

Jinghui FAN, Yuzhu ZUO, Tanqing LI, Xiaobo ZHANG

Preparation and physicochemical property of chicken yolk immunoglobulin (IgY) against porcine transmissible gastroenteritis virus (TGEV)

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Abstract Oral administration of immunoglobulin prepared from the egg yolk of hens immunized with porcine transmissible gastroenteritis virus (TGEV) has been demonstrated to reduce piglets mortality significantly in our previous studies. In the present study, we investigated the stability of chicken yolk immunoglobulin (IgY) specific to TGEV by measuring the remaining activity by ELISA. The results showed that the IgY was stable between pH 4 and pH 11. In the incubation with pepsin at pH 4 and pH 6, about 90% and 100% of the IgY activity remained, respectively. IgY activity could remain approximately 80% at 60°C for 30 min, suggesting that pasteurization can be applied to sterilizing the product. The stability of IgY at 25°C and freezing-thawing treatment indicated that the IgY was easy to be conserved. These results highlight the attractive potential application of IgY as the antibodies of oral administration for treatment of TGEV infections.

Keywords egg yolk immunoglobulin, physicochemical property, porcine transmissible gastroenteritis virus

1 Introduction

Transmissible gastroenteritis (TGE) is a highly contagious viral disease of swine characterized by vomiting, diarrhea, and dehydration. The causative agent has been demonstrated to be porcine transmissible gastroenteritis virus (TGEV). For piglets at the age of younger than two weeks, nearly 100% of them die when exposed to virulent TGEV (Pensaert, 1984; Saif and Wesley, 1999). TGE is of

considerable economic importance to large swine-breeding units. Since swine are born without immunoglobulin, the presence of TGEV-specific antibodies in the colostrum and milk of the sows is critical for the survival of the piglets (Tuboly and Nagy, 2001). This suggests that oral administration of specific antibodies is effective in preventing intestinal infection. However, oral administration of antibodies is prohibitively expensive when large amounts of antibodies are required (Shin et al., 2002). Therefore, it is important to search for effective methods as alternatives to maternal antibody for treatment of TGEV infections.

In 1960s, a large amount of immunoglobulin was found in egg yolk and later denominated IgY (Hatta et al., 1990). The IgY antibodies showed similar biological activities to colostrum antibodies in neonatal pigs (Yokoyama et al., 1992; Yokoyama et al., 1993; Murata and Namioka, 1977). Some studies indicated that IgY was effective, safe, and protective in intestinal infection (Sunwoo et al., 1996; Kuroki et al., 1997; Sarker et al., 2001; Hennig-Pauka et al., 2003). These studies demonstrated that IgY from immunized chickens was an effective means against specific bacterial and viral infections. In the previous study, we produced IgY against TGEV and investigated its immunoprophylactic effect in neonatal pigs (Zuo et al., 2008). Oral administration of the IgY antibodies has been demonstrated to reduce piglets mortality dramatically. In this study, we evaluated the potential application of IgY as an alternative method against TGEV in piglets by investigating its stability.

2 Materials and methods

2.1 Preparation of IgY

Isolation and purification of IgY were previously described in detail by Zuo et al. (2008). Briefly, the white leghorn

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Jinghui FAN (✉), Yuzhu ZUO, Tanqing LI, Xiaobo ZHANG
College of Animal Science and Veterinary Medicine, Agricultural University of Hebei, Baoding 071001, China
E-mail: jinghui76@163.com

hens, 25 weeks old, were immunized intramuscularly with TGEV emulsified with Freund's adjuvant. The crude antibody from yolk was extracted by the water-soluble fraction and further purified using 40% ammonium sulfate precipitation followed by dialysis. Purified IgY was filtered through 0.2 μm membrane filter and stored at -20°C before further experimentation.

2.2 ELISA

To assess the titers of IgY specific to TGEV, we performed the enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated overnight at 4°C with purified TGEV antigen and blocked with 0.5% poly vinyl alcohol. After incubation for 2 h at 37°C , the plates were washed, and then, 100 μL volumes of appropriately diluted IgY preparations were added. After incubation, the plates were washed and incubated with HRP-conjugated goat anti-chicken IgG (invitrogen) for 1 h. The plates were washed again, with substrate solution added (0.04% 3, 3', 5, 5'-tetramethylbenzidine in phosphate-citrate buffer containing 0.02% H_2O_2). After 10 min incubation at room temperature, the reactions were stopped with 50 μL of 2 $\text{mol}\cdot\text{L}^{-1}$ H_2SO_4 per well, and the absorbance at 492 nm was read with an Immunoreader BIO RAD 680. The antibody titer was expressed as P/N values, where P represents the OD_{492} nm of IgY from immunized hens at a given dilution and N the OD_{492} nm of IgY from nonimmunized hens at the same dilution.

2.3 Heat stability of IgY

IgY solutions were incubated at 4°C , 10°C , 25°C , 37°C , 60°C , 70°C , 80°C , and 90°C for 30 min. The heat treated IgY was cooled in a water-ice bath. The remaining antibody activity was measured by ELISA. Antibody activity was represented as a percentage of the untreated control.

2.4 Acid stability of IgY

For the pH stability test, the pH of IgY solutions was modulated to the desired pH 2 to 12 with NaOH or HCl. After being incubated at 37°C for 2 h, each IgY solution was neutralized. The remaining antibody activity was measured by ELISA.

2.5 Pepsin stability of IgY

For the pepsin stability test, the pH of IgY solution was adjusted to 2, 4, and 6, respectively. IgY solution of each pH was mixed with pepsin at a rate of 20:1 (m/m). The mixtures were incubated at 37°C for 1, 2, 3, and 4 h. After the incubation, each IgY solution was neutralized to inactivate the pepsin.

2.6 Trypsin stability of IgY

The procedure of trypsin stability test has no difference with the pepsin stability test but with the pH of IgY solution. In our test, the IgY solution was adjusted to pH 7.5.

2.7 Repeated freezing-thawing treat stability of IgY

The IgY solution was treated with five cycles of freezing and thawing. The remaining antibody activity was measured by ELISA.

2.8 Stability of IgY at room temperature

The IgY solution was preserved at 25°C for 1, 2, 3, 4, 5, and 6 month(s), respectively. The remaining antibody activity was measured by ELISA.

3 Results

3.1 Specific antibody production

Eggs were produced up to three months after the first inoculation of hens with TGEV. The IgY was extracted by the water-soluble fraction (WSF) and purified by ammonium sulfate precipitation. The titer of IgY solution was examined by ELISA. The result showed that the specific antibody started to increase in the egg yolk at the 10th day and became higher after each boosting. The IgY reached its peak at the eighth week and kept at a high level until the last week during our test (Fig. 1).

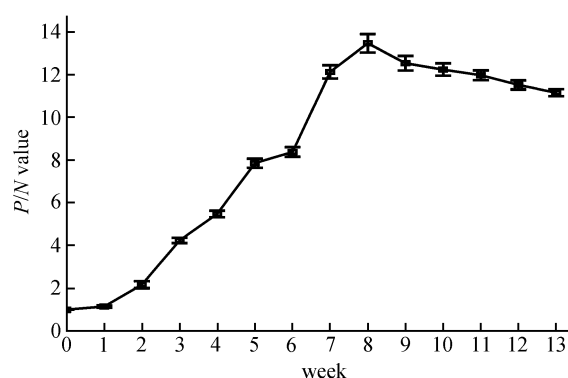


Fig. 1 Changes of IgY titers over time following immunization
Note: Immunization was done at 0, 2, 4, and 6 weeks. Each date point represents the average of three determinations and the error bars, the standard error of means.

3.2 Heat stability of IgY

The IgY solution was stable at 37°C . However, at 60°C for 30 min, the IgY lost approximately 20% of its antibody

activity. The antibody activity significantly decreased at 70°C and was almost completely lost at 80°C (Fig. 2).

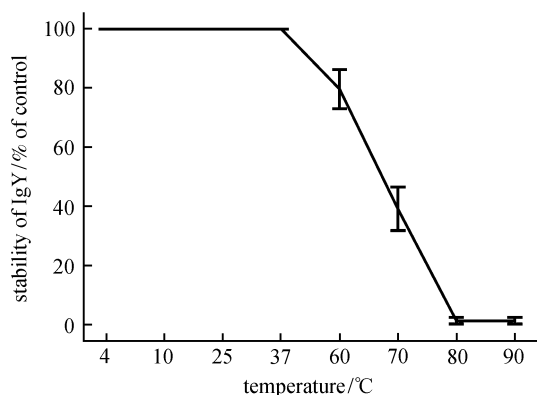


Fig. 2 Heat stability of IgY

Note: IgY was treated at various temperatures for 30 min. Remaining activities after the treatments were measured using ELISA and were expressed as a percentage of the initial activity.

3.3 Acid stability of IgY

IgY, specific to TGEV, showed a broad stability between pH 4 and pH 11. However, the antibody activity significantly decreased at pH 3 and pH 12. IgY lost 70% of its initial activity at pH 2 (Fig. 3).

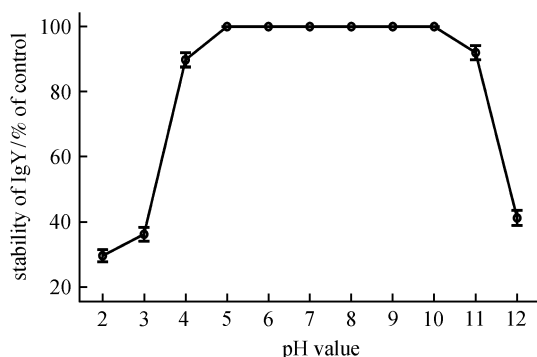


Fig. 3 Acid stability of IgY

Note: IgY was treated at various pH values for 2 h. Remaining activities after the treatments were measured using ELISA and were expressed as a percentage of the initial activity.

3.4 Stability of IgY in pepsin solution

The antibody activity of IgY was decreased rapidly when incubated with pepsin at pH 2. After 4 h of incubation, no active IgY almost existed. It can be deduced that the pepsin could facilitate the denaturation of IgY in an acidified environment (Figs. 3, 4). By contrast, about 90% and 100% of the activity remained at pH 4 and pH 6, respectively (Fig. 4).

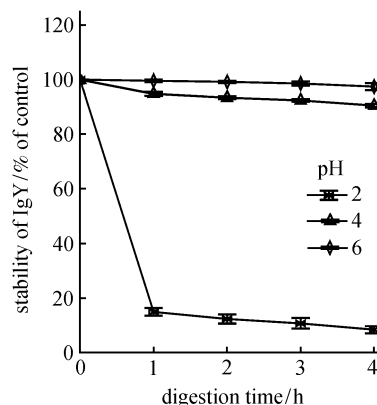


Fig. 4 Pepsin stability of IgY

Note: IgY was treated with pepsin at pH 2, 4, and 6 for 1 h, 2 h, 3 h, and 4 h. Remaining activities after the treatments were measured using ELISA and were expressed as a percentage of the initial activity.

3.5 Stability of IgY in trypsin solution

In trypsin stability test, IgY also showed a broad stability to trypsin. Although the antibody activity of IgY was somewhat decreased, approximately 80% of the antibody activity was still remained after 4 h of incubation (Fig. 5). These results accorded with those obtained by Jaradat and Marquardt (2000), who claimed that the remained IgY activity was 82%.

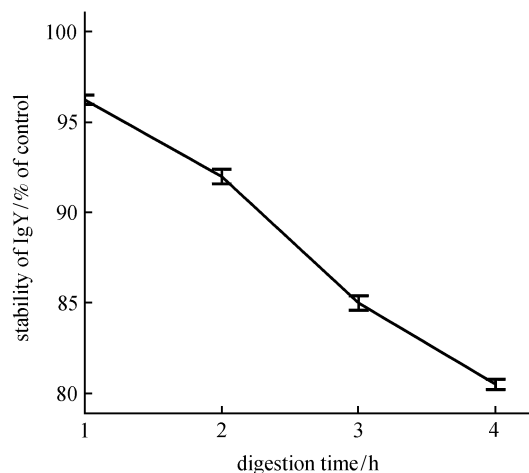


Fig. 5 Trypsin stability of IgY

Note: IgY was treated with trypsin at pH 7.5 for 1 h, 2 h, 3 h, and 4 h. Remaining activities after the treatments were measured using ELISA and were expressed as a percentage of the initial activity.

3.6 Repeated freezing-thawing treat stability of IgY

The IgY solution was treated with five cycles of freezing and thawing. The antibody activity of IgY, as determined by ELISA, had no change. It has been found that IgG from

mammals serum was significantly affected by freezing and thawing (Na et al., 1998). The result achieved in this test suggested the particular character of IgY.

3.7 Stability of IgY at room temperature

After being preserved at 25°C for six months, the remaining antibody activity of IgY was measured by ELISA. The results showed that antibody activity had no change compared with the initial activity. Similar results were obtained by Larsson et al. (1991), suggesting that the IgY was quite stable at normal temperatures.

4 Discussion

Transmissible gastroenteritis (TGE) is an acute enteric disease of swine, most often fatal to newborn piglets (Bohl et al., 1975). Economic loss in the swine industry resulted from intestinal diseases is extremely high. Since piglets can not mount efficiently active immunity, immunoprotection for newborn piglets is largely dependent on passive immunity through colostral immunoglobulin from the immunized dam (Cox et al., 1993; Lanza et al., 1995; Roth, 1999; Kweon et al., 2000). However, the maternal antibody level in piglets markedly reduced at three weeks old (Zhang and Lu, 2000). Therefore, it is necessary to enhance the passive immunity by administrating exogenous antibody (Li, 2003).

Native IgG purified from immunized mammal serum is of great cost, and its physicochemical property is unstable, which may limit their clinical application (Wang et al., 1997; Zhao et al., 2003). Tremendous efforts have been made to seek alternatives to IgG for a more widely available means of treating, suppressing, or preventing TGEV infections. IgY is the immunoglobulin that differs from IgG. Since it was found by Williams in 1962 (Williams, 1962), many researchers have paid special attentions to the advantages of IgY and made progress in IgY research (Hatta et al., 1993; Long et al., 1997; Na et al., 1998; You et al., 2006). However, conflict reports are also available about the stability of IgY. Indeed, some are contrary to others, especially the stability toward heat, pepsin, and trypsin.

In order to evaluate the potential use of IgY in the prevention and treatment of TGEV infection, the stability of IgY was investigated by measuring the remaining activity by ELISA. The results showed that the IgY was stable between pH 4 and pH 11. When we tested the stability toward pepsin and trypsin, it was demonstrated that the IgY stay active when incubated with pepsin at pH 4 and pH 6 and showed a broad stability to trypsin. These results indicated that IgY could increase in its resistant to the gastric acid of piglets and the digestion of pepsin and trypsin, and it could be used as the antibodies for oral administration. IgY activity could be remained

approximately 80% at 60°C for 30 min, suggesting that pasteurization can be applied to sterilize the product. The stability of IgY at 25°C and freezing-thawing treatment indicated that the IgY was easy to be conserved. All these encouraging results together with its higher productivity and effectiveness would make the IgY be a novel approach to the management of TGEV infection in piglets.

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References

- Bohl E H, Frederick T, Saif L J (1975). Passive immunity in transmissible gastroenteritis of swine: intramuscular injection of pregnant swine with a modified live-virus vaccine. *Am J Vet Res*, 36 (3): 267–271
- Cox E, Pensaert M B, Callebaut P (1993). Intestinal protection against challenge with transmissible gastroenteritis virus of pigs immune after infection with the porcine respiratory coronavirus. *Vaccine*, 11: 267–272
- Hatta H, Kim M, Yamamoto T (1990). A novel isolation method for hen egg yolk antibody, “IgY”. *Agric Biol Chem*, 54: 2531–2535
- Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T (1993). Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. *Biosci Biotechnol Biochem*, 57: 450–454
- Hennig-Pauka I, Stelljes I, Waldmann K H (2003). Studies on the effect of specific egg antibodies against *Escherichia coli* infections in piglets. *Dtsch Tierarztl Wochenschr*, 110(2): 49–54
- Jaradat Z W, Marquardt R R (2000). Studies on the stability of chicken IgY in different sugars complex carbohydrates and food materials. *Food Agric Immunol*, 12(4): 263–272
- Kuroki M M, Ohta Y, Ikemori F C, Icatlo J, Kobayashi C, Yokoyama H, Kodama Y (1997). Field evaluation of chicken egg yolk immunoglobulins specific for bovine rotavirus in neonatal calves. *Arch Virol*, 142: 843–851
- Kweon C H, Kwon B J, Woo S R, Kim J M, Woo G H, Son D H, Won H, Lee Y S (2000). Immunoprophylactic effect of chicken egg yolk immunoglobulin (IgY) against Porcine Epidemic Diarrhea Virus (PEDV) in piglets. *J Vet Med Sci*, 62(9): 961–964
- Lanza I, Shoup D I, Saif L J (1995). Lactogenic immunity and milk antibody isotypes to transmissible gastroenteritis virus in sow exposed to porcine respiratory coronavirus during pregnancy. *Am J Vet Res*, 56: 739–748
- Larsson A, Karlsson-Parra A, Sjoquist J (1991). Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. *Clin Chem*, 37(3): 411–414
- Li J T (2003). Egg yolk immunoglobulin (IgY) and its immunoprophylactic effect in piglets. *Swine Production*, 4: 9–11 (in Chinese)
- Long Z E, Zhong Q P, Zhu Y K, Xiong Y H, Yang R J, Yang N S, Liu H L, Xie J L (1997). Study on stability of yolk immunoglobulin (IgY).

- Chinese Journal of Biologicals, 10(4): 218–220 (in Chinese)
- Murata H, Namioka S (1977). The duration of colostral immunoglobulin uptake by the epithelium of the small intestine of neonatal piglets. *J Comp Pathol*, 87: 431–439
- Na H, Yang Y Z, Ou L, Yuan Q S, Zhu W (1998). Studies on properties of chicken egg yolk immunoglobulins (IgY). *Journal of East China University of Science and Technology*, 24(2): 172–175 (in Chinese)
- Pensaert M B (1984). Virus gastroenteritis in suckling pigs, *Rev Sci Tech Off Int Epizoot*, 3: 809–818
- Roth J A (1999). *Diseases of Swine*. 8th ed. Ames, Iowa: Iowa State University Press, 799–819
- Saif L J, Wesley R D (1999). Transmissible gastroenteritis and porcine re-spiratory coronavirus. In: Straw B E, D’Allaire S, Mengeling W L, Taylor D J, eds. *Diseases of Swine*. 8th ed. Ames, Iowa: Iowa State University Press, 295–325
- Sarker S A, Casswall T H, Juneja L R, Hoq E, Hossain I, Fuchs G J, Hammarstrom L (2001). Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. *J Pediatr Gastroenterol Nutr*, 32: 19–25
- Shin J H, Yang M, Nam S W, Kim J T, Myung N H, Bang W G, Roe I H (2002). Use of egg yolk-derived immunoglobulin as an alternative to antibiotic treatment for control of *Helicobacter pylori* infection. *Clinical and Diagnostic Laboratory Immunology*, 9(5): 1061–1066
- Sunwoo H H, Nakano T, Dixon W T, Sim J S (1996). Immune responses in chickens against lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. *Poult Sci*, 75: 342–345
- Tuboly T, Nagy E (2001). Construction and characterization of recombinant porcine adenovirus serotype 5 expressing the transmissible gastroenteritis virus spike gene. *Journal of General Virology*, 82: 183–190
- Wang J, Gong C M, Zhao S D, Song X (1997). Study on physicochemical characteristics of yolk immunoglobulin (IgY) to polio virus type III. *Chinese Journal of Biologicals*, 10(3): 140–143 (in Chinese)
- Williams J (1962). Serum proteins and the livetins of hen's-egg yolk. *Biochem J*, 83: 346–355
- Yokoyama H, Peralta R C, Diaz R (1992). Passive protection of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Infect Immun*, 60: 998–1007
- Yokoyama H, Peralta R C, Sendo S, Ikemori Y, Kodama Y (1993). Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. *Am J Vet Res*, 54: 867–872
- You Z H, Jiang Z Q, Wang N, Sun J H, Lu M E (2006). Study on the stability of chicken IgY against F18 + ETEC. *Chinese Journal of Veterinary Drug*, 40(2): 5–8 (in Chinese)
- Zhang H F, Lu Q P (2000). Development of piglets digestive and immunologic function. *China Feed*, 22: 15–17 (in Chinese)
- Zhao Y L, Zhang Y P, Cai Z M (2003). Studies on physicochemical characteristics of specific chicken egg yolk antibody against lipopolysaccharide. *Immunological Journal*, 19(4): 296–298
- Zuo Y Z, Fan J H, Fan H X, Li T Q, Zhang X B (2009). Immunoprophylactic effect of egg yolk immunoglobulin (IgY) against porcine transmissible gastroenteritis virus (TGEV) in piglets. *Front Agric China*, 3(1): 104–108