

Extraction and Purification of Anti-*Helicobacter pylori* IgY

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Abstract

The aim of this study was to establish an efficient procedure to produce anti-*H. pylori* urease IgY from the eggs of the laying hens. Four White Leghorn hens (52 weeks old) were immunized intramuscularly with the purified urease of *H. pylori* with aluminum hydroxide gel adjuvant. To increase the specificity and antibody titer, three boosters were given at 2-week intervals following the first injection. After the final immunization, the eggs were collected daily and stored at 4°C. The yield of yolk proteins were 47.52%, 41.07% and 51.35% for the methods of dextran sulphate, isopropanol and water-soluble fraction (WSF), respectively. Further, IgY was purified successively by ammonia sulfate, affinity chromatography and ion exchange chromatography for each crude protein. Ammonia sulfate salting produced the IgY with a purity of 37-53% and the titer of 22.6-29.5 unit/mg. The purity, relative purification folds and titer characteristics of the purified IgY were 85-90%, 19.63-34.61 and 223.8-273.4 unit/mg protein for affinity chromatography, and 89-92%, 25.33-47.14 and 288.8-372.4 unit/mg protein for ion exchange chromatography, respectively. This anti-*Helicobacter pylori* IgY was specific against *H. pylori* urease. Here, we established a simple and inexpensive method to obtain a yield of 90% purity of the anti-*H. pylori* IgY in combinations of the WSF method, ammonia sulfate precipitation, and affinity chromatography.

Keywords: white leghorn hens, extraction and purification, Anti-*Helicobacter pylori*, IgY

1. Introduction

Most commercial antibodies are produced from rabbits, rats or sheep. However, poultry is more cost-effective to produce antibody from egg yolk (100-150 mg per egg⁻¹) than from blood of mammals (Karlsson et al., 2004; Ko & Ahn, 2007). In mammalian, the IgG is the most abundant than other four antibodies including IgA, IgM, IgE and IgD. Similar to mammalian IgG in function and structure, IgY (immunoglobulin from yolk, so-called chicken IgG.) of poultry is transferred from blood to the egg yolk in a passive process. However, these two antibody types differ in molecular weight (180 Kda for IgY vs 150 Kda for mammalian IgG) (Tini et al., 2002; Zhang, 2003). However, properties regard lipidphilia, and the pH of isoelectric point revealed that IgY is more lipidphilic and lower pH than IgG (Davalos-Pautoja et al., 2000).

For the wide utilization of the IgY, several methods have been developed to improve the purity and quantity of the IgY purified from egg yolk in more simple and less chemicals (Kim & Nakai, 1998). In the IgY purification, high concentration of lipids and lipoproteins in egg yolk is one of the major obstacles (Verdolviva et al., 2000). Therefore, various strategies have been used to purify IgY firstly with salt precipitation i.e. ammonium sulfate ((NH₄)₂SO₄) or sodium sulfate (Na₂SO₄), followed by centrifugation, alcohol precipitation such as isopropanol (Bade & Stegemann, 1984), or water-soluble fraction (WSF) and chromatographic methods (Akita & Nakai, 1992). Further, a simple and automatic filtration system has been developed (Polson et al., 1985). However, gel filtration chromatography and affinity chromatography are still required for final purification (Kim & Nakai, 1996). With application of mammalian IgG in disease diagnosis or experiments e.g. ELISA, detoxification of toxic materials, and prevention and treatment of diseases (Reilly et al., 1997; Yokoyama et al., 1992), IgY can

prevent and cure intestinal infectious diseases such as enterotoxigenic *E. coli* and *Salmonella* (Yolken et al., 1988; Ikemori et al., 1992; Karlsson et al., 2004). Therefore, IgY is a potential substitute for antibiotics or vaccine.

Helicobacter pylori infection is frequently observed in adults worldwide and causes gastric and duodenal ulcers, gastric lymphoma, and possibly gastric carcinomas (Shin et al., 2002; Chu et al., 2003). Neutralization of highly acidic environment by secreted urease leads to *Helicobacter* spp. survival in stomach, which penetrates and damages the mucus (Shin et al., 2003; Yang et al., 2012). Therefore, the urease can be a potential diagnostic and therapeutic target by developing anti-*H. pylori* IgY to prevent and cure the *H. pylori* associated stomach diseases (Shin et al., 2004). Although multiple antibiotic treatments eradicate most *Helicobacter pylori* (*H. pylori*) infections, therapy fails in 10-15% of cases due to the development of drug resistance (Suzuki et al., 2004). Consequently, it is important that new, more broadly based therapies for the treatment of *H. pylori* infection should be identified. The aim of this study was to evaluate the efficacy of three methods for purification of anti-*H. pylori* IgY from the egg yolks produced by White Leghorn laying hens capable of a high production of eggs and then to establish the method for producing highly purity (> 90%) of anti-*Helicobacter pylori* IgY from White Leghorn hens.

2. Materials and Methods

2.1 Immunization

White Leghorn hens (52 weeks old, n= 4) were immunized with 10 mg purified urease of *Helicobacter pylori* with aluminum hydroxide gel adjuvant (Sigma-Aldrich) through wing muscle injections for 3 times at 2-week intervals following the first injection. Before and after antigen injection, the serum IgY titers were examined to confirm the increase of antibody titer against *H. pylori*. Then the eggs laid were collected daily for 6 months and stored at 4°C. During the period of egg collection, the urease antigen was continuing injected into each hen every month.

2.2 IgY Extraction

After separating the yolks from egg white and puncturing the yolk sac with a needle, the contents were allowed to drip through a nylon mesh into a measuring cylinder, and then diluted with 10-fold distilled water. IgY was precipitated by three methods. In the dextran sulphate method (Jensenius et al., 1981), the diluted yolk was then mixed with dextran sulphate (MW < 500,000) (Sigma-Aldrich) containing 1 M CaCl₂ and then centrifuged at 1,500 X g for 10 min at room temperature. The precipitate was dissolved with PBS and stored at -20°C. In the isopropanol method (Bade & Stegemann, 1984), 100 mL of isopropanol were mixed with the diluted yolk solution for 4 hours at 4°C. The mixture was centrifuged at 3,000 X g for 15 min. to separate yolk fat. The pellet was washed thrice until pellet became white from yellow. The PBS with 0.01% NaN₃ was added to dissolve the precipitate at room temperature for 1 h and the solution was centrifuged at 8,500 X g and 25°C for 30 mins. The supernatant was finally filtered through No.1 filter paper. The filtrate was collected and stored at -20°C. In the extraction of water-soluble fraction with modified method reported by Kim and Nakai (1996); the diluted yolk was adjusted to pH = 5.0 with 0.1 N HCl, and stood at 4°C for 24 h. The solution was centrifuged at 5,500 X g and 4°C for 1 hr. The supernatant was filtered through Whatman No. 52, followed by 0.45-µm filter (Millipore) at 4°C. The filtrate was collected and stored at -20°C.

2.3 Purification of IgY by Affinity Chromatography and Ion Exchange Chromatography

Total egg proteins of the previously prepared filtrates were precipitated by slowly addition of ammonium sulfate [(NH₄)₂SO₄] to reach final concentration about 40%. Next, the protein solution was centrifuged at 8,500 X g for 15 mins. and the pellet was dissolved in phosphate buffer (pH = 7.8). Then, centrifugation was performed at 2,100 X g for 15 mins. After dissolving the protein pellet with PBS, the protein solution in a membrane (Spectra/Por Cellulose Ester membrane MWCO: 100000) was dialyzed with PBS to remove the remaining salt (Hansen et al., 1998; Cook et al., 2001).

The IgY was purified by an affinity column (Econo-Pac Blue Cartridges) packed with DEAE Affi-gel with specific function group (Cibacron® Blue F3GA Cibacron Blue F3GA and (DEAE)-O(CH₂)₂ N(CH₂CH₃)₂HCl) or an Macro-Prep ion exchange column (Econo-Pac High Capacity Ion Exchange Cartridges) with specific function group (-SO³⁻). One mL of the protein solution was infused into the column and the IgY was eluted by buffer containing 1.4 M NaCl. In addition, the IgY was purified by an ion exchange column (Econo-Pac High Capacity Ion Exchange Cartridges) packed with Macro-Prep ion exchange supports with specific ligand. One ml of the sample was infused into the column and the IgY was then eluted with low salt buffer. The elutate from both methods was neutralized with 0.02 M Tris-HCl (pH = 8.0) immediately.

2.4 Quantification, Purity, and Titer Determination of IgY

The total protein amount of the sample was determined using either the Biuret method or Lowry method (Lowry et al., 1951). The IgY purity was determined by SDS-PAGE. Equivalent amounts of proteins were boiled in sample buffer (2.5 mL 0.5 M Tris-HCl, pH 6.8; 4.0 mL 10% SDS; 2.0 mL glycerol; 1.0 mL β -mercaptoethanol (2-ME); and 0.5 mL distilled water for 5 minutes. 30 μ g of total proteins were separated by 4-8% polyacrylamide gel at 70 volt. Commercial IgY (Sigma) was used as the standard. The concentration of each band was determined using a densitometer (Helena, Model Bfj 00105, USA). The IgY antibody titers were measured with the ELISA method and commercial IgY was used as the standard. The relative purity and recovery was determined as follows: The relative purity (fold) = the titer in steps/the titer in crude extract step. Recovery (%) = protein x titer in steps/(protein x titer in crude extract step).

The specificity of the IgY against the urease of *H. pylori* was evaluated by western blotting. When SDS-PAGE was finished, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MO, USA). The membranes were incubated in blocking buffer (5% (w/v) non-fat milk in PBS) at room temperature for 1 h. After blocking, chick anti-*H. pylori* IgY was added at 1/100 at 4°C with gentle shaking overnight. The membrane was washed with PBST (per liter containing 2.9 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , 0.2 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 mL Tween 20) for three times and then incubated with rabbit anti-chicken IgY antibody (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. Enhanced chemiluminescence (ECL) substrates reagents (PerkinElmer, MA, USA) was used to develop the fluorescent signals.

3. Results and Discussion

As major pathogen to cause gastric and duodenal ulcer and possibly gastric cancer, *H. pylori* can infect repeatedly more than 90% of adults (Chu et al., 2003). In this study, we established a procedure to produce the anti-*H. pylori* IgY through the eggs of White Leghorn hens. In egg, phospholipids and cholesterol of the fat accounts for about one-third of the yolk weight; proteins account for about 15-17% of the yolk weight and the IgY is major component in plasma protein (Powrie, 1976). Therefore, different methods, including extra-centrifugation (McBee & Cotterilly, 1979), polyethylene glycol (Polson et al., 1985), polyacryl acid resins (Hamada et al., 1991), sodium dextran sulfate (Jensenius et al., 1981), carrageenan and xanthan gum (Hatta et al., 1990) have been developed to purify the proteins from the fats. In this study, we compared the efficiency of three extraction methods in extraction of the total proteins from egg yolks. The yields were 47.52%, 41.07% and 51.35% for the methods of dextran sulphate, isopropanol and water-soluble fraction (WSF), respectively (Table 1). Apparently, water soluble fraction method is the best method to extract the total proteins from the egg yolk.

Further, avian antibodies can be concentrated using different salts, such as ammonium sulphate (Akita & Nakai, 1993), sodium sulphate (Wooley & Landon, 1995) or caprylic acid (McLaren et al., 1994). IgY could reach the purity of 98.3% and yield of 73% by sodium sulfate precipitation (Hatta et al., 1990). In this study, we obtained a purity of 37-53% and titer of 22.6-29.5 unit/mg protein among these three methods by the optimal ammonia sulfate ($(\text{NH}_4)_2\text{SO}_4$) 45~55% (W/W). This result indicates that extraction method also affect the protein concentration by the salting precipitation. Following, there are many methods to purify the IgY from yolk (Schwarzkopf & Thiele, 1996; Gee et al., 2003). Firstly, simple water dilution method is used to remove lipoproteins from WSF for the IgY purification (Jensenius et al., 1981; Kwan et al., 1991; Akita & Nakai, 1992; 1993). This water dilution method has demonstrated as the best method to recover and purify the IgY with the highest purity of 34% in comparison with the methods of polyethylene glycol, dextran sulphate and chloroform (Verdoliva et al., 2000). However, the purity (74-78%) of the IgY from WSF with water dilution method was lower than that (83-99%) of the IgY from WSF with filter paper (Kim & Nakai, 1998).

The IgY has been further purified from the WSF protein extracts by different processes, such as delipidation to extract the IgY from other livetins (Kim & Nakai, 1998). Generally, affinity chromatography and ion exchange chromatography are the best methods to purify the IgY. Verdoliva et al. (2000) obtained the highest yield of the IgY (10.2 mg/mL) and the best purity (> 90%) from the WSF using TG19318/Emphaze and TG19318/Emphaze affinity column. Additionally, affinity chromatography with synthetic ligand could gain the 92.1% purity and the 78.2% recovery of the IgY (Dong et al., 2008). In the present study, we used affinity chromatography to get a purity of 85% at least for three extraction methods, even up to 90% for the WSF method (Table 1). Further purification with ion exchange chromatography only increased slightly the purity of 2-4%. These results suggest that one step of affinity chromatography is enough to purify the IgY costly and efficiently. Further, we characterized the antibodies obtained between affinity chromatography and ion exchange chromatography. The purity, relative purification fold and titer were 85-90%, 19.63-34.61 and 223.8-273.4 unit/mg protein for affinity chromatography and 89-92%, 25.33-47.14, and 288.8-372.4 unit/mg for ion exchange chromatography

respectively (Table 1, Figure 1). Finally we found high specificity of the IgY against the urease of *H. pylori* by western blot analysis (Figure 2). Here, we established a procedure to extract and purify the anti-*H. pylori* IgY from the eggs of White Leghorn hens. This procedure combines sequentially water-soluble fraction (WSF) method, water dilution, ammonium sulfate salting and affinity column. Anti-*H. pylori* IgY can be used to prevent and cure the *H. pylori* infection.

Table 1. Titer and purity of IgY obtained from the egg yolk of White Leghorn hen after purified steps

Technique	Protein (mg)			IgY titer (U/mg protein)			Relative purity (fold)			Purification (%)			Recovery (%)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Crude extract	1687 (47.52%)	1458 (41.07%)	1823 (51.35%)	9.2	7.9	11.4	1	1	1	-	-	-	100	100	100
Salting out (NH ₄) ₂ SO ₄	58.33	55.70	61.24	26.2	29.5	22.6	2.85	3.73	1.98	45	53	37	9.85	14.3	6.66
Affinity Chromatography	2.65	2.26	2.80	247.1	273.4	223.8	26.86	34.61	19.63	87	85	90	4.22	3.02	3.02
Ion Exchange Chromatography	1.60	1.55	1.72	319.0	372.4	288.8	34.67	47.14	25.33	90	89	92	3.29	5.01	2.39

Egg contains protein amount of 3.55 g/yolk

Used 10 eggs in this analysis

Method 1: dextran sulphate

Method 2: isopropanol

Method 3: water-soluble fraction

Relative purity (fold) = the titer in steps/the titer in crude extract step.

Purification (%): The IgY purity was determined by SDS-PAGE and examined using a densitometer.

Recovery (%) = protein x titer in steps/protein x titer in crude extract step.

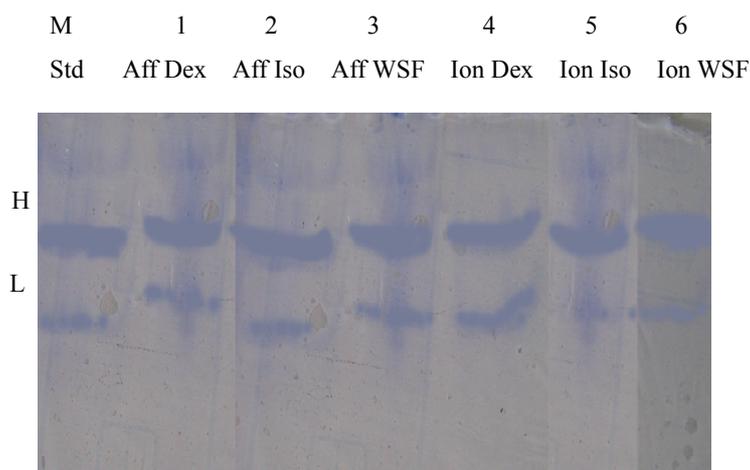


Figure 1. SDS-PAGE analysis of anti-*H. pylori* IgY purified by after affinity chromatography and ion exchange chromatography

After gel electrophoresis in reduced condition, IgY was separated into two polypeptides of ~67 Kda (heavy chain, H) and ~30 Kda (light chain, L). M: IgY standard. Lane 1: IgY purified by dextran sulphate and affinity chromatography; Lane 2: IgY purified by isopropanol and affinity chromatography; Lane 3: IgY purified by water-soluble fraction and affinity chromatography; Lane 4: IgY purified by dextran sulphate and ion exchange chromatography; Lane 5: IgY purified by isopropanol and ion exchange chromatography and Lane 6: IgY purified by water-soluble fraction and ion exchange chromatography.

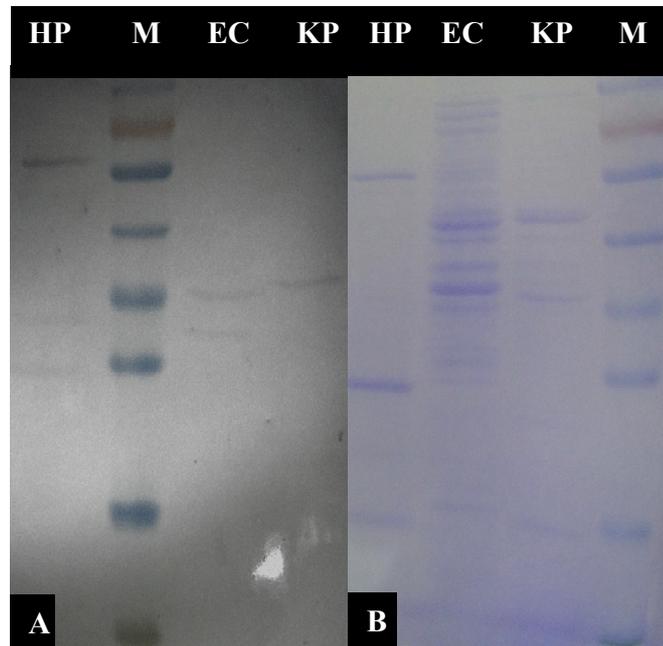


Figure 2. Characterization of chicken IgY against the urease of *Helicobacter pylori* by Western blot analysis (A) and SDS-PAGE gel analysis (B). M: Protein size marker, HP indicates the urease of *Helicobacter pylori*, EC represents *E. coli*, and KP means *Klebsiella pneumoniae*

4. Conclusion

We used White Leghorn hens to produce the anti-*H. pylori* IgY eggs. Among three extraction methods of dextran sulphate, isopropanol and water-soluble fraction (WSF), WSF method was the best method to recover and purify the total proteins. Further, high purity of the IgY could be obtained by ammonia sulfate precipitation and affinity chromatography and/or ion exchange chromatography.

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