and is not fully exposed to both B-cells in the host animal and the antibodies during the selection process (Fasciglione et al., 1996). Therefore, all or most of the isolated binders are non-specific.

Azoxystrobin (Fig. 1) with molecular weight of 403 Da and water solubility of 6 mg ml^{-1} is one of the largest selling fungicides worldwide (Bartlett et al., 2002) and is effective against the major groups of plant pathogenic fungi (Godwin et al., 1992). Although, this fungicide is safe against many non-target organisms, the possibility of runoff and drift of azoxystrobin from application sites into sensitive aquatic systems demands controlled use and effective detection methods (PMRA, 2000). With this in mind, a llama (*Lama glama*) was immunized with an azoxystrobin–BSA conjugate, after which we investigated the convIgG and HcIgG responses at different time points. An immune library was constructed from the blood lymphocytes. Three V_HHs specific to azoxystrobin were isolated and their amino acid sequences and binding characteristics were determined.

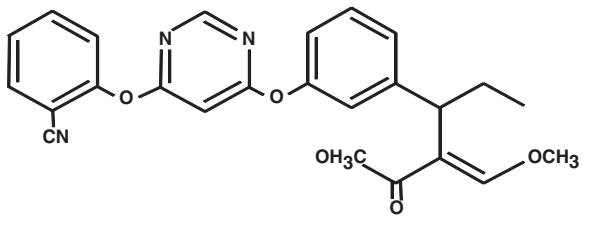

Fig. 1. Chemical structure of azoxystrobin (MW 403 Da).

2. Methods and materials

2.1. Llama immunization

A two-year-old male llama was immunized by subcutaneous, lower-back injection with 250 μg of azoxystrobin ([methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate]) (Syngenta, Guelph, ON, Canada) conjugated to bovine serum albumin (BSA) (Furzer et al., 2006) in 1.0 mL PBS emulsified with an equal volume of TiterMax adjuvant (Sigma-Aldrich Chemical Co., St. Louis, MO). The second injection was given three weeks later, and was followed by three more biweekly injections. Between immunizations (i.e., 7, 28, 42, 56 and 70 days after the first injection) 15.0 mL of blood was collected at biweekly intervals. Forty-nine days after termination of immunization (day 112) another 15 mL of blood was collected. To harvest the serum, the collected blood was incubated at 4 °C overnight, followed by centrifugation at 2700 × *g* for 10 min at 4 °C. The immunization protocol was approved by the University of Guelph Animal Care Committee (Guelph, Canada).

2.2. Assessment of convIgG and HcIgG sensitivity

To fractionate convIgG and HcIgG, 1 mL of the sera collected from days 0, 42, 70 and 112 was dialyzed against phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter of water, pH 7.5). The collected sera were fractionated by protein G chromatography (GE Healthcare, Piscataway, NJ) and eluted by acidic elution as described by Hamers-Casterman et al. (1993). The HcIgG fractions were subjected to a second round of fractionation to eliminate the possibility of convIgG contamination. These fractions were also dialyzed against PBS.

The purity and presence of convIgG and HcIgG were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and an immunoblot. The convIgG and HcIgG responses against azoxystrobin conjugated to ovalbumin (OVA) were monitored at different time points (i.e., days 0, 42, 70 and 112) by indirect ELISA. Wells of a 96-well microtiter plate were coated with 1 μg mL^{-1} of azoxystrobin–OVA in PBS. As a negative control, one row was coated with the same concentration of OVA. The coated plates were incubated at 4 °C for 16 h. After washing the wells three times with PBS, wells were blocked with 4% MPBS (4 g of skim milk in 100 mL of PBS) for 2 h at room temperature (22–24 °C). Wells were washed as described above and 100 μg mL^{-1} of either convIgG or HcIgG was added in the wells of the first column of the microtiter plate, followed by a serial dilution (1/4 v/v) across the plate with PBS. The polyclonal antibodies were allowed to bind to the conjugate for 1.5 h at room temperature. Wells were washed five times with PBST (0.05% Tween20 in PBS) and incubated for 1 h with 100 μL polyclonal goat anti-llama serum (1/1000 v/v; Bethyl Laboratories Inc., Montgomery, TX) followed by polyclonal swine anti-goat serum conjugated to horseradish peroxidase (1/3000 v/v in PBS; Bethyl Laboratories Inc.). TMB peroxidase substrate (Bio-Rad, Hercules, CA) was added (100 μL) to each well and the reaction was stopped after 30 min with 1 M