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In-Vitro and In-Vivo Binding Activity of Chicken Egg Yolk Immunoglobulin Y (IgY) against Gliadin in Food Matrix

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ABSTRACT: Chicken egg yolk immunoglobulin Y (IgY) is a promising alternative for the prevention of enteric gliadin absorption, the predisposing factor of celiac disease (CD). IgY antibody was produced from the egg yolk of Single Comb White Leghorn chickens during the immunization period for the development of an oral immunotherapeutic agent. Here, we report the potential use of spray dried IgY antibody formulation using sugar protectants (mannitol, sorbitol, or microcrystalline cellulose powder (MCCP)). The long-term stability of the spray dried egg yolk powder formulated with 37.5% mannitol (EYP-M) preserved IgY antibody activity at 99.9%, which was significantly higher than that with other protectants ($p < 0.05$). In a dissolution test, the EYP-M shows 82.4% IgY activity after 2 h in simulated gastric fluid (SGF). A competitive ELISA at 50% inhibition (IC_{50}) shows that 1.6 mg/mL EYP-M bound to 7.6 mg/mL and 10.5 mg/mL gliadin in SGF without and with food matrix conditions, respectively, whereas in simulated intestinal fluid, the formulation bound to 10 mg/mL gliadin, regardless of a food matrix. In-vivo study: BALB/c mice fed with EYP-M and gliadin at a ratio of 1:5 (w/w) demonstrated that gliadin absorption in the gastrointestinal tract was minimal at <1%. Thus, EYP-M containing IgY antibody may be used in CD patients to eliminate the effects of ingested toxic gliadin.

KEYWORDS: *celiac disease, ELISA, gliadin, immunoglobulin Y, mannitol*

INTRODUCTION

Celiac disease (CD) is induced by ingestion of gluten, which is derived from wheat, barley, rye, and possibly oats. The term gluten refers to the entire protein component enriched with proline and glutamine and commonly called the alcohol-soluble prolamins. Among prolamins, wheat gliadin peptides are known to be involved in the pathogenesis of CD. The gliadin fractions are resistant to digestion by gastrointestinal proteases, which increase their survival in the human intestine and thus remain in the intestinal lumen.[1] The ingestion of a 33-mer peptide from α-gliadin causes malabsorption syndrome,[2] which is known to express clinical symptoms of an autoimmune attack,[3] as well as inflammatory skin reactions.[4] To prevent celiac attack, CD individuals should have a life-long gluten-free diet which is the only known treatment available thus far.[5] Furthermore, it may be a frustrating challenge for CD individuals to completely avoid gluten since there is limited availability of gluten-free products, and gluten, being a common food ingredient, may contaminate other foods during food processing.

Oral antibody passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and the potential to treat localized conditions in the gastrointestinal tract.[6] Among antibodies, chicken egg yolk immunoglobulin (IgY) is ideal for passive immunotherapy, as it may be readily obtained in large quantities from egg yolk, presenting a more cost-effective, convenient, and hygienic alternative to mammalian antibodies.[7] Chickens can produce high titer of IgY against a wide range of proteins including highly conserved mammalian proteins which may not be as satisfactory as those produced in other experimental animals (mouse, rat, rabbit, horse, goat, etc.).

Evidence from a review of the literature reveals that IgY has been used as effective oral passive immunotherapy in preventing gastrointestinal infectious disease from rotavirus in suckling mice,[8] *S. typhimurium* and *S. dublin* in calves,[9] enterotoxigenic *Escherichia coli* in neonatal piglets,[10] and rotavirus in children.[11] However, there is still controversy regarding the stability of IgY in the gastrointestinal tract (GIT) due to low pH and enzymatic degradations. IgY has been found to be relatively resistant to intestinal trypsin and chymotrypsin digestion but sensitive to gastric pepsin digestion.[12] To improve antibody stability against harsh gastric conditions, a number of formulations have been suggested. Additives such as sucrose,[13] β-cyclodextrin, and gum arabic microencapsule with lecithin/cholesterol liposome,[14] sorbitol,[15] and pH-sensitive methacrylic acid copolymer,[16] as well as chitosan-alginate microcapsules,[17] have been evaluated. Selection and testing of additives to protect chicken IgY from gastric acid and enzymatic degradation remains the major focus of our research.

In this study, we have included 3 potential protectants for IgY: mannitol, sorbitol, and microcrystalline cellulose powder (MCCP), which are widely used in oral pharmaceutical formulations and food products since they are regarded as relatively nontoxic and nonirritant. Sorbitol and mannitol are sugar alcohols used in the food industry to improve texture, resist moisture, and prevent foods from browning effects. MCCP is soluble in dilute basic conditions (intestinal conditions) but practically insoluble in acidic gastric conditions. One of the processes used in the formulation of protective

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substances for antibodies involves a technique called spray drying. To our knowledge, no studies have been performed using these protectants during the IgY antibody spray drying process. Therefore, in this study, we make the first attempt to develop an effective antigliadin IgY capsule formulation using sugar protectants as well as to evaluate its potential use as an oral passive immunotherapy to neutralize and/or prevent gliadin absorption both in vitro and in vivo.

MATERIALS AND METHODS

Chemicals and Apparatus. Wheat (*Triticum aestivum*) flour was obtained from a commercially available source in a local store. 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 3,3′,5,5′ tetramethylbenzidine (TMB) were purchased from KPL, Inc. (Maryland, USA). D-Mannitol, sorbitol, and microcrystalline cellulose were purchased from PCCA (Ontario, CA). BCA protein assay kit was purchased from Thermo Scientific, Fisher Canada (Ontario, CA). Bradford protein assay kit was purchased from Bio-Rad Laboratory (Ontario, CA). Complete protease inhibitor was purchased from Roche (Rockville, MD, USA). Pepsin (P-7000), pancreatin (P-8096), gliadin (G-3375), rabbit antichicken IgG peroxidase, chicken IgG, TMB substrate for membrane, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mini-Protein III electrophoresis kit was purchased from Thermo Scientific, Fisher Canada (Ontario, CA), and Microtiter 96-wells plates were purchased from Corning, Costar Inc. (NY, USA). ELISA Vmax kinetic microplate reader instrument was obtained from Molecular Devices Corp (Sunnyvale, CA, USA). Buchi 190 mini-Spray Dryer instrument was obtained from Buchi AG (Flawil, Switzerland). USP dissolution Apparatus 1 (Vankel VK 7000) was obtained from Vankel (Edison, NJ).

Extraction of Gliadin. Gliadin was extracted from wheat flours according to the method previously described,[18] with slight modifications. Briefly, wheat (*Triticum aestivum*) flour was defatted with 10 volumes of butanol by stirring for 1 h prior to centrifugation at 3,000*g* for 30 min. The wheat pellets were then collected and further processed by removing other globular proteins with 10 volumes of 0.5 M NaCl followed by mixing and centrifugation. Ten volumes of aqueous solvent containing 1% acetic acid, 2% methanol, and 50% propanol were then added to the residual pellet and mixed for 1 h. After centrifugation at 3,000*g*, the supernatant was collected and diluted with two volumes of 1.5 M NaCl at 4 °C overnight. Wheat gliadin pellets were then collected after centrifugation at 3,000*g*. Protein content of gliadin was measured by BCA protein assay with Sigma gliadin as a reference at the absorbance of 562 nm. The isolated gliadin was dissolved in 2 M urea solution and used for the immunization of chickens and further studies.

Production of Antigliadin IgY Antibody. Ten 23-week-old Single Comb White Leghorn laying hens were cared for in accordance with the guidelines of animal welfare of the Canadian Council on Animal Care, approved by the Animal Care and Use Committee, University of Alberta (Protocol Number #097). Chickens were subcutaneously immunized (total volume 1 mL) with gliadin (500 μg of protein/mL) in phosphate buffered saline (PBS, pH 7.2) with an equal volume of Freund's incomplete adjuvant. Two boosters were given after 2 and 4 weeks of the initial immunization. Eggs were collected daily and stored at 4 °C until the extraction of the antibodies.

The egg yolk was physically separated from the egg white and first mixed gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give pH 4.0) to avoid possible disruptions of egg yolk granules due to the presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to a pH 5.0 to 5.2 and incubated at 4 °C for 12 h. The water-soluble fraction (WSF) was obtained by centrifugation at 3,125*g* at 4 °C for 20 min. The supernatant was collected as the IgY-rich WSF and titrated by indirect ELISA (mentioned below) using gliadin as a coating antigen. The IgY was further purified by ammonium sulfate precipitation (60%) followed by Sephacryl S-300 gel chromatography.

Indirect ELISA for Gliadin Specific IgY Activity. Unless indicated otherwise, all incubations were performed at 37 °C with four times washing with PBS containing 0.05% Tween 20 (PBS-T) in each step. Microtiter plates were coated with 100 μL of gliadin (10 μg/mL of 60% ethanol). Each well was blocked by 120 μL of 3% BSA solution (w/v) in PBS-T. One hundred microliters of WSF (diluted 1:1,000 in PBS-T) of hyperimmunized egg yolk or samples from the dissolution test at each time point and nonspecific IgY (diluted 1:1,000 in PBS-T) as a control were added to the plates. After washing, 100 μL of rabbit antichicken IgY-horse radish peroxidase (HRP) (diluted 1:3,000 in PBS-T) was added to each well followed by 100 μL of freshly prepared substrate solution, 2,2′-azino-bis -(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. Optical density (OD) at 405 nm was read after 30 min using a microplate reader. The specific IgY titer was determined by subtracting the value of the nonspecific IgY.

Total IgY Concentration. To estimate the total IgY in liquid egg yolk and spray dried egg yolk powder (EYP), ELISA was performed as mentioned above. A microtiter plate was coated with 100 μL per well of rabbit antichicken IgG at a final concentration of 2 μg. The WSF of liquid and dried egg yolk diluted 1:10,000 and 1:50,000 in PBS, respectively, were added to the plate. Two-fold serial dilutions of purified chicken IgG in PBS (0.5 to 0.031 μg/mL) were used as a reference antibody to prepare a standard curve on the same plate. Total IgY concentrations in WSF were determined using the standard purified chicken IgG.

Preparation of Egg Yolk Powder Capsules. Egg yolk was used alone or mixed with three protectants such as mannitol, sorbitol, or MCCP in proportions of 95:5, 90:10, 85:15, or 80:20 (w/w). A 100 mL sample of each egg yolk with protectants were dried by using a Buchi 190 mini-Spray Dryer ranged from 140 to 80 °C inlet temperatures and 110−60 °C outlet temperatures at a feed rate of 2 mL/min. Spray dried EYP formulated with mannitol (EYP-M), sorbitol (EYP-S), or MCCP (EYP-Mc) were collected and stored in airtight containers. Egg yolks without protectants were also dried as a reference. Four EYP formulations were capsulated into gelatin capsules ("00" size) at 500 mg of EYP per capsule.

Long-Term Stability of EYP. Using an indirect ELISA method, the long-term stability of capsules containing EYP were tested for total IgY content after 78 weeks of storage period in an airtight container protected against light at RT.

Dissolution Testing under Simulated Gastric Conditions. The stability of the EYP capsules to gastric conditions was evaluated using simulated gastric fluid (SGF). SGF was prepared as described in the United States Pharmacopoeia (USP32-NF27), consisting of 3.2 mg/mL pepsin (P7000, Sigma) in 0.03 M NaCl, at pH 1.2. A capsule ("00" size) of EYP, EYP-M, EYP-S, or EYP-Mc (500 mg/capsule) was placed in each basket of USP Apparatus 1 (VanKel, VK7000, Germany). The release profile of each EYP formulation was determined at 100 rpm in 500 mL of SGF media. At intervals of 15, 30, 45, 60, 90, and 120 min, 0.5 mL aliquots from each vessel was neutralized with 0.1 M sodium carbonate buffer, pH 9.6, and the IgY activity was assessed by ELISA as described above. Dissolution tests for each EYP formulation were performed in 6 vessels per test with three repeats. IgY activity was expressed as specific IgY concentration.

ELISA-Gliadin Inhibition Assays of EYP. A 1.6 mg/mL EYP-M was preincubated with serial dilutions of Sigma gliadin (ranging from 0 to 25.6 mg/mL) at 37 °C for 1 h in SGF or simulated intestinal fluid (SIF, containing 10 mg/mL pancreatin (trypsin, chymotrypsin, elastase, carboxypeptidase, amylase and lipase) in 0.05 M KH_2PO_4, at pH 6.8). The preincubations were performed under 2 conditions, one with gluten free food (boiled rice was used at 2 g/mL.) and the other without gluten free food. After preincubation, to neutralize enzymes, an aliquot of 0.5 mL from SGF was neutralized with 0.275 mL of 0.1 M sodium carbonate buffer (pH 9.6), whereas an aliquot of 1 mL from SIF was neutralized with 0.1 mL of Complete Protease Inhibitor solution in PBS (Roche; one mini-tablet dissolved in 5 mL of PBS, pH 7.2). Neutralized samples were kept frozen until analyzed. Microtiter plates were coated with 150 μL of Sigma gliadin in 70% ethanol at a concentration of 500 μg/mL and incubated at 37 °C for 1

Table 1. Antigliadin IgY Antibody Titer of Egg Yolks Collected during the Immunization Period

	weeks					
items	0	2	4	6	8	10
daily egg production[a]	9	10	9	10	10	9
IgY titer[b]	0.07 ± 0.00	0.21 ± 0.02	0.65 ± 0.05	0.72 ± 0.11	0.81 ± 0.14	0.60 ± 0.05

[a]Ten 23-week-old laying hens were immunized with gliadin (500 μg of protein/mL) at week 0, 2, and 4, and eggs were collected daily for measuring daily egg production. [b]Values of IgY titer are the mean of optical density ± SD (n = 10).

Table 2. Total IgY Content and Specific IgY Activity of Egg Yolk Powder without Protectants at Different Spray Dry Temperatures

	inlet/outlet temperatures (°C)			
items	140/110	120/100	100/80[a]	80/60[a]
physical appearance	partially burnt	pale yellow powder	pale yellow powder	pale yellow powder
total IgY (mg/g)	N/A	20.96 ± 1.79	25.16 ± 0.23	25.24 ± 2.03
anti-gliadin IgY (mg/g)	N/A	1.65 ± 0.01	1.98 ± 0.02	1.99 ± 0.03

[a]Significantly high IgY content as compared to higher temperatures (p < 0.05).

Table 3. Total IgY Content in Egg Yolk Powder with Different Concentrations of Three Protectants

	IgY content (mg/g)			
protectants	12.5%	25%	37.5%[a]	50%
EYP-M	27.20 ± 0.75	28.54 ± 1.05	31.13 ± 0.40	31.16 ± 1.11
EYP-S	27.96 ± 0.65	28.11 ± 0.87	31.17 ± 0.59	31.14 ± 0.94
EYP-Mc	29.22 ± 0.99	28.60 ± 0.77	31.12 ± 0.54	N/A

[a]Significantly higher IgY content at protectant concentration of more than 37.5% (p < 0.05). The total IgY content in the egg yolk powder without protectants was 25.24 mg/mL (see Table 2 for further details).

h. The plates were then washed four times with PBS. After blocking with 3% BSA solution (w/v) in PBS at 37 °C for 45 min, 150 μL of preincubated samples was added to the wells and incubated at 4 °C overnight. After washing the plates four times with PBS-T, 150 μL of rabbit antichicken IgY conjugated with HRP (diluted 1:3,000 in PBS) was added to each well and incubated at 37 °C for 1 h. The plates were washed again with PBS and developed with 150 μL of freshly prepared ABTS substrate. After 30 min, the OD was measured at 405 nm using a microplate reader. The 50% inhibition of control (IC_{50}) is defined as the concentration of Sigma gliadin that gives half of the maximum signal intensity of antigliadin IgY without gliadin (A_0). The standard curve was normalized by expressing experimental absorbance values (A) as A/A_0 X100.

In-Vivo Gastrointestinal Binding Activity of EYP. A total of 128 3-week old female BALB/c mice were cared for according to the animal welfare guidelines of the Canadian Council on Animal Care, approved by the Animal Care and Use Committee, University of Alberta (Protocol Number #074/10), during the 1 week housing period, on a normal diet. A day before the treatment, all the mice were kept on a 24 h fast and randomly divided into 4 feeding groups (A, B, C, and D) of 8 mice each: Group A, Sigma gliadin, 100 mg in 0.2 mL of water; Group B, EYP-M 4 mg and Sigma gliadin, 100 mg of powder reconstituted in 0.2 mL of PBS; Group C, EYP-M, 20 mg, and Sigma gliadin, 100 mg of powder reconstituted in 0.2 mL of PBS; and Group D, EYP-M, 100 mg reconstituted in 0.2 mL of PBS. The formulations were administered to the fasted mice by a micropipet. After 12 h, the mice were euthanized, and necropsy was immediately carried out to collect tissue samples from the stomach, small intestine, as well as large intestine contents. The lumen of the dissected stomach, small intestine, and large intestine were scraped and washed with 10 mL of PBS neutralized containing Complete Protease Inhibitor, mixed well under continuous shaking for 1 h, and centrifuged at 10,000g, 15 min. The supernatant was collected and analyzed for residual gliadin by the heterosandwich ELISA mentioned below.

Heterosandwich ELISA for Gliadin. Except as otherwise indicated, all incubation steps were performed at 37 °C. Four times washing was conducted by PBS-T between each step. The assay was carried out with the gliadin specific monoclonal antibody HYB-314 (mAb raised against synthetic peptide residues KLQPFPQPEL-PYPQPQ of α-gliadin peptide (58−73)) as capture antibody and purified gliadin specific IgY as detection antibody. Wells were coated with 100 μL of gliadin specific mAb (10 μg per well) in PBS at 4 °C overnight. Nonspecific binding sites were blocked with 120 μL of 5% BSA for 45 min. A 100 μL aliquot of sample (1:100,000 in PBS-T) and serially diluted Sigma gliadin standard (1.28 μg−0.625 ng) in PBS-T was added to triplicate wells and incubated at room temperature for 1 h. Then, 100 μL of gliadin specific IgY (diluted 1:2,000 in PBS-T) was added and incubated for 1 h. After washing the plates, 100 μL of chicken antimouse IgG conjugated with HRP (1:5,000 in PBS-T) was added to each well and incubated for 1 h. Each well was incubated with 100 μL of freshly prepared TMB substrate followed by optical density reading at 650 nm by an ELISA Vmax kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the control from that of specific antibody.

Statistical Analysis. The analysis of variance (ANOVA) and Student's t test was used to analyze for significant differences of gliadin specific IgY contents during the immunization period, total IgY or gliadin specific IgY contents at different spray dry temperatures compared to raw egg yolk (on dry basis), among IgY formulations with 0, 12.5, 25, 37.5, and 50% mannitol, sorbitol, or MCCP, long-term stability of EYP formulations, IgY survival during dissolution test in SGF, and % gliadin absorbed between different in vivo feeding conditions. The differences were considered statistically significant at p < 0.05.

RESULTS

Production of IgY. Table 1 illustrates the antigliadin antibody (IgY) titer from egg yolks collected from hyper-immunized chicken. The activity of antigliadin IgY detected on day 0, gradually increased at week 2 after the first immunization. The first booster immunization was given at week 2, illustrating a rapid increase of IgY titer with a peak at week 4. The second booster was given at week 4 to maintain

the high titer of gliadin specific IgY up to week 8 with a gradual reduction thereafter. The eggs from hyperimmunized hens between weeks 4 to 8 contained highly specific IgY against gliadin and was used for further studies. Total IgY contents were constant regardless of immunization, and daily egg production was not affected by the immunization.

Optimum Spray Dry Condition. Table 2 illustrates total IgY content and specific IgY activity of EYP at different spray dry temperatures. Depletion of IgY content is observed upon exposure to heat during the spray drying process. The optimum condition found for spray drying was at temperatures of 80 °C inlet and 60 °C outlet or 100 °C inlet and 80 °C outlet since they resulted in minimal IgY degradation.

Effect of Protectants. Table 3 illustrates IgY content in EYP with protectants. For spray dry processing, protectants (mannitol, sorbitol, and MCCP) were used up to their maximum soluble amount, which is 50% for mannitol and sorbitol and 37.5% for MCCP. With increasing concentration of protectants, IgY content was higher. Maximal IgY protection was achieved at 37.5% protectant (on dry basis) because above this concentration, no further increases of IgY content was observed ($p < 0.05$). Consequently, this protectant concentration was chosen for IgY stability and efficacy experiments.

Long-Term Stability of EYP Formulations. Figure 1 illustrates the long-term stability of the EYP formulation

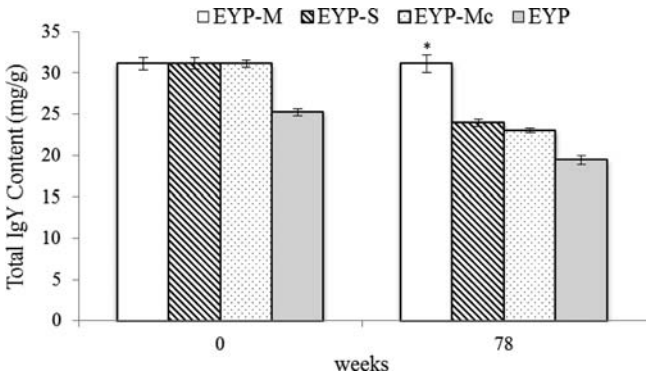

Figure 1. Long-term stability of EYP capsules without and with protectants (EYP, EYP-M, EYP-S, and EYP-Mc) at 37.5% (on dry basis) after 78 weeks of storage. EYP, egg yolk powder; EYP-M, egg yolk powder mixed with mannitol; EYP-S, egg yolk powder mixed with sorbitol; and EYP-Mc, egg yolk powder mixed with microcrystalline cellulose powder. *, EYP-M formulation was significantly stable after 78 weeks ($p < 0.05$).

without protectant and EYP with protectants (EYP, EYP-M, EYP-S, and EYP-Mc) at 37.5% (on dry basis) after 78 weeks of storage. The initial IgY content of EYP without protectants was significantly lower than other EYP with protectants by 25% ($p < 0.05$). Among four EYP formulations, the IgY content in EYP-M was the most stable for 78 weeks ($p < 0.05$), while in EYP-Mc and EYP-S, IgY content was decreased.

Dissolution Test of EYP Capsule under Simulated Gastric Conditions. Figure 2 illustrates survivability of IgY in EYP, EYP-M, EYP-S, and EYP-Mc in dissolution apparatus I, 500 mL of SGF medium, paddle speed of 100 rpm at dissolution times of 0, 15, 30, 45, 60, 90, and 120 min. Gastroresistance and the release of IgY under simulated gastric condition were assessed by USP dissolution testing of the EYP capsule formulations in SGF for 2 h. At each time interval, the samples were neutralized to inhibit pepsin activity, and IgY activity was determined by indirect ELISA (Figure 2). After 2 h of SGF exposure, the EYP formulation without protectant demonstrated a significant decrease (36.2%) of IgY activity as compared to the raw egg yolk (on dry basis) which contains 2.54 mg/g gliadin specific IgY. The incorporation of mannitol and MCCP protectants maintained significantly greater IgY activity of 82.4% and 86.3%, respectively, after 2 h in SGF.

ELISA-Gliadin Inhibition Assays of EYP. The EYP-M formulation showed the highest long-term and gastric stability; hence, it was selected to further evaluate the in vitro ability to bind free gliadin in SGF and SIF. Figures 3 and 4 illustrate in vitro binding study determined by competitive ELISA of EYP-M containing antigliadin IgY antibodies to gliadin ranging from 0 to 1.28 g after 1 h exposure in SGF and SIF at 37 °C under without food and with food conditions. The optimal concentration of EYP-M for SGF and SIF condition was found to be 1.6 mg/mL giving the maximal absorptivity signal. With increasing amounts of gliadin, the absorptivity signal was proportionally reduced. At 50% signal inhibition (IC_{50}), the given amount of EYP-M bound to 7.6 mg/mL gliadin without food (Figure 3a), while in the presence of food, the EYP-M bound to 10.5 mg/mL gliadin (Figure 3b). Under SIF conditions, the same amount of EYP-M bound to 10 mg/mL gliadin, regardless of a food matrix (Figure 4).

In-Vivo Gastrointestinal Binding Activity of EYP. The most stable formulation from the dissolution test (EYP-M) was fed to mice along with gliadin, followed by euthanization after 12 h of feeding. Residual gliadin upon ingestion was determined at different sites along the GIT (stomach, small intestine, and large intestine). Table 4 illustrates gliadin content in the gastrointestinal tract after 12 h of feeding measured by heterosandwich ELISA. The amount of residual gliadin in the stomach was undetectable but detectable in the small and large intestine. The highest gliadin appeared to be in the lumen of the large intestine and to a lesser extent in the small intestine.

Throughout the GIT, significantly more gliadin was retained in the lumen by increasing the amount of EYP-M fed. The mouse intake of 100 mg of gliadin alone caused 42.8% gliadin to be absorbed throughout the GIT. With the EYP-M intake weight ratio of 1:20 and 1:5 to gliadin, significantly lower amounts of gliadin were found to be absorbed in the GIT at 15.7% and 0.7%, respectively.

DISCUSSION

Gliadin peptides are components of a human diet, but are found to be highly immunogenic in laying hens,[19] due to high phylogenetic differences between avian and mammalian species. In this study, IgY polyclonal antibody against wheat gliadin is produced as a universal antibody to neutralize all CD causing prolamins (wheat, rye, barley, and possibly oat), due to its cross-reactivity with these prolamins.[20]

Spray drying was used to process the hyperimmunized egg yolks, as it is the most economical method for mass production of egg yolk powder. Since IgY as a protein source is susceptible to heat denaturation during the spray drying process, several studies have proven the use of high concentration of sugar and chitosan protectants to improve or increase IgY stability against heat.[13,17] Our result shows that IgY is vulnerable to heat denaturation over 100 °C similar to a report of spray dried egg yolk with β-cyclodextrin at 100 °C inlet temperature,[14] whereas another report showed that IgY titer was very stable under spray drying temperatures of 140 to 170 °C with 30% Eudragit L30D-55 as protectant.[10] In this study, mannitol, sorbitol, and

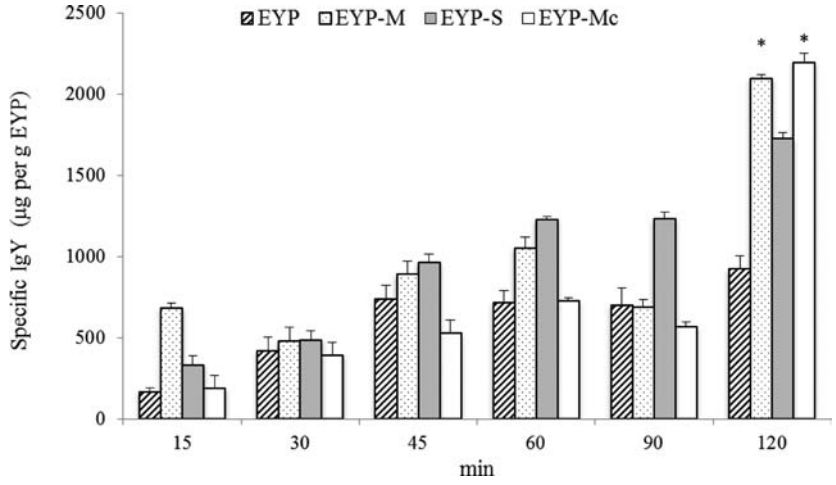

Figure 2. Survivability of IgY in EYP, EYP-M, EYP-S, and EYP-Mc in dissolution apparatus I. 500 mL of SGF medium, paddle speed of 100 rpm at dissolution times of 0, 15, 30, 45, 60, 90, and 120 min. *, EYP-M and EYP-Mc formulations had significantly protective effects on IgY antibodies in 120 min under simulated gastric fluid conditions ($p < 0.05$).

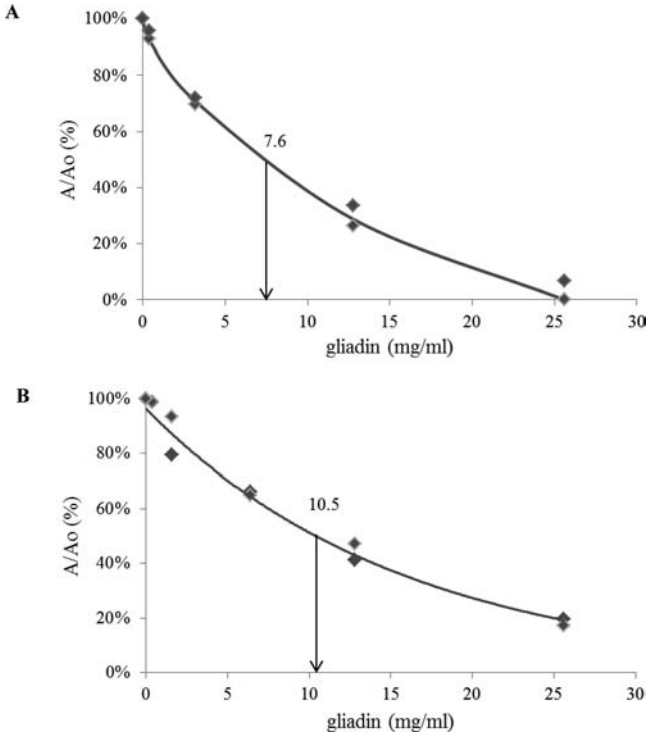

Figure 3. In-vitro binding study determined by competitive ELISA of 0.08 g of EYP-M containing antigliadin IgY antibodies to gliadin ranging from 0 to 1.28 g after 1 h exposure in SGF at 37 °C under (A) without food and (B) with food conditions. *A*, absorbance of sample containing antibodies and gliadin; A_o, absorbance of control containing antibodies only. The arrows indicate 50% inhibition of the control (IC_{50}).

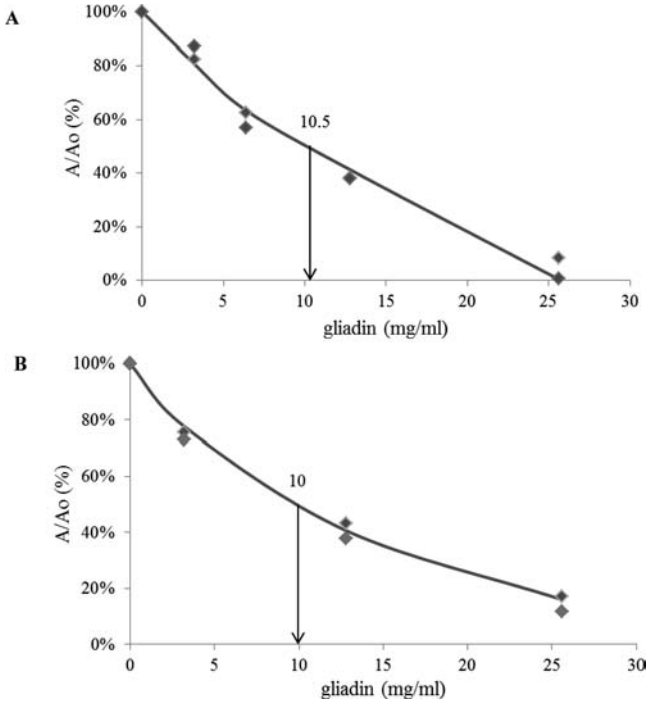

Figure 4. In-vitro binding study results determined by competitive ELISA of 0.08 g of EYP-M containing antigliadin IgY antibodies to gliadin ranging from 0 to 1.28 g after 1 h exposure in SIF at 37 °C under (A) without food and (B) with food conditions. *A*, absorbance of sample containing antibodies and gliadin; A_o, absorbance of control containing antibodies only. The arrows indicate 50% inhibition of control (IC_{50}).

MCCP at the concentration of 37.5% (on dry basis) significantly protected IgY from heat denaturation, while EYP without protectants lost 25% of IgY activity. Another study of the IgG/mannitol spray dry powder indicated a change in the protein environment in the solid state, due to alterations of the polar environment of the peptide groups, which is totally regained upon redissolution.[21] This protective effect of mannitol in the present study against heat denaturation may also be explained in the same manner as seen in lactose, by forming hydrogen bonds during the hot air spray drying process.[22]

Regarding the long-term stability of the antibody, there was a study performed with serum IgG with mannitol protection by spray drying that indicated an inhibition of aggregate formation in samples stored at 2−8 °C for 52 weeks.[21] In another study,[23] sorbitol was used during the spray drying process which resulted in a glassy matrix surrounding the antibodies due to its low glass transition temperature, maintaining the stability of IgG in its dry state. These studies were focused on the physical

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Table 4. Gliadin Content in the Gastrointestinal Tract after 12 h of Feeding[a]

	feeding conditions		average gliadin remaining after 12 h feeding (mg)			
groups	EYP (mg)	gliadin (mg)	stomach	small intestine	large intestine	absorbed gliadin (%)
A		100	N/A	22.2 ± 1.33	35.0 ± 2.10	42.8
B	4	100	N/A	19.5 ± 2.42	64.8 ± 4.44	15.7
C	20	100	N/A	20.7 ± 1.66	78.6 ± 4.82	0.7[b]
D	100		N/A	N/A	N/A	N/A

[a]The measurement of gliadin was assayed by heterosandwich ELISA. [b]Significantly reduced absorbed gliadin as compared to that in conditions A and B ($p < 0.05$).

properties of IgG and not the actual antibody activity during the storage conditions. In this study, IgY antibody content was determined during the 78 weeks of storage at room temperature. Among the 3 protectants, mannitol stabilized the IgY antibody long-term.

In order to effectively use antigliadin IgY as oral immunotherapy to prevent gliadin absorption in the small intestine region, it needs to be active in the stomach and the proximal part of the small intestine. Studies have shown that, purified IgY is extremely sensitive to gastric conditions and is rapidly inactivated,[12] which could be due to the acidic conditions and/or pepsin presence in the stomach. Pepsin digests IgY to Fab′ IgY fragments, having one antigen binding site. Fab′ IgY is as effective as the whole intact IgY since it has the capability to bind to the antigen and exhibit neutralizing activity.[24,25] The antibody inactivation under acidic conditions could also be due to the conformational changes of internal tryptophan residues when exposed to the hydrophilic environment.

Several strategies to prevent degradation of IgY have been mentioned earlier in the literature, such as microencapsulation with β-cyclodextrin and gum arabic and lecithin/cholesterol liposome,[14] and the employment of the pH-sensitive methacrylic acid copolymer as the enteric coating of IgY.[16] High concentration of sugar (30−50%)[13] or sorbitol (30% or more)[15] has also been proven effective to stabilize IgY activity under acidic conditions. In the present study, the use of mannitol protectant prevents irreversible IgY heat denaturation and enzymatic digestion in gastric fluid conditions.

In this study, in-vitro dissolution testing was employed to ensure the efficacy of IgY in our developed spray dried EYP capsule formulations; in terms of stability under harsh pH and proteolytic enzymes, as well as to determine the release profile of IgY in simulated gastric conditions. There are studies on the stability of purified IgY from egg yolk without protectants against pepsin enzyme at lower enzyme/substrate ratios such as 1:20,[26] and 1:250.[16] According to USP32-NF27, pepsin enzyme at the concentration of 3.2 mg/mL is required to represent the SGF. Since, the EYP spray dried formulations contained 20% protein, in this study the enzyme/total protein ratio for pepsin in the SGF was 16:1. With higher concentration of pepsin enzyme used in this study, IgY in EYP-M and EYP-Mc capsule formulations retained over 80% activity up to 2 h exposure in SGF. This finding is explained by the reduction of the exposed hydrophobic moiety of IgY in sugar solutions that occur because of increased interactions between hydrophobic groups inside the protein molecule.[27] Also, changes in preferential solvation of protein molecules[28] and structural blockage of conformational changes of internal tryptophan residues when placed in sugar solutions may be partly responsible for added protection.[13,15] The nonextracted egg yolk contains phospholipids and other proteins, which may have also contributed to the additional gastroprotection effect.

For the first time, the binding efficacy of antigliadin IgY is evaluated in simulated gastrointestinal conditions to estimate the dose of IgY required to neutralize gliadin in vitro. EYP-M, the most stable formulation found, was subjected to further in-vitro and in-vivo binding studies. In SGF, EYP-M formulation (1.6 mg/mL) demonstrated a better ability to bind gliadin (10.5 mg/mL) in the presence of food, as compared to that under without-food conditions (7.6 mg/mL). This result indicates that the EYP-M formulation should be administered during the fed state. Improved binding affinity may be due to the diluted acidic effect from food in the in-vitro gastric condition. However, this effect needs to be verified in vivo as there may be more acid production in the stomach due to the presence of additional food. Interestingly, in SIF, the presence of food had no additional effect on the binding efficacy of IgY in EYP-M bound to gliadin (10 mg/mL).

BALB/c mice were chosen for the in-vivo feeding study since mice have an overall GIT transit time similar to that of humans of 20−30 h.[29] The absorption of gliadin in experimental mice was found to achieve peak values between two to three hours after ingestion.[30] The transit time through the stomach is highly variable with an average time of 1 to 1.5 h[31] and an average of 10 h through the intestine for mice.[32,33] Hence, in this study we chose to determine the residual gliadin content in the mice GIT at 12 h after feeding. The EYP-M neutralized gliadin in the GIT, preventing gliadin from being absorbed. Our in-vivo feeding study proves that EYP-M intake at the weight ratio of 1:20 and 1:5 to gliadin, prevented 63.3% and 98.4% gliadin absorption, respectively, as compared to the ingestion of gliadin alone without administration of EYP-M.

The results demonstrate that the spray dried EYP formulation with mannitol as protectant can successfully protect the IgY antibody from gastric inactivation. The developed formulation may provide an effective means of preventing CD when coadministered with gliadin contained food. Further in-vivo studies need to be verified to prove this concept.

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Notes

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■ ABBREVIATIONS USED

ABTS, 2,2′-azino-bis -(3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; CD, celiac disease; ELISA, enzyme-linked immunosorbent assay; EYP, egg yolk powder; GIT, gastrointestinal tract; HPO, horseradish peroxidase; IC_{50}, 50% inhibition of control; IgY, egg yolk immunoglobulin; MCCP, microcrystalline cellulose powder; OD, optical density; PBS, phosphate buffered saline; SD, standard deviation; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TMB, 3,3′,5,5′ tetramethylbenzidine; WSF, water-soluble fraction

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