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Affinity purification of egg yolk immunoglobulins (IgY) with a stable synthetic ligand

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ABSTRACT

Chicken IgY (egg yolk immunoglobulin) is a functional equivalent of mammalian IgG. Traditional methods for IgY purification involve multi-step procedures that result in low recovery of IgY. After a large scale screening of our 700-member synthetic ligand library synthesized by epichlorohydrin and cyanuric chloride methods, a high efficiency ligand of IgY was found. By one-step purification with this ligand, the purity of IgY could reach 92.1%, and the recovery of IgY could reach 78.2%. This synthetic ligand had a higher binding capacity of 74.8 mg IgY/ml and had no negative effects on immunoreactivity. Remarkably, this ligand was also highly stable and could resist 1 M NaOH, thus having great potential for the industrial-scale production of IgY.

1. Introduction

Chicken IgY (egg yolk immunoglobulin) is the functional equivalent of mammalian IgG [1]. It is found in the serum of the chicken and is passed from the mother chicken to the embryo via the egg yolk, a process that results in a high concentration of IgY in the egg yolk [2]. The use of chicken IgY has many advantages compared to mammalian IgG [2] such as: (1) no sacrificing or bleeding of the animal, (2) high yield (100–150 mg IgY/each yolk), and (3) less cross-reactivity with mammalian proteins. Due to the difficulty in the removal of the lipid fraction of the egg yolk and the difficulty in purification of IgY, the IgY yield is less than 2% of the total polyclonal antibodies produced worldwide [3]. Traditional methods for IgY purification involve multi-step, complex, and time consuming procedures such as ammonium sulfate precipitation [4], polyethylene glycol precipitation [5], water dilution [6], ultra filtration [6], gel filtration [7], thiophilic gel chromatography [8], and ion exchange chromatography [9]. The abundant lipoprotein in egg yolk is usually denatured with chloroform and removed by centrifugation. The recovery of IgY via these methods is very low. According to United States Patent 5367054 [10], using the multi-step procedure of water dilution, ultra filtration, anion exchange, and ammonium sulfate precipitation, the obtained purity of IgY was 88%, and the total recovery of IgY was 84.4% × 75.1% × 72.5% × 68.8% = 31.6%.

Recently, Verdoliva et al. [3] reported a synthetic ligand for IgY affinity purification consisting of a tetrameric tripeptide (Arg-Thr-(Tyr)$_4$-K$_2$-K-G), named TG19318, synthesized by solid-phase peptide synthesis using a peptide synthesizer. Greater than 90% recovery and 90% purity of IgY were obtained by this ligand. Its binding capacity reached 65 mg IgY/ligand ml^{-1}. However, this ligand detaches from the matrix and its binding capacity is reduced by 30% after exposure to 0.1 M NaOH for 1 h [3]. Because the ligand column needs to be cleaned and used repeatedly in industrial production, this deficiency greatly limits this ligand's industrial scale application for IgY purification.

Since a non-peptide mimic of protein A was designed and synthesized for the affinity purification of IgG [11] by the synthetic pathway of epichlorohydrin and cyanuric chloride (2,4,6-trichloro-1, 3,5-triazine) as the scaffold, a ligand library with more than 700 compounds has been constructed in our lab [12]. Due to the covalent link between the gel matrix and ligand, all our ligands can resist chemical and biochemical degradation, display ease and low cost of production, and can withstand harsh sterilization without loss of performance. By performing a large scale screening from this ligand library, we found a highly stable affinity ligand, named as ligand 8-6, for the high efficiency purification of IgY.

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2. Materials and methods

2.1. Materials

The columns and amino compounds needed for ligand synthesis and packing were from Pierce, USA and Sigma–Aldrich Chemical Co., USA, respectively. Protein molecular mass markers and antibodies were from Promega Co., USA. Sepharose 4B and Sephacryl S-100 HR were from Pharmacia Biotech, Sweden. All other chemicals were of analytical reagent grade from local companies.

2.2. Immunization of chicken and preliminary extraction of IgY from egg yolk

According to the standard adjuvant method, laying chickens were immunized using formaldehyde-killed *Nitzschia closterium* as the antigen [13]. For the first dose, 200 μg antigen was mixed with an equal volume of Freund's complete adjuvant and was injected into each bird's cervical subcutaneous area. Two weeks later, 200 μg antigen was emulsified with an equal volume of Freund's incomplete adjuvant, and was injected into each bird's muscle under the wing. After another two weeks, the third immunization was made with 200 μg antigen without adjuvant. The eggs were collected daily, marked, and stored at 4 °C until use.

After being carefully broken, the egg white and egg yolk membrane were removed. The egg yolk was mixed fully with distilled water and chloroform at 1:4:1 (v/v/v) and was shaken at 60 rpm for 2 h. The mixture was centrifuged at 10,000 rpm for 25 min at 4 °C. The supernatant was used for further purification.

2.3. Screening of the optimal ligand and affinity purification of IgY

The design and synthesis of the ligand are referenced in the relevant article from our laboratory [12]. For the screening of the optimal ligand, 0.5 ml of gel immobilized synthetic ligand was packed into a small polystyrene column with porous discs at the bottom and the top of the gel. 240 columns of ligand linked gel were selected to bind the IgY according to the criterion of maximizing the difference of the ligands. Batch experiments were performed and each batch was composed of 24 ligand columns. The columns were equilibrated with a 10-fold column volume of buffer A (20 mM phosphate buffer, conductivity 2.2×10^3 μs/cm, pH 7.0) at 20 °C. The chloroform extract of egg yolk (8.4 mg/ml) was adjusted to pH7.0 and the conductivity was adjusted to 2.2×10^3 μs/cm with buffer A. After centrifugation for 25 min at 10,000 rpm, 3.0 ml of the supernatant was loaded onto the columns. Buffer A was used to wash these columns until the absorbance at 280 nm was less than 0.002. The flow rate was 3 ml/min. The bound proteins of the ligands were eluted with 0.1 M acetic acid. 0.1 M NaOH was used to clean and regenerate these columns. The protein concentration was determined with the Lowry method and referenced with bovine serum albumin [14]. The recovery of IgY was calculated by the contents of IgY in the eluate and loading sample via this Lowry method.After the optimal ligand of IgY, named as ligand 8-6, was chosen, a glass column (8 cm × 2.6 cm) (Pierce, USA) packing 40 ml ligand 8-6 was used for the scale-up purification. According to the purity and recovery of IgY, buffer A was optimized to 25 mM phosphate buffer, conductivity 3.0×10^3 μs/cm, pH 7.2. The chloroform extract of egg yolk was adjusted to pH7.2, and conductivity adjusted to 3.0×10^3 μs/cm with buffer A. After centrifugation for 25 min at 10,000 rpm, 270 ml of the supernatant was loaded into the glass column. The bound proteins of the ligand were eluted with buffer B (25 mM phosphate buffer plus 1.0 M NaCl, pH 7.2). 0.1 M NaOH was also used to clean and regenerate this column.

2.4. Gradient non-reduced SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An 8–15% gradient, non-reduced SDS-PAGE was carried out on a Miniprotean II system from Bio-Rad (Hercules, CA, USA). 10 μl samples were mixed with 10 μl loading buffer (4% SDS, 20% (v/v) glycerol and 0.02% Bromophenol Blue in 0.1 M Tris–HCl, pH 6.8). 8–15% gradient SDS-polyacrylamide gels were prepared according to the standard method. The electrophoretic buffer was 0.1% (w/v) SDS, 0.025 M Tris and 0.25 M glycine. Gels were stained and imaged with Coomassie® Brilliant Blue R. The purity and molecular mass of IgY were analyzed and calculated by Gelpro Analyzer 3.0 software (Media Cybernetics, Inc.).

2.5. HPLC analysis

The bound protein eluate of ligand 8-6 was submitted to HPLC analysis with a Knauer system of K501 pumps, K2501 UV monitor (Germany), and a TSK G3000SW column (TOSOH Co.). The solvent phase was 0.1 M PBS, 0.1 M Na_2SO_4, 0.05% NaN_3, pH 6.7. The flow rate was 0.6 ml/min.

2.6. Enzyme linked immunosorbent assay (ELISA)

The immunoreactivity of the purified IgY was determined by enzyme linked immunosorbent assay (ELISA) as described by Verdoliva et al. [3] with some modifications. Ninety-six well plates were coated with 5 ml sonicated suspension of formaldehyde-killed *Nitzschia closterium* (5000 cell per well) in 0.01 M phosphate buffer solution (PBS), and were incubated overnight at 4 °C. Plates were washed five times with 0.01 M PBS-0.05% Tween 20, followed by a blocking step using 200 μl/well of 1% BSA in 0.01 M PBS for 1 h at 37 °C. After incubation, the plates were washed five times with 0.01 M PBS-0.05% Tween 20. After dialysis and exchange with PBS buffer, the sample was adjusted to the final concentration of 1.0 mg/ml, and 50 μl wad added to each well. After incubation for 1.5 h at 37 °C, the plates were then washed five times with 0.01 M PBS-0.05% Tween 20, and then 100 μl of rabbit anti-chicken IgG peroxidase, diluted 1:1000 in 0.01 M PBS-0.05% Tween 20-1% BSA, were added to each well. After 1.5 h of incubation at 37 °C, the plates were washed with 0.01 M PBS-0.05% Tween 20 five times, and developed with a chromogenic substrate solution consisting of 0.4 mg/ml OPD (*o*-phenylenediamine) in 0.1 M sodium citrate buffer, pH 5.0, containing 5 mM hydrogen peroxide, and stopped by 2 M H_2SO_4. The absorbance at 492 nm of each sample was measured with a Shimadzu UV-260 UV-Visible Recording Spectrophotometer.

2.7. Determination of the stability of ligand 8-6

The stability of the synthetic ligand 8-6 was determined by treating with NaOH. Four columns (3.5 cm × 0.6 cm) packed with 0.5 ml ligand 8-6 were incubated in 0.1, 0.5, and 1.0 M NaOH for 14 days at 20 °C, followed by equilibration with buffer A (25 mM phosphate buffer, conductivity 3.0×10^3 μs/cm, pH 7.2). Then, the chloroform extract of the egg yolk was applied to the column, eluted with buffer A, and then buffer B (25 mM phosphate buffer plus 1.0 M NaCl, pH 7.2) according to the methods mentioned above.

2.8. Determination of the maximal binding capacity of ligand 8-6

The maximal binding capacity of ligand 8-6 to IgY was examined by overloading the affinity column with the purified IgY. Forty-five millilitre of purified IgY (2.3 mg/ml) was loaded onto the column containing 0.5 ml ligand 8-6 at a flow rate of 0.5 ml/min, and was eluted by buffer A and buffer B according to the method mentioned

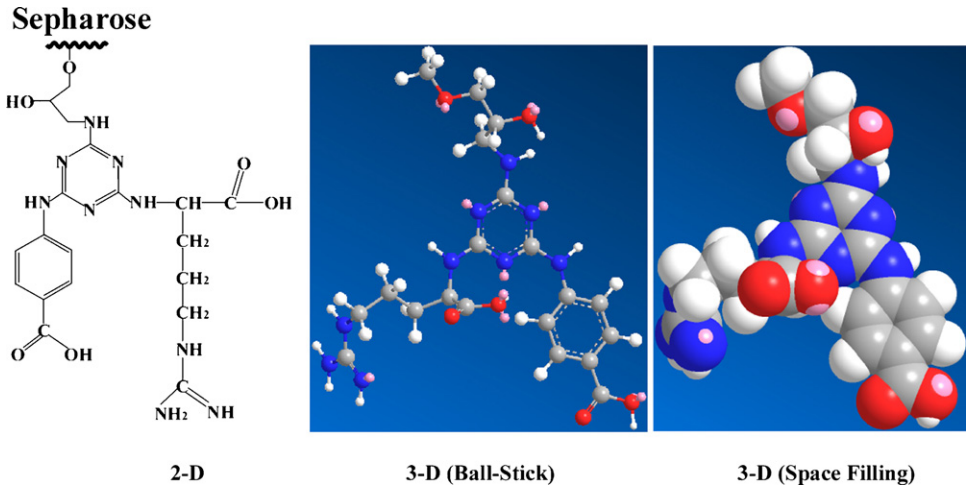

Fig. 1. 2-D and 3-D structures of synthetic ligand 8-6.

above. The binding capacity of ligand 8-6 to IgY was calculated according to the bound amount of IgY and the ligand volume.

3. Results and discussion

3.1. Screening of ligands

By screening 240 ligands featured in column packing, column equilibration, sample loading, flowthrough washing, bound protein eluting, column cleaning, gradient SDS-PAGE separating, gel staining, one optimal ligand for high efficiency purification of IgY, named as ligand 8-6, was found. The active groups of this ligand were *p*-aminobenzoic acid and arginine, respectively; 2-D and 3-D structures of this ligand are shown in Fig. 1.

3.2. Purification of egg yolk immunoglobulins (IgY)

By the scale-up purification of IgY with synthetic ligand 8-6 and 8–15% gradient non-reduced SDS-PAGE, the band profiles of egg yolk crude extract (lane 1) and the bound protein eluate (lane 2) of ligand 8-6 are shown in Fig. 2A. It can be clearly seen that the bound protein eluate of ligand 8-6 (lane 2) presented one single strong band. Further analysis confirmed that the band purity increased from 14.3 to 92.1%, and that the band recovery reached 78.2%. HPLC analysis indicated that the band purity could reach 94.3% (See Fig. 2B).

Sephacryl S-100 HR is a gel filtration medium that can be applied to remove salt and low molecular weight proteins less than 100 kDa. We used Sephacryl S-100 HR (5 cm × 30 cm, Pharmacia) to further purify the IgY (about 180 kDa) eluate after affinity chromatography with ligand 8-6. The results showed that the purity of IgY could be further increased from 92.1 to 96.5% by Sephacryl S-100 HR.

3.3. ELISA of IgY

The immunoreactivity of the purified IgY was determined by ELISA, using formaldehyde-killed *Nitzschia closterium* as the antigen. The obtained results are shown in Fig. 3. After immunization with *Nitzschia closterium*, the IgY activity increased significantly. The immunoreactive recovery of purified IgY via ligand 8-6 was higher than that of crude extract. This indicated that affinity purification of ligand 8-6 had no negative effects on the immunoreactivity of IgY and confirmed that the purified protein was exactly IgY.

3.4. The stability of ligand 8-6

One of the major concerns of applying affinity ligands for industrial scale procedures is the stability of the ligands under the conditions used for the cleaning and regeneration of the affinity columns. In particular, resistance to 1 M NaOH, used in depyrogenation, is essential, and was therefore used as the benchmark to assess the stability of the affinity adsorbent [15]. We used NaOH to test the stability of synthetic ligand 8-6. Results showed that the binding capacities of three different treatments (0.1, 0.5, 1.0 M NaOH) were 3.74, 3.64, and 3.64 mg IgY/ml moist weight gel, respectively, and were only reduced 0.68%, 3.43%, 3.43% compared to the water control (3.77 mg IgY/ml moist weight gel). The IgY purities of three different treatments were 92.2%, 92.7%, 92.3%, respectively, which were all very close to the control of 92.5%. These results indicated

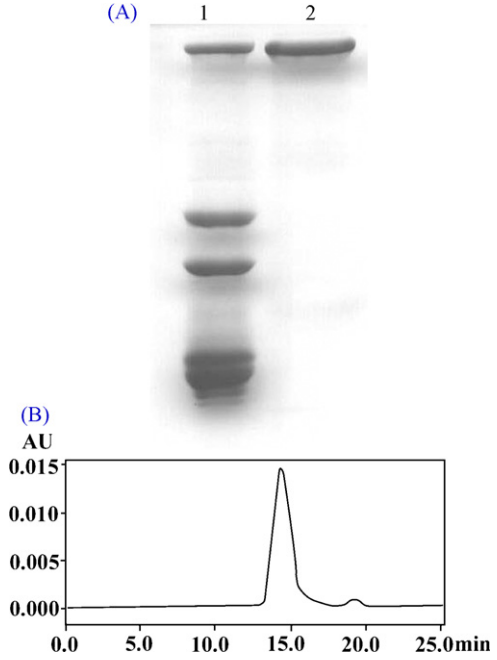

Fig. 2. Affinity purification of IgY with synthetic ligand 8-6. (A) The electrophoretic profile of crude extract (lane 1) of egg yolk and the bound protein eluate (lane 2) of ligand 8-6 by 8–15% gradient, non-reduced SDS-PAGE. (B) TSK G3000SW HPLC of the purified IgY. The flow phase was 0.1 M PBS, 0.1 M Na_2SO_4, 0.05%NaN_3, pH 6.7; the flow rate: 0.6 ml/min. AU: the absorbance at 280 nm.

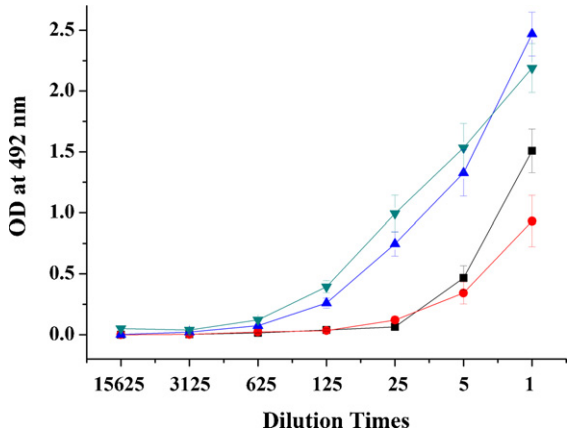

Fig. 3. ELISA of IgY. (●): The chloroform extract of non-immunized egg yolk, (■): the purified IgY of non-immunized egg yolk, (▲): the chloroform extract of egg yolk immunized with *Nitzschia closterium* and (▼): the purified IgY of egg yolk immunized with *Nitzschia closterium*. The initial concentration of IgY was adjusted to 1 mg/ml. Results were averaged in triplicate.

that the synthetic ligand 8-6 was highly stable, could resist 1.0 M NaOH, and had great potential for the industrial production of IgY.

3.5. The maximal binding capacity of ligand 8-6

By overloading the affinity column of ligand 8-6 with purified IgY in triplicate, the maximal binding capacity of ligand 8-6 to IgY was calculated as 74.8 mg IgY/ml moist weight gel, which is higher than the binding capacity of TG19318 (65 mg/ml) reported by Verdoliva et al. [3] and the commercial product HiTrap™ IgY Purification HP Column (20 mg/ml, http://www1.gelifesciences.com). This demonstrated that our synthetic ligand 8-6 was excellent for the affinity purification of IgY and could be used as a substitute for TG19318 and HiTrap™ IgY Purification HP Columns.

3.6. Affinity purification of ligand 8-6 with various antibodies

To extend the potential application of ligand 8-6, we tested its affinity purification abilities on the IgG of human serum, bovine serum, rabbit serum, as well as the IgY of duck yolk, pigeon yolk, and quail yolk. All the samples were diluted with distilled water, adjusted to pH 7.2 and a conductivity of 5.8×10^3 μs/cm, and centrifuged for 10 min at 10,000 rpm. A 10 ml volume of supernatant was loaded to the column of ligand 8-6 and washed with 5 ml buffer A. The bound fractions were eluted with 0.1 M HAc, and then with 0.1 M NaOH. All the eluates were resolved by 8–15% gradient, non-reduced SDS-PAGE. The recoveries measuring over 50% of IgG or IgY included human serum, duck yolk and pigeon yolk. The purity and recovery of human IgG could reach 86.4% and 67.4%, respectively; the purity and recovery of pigeon IgY could reach 75.4% and 85.2%, respectively; and the purity and recovery of duck IgY could reach 75.6% and 54.3%, respectively. This indicated that ligand 8-6 could be applied for the affinity purification of IgG or IgY in human serum, duck yolk, and pigeon yolk. In addition, ligand 8-6 could purify both IgY and IgY (ΔFc) of duck yolk according to the band profile of SDS-PAGE (data not shown).

4. Conclusion

Based on our experimental results that ligand 8-6 could withstand the harsh cleaning of 1 M NaOH without loss of performance, that we could one-step purify and recover 78.2% IgY from the chloroform extract of egg yolk, that the purity of IgY could reach 92.1%, and that its maximal binding capacity was higher than that of the TG19318 and HiTrap™ IgY Purification HP Columns, we believe that ligand 8-6 has great potential for the industrial scale purification of IgY.

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