Selection and Characterization of Recombinant Dromedary Antiovalbumin Antibody Fragments That Do Not Cross-React with Ovalbumin-Related Protein X: Use for Immunoaffinity

Gilles Bruneau,*[†,‡,§,||] Abdelhaq Anouassi,[⊥] Sophie Réhault-Godbert,[#] Sylvie Canépa,[†,‡,§,||,∇] and Michel R. Blanc[†,‡,§,||]

[†]INRA, UMR85 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France

[‡]CNRS, UMR7247, F-37380 Nouzilly, France

[§]Université François Rabelais de Tours, F-37041 Tours, France

[||]IFCE, F-37380 Nouzilly, France

[⊥]Institut Agronomique et Vétérinaire Hassan II, Rabat, Maroc & Veterinary Research Center, Abu Dhabi, United Arab Emirates

[#]INRA, UR83 Fonction et Régulation des Protéines de l'Oeuf, 37380 Nouzilly, France

[∇]Plateforme de Protéomique Analytique et Fonctionnelle, Laboratoire de Résonance Plasmonique, INRA de TOURS, 37380 Nouzilly, France

ABSTRACT: Ovalbumin-related protein X (OVAX) and ovalbumin are two very close ovalbumin-related serpins. As primary data on OVAX remain recent, information about possible cross-reaction of available antiovalbumin antibodies with OVAX is still missing. Using labeled purified OVAX and dot ligand blotting, we identified 49 recombinant dromedary antiovalbumin single domain antibody (sdAb) fragments that were unable to bind OVAX. Discrimination between OVAX and ovalbumin was confirmed for two of the corresponding sdAb fragments by surface plasmon resonance and Western ligand blotting (WLB) characterizations. Furthermore, they were covalently linked to Sepharose and used as an affinity matrix for ovalbumin depletion. At least 90% of the original ovalbumin was eliminated from the allantoic fluid of 14 day old chicken embryo in one step. These sdAb fragments, which bind ovalbumin with nanomolar affinity, should also contribute to a better characterization of ovalbumin preparations.

KEYWORDS: ovalbumin, OVAX, VHH, Camelus dromedarius, Western ligand blotting, plasmon surface resonance

■ INTRODUCTION

Serine protease inhibitors (serpins) share a common tertiary structure.[1,2] Ovalbumin is the archetype of the ovalbumin-related serpins or clade B serpins (ov-serpins). These ov-serpins are characterized by their short N and C termini, as compared to those of the prototype α1-antitrypsin, and by the absence of a conventional secretory signal peptide.[3] Analysis of the *Gallus gallus* genome has led to the identification of three ov-serpins, namely, ovalbumin, ovalbumin-related protein X (OVAX), and ovalbumin-related protein Y (OVAY).[4] Ovalbumin is secreted throughout the reproductive tract of the hen, mainly in the magnum, during egg formation.[5] OVAX and OVAY, recently detected in hen albumen,[6,7] are likely, as egg white proteins,[8,9] to be synthesized by the same tissue (magnum), but this remains to be proven. Ovalbumin is the principal egg white protein, and although its function remains unclear, it probably plays a major role as a nutrient supply for the developing embryo.[10] Along with maspin/serpinB5, ovalbumin is one of the few members of the serpin family without protease inhibitor activity.[4] It is not known whether OVAX and OVAY behave as protease inhibitors or not. OVAX has been recently purified.[11] The genes corresponding to these three proteins are located on chromosome 2, within a 40 kb region.[4] The predicted amino acid sequence of OVAX (XP_418984, gi|118086485, in NCBI database) is indicated as being "similar to ovalbumin-related protein Y" in databases.[7−9] The alignment of OVAX and ovalbumin reveals 61% amino acid sequence identity and 77% sequence homology (Figure 1A), suggesting that they have evolved from the same ancestor gene.[12,13]

As the three ov-serpins are closely related, their identification needs to dispose of immunological tools capable of discriminating between them. Now, the available information concerning cross-reactivity of commercial antiovalbumin antibodies with other ov-serpins is missing. Besides classic immunoglobulins (Igs) found in other mammals, camelids produce Igs that consist only of heavy chains and that are devoid of the CH1 domain (HC IgGs). The variable fragment of these HC IgGs (VHH) has a molecular weight of around 15 kDa and is the smallest known antigen-binding unit.[14] Several single-domain antibody (sdAb) fragments that bind ovalbumin with nanomolar affinity have been selected from a library expressing total VHHs using phage display technology, and produced in *Escherichia coli*.[15] The recent availibility of purified OVAX[11] allows us to characterize from these ovalbumin-binding sdAb fragments specific ones that do not recognize

OVAX. Furthermore, these ovalbumin binding sdAb fragments were used after immobilization onto Sepharose to very efficiently deplete ovalbumin from ovalbumin-containing chicken allantoic fluid in only one step.

■ MATERIALS AND METHODS

Animal Tissue. Nonfertilized egg whites were obtained from laying hens (ISA Brown, Hendrix Genetics, St. Brieuc, France). Fourteen day old chicken embryos were produced in the UEPEAT experimental unit of INRA (Nouzilly, France). After recovery, allantoic fluid from five embryos was centrifuged for 1 min at 5000g and stored at 4 °C until use. Magnum tissue (598 mg) from laying hens (UEPEAT, Nouzilly, France) was homogenized in 5 mL of phosphate-buffered saline (PBS) containing one tablet of protease inhibitor cocktail (#1697498, Roche Diagnostic, Mannheim, Germany) per 50 mL of buffer using an Ultra-Turrax homogenizer and centrifuged (37000g, 30 min, 4 °C). The supernatant was stored at −20 °C until use.

Purification of OVAX. Fresh nonfertilized egg whites were diluted 1:1 (v/v) in 50 mM Tris-HCl, 50 mM NaCl, and 0.02% NaN₃, pH 7.4, gently homogenized, and centrifuged (14000g, 10 min, 4 °C) to eliminate viscous or insoluble proteins. OVAX was purified from soluble proteins according to Réhault-Godbert et al.[11] Egg white proteins were fractionated according to their capacity to bind a heparin-coupled matrix by ionic interaction and/or through high-specific binding sites.[16] This step has enabled us to separate the heparin-binding proteins containing OVAX from the most abundant egg white protein ovalbumin, which did not bind heparin. A heparin-sepharose affinity chromatography using the batch method was performed according to the manufacturer's instructions (GE Healthcare Biosciences, Aulnay-sous-bois, France). Briefly, clarified egg white (see above) diluted 1:1 in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4, was incubated with heparin-sepharose beads overnight at 4 °C. The beads were then washed with 50 mM Tris-HCl and 150 mM NaCl, pH 7.4, until the absorbance at 220 nm reached zero. Elution of bound proteins was achieved with 50 mM Tris-HCl and 1 M NaCl, pH 7.4. Eluted samples were pooled and injected onto a Hiprep 16/60 column containing Sephacryl-S-100 HR (GE Healthcare Biosciences) using 10 mM sodium phosphate and 50 mM sodium chloride, pH 7.2, as the mobile phase. The major peak was collected and submitted to a biotin-sepharose affinity chromatography to remove traces of avidin. The protein concentration was determined using Protein Dc Assay (Biorad, Saint-Quentin, France) with bovine serum albumin (BSA) (Sigma-Aldrich, Saint-Quentin Fallavier, France) as a standard. The final preparation ("OVAX") was around 93% pure [sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie brilliant blue staining, Figure 3A, lane 3]. The protein was definitively identified as OVAX based on the identification of peptides by tandem mass spectrometry (MS/MS) after trypsin hydrolysis.[11] The protein yield was around 7.5 mg per 50 mL egg white.

Identification of OVAX-Binding Phages among Ovalbumin-Binding Phages. The 49 ovalbumin-binding phages previously obtained[15] were analyzed by dot ligand blotting with ¹²⁵I-labeled OVAX and processed as indicated below.

¹²⁵I-Labeling of OVAX and Ova41. OVAX (10 μg) was labeled with ¹²⁵I in the presence of chloramine-T and purified as described.[15] One of the sdAb fragments thus obtained and purified (ova41) was labeled with ¹²⁵I in the presence of either chloramine-T as indicated above ("¹²⁵I-tyrosine-labeled ova41") or ¹²⁵I-Bolton-Hunter reagent (#NEZ 120, PerkinElmer, Villebon sur Yvette, 91140, France) as per Bolton and Hunter[17] ("¹²⁵I-Bolton-Hunter-labeled ova41"). For the latter, 10 μg of ova41 diluted in 10 μL of 220 mM borate buffer, pH 9.5, was incubated with 100−150 μCi of evaporated NEZ 120 for 15 min at room temperature (RT). The reaction was stopped by the addition of glycine in large excess (0.2 M, 50 μL, 10 min). Potassium iodide was added as a carrier before purification on a PD10 column (GE Healthcare) equilibrated with PBS containing 0.1% gelatin. The specific activity of OVAX was 58 μCi/μg. The specific activity was similar for the ¹²⁵I-tyrosine-labeled ova41 and the ¹²⁵I-Bolton-Hunter-labeled ova41 (0.2−0.9 μCi/μg).

SDS-PAGE, Western Ligand Blotting (WLB), and Western Blotting (WB). SDS-PAGE and transfer to nitrocellulose membranes were performed as previously described in detail.[15] For both WB and WLB, transfer membranes were then saturated with skimmed milk and washed with Tris-buffered saline containing 0.05% Tween 20 (TBST). All washes were performed with TBST. For WLB, membranes were incubated with ¹²⁵I-labeled ova41 (see the 125I-Labeling of OVAX and Ova41 section and ref 15) and then exposed to a Phosphor screen (GE Healthcare), which was read and analyzed using a Phosphor-Imager apparatus (Molecular Dynamics, GE Healthcare). For ovalbumin WB (Figure 3), membranes were first incubated with a rabbit antichicken ovalbumin antiserum (C6534, Sigma-Aldrich) and, after washings, with a horseradish peroxidase-labeled donkey antirabbit IgG polyclonal IgGs (H+L) (#711-035-152, Jackson Immunoresearch, West Grove, United States) as a secondary antibody. The membranes were washed, and binding was revealed with 4-chloro-α-naphthol. Alternatively, for ovalbumin WB in ovalbumin depletion experiments (Figure 4), membranes were revealed with diaminobenzidine (DAB) as a chromogen and H₂O₂ as a substrate. Ovalbumin preparation was ovalbumin A5503 (Sigma-Aldrich). Molecular weight markers were Precision Plus Protein All Blue Standards (BioRad Life Science, Marnes la Coquette, France).

Analysis of Ovalbumin-Binding sdAb Fragments by Surface Plasmon Resonance (SPR). Two sdAb fragments, which gave the best dot ligand blot signal as phages (ova41 and ova82) were used. Their binding affinities were analyzed by SPR, using a Biacore T100 apparatus (GE Healthcare). Ovalbumin, OVAX, or BSA was covalently coupled by amine coupling to a CM5-chip according to the manufacturer's instructions. A high-level immobilization chip [HL-chip; OVAX, 3030 resonance units (RU); ovalbumin, 2220 RU; and BSA, 3250 RU] was designed to analyze sdAb fragments binding to OVAX with ovalbumin and BSA as controls, while a low-level immobilization chip (LL-chip; ovalbumin, 193 RU; and BSA, 270 RU) was designed to determine K_d values for ova41 and ova82 . sdAb fragments were injected for 2 min at concentration ranges of 200−400 nM (HL-chip), 1.6−100 nM (ova41), and 6.25−200 nM (ova82) for the LL-chip, in duplicate with a 30 μL/min flow. For affinity studies, dissociation was studied over 10 min. Regeneration was carried out using 100 mM H₃PO₄. Data were analyzed using Biacore T100 evaluation software, version 1.1 (GE Healthcare), with the Langmuir 1:1 type calculation model.

Immunodepletion of Ovalbumin from Chicken Allantoic Fluid. Recombinant ova41 and ova82 (in the ratio of 1:1, 0.7 mg/ sdAb fragment) in 0.2 M NaHCO₃ and 0.5 M NaCl, pH 8.3, were coupled to a 1 mL HiTrap NHS-activated column (GE Healthcare) according to the manufacturer's manual. The column was washed alternatively with buffer A (0.5 M ethanolamine and 0.5 M NaCl, pH 8.3) and buffer B (0.1 M sodium acetate and 0.5 M NaCl, pH 4) three times each and then equilibrated with PBS, pH 8.3. One milliliter of allantoic fluid from 14 day old chicken embryos (n = 5) was loaded on the sdAb fragments-immobilized column. Unbound proteins were washed with PBS (flow-through fraction). Ovalbumin was eluted with 1 mL of 0.1 M glycine, pH 2.7, and immediately neutralized with 50 μL of 1 M Tris-HCl, pH 9.5. Flow-through and eluted products were collected as 1 mL fractions. Ovalbumin was quantified in duplicates, in allantoic fluid and flow-through fractions, using the Serazym Ovalbumin-ELISA kit (Seramun, Diagnostica Gmbh, Heidesee OT Wolzig, Germany) as recommended by the manufacturer. Furthermore, loaded samples, flow-through fractions, and eluted samples were analyzed using SDS-PAGE experiments using reduced conditions, followed by Coomassie blue staining. Ovalbumin was detected by WB using a rabbit antiovalbumin polyclonal antibody (see higher).

■ RESULTS

Screening of Ovalbumin-Binding Phages Using ¹²⁵I-Labeled OVAX by Dot Ligand Blotting (DLB). Among the 49 ovalbumin-binding phages previously identified,[15] none was able to bind ¹²⁵I-labeled OVAX at a greater level than background (data not shown). The amino acid sequence of

A

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OVA   gi|129293     1  MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF
OVAX  gi|118086485  1  MGSISAANAEFCFDVFNELKVQHTNENILYSPLSIIVALAMVYMGARGNTEYQMEKALHF

OVA   gi|129293    61  DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ
OVAX  gi|118086485 61  DSIAGLGGSTQTKCGKSVNIHLLFKELLSDITASKANYSLRTANRLYAEKSRPILPIYLK
                                                                      - - -

OVA   gi|129293   121  CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV
OVAX  gi|118086485 121  CVKKLYRAGLETVNFKTASDQARQLINSWVEKQTEGQIKDLLVSSSTDLDTTLVLVNAIY

OVA   gi|129293   181  FKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM
OVAX  gi|118086485 181  FKGMWKTAFNAEDTREMPFHVTKEESKPVQMMCMNNSFNVATLPAEKMKILELPFASGDL
                                                                       - - -

OVA   gi|129293   241  SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA
OVAX  gi|118086485 241  SMLVLLPDEVSGLERIEKTINFEKLTEWTNPNTMEKRRVKVYLPQMKIEEKYNLTSVLMA
                                                                       - - -

OVA   gi|129293   301  MGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGS--AEAGVDAASVSEE
OVAX  gi|118086485 301  LGMTDLFIPSANLTGISSAESLKISQAVHGAFMELSEDGIEMAGSTGVIEDIKHSPELEQ
                                                                - - -

OVA   gi|129293   359  FRADHPFLFCIKHIATNAVLFFGRCVSP
OVAX  gi|118086485 361  FRADHPFLFLIKHNPTNTIVYFGRYWSP
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B

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               1        10  15 16         26 27          38 39     46 47      55 56
               |         |   |  |          |  |           |  |      |  |       |  |
OVA41  EVQLQESGG.GKVQP GGSLRLACTIS  GFTI....NTYY  MTWARQVS GKGLEWVAG  ITRD..GS
OVA82  EVQLQESGG.GSVQA GGSLRLSCAAS  GDT......VRT  MAWFRQAP GKEREGVAG  FNLP..IS
               |         |   |  |          |  |           |  |      |  |       |  |
               1        10  15 16         26 27  CDR1     38 39     46 47      55 56  CDR2

               65 66      74 75      84 85        96 97   104 105        117
               |  |       |  |       |  |         |  |    |   |          |
OVA41  RT DYID.SVKG RFTVSRDDAK NTVYLQMDNLKS EDTAVYYC GTGRRWA...TNPTY
OVA82  RP YYAD.GMKA RFTISGDKSK NTVTLQMDNLAP EDTANYYC AATRYTLDLS...SRIFQGDFDH
               |  |       |  |       |  |         |  |    |   |    |       |        |
               65 66      74 75      84 85        96 97   104 105 111.3   112.4    117
                                                                        CDR3

               118
               |
OVA41  QGKGTQVTVSS
OVA82  WGHGTQVTVSS
               |
               118
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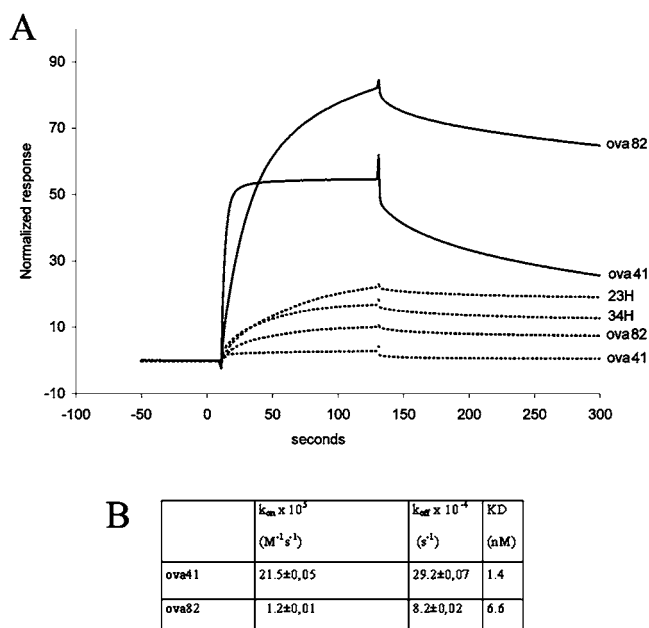
Figure 1. Amino acid sequence alignments of ovalbumin (OVA) and OVAX (A) and of ova41 and ova82 (B). (A) Identical residues and homologous residues are shaded in black and gray, respectively. The N-glycosylation site in the ovalbumin sequence[18] is indicated by stars. The five putative N-glycosylation sites in the OVAX sequence are underlined by dotted lines. The alignment was carried out using CLUSTAL W (1.8) and BOXSHADE (3.21). (B) Unique numbering of the Ig amino acid sequence for V-DOMAIN and CDR delimitation (bold), according to the ImMunoGeneTics information system (IMGT; http://imgt.cines.fr).

two of the sdAB fragments chosen for their high ovalbumin-binding capacity (associated with an absence of binding to OVAX) is shown in Figure 1B (Genbank accession numbers: FJ851189 for ova41 and FJ851188 for ova82).

Analysis of Affinity of Two Ovalbumin-Binding sdAbs by SPR. In accordance with the DLB data, resonance signals recorded for ova41 and ova82 bindings on immobilized OVAX

flow cell using HL-chip were low and lower than those recorded with two purified nonspecific VHHs directed against a protein unrelated to ovalbumin, at the same high concentrations (23H and 34H, Figure 2A). The resonance signals recorded with a polyclonal antiovalbumin IgG antiserum that cross-reacted with OVAX revealed that the immobilized OVAX was accessible to IgGs (not shown). Finally, the resonance

A

B

|	$k_{on} \times 10^5$ $(M^{-1}s^{-1})$	$k_{off} \times 10^{-4}$ (s^{-1})	KD (nM)
ova41	21.5±0.05	29.2±0.07	1.4
ova82	1.2±0.01	8.2±0.02	6.6

Figure 2. (A) Normalized sensorgrams observed with purified ovalbumin-binding sdAb fragments on immobilized OVAX (dotted line) or ovalbumin (solid line) by SPR. Ova41 and ova82 were used at a concentration of 400 nM (HL-chip; OVAX, 3030 RU; ovalbumin, 2220 RU; and BSA, 3250 RU). For normalization, RU levels measured for OVAX, ovalbumin, and BSA were divided by the corresponding RU levels after immobilization, the BSA sensorgram was then subtracted from the OVAX or ovalbumin sensorgrams, and finally, signals were multiplied by 1000. Two nonspecific sdAb fragments (23H and 34H) were used as controls. For other details, see the Materials and Methods. (B) Kinetic and affinity constants of the binding of two ovalbumin-binding sdAb fragments, ova41 and ova82, to immobilized ovalbumin on LL-chip (ovalbumin, 193 RU; and BSA, 270 RU). Experiments were performed in duplicate, and binding on the immobilized BSA flow cell (control) was subtracted.

signals recorded with ova41 and ova82 bindings to immobilized ovalbumin on the HL-chip (Figure 2A) were higher than those recorded with the two nonspecific VHHs under identical conditions (data not shown). Thus, taken together, the resonance signals observed with ova41 and ova82 on immobilized OVAX may be considered as nonspecific. When ovalbumin was immobilized at low RU levels (LL-chip), Biacore analysis indicated a nanomolar affinity for both sdAb fragments (Figure 2B).

Use of ^{125}I-Labeled Ova41 To Reveal Ovalbumin by WLB. ^{125}I-Bolton-Hunter-labeled ova41 was used to identify ovalbumin either purified or in egg white and magnum tissue extracts (Figure 3B, lanes 2, 1, and 4, respectively). The signals observed were of the expected apparent molecular weight (45 kDa) and were considered as specific as they disappeared when labeled ova41 was coincubated with nonradioactive ova41 in excess (not shown). As expected from DLB and plasmon resonance data, the labeled ova41 did not reveal OVAX (Figure 3B, lane 3). No signal was observed when using ^{125}I-tyrosine-labeled ova41 instead of ^{125}I-Bolton-Hunter-labeled ova41 (Figure 3C vs B) even after a long exposure. As a control, a rabbit polyclonal antibody against ovalbumin was able to reveal purified ovalbumin or ovalbumin in egg white and magnum extracts (Figure 3D, lanes 2, 1 and 4, respectively) and OVAX (Figure 3D, lane 3) on the same blot used for WLB (Figure 3B). However, the intensity of the signal observed with OVAX
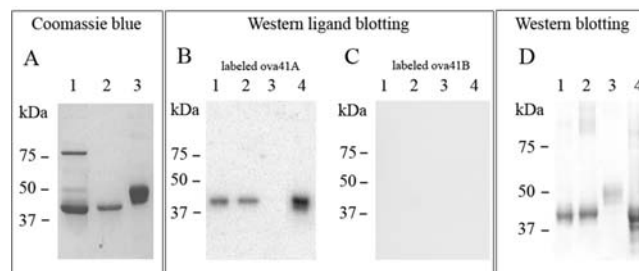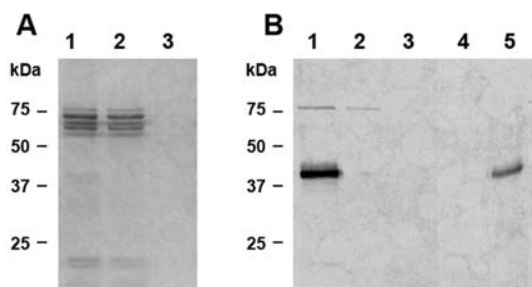

Figure 3. Analysis of ovalbumin and OVAX by SDS-PAGE (A) followed by WLB (B and C) or WB (D). In each panel, MW standards, egg white proteins (lane 1: A, 4 μg; B−D, 0.75 μg), purified ovalbumin (lane 2: A, 4 μg; B−D, 0.75 μg), purified OVAX (lane 3: A, 4 μg; B−D, 1.5 μg), and magnum tissue extracts (lane 4: B−D, 1.5 μg) were electrophoresed under reducing conditions. (A) Gel was stained by Coomassie blue. (B and C) After transfer, membranes were either incubated with ^{125}I-Bolton-Hunter-labeled ova41 ("labeled ova41A", B) or ^{125}I-tyrosine-labeled ova41 ("labeled ova41B", C). Note the absence of binding in C when compared with B. This absence was also observed after longer exposure. (D) The transfer membranes were then incubated with a rabbit polyclonal antiovalbumin serum (C6534, Sigma-aldrich, 1:2000), followed by a peroxidase-labeled donkey antirabbit IgG antibody (711-035-152, Jackson Immunoresearch, 1:10000), and binding was revealed with 4-chloro-α-naphthol. When ovalbumin in excess was incubated with antiovalbumin serum, signals were absent for egg white and ovalbumin lanes and almost totally attenuated for magnum extracts lane (not shown).

was 7.9 times lower than that observed for ovalbumin (Figure 3D, lane 3 vs 2) using the same amount of loaded proteins.

Immobilized Antiovalbumin sdAb Fragments To Deplete Ovalbumin. One milliliter of allantoic fluid from each of the five 14 day old chicken embryos was loaded to the sdAb fragments-immobilized matrix. The content of ovalbumin in the loaded sample and its recovery in the flow-through fractions were both quantified by ELISA. The depletion (ovalbumin amount in the loaded sample − amount in flow-through expressed as percent of the amount in the loaded sample) was estimated at an average of 90.5% ± 1.95 (n = 5). In the excluded proteins retrieved only in the first milliliter of flow-through (Figure 4A, lane 2 vs lane 3, Coomassie blue stained SDS-PAGE), ovalbumin was undetectable (Figure 4B, lane 2 vs lane 1). The eluted product (glycine, pH 2.7) was confirmed as ovalbumin by ELISA (data not shown) and Western blot (Figure 4B, lane 5) using antiovalbumin polyclonal antibodies different for the two methods.

DISCUSSION

We have developed specific antiovalbumin sdAb fragments capable of discriminating between ovalbumin and OVAX, as shown by DLB, SPR, and WLB characterization. None of the 49 phages selected on the basis of binding to ovalbumin was able to bind OVAX, although the amino acid sequences of ovalbumin and OVAX are 61% identical (Figure 1A). These data are open to many interpretations. First, the amino acid sequences common to both proteins could be nonantigenic or at least nonimmunogenic, which may explain the lack of sdAb fragments directed against them. Second, such sdAb fragments could be significantly under-represented because of the potential immunodominance of the OVA-specific epitopes as compared with those common to OVA and OVAX. Lastly, the common amino acid sequences could be inaccessible during the process of phage display selection. Altogether, this might be

Figure 4. Analysis of ovalbumin affinity purification step by SDS-PAGE in reduced conditions with Coomassie blue staining (A) and WB (B). One milliliter of allantoic liquid from 14 day old chicken embryos was loaded on an antiovalbumin sdAb fragments immobilized matrix. (A) Loaded sample (10 μL, lane 1), first flow-through fraction (10 μL, lane 2), and second flow-through fraction (10 μL, lane 3). (B) Lanes 1−3: identical to A, first elution fraction (10 μL, lane 4), and second elution fraction (10 μL, lane 5). After transfer, the membrane was incubated with a rabbit antiovalbumin polyclonal antiserum (C6534, Sigma-Aldrich, 1:5000), followed by a peroxidase-labeled donkey antirabbit IgG antibody (711-035-152, Jackson Immunoresearch, 1:10000), and binding was revealed with DAB and H_2O_2.

due either to the presence of post-translational modifications or to the specific folding of one of the proteins. The apparent 50 kDa molecular weight OVAX (Figure 3A, lane 3) was higher than the one deduced from the amino acid sequence at 43.8 kDa. Moreover, OVAX migrated as a broad band on SDS-PAGE with reduced conditions (Figure 3A, lane 3, and Figure 3D, lane 3). This suggests that OVAX could be glycosylated like its homologues ovalbumin[18] and OVAY.[6] Only one of the two potential N-glycosylation sites is glycosylated in ovalbumin (Figure 1A). Five potential sites of N-glycosylation (Asn-X-Ser/Thr) were predicted in OVAX (Figure 1A). The determination of the OVAX glycosylation pattern and a comparison of the 3D structure of ovalbumin and OVAX may yield an explanation for the present data. The observation that polyclonal antibodies raised against ovalbumin have a much higher affinity for ovalbumin than for OVAX is suggested by their binding capacities illustrated by the WB data (Figure 3D, lane 3 vs lanes 1 or 2) and evidenced by SPR (not shown). This leads to a similar interpretation. Nevertheless, it cannot be excluded that polyclonal antibodies developed before the first characterization of OVAX could have been produced using an insufficiently purified ovalbumin, containing OVAX traces, as immunogen. In this case, OVAX-specific Igs might be under-represented as compared to the ovalbumin-specific ones. The analysis of a higher diversity phage display might have generated phages capable of binding both ovalbumin and OVAX.

Antiovalbumin sdAb fragments can be used to develop immunoaffinity purification system. VHHs and to a lesser extent VHs are well-known to be stable and resistant proteins suitable for coupling on immobilized matrix .[19,20] Verheesen et al.[21] have shown, for anti-ice structuring protein VHH, that such VHHs linked matrix can support various hundreds of regenerations without loss of binding capacity. We report the depletion of around 90% of ovalbumin from 14 day embryos allantoic fluid on an affinity matrix made with ova41 and ova82. The depletion rate was estimated with the SERAZYN ELISA kit for which the specificity of the antiovalbumin polyclonal antibody, notably concerning the possible cross-reaction with other ov-serpins, was not specified. Moreover, an approximately 75 kDa contaminant protein was detected by WB in both the loaded sample and the flow-through fractions (Figure 4B, lanes

1 and 2). These reasons could explain why the mean depletion rate estimated by ELISA was only 90%, although no 45 kDa ovalbumin was detected in the flow-through fraction using WB (Figure 4B, lanes 2 and 3). This depletion rate was similar in all five allantoic fluids analyzed. Antiovalbumin sdAb fragments-mediated affinity purification could be used in vaccine preparation from embryonated eggs, as an alternative for removal of ovalbumin, one of the major contributors to allergic reactions. It could also be applied to remove a large part of ovalbumin from egg white proteins before proteomic or biochemical studies of rare proteins. Alternatively, OVAX-free ovalbumin could be purified by elution from such a matrix.

If ova82 exhibited all of the hallmarks of VHHs,[22] on the contrary, the ova41 sdAb fragment exhibited VH hallmarks, although it was isolated from a library expressing total VHHs. Several studies showed similar typical conventional VHs with VHH properties, in llama and camel sdAb libraries.[23,24] While the characteristic hydrophobic stretch G44L45W47 (Kabat numbering,[25] IMGT, G49L50W52, Figure 1B) considered as hallmark for the VHs was present in the ova41 sequence, three other positions (K11, A37, and Q103, Kabat numbering) showed noncanonical aminoacid for VH. The conserved Leu at position 11 (IMGT L12, Figure 1B) in VHs was replaced by Lys in ova41. L11 was reported to interact with F149 and P150 and participate to the elbow motion of classical IG via the connection between the VH domain and the CH1 domain.[26] The substitution L11K may decrease the interaction with F149 and P150, two apolar aminoacids. The replacement of L11 by a more hydrophilic residu could also contribute to make ova41 more soluble than a classic VH. At position 37 (IMGT 42, Figure 1B), Val was substituted by Ala. This could affect the thermostability of ova41,[27] even if Val is replaced by another non polar aminoacid. Tryptophan103 (IMGT 118, Figure 1B) in VH domains is involved in the association with the VL domain.[22] In ova41, Trp was substituted by Gln, a polar amino acid. W103 is a hallmark of VHs, but it was replaced by more hydrophilic aminoacids in some llama VHHs. Such a change in the hydrophobicity of the VH surface might make an association VH-VL less efficient. Even if ova41 has numerous VH characteristic hallmarks (G44L45W47), the substitutions at position 11, 37, and 103 could modify its structural properties.[23,24] Ova41 had a nanomolar affinity against OVA (Figure 2B) without the need for VL association and so can be described as a VHH-like VH. Thus, we used a ^{125}I-labeled ova41 to reveal the corresponding antigen in WLB. A ^{125}I-labeled ova41 with nanomolar affinity allowed ovalbumin, either purified or from egg white proteins/magnum tissue extracts, to be revealed (Figure 3B).

Labeling with the ^{125}I-Bolton-Hunter reagent clearly preserved binding ability or, at least part of it, whereas "chloramine-T-labeling" did not (Figure 3B vs 3C). Chloramine-T-labeling induces preferential covalent linking of the label onto tyrosine residues.[28] Furthermore, CDR3 are strongly implicated in the affinity of sdAbs. Altogether, these two data and the presence of a tyrosine residue in the CDR3 of ova41 (Figure 1B) that is potentially affected by "chloramine-T-labeling" could explain the results. A similar loss of binding activity has been observed after chloramine-T labeling technique by Bolton and Hunter,[17] although the label was carried out onto some proteins or peptides different from sdAb fragments. Conversely, with their [125]I N-hydroxysuccinimide labeling method,[17] the binding activity was preserved as we observed here for ova41. To our knowledge, these are the first

published data concerning the loss of binding capacities after chloramine-T sdAb fragment labeling.

We showed that ova41 and ova82 do not present affinity for OVAX, but the lack of available purified OVAY prevented us from concluding on the specificity of these sdAb fragments toward all of the ov-serpins. OVAY is a glycosylated protein that migrates around 52–53 kDa in SDS-PAGE,[29,30] slighty higher than the apparent molecular weight of OVAX (around 50 kDa, Figure 3A, lane 3, and Figure 3D, lane 3). Nevertheless, the two ov-serpins may not be easily distinguishable by SDS-PAGE in the same sample, because of the heterogeneous migration of glycosylated proteins. OVA and OVAY have been only separated by electrofocalization.[31] Evidently, the availability of purified OVAY will be necessary to determine the cross-reactivity of our antiovalbumin sdAb fragments with OVAY.

In conclusion, we have developed and characterized ovalbumin-binding sdAbs fragments with no affinity for OVAX. These sdAb fragments should contribute to better purification and characterization of ovalbumin preparations and also to distinguish between ov-serpins in addition to other existing tools. Furthermore, these sdAb fragments appeared to be able to efficiently deplete ovalbumin from ovalbumin containing media in only one step.

■ AUTHOR INFORMATION

Corresponding Author
*Tel: 33 247 427 967. Fax: 33 247 427 743. E-mail: Gilles.Bruneau@tours.inra.fr.

Notes
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■ ABBREVIATIONS USED

BSA, bovine serum albumin; DLB, dot ligand blotting; Ig, immunoglobulin; MS/MS, tandem mass spectrometry; OVAX, ovalbumin-related protein X/similar to ovalbumin-related protein Y; OVAY, ovalbumin-related protein Y; PBS, phosphate-buffered saline; RU, resonance unit; sdAb, single-domain antibody; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; WB, Western blotting; WLB, Western ligand blotting

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