# University of Alberta

## ANTIBODY-BASED DIAGNOSTIC AND THERAPEUTIC APPROACHES ON GLUTEN-SENSITIVE ENTEROPATHY

by

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## *DEDICATION*

*I dedicate this thesis to my beloved family with all my love respect, and gratitude.*

**ABSTRACT**

Gluten-sensitive enteropathy, called Celiac disease (CD), is one of the most frequent autoimmune diseases, occurring in 1% people worldwide, upon gliadin ingestion. Currently, the only treatment available for CD individual is a strict life-long gluten-free diet. Chicken egg yolk immunoglobulin Y (IgY) is produced and examined for its efficacy *in vitro*, *ex vivo*, and *in vivo* to prevent enteric absorption of gliadin. This antibody was also used to develop sensitive and rapid detection kits for gluten.

The extracted toxic gliadin was immunized into chickens inducing humoral immune response to produced gliadin-specific IgY antibodies. The IgY antibody was separated from non-protein component in egg yolk and was purified by gel chromatography, showing > 95% purity on electrophoresis. One gram of purified IgY antibody contains 79 mg of specific anti-gliadin IgY antibody based on quantitative ELISA technique. Under *in vitro* simulated gastric and intestinal conditions, competitive ELISA demonstrated that 1.5 mg anti-gliadin IgY completely neutralized 6.6 g gliadin in test tube. Using Caco2 cell culture as *ex vivo* test, anti-gliadin IgY prevented gliadin absorption (at a ratio of 1:3,000), resulting in no pro-inflammatory response (TNF-α and IL-1β). In-vivo BALB/c mice study showed that 31 μg specific anti-gliadin IgY antibody prevented 100 mg gliadin absorption in the gastrointestinal tract when both antibody and gliadin were orally fed.

The developed antibody was used to also develop sensitive double antibody sandwich ELISA (DAS-ELISA) Immunoswab and Immunostrip assay based on anti-gliadin IgY and biotinylated monoclonal antibody (mAb) showing a detection limit of 4 ng/mL, 1.25 μg/mL and 0.25 μg/mL, respectively.

Anti-gliadin IgY has potential to be used as an oral passive antibody therapy to treat CD. This CD therapeutic candidate may provide an effective means of preventing

CD when co-administered with gliadin contained food. Further clinical studies are warranted to test anti-gliadin IgY formulation in CD subjects exposed to gluten.

The combination of anti-gliadin IgY and biotinylated mAb provided reliable, sensitive and inexpensive tools for the detection of gluten in gluten-free and gluten-contained food products.

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| | |
|---|---|
| °C | degrees centigrade |
| ABTS | 2,2'-Azino-bis -(3-ethylbenzthiazoline-6-sulfonic acid) |
| AGA | anti-gliadin antibodies |
| BSA | bovine serum albumin |
| CD | celiac disease |
| CDAT | celiac dietary adherence test |
| CFU | colony forming units |
| $C_H$ | constant domain of the heavy chain |
| $C_L$ | constant domain of the light chain |
| CTB | Cholera toxin B |
| DAS-ELISA | double sandwich enzyme-linked immunosorbent assay |
| DDA | dimethyl dioctadecyl ammonium bromide |
| DGP | deamidated gliadin peptide |
| ED | effective dose |
| ELISA | Enzyme-linked immunosorbent assay |
| EMA | anti-endomysial antibody |
| EMSAs | electrophoretic mobility shift assays |
| ESPGHAN | European Society of Paediatric Gastroenterology, Hepatology, and Nutrition |
| ETEC | Enterotoxigenic Escherichia coli |
| EYP | egg yolk powder |
| Fab | antigen binding site of the antibody molecule |
| Fc | Crystalizable (constant) region of the antibody molecule |
| GCHV | grass carp hemorrhage virus |
| GFD | gluten-free diet |
| GIT | gastrointestinal tract |
| HAGP-A | high-molecular weight mucin-like glycoprotein-A |
| HBSS | Hank's balanced salt solution |
| HIF-1α | α-subunit of hypoxia inducible factor-1 |
| HLA | human leukocyte antigen |
| HR | hinge region |
| HP | horseradish peroxidase |

| | |
|---|---|
| HRV | human rotavirus |
| $IC_{50}$ | 50% inhibition of control |
| IDDM | Insulin Dependent Diabetes Mellitus |
| IECs | intraepithelial cells |
| IELs | intraepithelial lymphocytes |
| IgA | immunoglobubin class A |
| IgE | immunoglobubin class E |
| IgG | immunoglobubin class G |
| IgM | immunoglobubin class M |
| IgY | egg yolk immunoglobulin |
| IP | intraperitoneal |
| kDa | kilo Dalton |
| LD | lethal dose |
| M6P/IGFII-R | mannose-6-phosphate/insulin-like growth factor-II receptor |
| mAb | monoclonal antibody |
| MCCP | microcrystalline cellulose powder |
| MDCK | Madin-Darby Canine Kidney |
| mg | milligram |
| MHC | major histocompatibility complex |
| MIC | MHC class I–related ligands |
| MICA | MHC class I chain-related gene A |
| min | minute |
| MWCO | molecular weight cut-off |
| ND | not detectable |
| NKG2D | homodimeric natural killer-activating receptor |
| NT | neutralization titer |
| OD | optical density |
| P(HEMA- co -SS) | poly (hydroxyethylmethacrylate-co-styrene sulfonate) |
| PA | *Pseudomonas aeruginosa* |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pDCs | plasmacytoid dendritic cells |
| PEPs | prolyl endopeptidases |

| | |
|---|---|
| PKCa | protein kinase Ca |
| PLG | poly (lactide-co-glycolide |
| RT | room temperature |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SGF | simulated gastric fluid |
| SIF | simulated intestinal fluid |
| T1D | type 1 diabetes |
| TGA | anti-tissue transglutaminase antibody |
| TGF-β | transforming growth factor beta |
| TK | thymidine kinase |
| TMB | 3,3´,5,5´ tetramethylbenzidine |
| TNF | tumor necrosis factor |
| Tregs | regulatory T cells |
| tTG | tissue transglutaminase |
| USP | United States Pharmacopoeia |
| $V_H$ | variable domain of the heavy chain |
| $V_L$ | variable domain of the light chain |
| WSF | water-soluble fraction |
| µg | micro gram |
| µL | micro liter |

# CHAPTER 1: Introduction

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## 1.1 Definition of celiac disease

Celiac disease (CD) is an inherited autoimmune disorder of the small intestine caused due to ingestion of gliadin in genetically predisposed individuals.CD has been classified as: classic, atypical, silent and latent [1, 2]. Classic CD patients are characterized by gastrointestinal symptoms including diarrhea, abdominal pain, vomiting, bloating and distention, constipation and weight loss. Atypical CD is the most common form of the CD, where patients have few or no gastrointestinal symptoms. They are instead present with extraintestinal symptoms, such as dermatitis herpetiformis, anemia, delayed puberty or osteoporosis. Silent CD patients have no symptoms, but affected patients have villous atrophy evident on small bowel mucosal biopsy. They may have associated atypical features of CD, such as osteoporosis or anemia. Latent CD is known as potential CD, having no symptoms. Patients with latent CD show the same blood abnormalities as do patients with CD, but their small intestine biopsy findings do not suggest autoimmune disease.

## 1.2 Prevalence

CD originally thought to almost exclusively affect white Europeans, is now known to be widely distributed worldwide [3]. Epidemiological studies conducted in areas supposedly free of CD, including Africa, the Middle East, Asia, and South America, show that the disease was previously underdiagnosed [4]. This provides evidence that CD is one of the most common genetic diseases, resulting from both environmental (gluten) and genetic (human leucocyte antigen (HLA) DQ2/8 and non-HLA genes) factors.

The world distribution of CD seems to have followed the mankind wheat consumption and the migratory flows. Man originally fed on meat, fruit and vegetables, with no exposure to gluten-containing cereals. It was only about 10,000 years ago in a small region called the "*Fertile Crescent*" of the Middle-East (including Anatolia (Southern Turkey), Lebanon, Syria, Palestine and Iraq) where wild wheat and barley grains successfully cultivated due to favorable environmental conditions. In the *Fertile Crescent* some tribes changed from nomadic to stable settlement style of living because land cultivation permitted food storage, and later migrated westwards to obtain new lands for cultivation. These persons spread through the Mediterranean area (Northern Africa, Southern Europe) and Central Europe. The expansion continued from 9,000 to 4,000 BC by which time the cultivation of wheat and barley had spread all over the Old Continent, also reaching Northern Europe (Ireland, Denmark and the Scandinavian countries). This expansion in farming was due to the diffusion of agricultural practices and replacement of

local inhabitants by descendants from the Middle-East [5]. Hence, the European and North-African populations share genetic background with the peoples of Middle-East origin.

In the past, the prevalence of CD had been underestimated, but it is now regarded one of the most common genetic disorders in the West with 1% prevalence [6-8]. Interestingly, there is increased prevalence of CD amongst women compared to men with male:female ratio of 1:2.8 [9]. This could be due to the finding that men with CD were diagnosed at an older age [10]. Indeed, there have been reported CD cases among immigrant children native of Eastern Europe, Northern, West and East Africa, the Middle East and Southern Asia, according to their acquisition of Western dietary practices (i.e., short period or lack of breast feeding and early weaning with a great amount of gluten intake) [11]. This suggests that many persons may have the genetic predisposition to CD but the clinical presentation only occurs when there is sufficient gluten in the diet.

### 1.2.1 Normal at-risk persons

In several parts of the world, the presence of the combination of antibody (serum tissue transglutaminase and endomysial autoantibodies) positivity and an HLA haplotype associated with CD is predictive of small-bowel abnormalities indicative of CD. For the majority of countries, the CD prevalence is unknown. Figure 1-1 shows a range of estimated normal at-risk CD prevalence in continents and nations around the globe. It must be noted that some studies report prevalence of CD based on serology, others on celiac compatible small bowel biopsies and a few on serology, biopsy and response to gluten challenge.

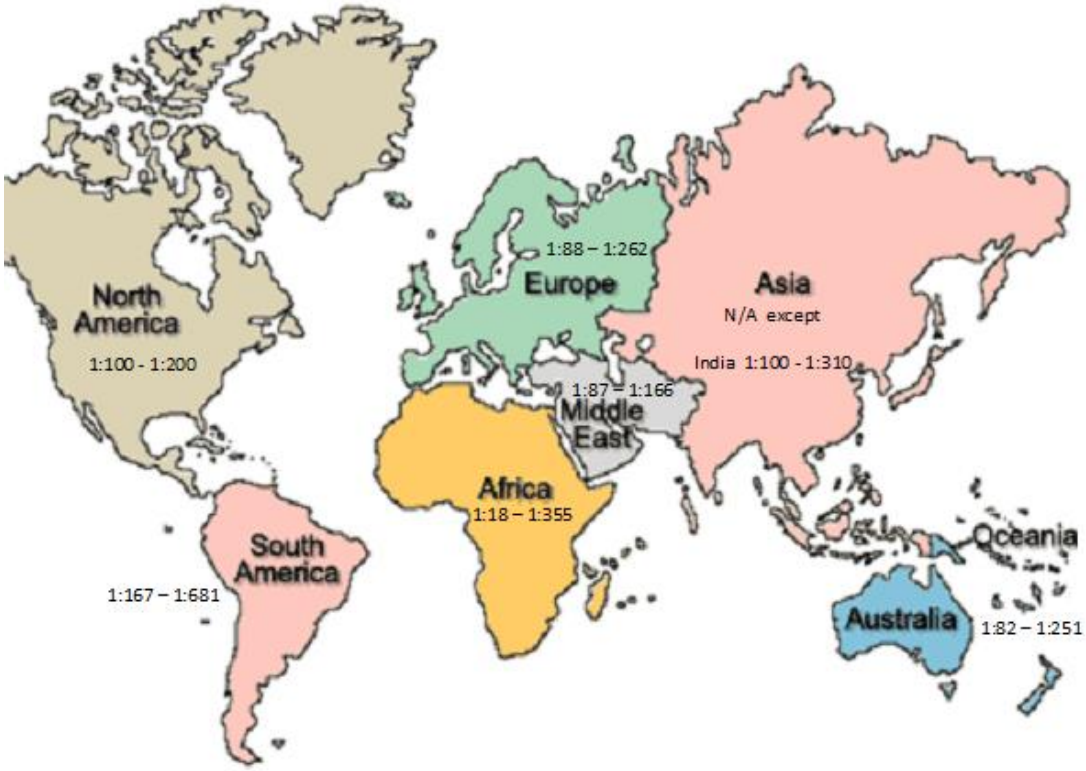



**Figure 1-1** Prevalence of CD worldwide.

### 1.2.2 High at-risk persons

In the general celiac population (without classical CD symptoms, e.g. diarrhea or weight loss), there are high risk groups that may have higher CD prevalence rates (Table 1-1). Among factors that denote a higher risk for CD, the most important factor is familial history of biopsy proven CD with an estimate of 20% or more of first-degree relatives having CD [12]. Some authors observed a higher prevalence in CD siblings as compared to parents [13-15]. A study in Swedish youth (< 20 years old) diagnosed with Type 1 Diabetes confirmed the low prevalence (0.7%) of symptomatic CD at initial onset of diagnosis, but an overall prevalence reached 10% during a 5-year follow-up [16]. Thus, the prevalence of an association with CD in high risk groups may increase over time.

**Table 1-1** High risk populations for CD [12]

| |
|---|
| Relatives, especially first-degree |
| Anemia, especially iron deficiency |
| Osteopenic bone disease |
| Insulin-dependent diabetes (type 1), especially children |
| Liver disorders, especially Autoimmune hepatitis and Primary biliary cirrhosis |
| Genetic disorders, including Down and Turner's syndrome |
| Autoimmune endocrinopathy, especially thyroid disease |
| Skin disorders, particularly dermatitis herpetiformis |
| Neurological disorders, including ataxia, seizures, Myasthenia gravis |
| Others, including IgA nephropathy |

The overall prevalence of CD is highly dependent on the HLA DQ2/DQ8 typing and gluten consumption. The population with positive HLA typing for celiac have high chances of developing celiac symptoms when on high gluten consumption. However, the population with diabetes, autoimmune disorder or relatives of CD individuals have even higher risk for the development of CD, since they share the same HLA typing.

### 1.3 CD causing factors

It is found that 30% of the Caucasian populations carry HLA-DQ2 and most will eat wheat, while only 1 in 100 will develop disease [17]. The remaining susceptibility is thought to be due to a combination of genetic and environmental factors (Figure 1-2).

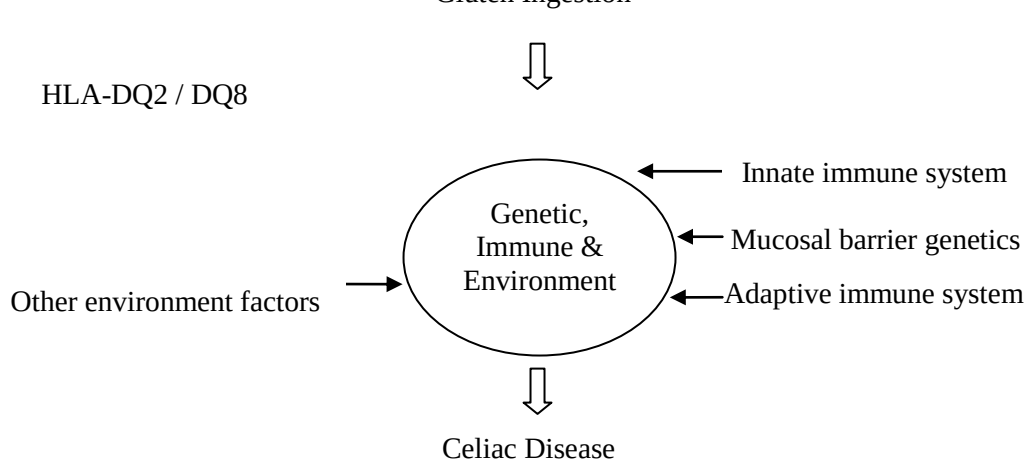



**Figure 1-2** Factors necessary for CD development [18].

HLA-DQ2.5 carriage is necessary for disease development, but it is not sufficient by itself. A combination of other genetic factors influencing the mucosal barrier, the adaptive and the innate immune system also impact the likelihood of disease development. Wheat ingestion is a known environment factor that is necessary for disease development but on top of this, a number of factors such as timing of gluten ingestion and breast feeding cessation may influence disease development [18].

### 1.3.1 Genetic factors

Genetics play a strong role in CD, indicated by the high disease concordance in monozygotic twin [19]. The CD prevalence rose from 1% to 17.6% in sisters, 10.8% in brothers, and 3.4% in parents [20]. CD is associated with HLA alleles as well as more than 250 other major histocompatibility complex (MHC) and non-MHC genes. The main genetic factor is the given HLA-DQ genes, i.e., the genes encoding DQ2 or DQ8 in the HLA complex on 6p21.

HLA genotype contributes to the genetic risk for CD at 30–50% [20, 21]. Non-HLA genes contribute more evidence to the CD genetic background than the HLA genes, but each by itself contributes modestly to the disease development. Hence, it is reasonable to assume that the susceptibility to CD involves polymorphic genes that influence the immune response to gluten, as shown for the HLA-linked genes [22].

Ninety percent of European patients with CD carry the human leucocyte antigen (HLA)-DQ2 molecule, encoded either in cis on the HLA-DQA1*0501-DQB1*0201 haplotype (HLA-DQ2.5cis) or in trans on the HLA-DQA1*0505 DQB1*0301/DQA1*0201-DQB1*0202 haplotypes (HLA-DQ2.5trans). Approximately 5% express HLA-DQ8, encoded by HLA-DQA1*0301-DQB1*0302. The majority of the remainder carry the HLA-DQA1*0201-DQB1*0202 haplotype [21]. With genetic testing, DQ2 is almost synonymous with DQB1*02, a gene with two common alleles designated DQB1*0201 and DQB1*0202. The DQ2 frequency in Caucasian in Western Europe populations has been estimated at 20–30%, and relatively high frequencies also occur in Northern and Western Africa, the Middle East and central Asia [23].

Thereafter, the overall frequency of DQ2 declines from West to East with low frequencies in populations in South-East Asia and the virtual absence of DQ2 in Japan (Table 1-2). DQ8 frequency has a worldwide distribution, whereas DQ2.5, is common in South and Central America; approximately 90% of Amerindian populations carry DQ8 and may display the celiac phenotype [24]. The frequency of DQ8 population is shown in Table 1-

**Table 1-2** Frequency of HLA-DQ2, encoded by HLA-DQB1*02 and HLA-DQ8, encoded by HLA-DQA1*0301-DQB1*0302

| < 5% | 5%-20% | 20% |
|---|---|---|
| **HLA-DQ2** | | |
| Albania | Belarus Algeria | Algeria |
| Canada BC (Athabaskans) | Cameroon | Australia |
| Cook Islands | Congo | Belgium |
| Indonesia | Costa Rica | Central African |
| Japan | China | Republic |
| Jordan | Cuba | Croatia |
| Papua New Guinea | Ecuador Africans | England |
| Philippines | France | Equatorial Guinea |
| Samoa | India | Bioko Island |
| | Malaysia | Ethiopia |
| | Mexico | Germany |
| | Poland | Greece |
| | Russia | Iran |
| | Singapore | Ireland South |
| | South Korea | Israel |
| | Spain | Italy |
| | Sri Lanka | Mongolia |
| | Sweden | New Zealand |
| | Taiwan | Pakistan |
| | Thailand | Saudi Arabia |
| | Turkey | Slovenia |
| | Uganda | Tunesia |
| | Ukraine | USA |
| | Vietnam | |
| **< 5%** | **5%-20%** | **20%** |
| **HLA-DQ8** | | |
| Australia | Algeria | Argentina |
| China | Belgium | Ecuador |
| Georgia | Brazil | Ethiopia |
| Greece | Canada BC (Athabaskans) | Mexico |
| North India | Croatia | Venezuela |
| Spain | England Caucasoid | |
| Uganda | France | |
| | South India | |
| | Israel | |
| | Italy | |
| | Japan | |
| | Russia | |
| | South Korea | |
| | Tunisia | |
| | Turkey | |
| | Ukraine | |
| | USA European American | |

Estimates are based on studies included in a comprehensive Internet website [24]. In several countries, the frequency is not known.

Studies using monozygotic twins, which are assumed to share environmental factors, have estimated the percentage of non-HLA genetic variants which predispose to CD of approximately 60% [25]. The incidence of the CD in siblings was very close to that observed in dizygotic twin (11%). These data suggest that shared environment (aside from gluten) has little or no effect on the concordance of dizygotic twins reared together [26]. To date a large list of variants have been suggested to predispose to CD through a combination of linkage and association studies, a large number of variants, however, do not stand up to further scrutiny. Only those that have been validated with convincing evidence in multiple populations are mentioned here.

In CD, like many common diseases, this genome wide linkage approach has been fairly unsuccessful at locating variants. Linkage was found to various regions including 5q and 19p, however, the only genomic region that was replicated with some reliability in other populations was 2q33, a region that contains the *CTLA4* [27], *ICOS* and *CD28* genes [25]. *CTLA4* is an excellent candidate gene for involvement in CD not only due to its integral involvement in the suppression of immune responses but also because it has been implicated as a genetic variant that increases susceptibility to Type 1 diabetes (T1D) [27].

The prevalence of CD in patients diagnosed with T1D has been estimated at up to 15% in children and 6% in adults [28]. The reason for this association has never been fully elucidated, but common mechanisms within the pathogenesis and genetics of the two conditions may provide some insights. IL21 region displays CD associations to T1D [29], rheumatoid arthritis [30], Grave's disease [31], and psoriatic arthritis [32]; but genetic involvement in all these conditions is not currently understood. There is possibility of shared genetic susceptibility to autoimmunity through *IL-2*, *IL-21* locus, both inside and outside of the HLA region, with almost no function identified thus far [17]. Like the studies associated with the HLA-DQ2.5 variant, further identification of the causal variant and its function will provide a unique insight into CD and other autoimmune disease biology.

### 1.3.2 Dietary gluten factor

Gluten is a protein that appears in wheat, barley, rye and oat, compositing of prolamin and glutelin. The majority of the proteins in food responsible for the immune reaction in CD are the prolamins. Prolamins are found in several grains, such as wheat (gliadin), barley (hordein) and rye (secalin), and possibly avenin in oats. Different oat cultivars contain different protein sequences, therefore varieties of oats are toxic for CD patients and other not [33]. Because of high proline and glutamine content and specific

sequence patterns, prolamins are resistant to gastrointestinal proteolytic enzymes and are excellent substrates for deamidation by tissue transglutaminase.

The incomplete gastrointestinal digestion of gluten leads to the appearance of gluten-derived gliadin peptides such as 33mer (LQLQPFPQPQLPYPQPLPYPQPQL PYPQPQPF) with a variety of characteristics [34]. It contains overlapping T-cell epitopes, and its deamidated form is a potent T-cell stimulator, generating the glutamic acid residues essential for binding to HLA-DQ2 in celiac patients [35]. The ingestion of prolamins from wheat, barley, rye and possibly oats causes histological changes in the small intestine mucosa of celiac patients, leading to a malabsorption syndrome [36]. Clinical symptoms of an autoimmune attack after ingestion of the gluten containing food include digestive symptoms and skin reactions.

Gliadin peptides cause stimulation of the innate and adaptive immune system [37-39]. The prototype of peptides effective on innate response is 19-mer peptide (p31–43/49, amino acid sequence PGQQQPFPPQQPY), which has been proved both *in vitro* and *in vivo* to be harmful for CD patients [40, 41]. Peptide 31–43 stimulates the synthesis and release of IL-15, a proinflammatory cytokine that promotes the adaptive immune response [38], involving CD4+ T cells that recognize several deamidated gliadin peptides [42]. Unlike p31–43 which is not immunogenic for T cells, peptide 57-68 (p57–68), which binds to HLA-DQ2/8 molecules, is one of the dominant epitopes recognized by T cells isolated from the intestine of CD patients [42]. The so-called toxic peptides, of which p31-43 is probably the most fully studied, modulate the small-intestinal mucosal biology via an innate immune mechanism.

### 1.3.3 Other causing factors

Genetic predisposition association (HLA, MYO9B), exogenous trigger (gluten), pro-autoimmune genetic background, viral infections, tissue damage, early termination of breastfeeding and gender contribute to the development of CD (Table 1-3) [43].

Apart from introduction of gluten during the first year of life, infectious agents may play a role in development of CD. Several studies have implicated infections with Adenovirus type 12 (Ad12) [44], HCV [45, 46], *Campylobacter jejuni* [47], *Giardia lamblia* [48], Rotavirus [49] and Enterovirus infection [50], as triggers for the development of CD. The immunologic response in persons genetically susceptible to CD may be activated due to a shared Adenovirus sequence of 8 to 12 amino acids with the toxic gliadin fraction [51]. Other factors such as timing of gluten ingestion and breast feeding cessation may involve

in the pathogenesis and disease development of CD [18]. Some initiating factors, such as gluten overload, gastric surgery "unmasking", giving up smoking, and infections can also trigger the disease, which can become apparent in an abrupt manner [52, 53].

**Table 1-3** The most important factors contributing to the development of CD [43]

| Factors contributing to onset of CD | Mechanism |
|---|---|
| Gluten | Elicit T cell responses |
| | Induces cytokine production and intestinal lesion |
| Age of introduction of gluten | Weak gut immunity during early childhood |
| HLA-DQ2 or HLA-DQ8 | Gluten presentation |
| MYO9Bo | Increased permeability of the intestine |
| Pro-autoimmune genetic background | Shift in Th1/Th2 balance towards Th1 |
| | Defect in generation of active tolerance (e.g., regulatory T cells) |
| Viral infections | IFN production |
| | Tissue damage |
| Tissue damage | Increased level of tTG |
| | Danger signals |
| Early termination of breastfeeding | Decreased protection against infections |
| Gender | Hormone-related pro-autoimmune status |

### 1.3.4 Time of trigger (onset of gluten intolerance)

All CD individuals are born with a genetic predisposition, but the age of onset may vary from infancy to old age. Some are diagnosed at birth or during childhood, but others may remain dormant until it is triggered later in life.

Several studies related the rise in childhood CD to infant feeding practices [18, 54]. Consumption of wheat, barley, and rye in the first 3 months children have significantly increased the risk of developing CD-associated autoantibodies, as compared with exposure during first 4 to 6 months [54, 55].

Although CD can be diagnosed at any time of life, it is present mostly in either early childhood (between 9 and 24 months) or in the third or fourth decade of life [3, 41, 56, 57]. In contrast to the 1/1 sex ratio in children, in adulthood it is diagnosed twice as frequently in females. Interestingly, CD is also becoming more frequently recognized in

the elderly, and in this population, a 1/1 sex ratio has also been noted [58]. Although the "classical" gastrointestinal malabsorption syndrome characterized by diarrhea, steatorrhoea, weight loss, fatigue, and anemia may occur in severe cases, most patients have a milder symptoms such as abdominal discomfort, bloating, indigestion, or non-gastrointestinal symptoms (or no symptoms at all) [3, 41, 56, 57]. Maki et al. reported a shift of 5–6 years of age at diagnosis in Finland, with less than 50% of new cases presenting typical gastrointestinal symptoms [59]. In England [60], Scotland [61], Canada [62], and the United States [8], reports have also shown that almost 50% of newly diagnosed CD patients do not present with gastrointestinal symptoms.

### 1.4 Pathogenesis

CD is an autoimmune disease associated with the genetic predisposition HLA and tissue transglutaminase (tTG) autoantigen. TTG is a calcium dependent enzyme that plays a crucial role in CD pathogenesis [63]. TTG mediates ordered and specific deamidation of gliadins, creating an epitope that binds efficiently to DQ2 and is recognized by gut-derived T cells [64]. This causes activation of T-cells, production of high levels of pro-inflammatory cytokines, apoptotic death of enterocytes, and induction of clonal expansion of B-cells. These B-cells differentiate into anti-gliadin and anti-tTG antibody secreting plasma cells. Interaction between with the extracellular tTG (mtTG) and anti-tTG-autoantibody may further contribute to epithelial damage (Figure 1-3).

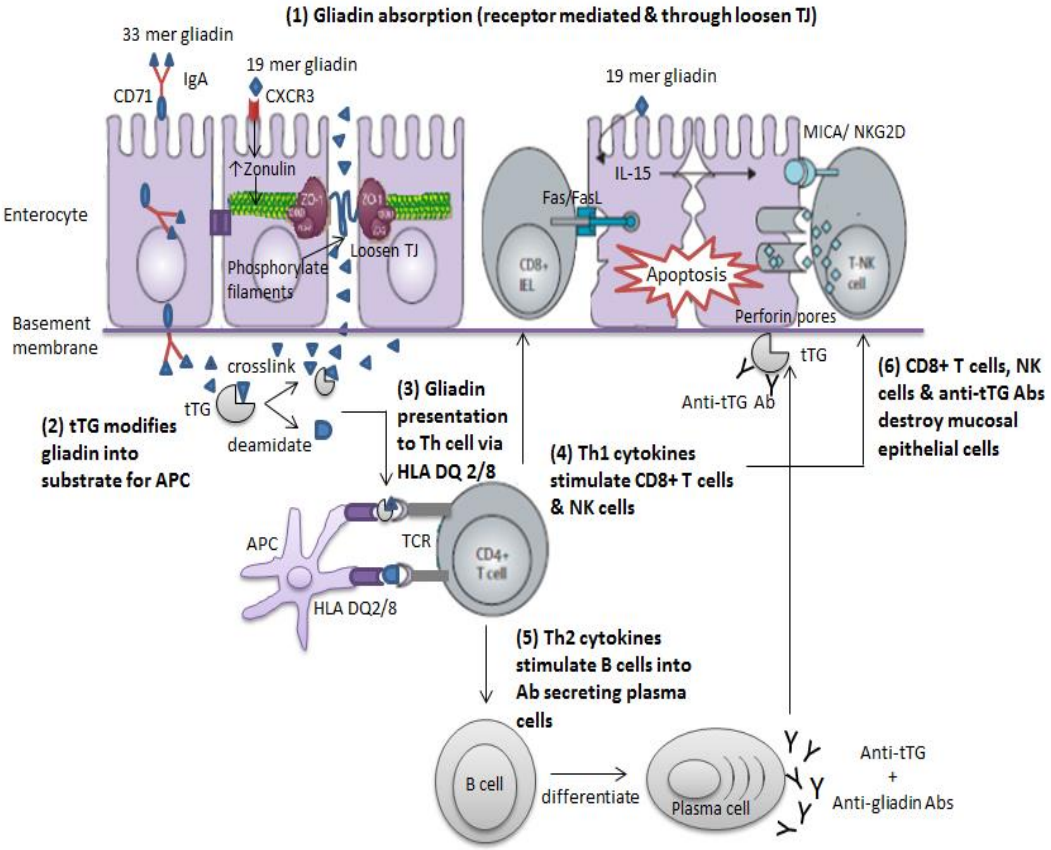



**Figure 1-3** Mechanisms of mucosal damage in CD [65]. Gliadin peptides crosses the enterocyte by paracellular tight junctions (TJ) as a consequence of increased release of zonulin leading to impaired mucosal integrity upon 19 mer gliadin binding to CXCR3 receptor, or via transcytosis, or retrotranscytosis of secretory IgA through transferrin receptor CD71. Tissue transglutaminase (tTG) deamidates or crosslinks 33 mer gliadin which is then recognized by HLA-DQ2 or -DQ8 molecules of antigen presenting cell (APC). APC presents the toxic peptide to CD4+ T (αβ) cells. Activated gluten-reactive CD4+ T-cells produce high levels of pro-inflammatory cytokines. Th1 cytokines promote increased cytotoxicity of intraepithelial lymphocytes (IELs; γδ T cells) and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand (FasL) system, or interleukin 15 (IL-15)-induced perforin/granzyme and NKG2D–MICA signaling pathways. The production of Th2 cytokines activate and induce clonal expansion of B cells, which differentiate into anti-gliadin and anti-tTG antibody secreting plasma cells. Interaction between with the extracellular tTG (mtTG) and anti-tTG-autoantibody may induce epithelial damage.

### 1.4.1 Site of CD

During gluten consumption, these tTG autoantibodies are produced by the mucosa of the small intestine, and detected in patients' serum but disappear slowly from the patient's circulation on a gluten-free diet (GFD) [66]. Extraintestinal CD symptoms may be associated with IgA deposits on extracellular tTG in the liver, kidney, lymph nodes and muscles of CD patients [66]. The mucosa is expanded by increased numbers of lymphoid cells both in the intraepithelial compartment, in which there is an increase in γδ T cells, and in the lamina propria, which is expanded by lymphocytes and plasma cells. The intestinal crypts are elongated because of an increase in dividing epithelial cells, and villi are shortened or even completely absent because of rapid loss of mature epithelial cells from the villus tip.

### 1.4.2 Intestinal permeation of gliadin

Intestinal epithelium plays a central role in CD disease pathogenesis. It modulates the intestinal immune system that is acutely altered by gliadin. This indicates that gliadin can gain access to the basal surface of the epithelium, and therefore interact directly with the immune system, via both trans- and paracellular routes of absorption.

#### *1.4.2.1 Retrotranscytosis of gliadin*

The protected retrotransport of secretory IgA into the intestinal lumen via the transferrin receptor CD71, allows the entry of intact and thus harmful gliadin peptides into the intestinal mucosa by a transcellular route. The overexpression of the transferring receptor CD71 in patients with active CD by transportation of gliadin across the intestinal mucosa through retrotranscytosis of secretory immunoglobulin –gliadin complexes is shown in Figure 1-4 [67, 68].

Transcytosis of α2-gliadin-33 mer (an important trigger of CD) by apical-to-basal is stimulated by interferon-γ, which is a key cytokine involved in CD immunopathogenesis [69].

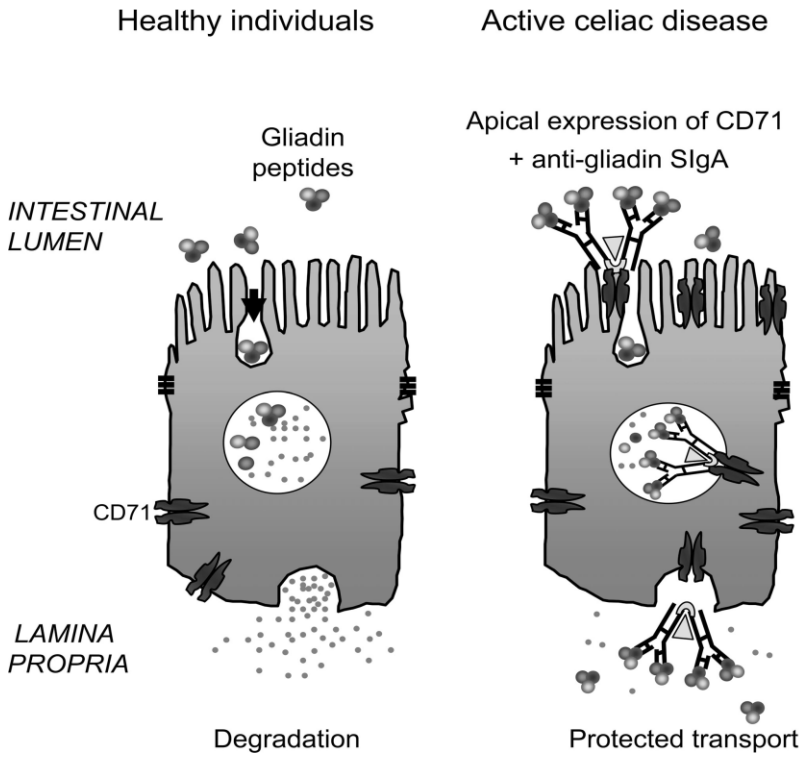



**Figure 1-4** CD71 receptor–mediated transport of IgA–gliadin complexes in CD [68]. Gliadin bound to apically expressed CD71 receptor in active CD individual allows protected transport of gliadin into the lamina propria.

#### *1.4.2.2 Paracellular pathway of gliadin*

There have been recent hypotheses that the non-digested gliadin in the intestinal lumen cause CD pathogenesis by stimulation of the innate and adaptive immune systems [38, 39, 70]. Zonulins provide information on the regulation of intercellular tight junctions (TJs) and increased intestinal permeability [71-75]. It is released by the enterocyte upon apical exposure to α-gliadin digests [76, 77]. Lammers et al. have identified that MyD88 induces release of zonulin upon gliadin binding to CXCR3 on enterocytes, as a result inducing greater epithelial permeability and subsequent paracellular gliadin passage to the gut mucosa [77].

After binding to its surface receptor, gluten is internalized [78], and subsequently triggers a series of intracellular events including phospholipase C and Protein kinase Ca (PKCa) activation and actin polymerization, which lead to the opening of TJs [72, 79], through Zot/Zonulin receptor (Figure 1-5).

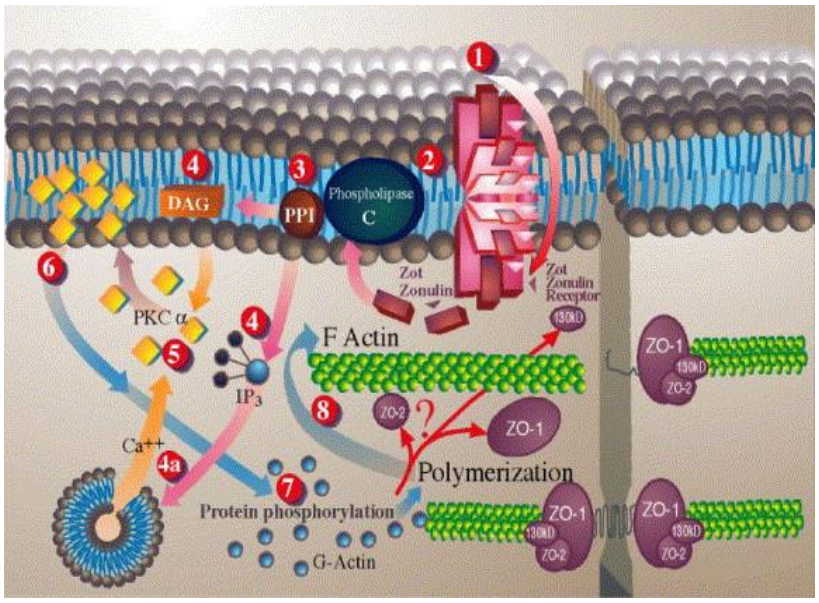



**Figure 1-5** Proposed Zot intracellular signal mediated opening of intestinal TJ [80]. (1) Zot interacts with a specific Zot/Zonulin intestinal surface receptor; (2) leading to protein internalization and; (3) activation of phospholipase C; (4) that hydrolyzes phosphatidyl inositol to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG). (5) PKCa is then activated; (4) either via DAG or (4a) through the release of intracellular $Ca^{2+}$ via PPI-3. (6) Membrane-associated, activated PKCa catalyzes the phosphorylation of target protein(s); (7) with subsequent polymerization of soluble G-actin in F-actin. (8) This polymerization causes the rearrangement of the TJ filaments and displacement of proteins (including ZO-1). As a result, intestinal TJ becomes loosened.

The toxic peptides, such as the p 31-49, trigger an innate immune response [38], characterized by the production of IL-15 by epithelial cells and lamina propria dendritic cells [64]. There is some evidence that this response is a generalized response in all individuals, but is amplified in CD patients (possibly due to a lower threshold to IL-15) who only get disease as a result of adaptive immune system involvement [81]. IL-15 affects the epithelial barrier, both by increasing the permeability through disruption of the tight junctions [76, 82], and acting on intraepithelial lymphocytes (IELs) promoting IFN-γ production as well as a potent cytotoxic activity particularly by NKG2D+ cells [83, 84]. Therefore, immunoadaptive peptides, like the 33-mer, can now reach the lamina propria, where they are presented by dendritic cells to gluten-specific T cells [85, 86].

#### *1.4.2.3 Other pathways*

There are several pathways including cellular signals that may be involved in the mucosal damage in CD. Deamidation of gluten peptides by tissue tTG reinforces presentation of gluten peptides by HLA-DQ2 or HLA-DQ8 molecules of plasmacytoid dendritic cells (pDCs) to T cells, which activate gluten-reactive Th1 cells and produce high levels of proinflammatory cytokines. IL21 is overproduced in the mucosa of CD patients, where it helps sustain T-bet expression and IFNγ production [80]. Th1 cytokines promote increased cytotoxicity of intraepithelial lymphocytes (IELs) and natural killer (NK) T cells which cause apoptotic death of enterocytes by the Fas/Fas ligand (FasL) system, or interleukin 15 (IL-15)-induced perforin/granzyme and NKG2D–MICA signaling pathways. IFN-α released by activated pDCs perpetuates the inflammatory reaction by inducing Th1 cells to produce IFN-γ. IL-21 and IL-15 produced by DCs and intraepithelial cells (IECs) also inhibit transforming growth factor beta (TGF-β) signaling and regulatory T cells (Tregs) function. Additionally, the production of Th2 cytokines, the Th2 cells driven activation and clonal expansion, the differentiation of B cells into plasma cells secreting anti-gliadin and anti–tissue transglutaminase antibodies [87], may induce epithelial damage. Hence in CD, there is impaired suppressor activity of Tregs. This defect in Tregs function could play a role in the pathogenesis of CD and in CD autoimmunity [88].

### 1.5 Celiac symptoms

CD commonly appears in early childhood, with severe symptoms including chronic diarrhea, abdominal distension, and failure to thrive. In many patients, symptoms may not develop until later in life, when the disease symptoms include fatigue, diarrhea, and weight loss due to malabsorption, anemia, and neurological symptoms (Table 1-4). Celiac disease is a life-long disorder, and if untreated, it is associated with increased morbidity and mortality [56, 89].

**Table 1-4** Intestinal and extraintestinal conditions associated with CD

| Typical symptoms | Atypical symptoms | Associated conditions |
|---|---|---|
| Chronic diarrhea | Secondary to malabsorption | Possible gluten dependent |
| Failure to thrive | Sideropenic anemia | IDDM |
| Abdominal distention | Short stature | Autoimmune thyroiditis |
| | Osteopenia | Autoimmune hepatitis |
| | Recurrent abortions | Sjogren syndrome |
| | Hepatic steatosis | Addison disease |
| | Recurrent abdominal pain | Autoimmune atrophic |
| | Gaseousness | gastritis |
| | Independent of malabsorption | Autoimmune |
| | Dermatitis herpetiformis | emocytopenic diseases |
| | Dental enamel hypoplasia | Possible gluten independent |
| | Ataxia | Down syndrome |
| | Alopecia | Turner syndrome |
| | Primary biliary cirrhosis | Williams syndrome |
| | Isolated hypertransaminasemia | Congenital heart |
| | Recurrent aphthous stomatitis | defects |
| | Myasthenia gravis | IgA deficiency |
| | Recurrent pericarditis | |
| | Psoriasis | |
| | Polyneuropathy | |
| | Epilepsy | |
| | Vasculitis | |
| | Dilatative cardiomyopathy | |
| | Hypo/hyperthyroidism | |

## 1.6 Diagnosis

In 1970, the European Society of Paediatric Gastroenterology laid down criteria for the diagnosis of CD in children, entailing three biopsies of an initial flat mucosa in the upper small intestine, restoration of the mucosa to normal on a GFD, and a deterioration of the mucosa after gluten challenge [90]. Given the current availability of serological tests being highly sensitive and specific, the European Society of Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) has proposed a revised CD diagnostic protocol [91]. Based on this protocol, if the symptoms (either "classical" or "atypical") and serological tests are suggestive of CD, small bowel biopsy followed by a favourable clinical and serological response to the GFD is now considered sufficient to definitely confirm the diagnosis. In asymptomatic patients improvement in mucosal appearance may be required to confirm the diagnosis, but in majority of symptomatic patients, continual abnormality of mucosa at the second biopsy is more likely to indicate slow /partial mucosal recovery [92]. This may also reflect that the site of re-biopsy (proximal small intestine) is often the last site to improve. In some patients the histological changes of CD may be patchy and/or diffuse along the length of the small intestine. Also, insert a table of the pathological changes in other parts of the GI tract and liver in CD.

The current approach to evaluating CD has been modified by the advent of highly sensitive and specific serological tests. An algorithm for diagnosing CD is given in Figure 1-6. Assays for IgA anti-tissue transglutaminase (TGA) and IgA anti-endomysial (EMA) have both the highest specificities and sensitivities, and are therefore regarded as being superior serological screening tools for diagnosis of CD [93].

Initial CD evaluation is based on a combination of positive CD-specific serological tests, histological findings in the intestinal biopsy, CD-predisposing gene encoding human leukocyte antigens (HLA) DQ2 or DQ8, family and medical history of CD, and clinical or histological response to GFD [6, 65]. However, CD diagnosis can be challenging in some non-responsive patients to GFD [94]. Practically all patients with CD carry HLA-DQ2 or HLA-DQ8. Thus the absence of these gene pairs reflects a very high negative predictive value for CD and should prompt consideration of other causes of small bowel-related symptoms and pathological changes [95, 96]. Positive TGA or EMA at initial diagnosis of CD or at any time in the clinical course of the disease helps to confirm the diagnosis of CD because of their excellent specificities of over 99% when small bowel villous atrophy is present on biopsy [97].

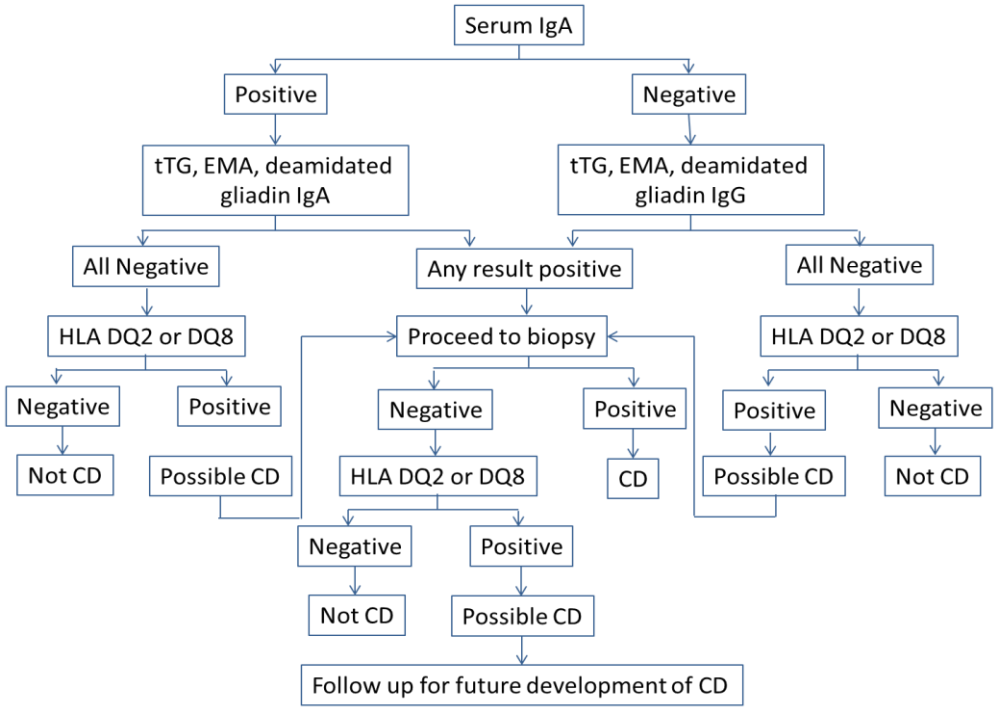



**Figure 1-6** Celiac disease diagnostic testing algorithm (from Mayo Medical Laboratories, Mayo Foundation for Medical Education and Research (MFMER).

However, false positive serological assays may also occur [98], in liver disease and small-bowel inflammation [99], so documentation of gluten sensitivity is important. A combination of biopsy and serological antibody can also be used to support diagnosis to reduce false positive results. A validated subjective Celiac Dietary Adherence Test (CDAT), a patient-completed tool, can also be used in conjunction with biological markers to assess dietary adherence and disease activity in individuals with CD [100].

There are several clinical conditions that are suggestive of CD, hence patients with indications as mentioned in Table 1-5 should be screened using serological tests for CD [101].

**Table 1-5** Clinical indications for serological testing for CD

| |
|---|
| Positive family history of CD |
| Autoimmune endocrine disorders |
| Insulin-dependent diabetes mellitus |
| Autoimmune thyroid disease |
| Autoimmune adrenal disease |
| Autoimmune connective tissue disorders |
| Sjogren's syndrome |
| Rheumatoid arthritis |
| Systemic lupus erythematosus |
| Hepatobiliary conditions |
| Primary sclerosing cholangitis |
| Primary biliary cirrhosis |
| Autoimmune cholangitis |
| Elevated transaminases |
| Other gastrointestinal disorders |
| Lymphocytic gastritis |
| Microscopic colitis |
| Miscellaneous conditions |
| IgA deficiency, IgA nephropathy |
| Down syndrome, Turner's syndrome |

### 1.6.1 Diagnostic tests

#### *1.6.1.1 Serological tests*

**HLA typing:** The contribution of HLA type to the genetic risk for CD has been variously estimated at 30%–50% [20, 21]. Many of the polymorphic genes are involved in susceptibility to CD encode products that influence the immune response upon gluten ingestion, as shown for the HLA-linked genes [102]. Although more non-HLA genes than

HLA genes contribute more to the genetic background of CD, each of them adds only a minor contribution to the disease development.

There is strong association between CD and the presence of human leukocyte antigen (HLA) DQA1*0501-DQB1*02 (DQ2) and DQA1*0301-DQB1*0302 (DQ8) haplotypes. Approximately 90% to 95% of patients with CD carry DQ2 and those patients that are negative for HLA-DQ2 are usually positive for HLA-DQ8 [103, 104], indicating a strong genetic risk for the disease. Several studies also have confirmed that the absence of HLA-DQ2, HLA-DQ8, or both virtually excludes the diagnosis of CD [105-107]. However, the modest sensitivity (HLA-DQ2, 70-99.8%; HLA-DQ8, 1.6-38%) and specificity (HLA-DQ2, 69-77%; HLA-DQ8, 77-85%) of the test means that a positive result is not sufficient to diagnose the disease (having a low positive predictive values[HLA-DQ2, 6.3-18; HLA-DQ8, 0.28-8.1] and likelihood ratios [HLA-DQ2, 2.25-4.33; HLA-DQ8, 0.07-2.53]) [108]. Even the presence of HLA-DQ2 or HLADQ8 in patients with positive serologic test results is strongly suggestive but not pathognomonic for CD. Antibody screening to identify participants with preclinical CD may be reduced by preselecting HLA risk group from the large populations with long-term follow-up for CD [109]. Hence HLA-DQ genotyping could be included in the algorithm of selecting large populations prospectively screened for CD.

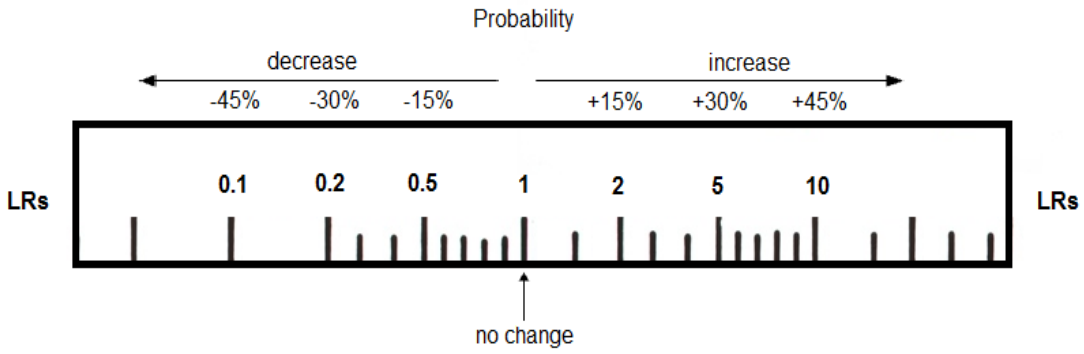
**Antibody level:** Several serum antibodies have been used to initially evaluate patients with suspected CD, monitor adherence and response to GFD, and screen asymptomatic individuals. Anti-gliadin antibodies (AGA) detection has low sensitivity and specificity, leading to high false-positive rate in patients [110]. Recent reports of deamidated gliadin peptide AGA (DGP-AGA) have suggested a much improved accuracy [111]. The sensitivity and specificity for IgA DGP-AGA is 84.3% and 79.8%, whereas for IgG DPG-AGA the sensitivity and specificity are 82.3% and 98.9%, respectively [112]. As shown in Table 1-6, EMA and TGA have been found to be superior to AGA and gives highest sensitivity and specificity of greater than 95% when used in combination [110, 113, 114]. EMA testing, however, produces a subjective and highly observer-dependent result, whereas TGA testing is quantitative.

**Table 1-6** Operating characteristics of serological markers to detect the CD in adults [115].

| Serological Tests | Sensitivity (%)* | Specificity (%)* | Predictive Value | | Likelihood Ratio | |
|---|---|---|---|---|---|---|
| | | | Positive | Negative | Positive | Negative |
| IgG AGA | 57–78 | 71–87 | 0.2–0.9 | 0.4–0.9 | 1.96-6 | 0.25-0.61 |
| IgA AGA | 55–100 | 71–100 | 0.3–1.0 | 0.7–1.0 | 1.89-∞ | 0-0.63 |
| IgA EMA | 86–100 | 98–100 | 0.98–1.0 | 0.8–0.95 | 43-∞ | 0-0.14 |
| IgA TGA | 77–100 | 91–100 | >0.9 | >0.95 | 8.55-∞ | 0-0.25 |
| IgA TGA and EMA | 98–100 | 98–100 | >0.9 | >0.95 | 49-∞ | 0-0.02 |

*95% CI

IgG: Immunoglobulin G; IgA: Immunoglobulin; AGA: Anti-gliadin antibodies; EMA: Anti-endomysial antibodies; TGA: Anti-tissue transglutaminase antibodies.




Abbreviations: LRs, likelihood ratios (the greater the LRs value, the more probability of disease increases; the closer the LR number is to zero the more the probability of disease decreases)

### *1.6.1.2 Small intestinal mucosal biopsy*

**Histopathological analysis:** Although the diagnosis of CD can be suspected on clinical or laboratory grounds, or as a result of serological tests, histology of the proximal small intestinal mucosa is still the diagnostic gold standard and must always be performed. Small intestinal histopathology of CD biopsy samples are characterized by typical architectural abnormalities. Marsh [115], has pioneered the theory of a sequence of progression of the CD lesion in the small intestinal mucosa.

According to the modified Marsh classification: normal mucosa is classified *Marsh 0*, intraepithelial lymphocytosis as *Marsh I*, intraepithelial lymphocytosis and

crypt hyperplasia as *Marsh II*, and intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy as *Marsh III* [116]. Later the Marsh-Oberhuger system was developed, where stage 3 was split into three sub stages (a, b and c) [115, 117]. The Marsh–Oberhuber classification was based on a 6-stage grading, namely (1) type 1 infiltrative lesions, characterized by normal mucosal architecture with an increased number of IELs; (2) type 2 hyperplastic lesions, characterized by an increase in depth without villous flattening; (3) type 3a, 3b, and 3c destructive lesion, characterized by mild, marked, and complete villous flattening, respectively; and (4) type 4 hypoplastic lesions, characterized by villous atrophy with normal cryptcryp height and IEL count.

Considering the broad spectrum of lesions possibly present in CD, the Marsh-Oberhuber system is undoubtedly valid under optimal clinical conditions, but the considerable number of diagnostic categories involved makes it prone to a low inter-observer and intra-observer agreement.

False-positive and false negative test results may occur due to patchy mucosal damage, inter-observer variability, low-grade histopathological abnormalities and technical limitations. It generally accepted that biopsy is sometimes not necessary, but 4-6 distal duodenum biopsies is recommended to diagnose CD, as a standard of care [6]. Several other limitations may be evident in high-volume, service-oriented laboratories with limited attention to quality control. Poorly oriented biopsies fixed in the endoscopy suite may be difficult to interpret. Inter-observer variation in pathological interpretation may occur, especially if there is limited access to a pathologist with expertise focused on interpretation of small intestinal biopsies. Some patients with low-grade histopathological abnormalities (Marsh I / Marsh II) can present with gluten-dependent symptoms or disorders before overt villous atrophy occurs. Furthermore, some patients with isolated intraepithelial lymphocytosis (Marsh I), who are not clinically suspected of having CD, may develop CD during follow-up [118]. Although the mucosal changes in CD are thought to develop gradually, whether minor mucosal lesions in asymptomatic patients indicate CD in an early stage is not yet clear [119].

In case of strong clinical suspicion of CD, duodenal biopsy must be performed regardless of serological analysis [120]; in cases of low suspicion of disease or screening, duodenal biopsy probably only needs to be performed in seropositive patients. Hence, the new system for routine use of simplified grading system with uniform diagnosis and increase validity of the pathologic diagnosis of CD was developed by using only three categories (A, B1 or B2) with A representing normal villous with lymphocytic infiltration

and B1 and B2 representing partial and complete villous atrophy, respectively [121]. The new proposed grading system classified the CD lesions into non-atrophic (grade A) and atrophic (grade B) [122]. Grade A was characterized by the isolated increase of IELs (>25/100 enterocytes) [123], whereas grade B was split into B1, in which the villous/crypt ratio is less than 3/1, with still detectable villi, and B2, in which the villi are no longer detectable. A comparison between the Marsh–Oberhuber and the new grading criteria is shown in Figure 1-7 b. Figure 1-7c represents pictures of the grades proposed in the new histologic grading criteria.

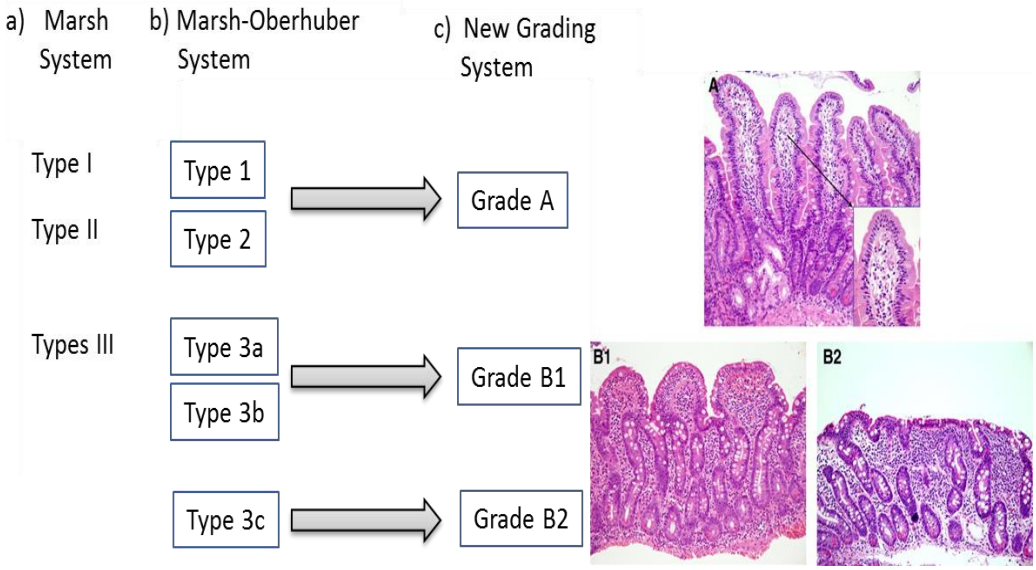



**Figure 1-7** A Comparison between the Mash classification for CD. a) and Marsh–Oberhuber; b) grading system for CD, and the new grading system (established in 2007) c) Representative pictures of the grades A (original magnification, 20×; *insert*, 60×), B1(20×), and B2 (20×), proposed in the new grading system. An alternative classification may simply describe "mild", "moderate" or "severe (flat)" architectural changes [122].

Recently, quantitative measurements of villous height, apical and basal villous widths, and crypt length (morphometry) have been used to determine changes in duodenal morphology, particularly after the introduction of a GFD, in correlation with Marsh grade, self-reported adherence to GFD, and changes in serology. GFD resulted in increase in villous area and a progressive decrease in crypt length, with a plateau after 6–12 months and mean villous area half that of control subjects [124].

Other conditions which may be associated with a "sprue-like" lesion are mentioned in Table 1-7, indicating the histological features which may be used to distinguish the conditions from CD. Other small bowel disorders, including Crohn disease in the duodenum, may cause mucosal scalloping. The difference between these conditions and CD is the lack of response to gluten withdrawal.

**Table 1-7** Conditions associated with a "sprue-like" lesion, and their histological features [125]

| Conditions | Histological features |
|---|---|
| Collagenous sprue | Collagenous band below atrophic epithelium |
| Mycobacterium-avium complex infection | Acid-fast bacilli, foam cells, PAS positive macrophages |
| Amyloidosis | Congo red-stained deposits with apple-green birefringence in polarized light |
| Crohn disease | Epitheloid granulomas and characteristic focal inflammation |
| Eosinophilic gastroenteritis | Eosinophilic infiltration |
| Lymphangiectasia | Ectatic lymph vessels, fat in lymphatics |
| Lymphoma | Clonal expansion of lymphocytes |
| Mastocytosis | Diffuse infiltration with mast cells |
| Infection | Organism seen on histological examination (eg. giardia lamblia, strongyloides, TB, HIV) |
| Whipple | Acid-fast bacilli, foam cells, PAS positive, diastase resistant staining in macrophages |
| Abeta-lipoproteinemia | Large lipid droplets |

## 1.7 Treatment

### 1.7.1 Gluten free diet

Currently, the only effective treatment available for CD individuals is a strict life-long GFD. In reality, total avoidance of gluten intake is extremely difficult, due to hidden gluten from food contamination [126]. For safety purposes, US Food and Drug Administration has set the limit (August 2011) of <20 ppm gluten (equivalent to 10 ppm gliadin) for gluten-free foods. The total daily consumption of gluten-free foods must be taken into account as it may exceed the tolerable limit of each celiac individual. It has been estimated that the threshold of prolonged gluten ingestion in some CD individuals may be lower than 50 mg per day [127].

However, some CD individuals can conceivably be more sensitive. The presence of hidden gliadin in contaminated food products represents an imminent risk for celiac consumers, because of long-term effect of regular ingestion of small amounts of gliadin [128], on causing positive tTG and characteristic small bowel biopsy.

### 1.7.2 Possible pharmaceutical therapies

Approaches to modify dietary gluten have been made to render gliadin non-toxic, since it is a non-invasive approach to CD patients. This approach has been less appealing due to loss of baking characteristic, public refusal for genetically modified crops, contamination of genetically modified crops with gluten contained crops grown nearby and heterogeneous uncharacterised immunostimulatory epitopes in gluten, and difference among patients response to epitopes and gluten levels [129].

A greater understanding of the pathogenesis of CD allows alternative future treatments to be designed. A number of preliminary studies have been published that illustrate from a conceptual perspective future possible approaches that may be pursued in more detail (Table 1-8). CD has been kept in the dark for decades with very little known about what is a relatively common medical condition. It is only recently that we have greater understanding of its prevalence, diagnosis and pathogenesis, which has supported the development of new therapeutic approaches to treat CD. There are several future directions to follow to treat CD, which if successful will supplement or even replace the current only effective treatment, the use of a GFD. A greater understanding of the pathogenesis of CD allows alternative future CD treatments to hydrolyse toxic gliadin peptide, prevent toxic gliadin peptide absorption, blockage of selective deamidation of specific glutamine residues by tissue, restore immune tolerance towards gluten, modulation of immune response to dietary gliadin, and restoration of intestinal architecture (Table 1-8).

**Table 1-8** Development of Pharmaceutical Therapies of CD

| Mechanism | | Therapeutic agent | Stage of study |
|---|---|---|---|
| Hydrolysis of toxic gliadin | ALV003 | Cysteine endoprotease B-isoform 2 (from germinating barley seeds) and SC-PEP (prolyl endopeptidase from *Sphingomonas capsulate*) | Phase II [130, 131] |
| | AN-PEP | *Aspergillus niger* prolyl endopeptidase | Phase II [132-134] |
| | | Lactobacilli | Discovery[135, 136] |
| | VSL#3 | Lyophilised bacteria, including Bifidobacteria, Lactobacilli and *Streptococcus salivarius* | Discovery[137] |
| Prevention of gliadin absorption | Larazotide | Hexapeptide derived from Zonula Occludens toxin of Vibrio cholera | Phase II[138, 139] |
| | | Synthetic polymer poly (hydroxyethylmethacrylate - co -styrene sulfonate) (P(HEMA- co -SS)) | Discovery[140-142] |
| | | Anti-gliadin IgY | Discovery[143] |
| tTG2 inhibitor | | Dihydroisoxazoles | Discovery[144] |
| | | Cinnamoyltriazole | Discovery[145] |
| | | Aryl [beta]-aminoethyl ketones | Discovery [146] |
| Peptide vaccination | Nexvax2 | Three deamidated peptides derived from wheat α-gliadin, ω-gliadin and B-hordein | Phase I[147-149] |
| | | Human hookworm (Necator americanus) inoculation | Phase II [150, 151] |
| Modulate immune response | | HLA-DQ2 blocker | Discovery[152-155] |
| | | Interleukin blocker | Discovery[156-158] |
| | | NKG2D antagonist | Discovery[159] |
| | | NKG2D antibodies | Discovery[160] |
| Restore intestinal architecture | | R-spondin-1 | Discovery[161] |

### 1.7.3 Oral passive antibody therapy

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 [162]. 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 [163]. Chickens can produce high titre 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.).

## 1.8 Antibody

The avian egg is a reserved life form to the next generation which turns into a bird. An egg is a storehouse of all the substances necessary for a potential new life. Chickens produce immunoglobulin in blood against almost all kinds of antigens including bacteria, virus, allergens, and foreign substances in host defense. As described more than 100 years ago, avian maternal antibodies are transferred to egg yolk to protect embryos and neonates [164].

The major antibody, immunoglobubin class G (IgG), in both blood serum and egg yolk has been termed IgY [165] because it is distinct from mammalian IgG in structural and functional properties. Circulating IgG from the hen plasma is first sequestered in the yolk of maturing oocytes in the ovarian follicle via a receptor mediated process which recognizes intact Fc and the hinge region of IgG [166]. Yolk is incorporated into the egg white in the oviduct along with the egg albumen secretion. As an egg is oviposited, as much as 200 mg of serum IgG are present in the egg yolk, hence the term immunoglobulin of egg yolk (IgY) [167, 168].

Maternal IgA and IgM, present in oviduct secretions, are acquired by the egg as it passes down the oviduct where the egg white is laid down. The concentrations of IgM and IgA in egg white and IgG in egg yolk are about 0.15 mg, 0.7 mg, and 25 mg per mL, respectively [169].

Using chicken as an antibody producer brings a number of advantages over conventional mammalian antibody and recombinant antibody production and serves as an alternative to antibody sources. Combined with the egg industry's capacity to produce thousands of eggs per day and an existing technology for the efficient fractionation and purification of IgY, it is conceivable that kilogram quantities of antibodies could be

produced on a daily basis. Several advantages in the preparation of antibody using hens over using animals are summarized as follows [170] :

- The conventional method inevitably sacrifices animals which have produced the specific IgG in their circulating blood. On the other hand, the method of using hens is sufficient only to collect the eggs laid by super immunized hens. For separation IgY, a large scale method is now applicable by automatic separation of the egg yolk with a machine.
- As egg yolk contains only IgY, the isolation of IgY from the yolk is much easier than that of IgG from animal blood sera.
- Large-scale feeding of hens for egg production now being carried out is also a merit for collecting the source of a specific antibody.
- Also, immunization of hens (vaccination) has long been applied to prevent hens from infectious diseases, indicating that immunization of hens is much more systematized to be effective than doing it for animals.
- Egg yolk as the source of IgY is much more hygienic than mammal's sera from which IgG is separated.
- Because of a taxonomical difference, the hen has the possibility to produce the antibodies whose formation is difficult or impossible in mammals.

Thus, IgY has been widely used as a passive immunization therapy to treat enteric infections in humans and animals. Another application is the use of IgY as an immunological tool in the field of diagnostics as well as biomedical research. In this review, we summarize published data on properties and applications of IgY for prophylactic, therapeutic and diagnostic uses and suggest directions for its future use.

### 1.8.1 Characteristics of IgY

#### *1.8.1.1 Structures of IgY*

IgY is the major serum immunoglobulin of birds, reptiles and amphibians and is capable of providing maternally derived passively acquired immunity to young, as well as mediating anaphylactic reactions in some of these species [171]. On the contrary to the five different classes (IgG, IgA, IgM, IgD, and IgE) of immunoglobulin molecules found in most higher mammals, lymphatic system of domestic avian species such as chickens, turkeys, and ducks consists of three classes of immunoglobulin, IgG, IgA, and IgM.

The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains.

Despite the similarities between IgY and IgG antibodies, there are also some profound differences in their structure. IgY consists of two identical heavy (H) chains and two identical light (L) chains, which are linked by a disulfide bridge. IgY has a molecular mass of ~180 kDa which is heavier than that (~150 kDa) of mammalian IgG. The H chains of IgY possess one variable domain (V), four constant domains (C) and no genetic hinge, unlike mammalian IgG which has three C domains and a hinge region. The molecular structure of IgY, therefore, is similar to mammalian IgM or IgE, which consist of four C domains. Comparisons of C-region sequences in IgG and IgY show that the $C_H2$ and $C_H3$ domains of IgG are most closely related to the $C_H3$ and $C_H4$ domains of IgY, respectively, and that the equivalent of the $C_H2$ domain is absent in heavy chains of IgG (Figure 1-8). The $C_H2$ domain was probably 'condensed' to form the structure similar to the IgG hinge region [172-174].

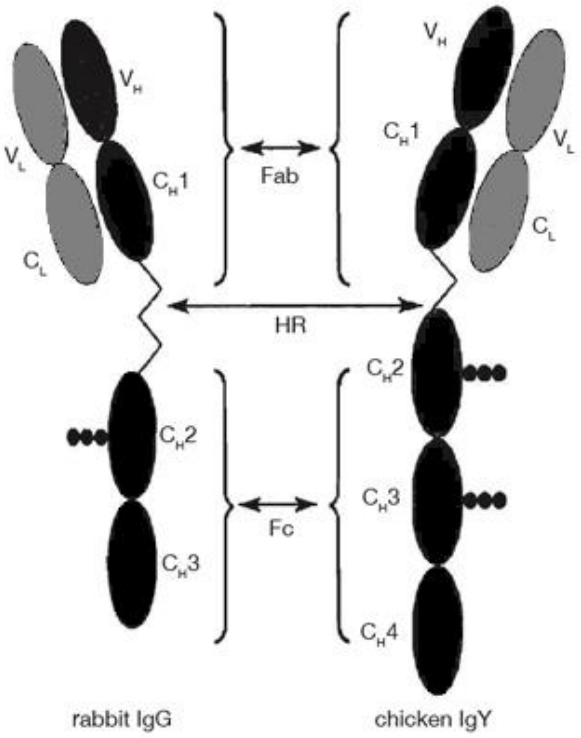



**Figure 1-8** Comparison of the molecular structure of chicken IgY and rabbit IgG [175].

V : variable domain of the light chain ($V_L$) and the heavy chain ($V_H$)

C: constant domain of the light chain ($C_L$) and the heavy chain ($C_H$)

HR: hinge region, the black points symbolize carbohydrate chains

Fab: Antigen binding site

Fc: Crystallisable (constant) region of the antibody molecule

In IgY, there are regions near the boundaries of the $C_H1$- $C_H2$ and $C_H2$- $C_H3$ domains that contain proline and glycine residues. These regions have the potential to confer limited flexibility on the molecule, in a manner analogous to the 'switch' regions in some mammalian Igs [176]. The content of β-sheet structure in C domains of IgY is lower than that of mammalian IgG; therefore, the conformation of IgY domains is more disordered in comparison to mammalian IgG. Intramolecular force as well as non-covalent interaction between H and L chains is an important factor to support the protein conformation. Intrachain disulfide linkage between the V region and C region of L-chain to stabilize the structure of the mammalian IgG L-chain is not present in the IgY L-chain and thus intramolecular forces of IgY are weaker than those of mammalian IgG [177].

The structural properties of IgY (e.g. molecular size, lower flexibility, conformation of domains, intramolecular bonding) are considered to influence the overall properties of IgY molecule. They also might be structural factors that have bearing on the lower molecular stability of IgY compared to mammalian IgG [177].

#### *1.8.1.2 Differences between IgY and IgG*

With regard to function, four important differences between IgY and IgG need to be considered. Firstly, IgY does not bind to protein A or G, an important feature of IgG that allows simple IgG isolation. However, there are several procedures for equally simple IgY isolation to compensate. Secondly, IgY does not bind the rheumatoid factor. IgG molecules often cause false positive results by interaction with RF in immunoassay. Thirdly, chicken egg-yolk immunoglobulin do not interfere with mammalian IgG; and finally, they do not activate mammalian complement and show no interaction with mammalian Fc receptors, which could mediate inflammatory response in the gastrointestinal tract, makes IgY antibodies very attractive for oral immunotherapy [163]. These differences bring great advantages to the application of IgY technology in many medical areas, such as xenotransplantation which is inhibition of xenograft rejection [178], diagnostics and antibiotic-alternative therapy.

Davalos-pantoja et al (2000) [179], showed that the IgY surface is more hydrophobic than that of IgG. This statement agrees with the structural differences between both proteins: the Fc portion, which is the most hydrophobic moiety in antibody molecules, is larger in the IgY molecule. IgY, is different from the IgG of mammals in molecular weight, isoelectric point, binding behavior with complements, etc. as summarized in (Table 1-9). IgY has a valency of ~2.0, reflecting large antigen-binding

sites, and binds an antigen strongly. However, IgY displays precipitating or agglutinating properties only at raised salt concentrations [180].

**Table 1-9** Comparison of properties of IgY and mammalian IgG

| Characteristics | Avian IgY | Mammalian IgG |
|---|---|---|
| Molecular weight | 180 kDa | 150 kDa |
| Isoelectric point | 5.7-7.6 | 6.4-9.0 |
| Heat stability | $\leq 65^0$C | $\leq 70^0$C |
| pH stability | pH 4-9 | pH 3-10 |
| Mammalian $F_c$ receptor binding activity | No | Yes |
| Protein A/G binding | No | Yes |
| Interference with mammalian IgG | No | Yes |
| Interference with rheumatoid factor | No | Yes |
| Activation of mammalian complement | No | Yes |

Sun et al (2001) [181] by studying the IgY against Rabies virus concluded that IgY has two structural differences when compared with mammalian IgG. First, the molecular weight of the heavy chain of IgY was larger than of mammalian IgG (65 kDa for IgY vs. 50 kDa for mammalian IgG) and the molecular weight of the light chain of IgY was smaller than that of mammalian IgG (19kDa for IgY vs. 23kDa for IgG). Second, pepsin digestion of mammalian IgG at pH 4.0 is reported to produce $F(ab)_2$ , but anti-rabies virus (aG) IgY was digested under the same condition, producing Fab. The two Fab arms may be so closely aligned that steric hindrance precludes the cross-linking of epitopes on two large antigens. The effect of conditions that permit those properties (e.g., salt or low pH) would be to release the Fab arms, providing functional independence of the binding sites [182].

The differences of Fc regions between IgY and mammalian IgG, which include number and nature of carbohydrate chains, flexibility of switch region and the number of constant regions, lead to the different interaction of IgY with molecules as an antigen in comparison to that of mammalian IgG (Table 1-8). The hinge region of IgY is much less

flexible compared to that of mammalian IgG [171]. It has also suggested that IgY is a more hydrophobic molecule than IgG [179].

Antibody diversity is achieved differently in chickens than in mammals [183, 184]. The IgY light chain locus consists of a single J-C unit to which the same V gene becomes rearranged in nearly all bursal cells. The chicken light chain repertoire thus appears to be of an extremely somatic type [183]. Consequently, the overall antibody diversity of IgY is lower than that of mammalian IgG, which is indicative of the restricted diversity of IgY specificities.

#### *1.8.1.3 Advantages over mammalian antibodies*

These unique biological attributes make IgY an effective natural food antimicrobial system as nutraceuticals and a potent biotechnology application as an immunotherapeutic agent. The feasibility of IgY use results in advantages with low cost and mass production, because large amounts of IgY should be administered to individuals for the neutralization of pathogens. Chickens are potent antibody producers that can serve as a successful substitution of mammals. (i) chicken housing is inexpensive; (ii) yolk/hen weight ratio is particularly good; (iii) eggs can be stored at 4°C for at least 1 year; (iv) large quantities of highly active IgY can be collected and produced on a daily basis for more than 2 years from the same animal at condition of regular interval immunizations and last but not least; (v) IgY are easily separated from IgM and IgA, produced in the egg white, due to the natural compartmentalization of eggs. Moreover chicken eggs are relatively low cost 'green source' of polyclonal antibodies.

Krief et al (2002) [185] have reported that antibodies harvested from hens (IgY) possess a recognition aptitude, for various conjugates, very closely related to antibodies harvested in a different organism (mice, IgG). All these antibodies proved to be highly specific to the whole structure of the hapten including the side chain. This is quite remarkable and also quite unusual.

#### *1.8.1.4 Affinity/specificity/avidity*

The antibody titres of concentrated IgY and the initial yolk-water soluble protein fraction are stable over a half year period; a long duration of a high immune response in the hen against bovine rotavirus antigen was noted [186].

Another advantage is that very low quantities of antigen are required to obtain high and long-lasting IgY titres in the yolk from immunized hens [187, 188].

Davalos-pantojai et al (2000) [179] in an experiment to develop a diagnostic test based on IgY–latex agglutination concluded that latex particles sensitized by IgY molecules do not aggregate by means of the rheumatoid factor. This unspecific agglutination takes place if particles are coated by mammalian antibodies. Also colloidal dispersions of IgY-covered latex particles were more stable (at pH 8) than those sensitized by IgG. These advantages make IgY an interesting antibody to be employed extensively in the development of particle enhanced immunoassays.

#### *1.8.1.5 Economy*

Chickens offer several advantages in terms of economical and practical aspects. Maintenance of a large flock of laying hens is economical because of large-scale feeding of hens [189]. Vaccination of laying hens to control various avian infectious diseases is already a common practice, which is well accepted by both poultry farmers and the consuming public [170]. The number of chickens used for antibody production can be reduced due to their capabilities of producing larger amounts of antibodies as compared to the number and capability of mammals [190] (Table 1-10).

**Table 1-10** Productivity of IgY and IgG

| Immunized animal | Rabbit | Hen |
|---|---|---|
| Source of antibody | Blood serum | Egg yolk |
| Kind of antibody | Polyclonal | Polyclonal |
| Quantity of antibody | 1,400 mg/rabbit | 40,000 mg/hen |
| (Quantity of specific antibody) | | |
| Anti-HRV (MO) antibody | $5.6 \times 10^6$ NT | $600 \times 10^6$NT |
| Anti-HRV (Wa) antibody | $37.8 \times 10^6$ NT | $520 \times 10^6$ NT |
| Anti-Mouse IgG antibody | 700 mg | 11,200 mg |
| Anti-Insulin antibody | 0 mg | 2,000 mg |

Abbreviations: HRV: Human rotavirus; NT: Neutralization titer

The maintenance of a large flock of layers is more economical than keeping large numbers of cows for antibody production. This makes IgY more feasible for large scale-production [189]. Another advantage of IgY over colostrums antibody is that a high

neutralizing antibody titre remains stable for a long period as compared to that of colostrums which decreases a few days after initial harvest [191].

#### *1.8.1.6 Immunological property of IgY*

The structural characteristics of IgY is relevant to the immunological properties. The differences of Fc regions between IgY and mammalian IgG, which include number and nature of carbohydrate chains, flexibility of switch region and the number of constant regions, lead to the different interaction of IgY with molecules as an antigen in comparison to that of mammalian IgG.

Most biological effectors functions of Igs are activated by the Fc region. Such a role of Fc region of IgY is very poor in secondary effectors capabilities in opsonization and complement fixation, although IgY is capable of binding to antigen strongly. IgY does not bind to protein A or G which are present on the surface membrane of *Staphylococci* and *streptococci* other than mammalian IgG. The role of Fc region of IgY still remains unclear, but it is sure that chicken antibodies do not activate the mammalian complement system and show no interaction with mammalian Fc receptors. Likewise, the reaction of antibody to cellular components may mediate inflammatory responses in the gastrointestinal tract. The vulnerable point makes IgY antibodies very attractive for oral immunotherapy [163].

Another property in Fc region of IgY is no interaction with rheumatoid factor which causes disease associated with rheumatoid arthritis resulting from inflammatory responses by reacting with the Fc region of mammalian IgG. Due to the phylogenetic difference, IgY antibodies do not cross react with mammalian IgG and show no interference with human anti-mouse antibodies. These differences bring great advantages to the application of IgY technology in many medical areas, such as xenotransplantation which is inhibition of xenograft rejection [178], diagnostics, prophylaxis of pathogens and antibiotic-alternative therapy.

#### *1.8.1.7 Animal welfare friendly non-invasive method*

Egg yolk can be separated automatically by using a machine, which enables IgY production to be largely scaled-up. Egg yolk contains only IgY while mammalian blood contains IgG together with other Ig classes [170]. Therefore, the isolation of IgY from egg yolk can be more simply carried out than that of mammalian IgG. These, combined with all the above advantages, characterize IgY production as less expensive and more convenient [192].

Polyclonal antibody production in mammals involves three procedures: immunizing with antigens, blood sampling, and purification of antibodies. In chickens, egg yolk serves as an antibody source, replacing blood sampling. The collection of egg yolk is less labor intensive and more hygienic than blood sampling [170]. In addition, chickens are not distressed by the non-invasive sampling method, which is compatible with modern animal protection regulations [193].

To eliminate invasive stress that results from antigen injection in hens, oral administration could be a good alternative. Hedlund, and Hau (2001) [194], have evaluated the oral administration of human immunoglobulin G with different adjuvants including Poly(lactide-co-glycolide) (PLG) microspheres, Cholera toxin B-subunit (CTB), CTB conjugated with glutaraldehyde, Dimethyl dioctadecyl ammonium bromide (DDA), and Softigen(R), pegylated C8/C10 mono/di glyceride and saline as positive control. High titres of immunospecific IgY antibodies against human IgG were recorded in the eggs from the chickens immunized orally, with the antigen combined with glutaraldehyde conjugated CTB and in the chickens immunized with the antigen combined with Softigen.

#### *1.8.1.8 Phylogenetic distance*

Another advantage is the enhanced immunogeneity conserved mammalian proteins exhibit in birds due to their phylogenetic distance [195]. This makes production of antibodies against conserved mammalian proteins usually more successful in chicken than in other mammals. Chickens display strong and stable immune responses for a long laying period, which is indicative of long-term use of chickens for antibody production [186]. Chickens can also produce more specific antibodies against mammalian antigens because of the phylogenetic distance between avian and mammals [196]. That is, IgY as a polyclonal antibody can frequently recognize more sites on a mammalian antigen as foreign. The restricted diversity of IgY, combined with this attribute, can make it more possible to produce specific antibodies against antigens that are well-conserved and rarely immunogenic in mammals [187]. There have been numerous studies that succeeded in producing IgY specific to low immunogenic antigens against mammals (Table 1-11).

**Table 1-11** Production of IgY specific to less immunogenic antigen against mammals.

| Antigen | Reference |
|---|---|
| Proliferating cell nuclear antigen of calf thymus | 195 |
| Heat-shock protein (Hsp 70) | 197 |
| Human insulin | 198 |
| Rat glutathione peroxide | 199 |
| Von Willebrand factor | 200 |
| Platelet glycoprotein IIb-IIIa | 200 |
| Parathyroid hormone related protein | 201 |
| Mouse erythroprotein receptor | 202 |

One study showed that the largely phylogenetic difference between avian host animals and the antigens of mammalian sources increases the immune response; thus, avian IgY recognized more epitopes than the corresponding mammalian IgG and was relatively more reactive to the highly conserved proteins [195]. It has been proposed that more widespread use of avian antibodies be promoted [190].

#### *1.8.1.9 High yield*

A laying hen produces approximately five to six eggs per week with a yolk volume of approximately 15 mL, the antibody concentration of which is higher than in the serum. Therefore, in 1 week a hen produces egg antibodies equivalent to 90 to 100 mL of serum or 180 to 200 mL of whole blood. This could be compared to an immunized rabbit, which yields approximately 20 mL whole blood/week when repeatedly bled and thus comparatively limited amounts of antibody can be produced by each rabbit. Only large mammals such as cows or horses can produce more antibodies than a laying hen. The blood collection procedure is time consuming and painful for the rabbit. Furthermore, the cost of feeding and handling is considerably lower for a hen than for a rabbit.

## 1.9 Production of IgY

### 1.9.1 Yield of IgY

The yield of IgY antibodies can be compared to that of IgG antibodies obtained by conventional immunization methods; 200 mg of IgG can be obtained monthly, with approximately 5% constituting the specific antibody. In the case of chicken,

approximately 3000 mg of IgY can be harvested each month, and between 5 and 10% is the specific IgY [203]. Compared to antibody production in rabbits, the IgY offers several advantages: i) no bleeding, only egg collection is required upon immunization; ii) IgY isolation is fast and simple; and iii) very low quantities of antigen are required to obtain high and long-lasting IgY titres in the yolk from immunized hens [167].

**Table 1-12** IgY isolation methods

| Method | Reference |
|---|---|
| Water dilution technique | 204 |
| Organic solvents extraction | |
| Choloroform | 192, 205 |
| Isopropanol and acetone | 206 |
| Other chemical compounds: | |
| Caprylic acid | 207 |
| Guanic acid | 208 |
| Hydroxypropylmethylcellulose phthalate | 209 |
| Phosphotungstic acid | 210 |
| Magnesium chloride | 210 |
| Ammonium sulphate | 211 |
| Sodium sulphate | 212 |
| Sodium dextran sulfate | 196 |
| Natural gums | |
| Xanthan and carrageenan | 213 |
| Alginates | 214 |
| Synthetic gum | |
| Hydroxypropylmethylcellulose phthalate | 213 |
| Ultracentrifugation | 215 |
| Polyacryl acid resins | 189 |
| Polyethylene glycol precipitation | 192, 216 |
| Anionic polysaccharide precipitation | 217 |
| Synthetic ligand (epichlorohydrin and cyanuric chloride) methods | 218 |

An antigen-specific IgG has been conventionally isolated from sera of animals, such as rabbits, which have been super immunized with an aimed antigen. IgY is also to

be isolated from the egg yolk laid by the hen super immunized previously. The preparation methods of IgY are shown in Table 1-12.

### 1.9.2 IgY purification

Lipids and proteins are the major constituents of egg yolk. The lipid fraction, including triglycerides, phospholipids, and cholesterol, constitutes approximately one-third of the yolk and mainly egg yolk solids. Proteins consist of 15-17% of the yolk, which can be separated by centrifugation into particles, 'the granules' and a clear fluid supernatant, 'the plasma' [219]. Granules are composed of 70% α- and β- lipovitellins, 16% phosvitin, and 12% low-density lipoproteins (LDL) [220]. Plasma is about 78% of the total yolk proteins and composed of a lipid-free globular protein, livetin (α-, β-, and γ-) and low-density lipoproteins [221]. IgY is known as γ- livetin and exists in egg yolk plasma proteins together with two other water-soluble proteins, α- and β-livetin, and lipoprotein; therefore, separation of IgY or γ-livetin requires extraction of the water-soluble fraction (WSF) from yolk lipoprotein followed by purification from other livetins [192]. The WSF can be obtained by using the water dilution method based on the aggregation of yolk lipoproteins at low ionic strengths as reported by Jensenius et al (1981) [196]. Centrifugation or filtration is subsequently used to fractionate the WSF from water-insoluble components of egg yolk [204]. For extraction of the WSF from egg yolk with water, two factors are of critical importance: the pH and the extent of egg yolk dilution [204].

The large amount of lipid in egg yolk [219] presents problems in the purification of antibodies. Several methods have been described for purifying IgY based on the strategy of delipidation with organic solvents, such as chloroform [205] or precipitation with PEG-6000 [216] or dextran sulphate [196]. Avian antibodies were then concentrated using ammonium sulphate [211], sodium sulphate [212] or caprylic acid [222]. Bizhanov and Vyshniauskis (2000) [223] compared dextran blue, chloroform and PEG methods to extracting IgY from egg yolk showing IgY isolated by the use of chloroform contained more total protein than that isolated with the other two methods and this difference was statistically significant and the proportion of IgY in the protein isolated by PEG was greater than that in the samples obtained using dextran blue or chloroform.

Due to increasing demands for food use, IgY has become increasingly important in food processing, and therefore, there is a need for developing efficient and simple techniques for IgY production. Some methods, such as gel permeation chromatography

[224], addition of polyethylene glycol [211, 216] and dextran sulfate [196, 211], and the [206] combination of ethanol precipitation with DEAE-cellulose ion-exchange chromatography have been investigated. However, most of these methods suffer from major drawbacks, i.e., low recovery rates of IgY or the complexity of the procedures, and they appear impractical for routine use in food items. Thus, an efficient, economic, and rapid method for separation of IgY from yolk is needed. Recently, *ì*-carrageenan [213], alginate [225], and hydroxypropylmethylcellulose phthalate [226] have been used to recover IgY from adequately diluted yolks in a single step process, and those polysaccharides exhibit satisfactory yields of IgY [217].Recently there are many efforts to purify IgY by using chromatography methods. The best delipidation and extraction method included yolk dilution with water under acidic conditions and $(NH_4)_2SO_4$ 60% precipitation from which 43 mg protein/yolk were recovered. Among the chromatographic methods, thiophilic chromatography permitted the recovery of a substantial quantity of pure IgY (10.4 mg IgY/yolk) [227]. The purification of IgY by lithium sulfate results in very pure IgY in high quantities (94% $\pm$ 5% of total egg yolk protein) [228].

A high efficiency ligand of IgY was found by synthetic ligand library synthesized by epichlorohydrin and cyanuric chloride methods, By one-step purification with this ligand, the purity of IgY could reach 92.1%, and the recovery of IgY could reach 78.2%, having a higher binding capacity of 74.8 mg IgY/mL and had no negative effects on immunoreactivity [218].

### 1.9.3 Egg yolk IgY powder processing

Nilsson (2007) [229], reported that IgY freeze-dried with sugar in this study is very stable for more than a year at RT and for at least three months in the absence of sugar. Lactose stabilized the IgY antibodies to a slightly lesser extent than sucrose and trehalose, but still the activity was higher than without the stabilizing agent, with the lowest concentration tested (0.012 M).

Jaradat et al (2000) [230], studied the effect of non-reducing sugars (sucrose, lactose and trehalose), complex carbohydrates (cyclodextrin and dextran) or egg yolk on the stability of purified chicken IgY was evaluated under different conditions. Regardless of the protectant that was used, about 20% of the activity of IgY was lost during freeze-drying. Trehalose was the best protectant followed by cyclodextrin and infant formula when IgY was stored for 6 or 14 weeks at different temperatures. IgY activity was completely lost after pepsin treatment in the presence of sugars or complex carbohydrates

while 34 and 40% of its activity was recovered when treated in the presence of egg yolk. IgY was fairly stable after trypsin treatment with the recovery of residual activity being between 75-100% depending on the protectant.

### 1.10 IgY stability

It has been reported by several workers that the activity of IgY is not adversely affected by pasteurization conditions [188]. The neutralization activities of both IgY and its Fab fragments were not reduced by heating at 65 °C for 15 min. The implication of this finding is that possible concerns with transmission of Salmonella or other bacterial contamination associated with egg products may not be a major concern. Consequently, pasteurization could be used to eliminate pathogenic contaminants, with the exception of heat-resistant spores, without adverse effect on antibody activity [231].

It was demonstrated that cooking eggs did not denature or functionally inactivate IgY. Yolken et al (1988) [232], showed that antibodies to Rotaviruses persisted in commercially pooled egg preparations that had been pasteurized. The activity of IgY was found not to be affected by pasteurization at 60 °C for 3.5min. This fact together with the higher productivity and the mass production at industrial scale strongly suggests the possibility for IgY as a practical reality to be applied by oral administration to prevent infectious disease [188].

Kyong et al (2002) [233], showed good stability at pH 5~7 after incubating 24 hr at each respective pH. IgY was inactivated irreversibly at pH below 4. IgY inactivation was irreversible. When incubating at each pH for 4 hr, IgY lost antibody activity at pH 4 or below. The antibody activity was significantly reduced (up to 50%) at pH below 3. IgY almost completely lost its activity by incubating for 4 hr at pH 2. The most noticeable loss in antibody activity was observed during the first 30 min. IgY still had partial activity after staying at room temperature for four months. When pH ranged from 5 to 9, IgY still had partial activity after staying in 37 °C for 3 hr. If pH was lower than 5 or higher than 9, it lost the whole activity in above condition [234].

#### 1.10.1 Heat

IgY has been thermally treated at various temperatures for different periods of time. The binding activity of IgY with antigen decreased with increasing temperature and heating time. The activity of IgY decreased by heating for 15 min at 70°C or higher [177] and IgY denatured seriously when thermally treated at temperatures higher than 75°C [235].

IgY is relatively stable to pressure as reported with no detectable inactivation of IgY by pressure up to 4,000 kg/cm$^2$ [236].

### 1.10.2 Cold

Freeze-drying and reconstitution of antibodies and biological materials can have a detrimental effect on protein structure and function [237]. Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive. However, proteins may suffer loss of activity as a result of conformational changes, aggregation or adsorption [238]. There have been some reports on the stability of IgY in regards to these methods. Chansarkar (1998) [239], however, showed that frozen or freeze-dried IgY resulted in some loss of antigen-binding activity and a significant drop in the solubility under the conditions of high salt and protein concentrations.

### 1.10.3 pH

The conformation stability of IgY is lower than that of mammalian IgG in any type of treatment such as acid, heat, and proteolytic enzyme, which suggests that the overall stability of IgY molecule is lower than that of mammalian IgG molecule [177, 188, 236]. The stability of IgY to acid and alkali has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3.0. Rapid decrease of the IgY activity at low pHs indicated conformational changes and damage in the Fab portion including the antigen-binding site. Under alkaline conditions, the activity of IgY did not change until the pH increased to 11; however, it was markedly diminished at pH 12 or higher [177].

### 1.10.4 Heat and pH combination

The heat and pH stability of IgY and rabbit IgG specific to human rotavirus were compared by measuring the antibody activity by ELISA [188]. IgY is more sensitive than that of IgG at temperatures higher than 70 °C. The study also showed that the temperature corresponding to the maximum of the denaturation endotherm ($T_{max}$) of IgY was 73.9 °C while that of IgG was 77.0 °C according to differential scanning calorimetry. The IgY activity under various acidic conditions (pH 2 and 3) was more sensitive than that of rabbit IgG. These observations were supported by Otani et al (1991) in studies comparing anti-$\alpha_{S1}$ casein IgY and rabbit IgG specific to mouse IgG. These differences in heat and

acid sensitivity between IgY and IgG may be attributed to the variations in their protein structures.

Shimizu et al (1988, 1992, 1993) [177, 236, 240] reported on the molecular stability of IgY antibodies in comparison with that of mammalian IgG antibodies and found that heat (> 75 °C) or acid (< pH 3.0) treatment reduced the antibody activity of IgY. The activity of IgY was decreased by incubating at pH 3.5 or lower and almost completely lost at pH 3.0. The activity of IgY was decreased by heating for 15 min at 65 °C or higher. Under alkaline conditions, changes in the activity of IgY did not occur until the pH increased to 11 but was markedly diminished by incubation at pH 12 or higher.

### 1.10.5 Proteolytic enzymes

IgY antibody is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. However, the IgY is more susceptible to pepsin, trypsin or chymotrypsin digestion than the rabbit IgG antibody. Otani et al (1991) showed the digestion profiles of egg yolk IgY and rabbit serum IgG antibodies specific to -$\alpha_{S1}$ casein by determination of a percentage decrease in undigested heavy chain over a time period by pepsin, trypsin or chymotrypsin digestion.

Susceptibility of IgY against *E.coli* [241], and human rotavirus [188] was also examined and the results were also demonstrated. SDS-PAGE profiles of IgY after incubation with pepsin revealed that IgY at pH 2.0 was hydrolyzed into small peptides, and no bands corresponding to IgY were detected in linear-gradient polyacrylamide gel electrophoresis [188]. On the contrary, incubation with pepsin at pH 4.0, heavy (H)-and light (L)-chain were clearly observed after 4 hr, although certain new bands appeared between H- and L-chains. Upon pepsin digestion, anti-rabies virus IgY is degraded into Fab, in contrast to mammalian IgG, which has been reported to be degraded into $F(ab')_2$ under the same conditions [181].

The behavior of IgY with trypsin and chymotrypsin was also examined. Changes in the neutralization titre of IgY were almost the same for the incubation with trypsin and with chymotrypsin. After 8 hr incubation, the activity of IgY in neutralization titre remained 39% and 41% for the mixtures with trypsin and chymotrypsin, respectively. On incubation with trypsin, the IgY H-chain disappeared, and several bands between H- and L- chain appeared on SDS-PAGE. In the case with chymotrypsin, both H- and L-chains of IgY remained unchanged, although a small band below H-chain was observed.

The high stability of IgY in the presence of trypsin indicates that once it passes the acidity of the stomach, it retains most of its activity and therefore, can combat or minimize the effect of intestinal pathogens [230].

IgY is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. The tryptic digestion of IgY retained its antigen-binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digests [240]. Hatta et al (1993) [188] also demonstrated the behavior of IgY against trypsin and chymotrypsin, showing that 39% and 41% of the activity by neutralization titre remained for the mixture with trypsin and chymotrypsin, respectively, after 8 hr of incubation. The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen–binding and cell-agglutinating activities. However, at pH 4.5 or below, both activities were lost [240]. The neutralization titre of IgY with pepsin under the different incubation time and pH was observed by Hatta et al (1993) [188]. The results confirmed the susceptibility of IgY to pepsin at low pH, which showed the loss of IgY activity at pH 2 and 91% and 63% of activity at pH 4 after 1 hr and 4 hr incubation time, respectively.

IgY activity is greatly reduced by gastric acid and pepsin, and therefore, even at high inclusion rates, egg-yolk antibodies was not found to be efficacious in post-weaning pigs as old as 3 to 4 weeks of age supplemented with chicken egg-yolk-antibody products in controlling *E. coli* induced diarrhea [242].

### 1.11 Protectants and IgY formulation

The IgY technology offers great future opportunities for designing prophylactic strategies against infectious GI diseases in humans and animals. However, there is still controversy regarding the stability of IgY through the GI tract. Finding an effective way to protect the antibodies from degradation in the GI tract would open the door for significant advances in IgY technology and nutraceutical applications. An alternative to improve or increase IgY stability in different condition is use of protectants. The effects of different protectants on IgY stability including non-reducing sugars (sucrose, lactose and trehalose), complex carbohydrates (cyclodextrin and dextran), infant formula or egg yolk on the stability of purified chicken IgY was evaluated under different conditions [230]. When food matrices such as egg yolk, egg white, and infant formula are used as

protectant, the extraction of IgY from egg yolk is unnecessary and we could directly incorporate them in feed, and subsequently its recovery increase and IgY production expenses decrease. Shimizu et al (1994) [236], studied the stability of IgY in the presence of high concentrations of sugar. A sucrose concentration of 30–50% was found to be highly effective in protecting the IgY from heat or acid denaturation and against high pressure as well [230]. Jaradat et al (2000) [230] showed that the activity of IgY after pepsin treatment was almost completely lost in the presence of the three sugars, dextran or cyclodextrin as well as when no protectant was used. In contrast, the recovery of IgY when incubated with pepsin in the presence of infant formula or egg yolk was 39 and 34%, respectively. They proposed that infant formula be fortified with specific IgY to protect infants against gastrointestinal pathogens. On the other hand, egg yolk is edible and therefore, can be used directly in certain foods without the need for the antibody purification.

Microencapsulation may been used as an effective method for protecting IgY from gastrointestinal inactivation [243, 244], but it will induce additional production cost. Optimum physical factors and encapsulation efficiency were established for preparation of homogeneous, spherical, and smooth chitosan-alginate microcapsules. [245].

Trehalose, cyclodextrin and infant formula were found to be the best protectants with over 80% of the original IgY activity recovered when stored at different temperatures (21, 37 and 50 °C) for 6 weeks and egg yolk, sucrose, lactose and dextran provided slightly lower protection. However, IgY lost almost half of its activity at 21 and 37 °C and all activity at 50 °C in the absence of any cryoprotectants [230].

Kyong et al (2002) [233] studied the IgY activity at pH 3 in the presence of polyols. Sorbitol and xylitol were used as polyols. Sorbitol suppressed IgY inactivation in a concentration-dependent manner. The IgY activity was significantly increased in the presence of 30% sorbitol or above. Fifty percent sorbitol showed almost complete suppression of acid-induced inactivation at pH 3; however, xylitol caused no increase in IgY activity. At larger intakes, polyols generally have a laxative effect and are usually used in limited amounts, or in combination with sucrose.

IgY showed almost the same activity as native IgY when sorbitol was replaced by sucrose. However, the acid induced inactivation of IgY was not enhanced by xylitol replacement with sucrose. They conclude that polyols are often used for stabilizing protein. Sorbitol and xylitol (among polyols) are widely-used sugar substitutes, due to their usefulness for diabetics, reduced cariogenecity, and inhibition of bone resorption [246, 247]. As indicated by several researchers [248], the preferential hydration of IgY may occur in

an aqueous sorbitol solution; therefore, hydrophobic interactions around tryptophan residues would be strengthened. These changes might protect the partial exposure of tryptophan residues, which would result in stabilization of the IgY structure. However, xylitol could not effectively stabilize hydrophobic interactions. Gekko et al (1999) [248] reported that the stabilizing effects of polyols would increase if the polyols chain length was increased. Xylitol shows less viscosity than sorbitol at the same concentration [249]. For these reasons, xylitol may not suppress the acid-induced inactivation of IgY. In conclusion, the IgY stability at pH 3 was considerably improved using high concentrations of sorbitol in solution. Sorbitol prevented the partial exposure of tryptophan residues, which resulted in the enhancement of IgY stability. However, xylitol could not suppress the acid-induced inactivation. These results suggest that sorbitol may be useful in stabilizing IgY at low pH. Therefore, IgY could potentially be applied in high acid food products and extensively used for protection against *H. pylori* infection.

## 1.12 Applications of IgY

IgY has distinguished application in the field of immunology. It has been reported in literature as therapeutic passive /active immunizations, diagnostics reagents and ligand of an immunoadsorbent. There have been numerous reports of IgY use to inhibit bacterial growth.

### 1.12.1 Passive immunization

An important application of IgY is for passive immunization therapy in which the specific binding ability to the antigens (pathogens, venoms, etc.) serves to neutralize the biological activities of those antigens. Passive immunization seems to be one of the most valuable applications of antibodies in which pathogen-specific IgG is administered to individuals to result in prevention of infectious diseases. Passive immunization differs from active immunization (vaccination) in that the former employs an antibody obtained from other animals. The administration of this antibody specific to certain antigens (bacteria, virus, toxin, etc.) to individuals orally or systemically works to neutralize infectious activity or toxicity of the antigens. For practical application of passive immunization, an effective method of preparation of the antibody will be necessary, because large amounts of antibody may be required to administer the antibody for the passive immunization.

Passive immunization differs from active immunization (vaccination) in that the former employs an antibody obtained from other animals. The oral administration of

pathogen-specific antibodies is considered to be one of the most valuable applications of antibodies to result in prevention of infectious diseases. Such an important application requires antibodies to be made available in large quantities, at an acceptable cost and with high affinity for their targets. Thus, the laying hens may be alternative, as IgY from hen plasma is actively accumulated to egg yolk in daily basis and is present in high concentrations. The specific IgY preparations against enteric pathogens such as viruses, bacteria and parasites have been prepared on an industrial scale from eggs laid by hens immunized with selected pathogens and have previously shown to be effective as prophylaxis against infections.

#### *1.12.1.1 Human passive immunization.*

Rotaviruses are a major cause of diarrhea illness in human infants and young animals, including calves and piglets. Infections in adult humans and animals are also common. In a randomized, double-blind study, children with proven rotavirus diarrhea were treated with specific IgY for human rotavirus strains, indicating the effect of IgY in the treatment of rotavirus-induced diarrhea in children [250]. Improved therapeutic effect can be achieved by increasing the dose in the clinical setting [251].

*Streptococcus mutans* is a major etiologic agent of human dental caries. It has been shown in an experimental animal model that oral passive immunization using IgY specific to *S. mutans* was effective in protecting dental caries. The oral administration of IgY specific to *S. mutans* glucan binding protein B resulted in a statistically significant reduction in caries development in an experimental rat model [252]. Furthermore, the effects of a mouth rinse containing IgY to *S. mutans* by the treatment of specific IgY powder prevented the establishment of this bacterium in dental plaque of humans *in vitro* and *in vivo*. The results supported the effectiveness of IgY with specificity to *S. mutans* grown in the presence of sucrose as an efficient method to control the colonization of *S. mutans* in the oral cavity of humans [170]. Therefore, these studies provide evidence for the potential advantages of using IgY with specificity to *S. mutans* for controlling plaque levels and subsequent oral health problems associated with plaque accumulation.

Smith et al (2001) [252] in an experiment with mice showed that passive administration of egg yolk antibody to *Streptococcus mutans* Glucan Binding Protein B (GBP-B) can inhibit the accumulation of *S. mutans* in the dental biofilm and can provide a level of protection from dental caries caused by these organisms. They also showed a long-term protective effect after limited (9- or 24-day) exposure to IgY antibody against

*S. mutans* GBP-B, another component putatively involved in the molecular pathogenesis of dental caries. They conclude that these studies support the use of oral passive immunization to interfere with *S. mutans* accumulation and subsequent development of dental caries. Furthermore, *S. mutans* GBP-B is identified as a new target, thus increasing the number of pathways by which passive immunization can achieve a protective effect.
Shin et al (2003) [253] have identified the immunodominant *Helicobacter pylori* proteins with reactivity to *H. pylori*-specific egg-yolk immunoglobulin. They identified that the urease α- and β-subunits and HSP60 were as immunodominant proteins with reactivity to IgY-Hp. Also, they found other immunodominant proteins, probable peroxiredoxin and probable thiol peroxidase. They concluded that these five immunodominant proteins strongly reactive to IgY-Hp and the immunization of hens with selective antigens with high immunocompetence will enable the production of highly specific IgY against *H. pylori*. In addition, the proteins identified may serve as potential targets for antimicrobial agents for the prevention of *H. pylori* infection.

Kollberg et al (2003) [254] studies by use of specific egg-yolk antibodies (IgY) against *Pseudomonas aeruginosa* (PA) in cystic fibrosis patient showed that oral passive immunotherapy with IgY Prolongs the time from first to next colonization with PA, delays chronic infection with PA in CF patients and reduces the intermittent colonization rate and consequently decreases the need for antibiotic treatments. Of course, they used a small number of patients so the statistical significance must be interpreted with caution.

#### *1.12.1.2 Animal passive immunization*

IgY can be used to prevent and reduce bacterial contamination when being used as an oral passive immunization particularly in poultry [255]. This binding may lead to the impairment of the biological functions of those components that play an essential role in the bacterial growth [167] and attachment to the intestinal cells [256]. In this way, the antibodies protect against adhesion of bacteria at the intestinal cells [257-259] and prevent the invasion into epithelial cells [257]. The powder preparation of egg yolk IgY, specific to *Salmonella enteritidis* and *Salmonella typhimurium* outer membrane proteins blocked the Salmonella sp. adhesion in a concentration-dependent manner. The antibodies led to decrease in transepithelial electrical resistance of the infected Caco-2 cell monolayers and inhibited the bacterial growth, markedly during the late exponential phase in the *in vitro* system [255]. IgY specific against 14 kDa fimbriae of *S. enteritidis* was orally administered to mice infected with the corresponding bacteria. The result showed decrease of bacterial

virulence. The passive immunization with IgY specific for *S. typhimurium* and *S. dublin* could prevent fatal Salmonellosis in calves [260]. This indicates that IgY against the microbial pathogen may be used for the feed additives, which provide prophylactic and therapeutic function.

Ikemori et al. (1997) [261]showed that significant protection against bovine corona virus (BCV) using specific IgY was achieved in calves treated with high titres of the IgY and colostrums antibodies against the challenge strain. Survival against BCV-induced mortality was 100% when 1:2,560 antibody titres were used for treatment of calves with the egg yolk powder. The calves treated with the IgY did not have severe diarrhea and had higher weight gains. About 8 g of egg yolk powder were obtained from one chicken egg and only one egg yolk was needed to protect one calf from diarrhea over a 7-day course of treatment. This result collaborates an earlier protection trial using IgY specific for ETEC in ETEC-infected calves [226].

The effect of IgY against ETEC on the reduction of intestinal colonization was dose-dependent. A daily intake of 5.5 g of egg powder was protective, whereas 3.5 g was insufficient for homologous protection.

In the study of oral administration of IgY, pigs did not survive from infection despite the high serum chicken IgG concentration detected. It can be assumed that most of the antibodies might have been transferred to the blood, with only a few antibodies remaining in the intestinal tract to fight the infection. To achieve protective antibody activity in the intestine, the supply of antibodies in the area must be sufficient to bind with the infective antigens. Good efficacy was achieved after daily treatment of susceptible pigs by oral administration of antibodies [209]. Actively acquired local anti-fimbrial immunity has been shown to be cross-protective against the other antigenic variant of fimbriae F18 [262], such cross-protection with a high dose of anti-fimbrial IgY [263].

Shin et al (2002) [264] in an study to evaluate the potential of IgY to control of swine respiratory disease in a mouse model showed that IgY could be a potential immunoprophylactic candidates against *P. multocida* 3A and 4D, *B. bronchiseptica* and *A. pleuropneumoniae* serotype 2 in swine.

*P. chinensis* infected shrimp was used in white spot syndrome virus neutralization assays, by treating with 0.01 mg/10 μL of IgY against TrVP28:19, exhibit a survival rate of 50% at day 15 post-challenge with white spot syndrome virus while those treated with 0.1 and 0.5 mg/μL exhibited survival rates of 85% and 83%, respectively [265]. Lu, Y. J. et al. (2009) [266] showed that Passive immunization of specific IgY antibodies

against White spot syndrome virus (a major cause of mortality in shrimp lacking a true adaptive immune response) through intramuscular injection, oral administration, and immersion have was an effective protection on crayfish by reduced mortality rate of 20%, 53.3-67.7%, 46.7%, respective, as compared to the 100% mortality of positive control group.

Therefore, anti-bacterial properties of IgY demonstrated a protective role in foods or feeds, preventing contamination by pathogenic bacteria and consequently reducing the risk of pathogens-causing infection in humans or animals. To date there have been efforts to develop effective means for controlling or preventing foodborne diseases, which are mainly caused by pathogenic bacteria contaminating foods. IgY, as a food-based deterrent, may serve as a novel protective measure characterized by being economical, efficacious and safe.

### 1.12.2 Active immunization

The protective efficacy of post-immunization IgY, tested by injecting groups of BALB/c mice with a lethal dose of *C albicans* and a variable regimen of pre- or post-immunization IgY, indicated that: 1) all the mice that received either pre- or post-immunization IgY survived at 7 days post-challenge compared with 20% of those in the untreated controls; 2) mean candidal colony forming units (CFU/mg) of kidney tissue were $25 \times 10^8 \pm 5.4 \times 10^8$ in untreated mice and $10 \times 10^3 \pm 1.4 \times 10^3$ in mice treated with pre-immunization IgY; no *C albicans* colonies could be detected in all challenged mice that were treated with post-immunization IgY; 3) multiple abscesses were observed in kidneys obtained from mice treated with pre-immunization IgY. No abscesses were seen in kidneys obtained from mice injected with post-immunization IgY [267].

Adachi et al (2008) showed that the antibodies produced by the H5-immunized ostrich strongly inhibited cytopathic effects in MDCK cells and prevented the death of an embryonated chick after a viral inoculation, indicating strong neutralization activity against H5N1 infections.

The water-soluble fraction (WSF) of the egg yolks containing anti-Vibrio anguillarum IgY via intraperitoneal (IP) route, oral intubation, or feeding of rainbow trout, showed that IP-injected anti-Vibrio IgY was transferred into the trout system in highest levels to confer protection against vibriosis in an experimental challenge [268].

The antibodies from duck eggs efficiently protected mice from envenomations with cobra venom and krait venom in a mouse model, was effective in neutralizing

lethality in mice injected at 4xLD50 of venoms [269]. The antibodies raised in chicken could effectively neutralize the pharmacological effects induced by venoms and chickens for Russell's viper venom which has an effective dose ($ED_{50}$) of 0.96 mg / $2LD_{50}$ / 18 g mice and 1.28 mg / $2LD_{50}$ / 18 g mice for Saw-scaled viper venom and 1 mL of specific anti-venom was effective in neutralizing 0.110 mg of Russell's viper and 0.137 mg of Saw-scaled viper venoms respectively ($PD_{50}$) [270]. Brazilian IgY-Bothrops antivenom was proved to be capable of neutralizing lethal toxic activity of the pool of Bothrops sp venoms from five species, with an effective dose (ED50) of 365 μL / 2 LD50 and, 1.0 mL of IgY antivenom could neutralize 0.154 mg of venom [271].

### 1.12.3 Growth inhibition of bacteria *in vitro*

Characteristics of pathogenic bacteria include the initiation of the infectious process and mechanisms such as transmissibility, adherence to host cells, invasion of host cells and tissues, ability to evade the host's immune system and symptoms of disease. Once pathogenic bacteria reside in the body, they must attach or adhere to host cells, usually epithelial cells. After the bacteria have established a primary site of infection, they multiply and spread directly through tissues or via the lymphatic system to the bloodstream. Outbreaks of *E. coli O157:H7* have been attributed to food-borne contamination in countries around the world. To cause disease, *E. coli* must first adhere to host intestinal epithelium, followed by bacterial colonization. Antibiotic therapy is not recommended early in the infectious process, because of disruption of the bacteria in the gut releasing Shiga-like toxins. Antibodies can, in principal, bind the bacterial surface and then inhibit the bacterial adhesion to host intestinal epithelium. The complex of antibody and bacteria can be eliminated as a waste, so could antibodies replace antibiotics? The effectiveness of IgY in suppressing the activity of *E. coli O157:H7* has been demonstrated by our study [203]. The specific binding activity of IgY leading to the inhibition of bacterial growth was explored by using immunoelectron microscopy by a negative staining and ultrathin sectioning method. These studies could visualize the interaction of bacteria with IgY in more detail than the ELISA technique and growth inhibition assay. The observation of immunogold particles labeling bacteria, furthermore, revealed the distribution of gold particles on and structural alterations of the bacterial surface. A similar result was also obtained from the growth inhibition study of IgY against salmonella [272].

Salmonellosis is known to be a non-host restricted serotype causing diseases syndrome like gastroenteritis and systemic infections in human and animal species. The

immune response of chickens against lipopolysaccharide [273], 14 kDa fimbriae [274] and whole cell [272] of salmonellae has been investigated on the possible control of Salmonellosis. Lee et al (2002) [272] studied the chicken egg yolk antibody (IgY) against *Salmonella enteritidis* and *Salmonella typhimurium* and conclude that, the binding activity of chicken egg yolk antibody (IgY) against these organisms resulted in inhibiting bacterial growth *in vitro*. Microscopic observation revealed the structural alterations of *Salmonella* surface bound by IgY. *In vitro* IgY studies may suggest that IgY binds to *Salmonella* surface molecules, which are critical for bacterial growth, and leads to the functional impairment of those components.

Recent investigations, concerning the passive immunity by oral administration of IgY, have increased interest in IgY. IgY decreased the incidence of diarrhea, due to the rotavirus or *E.coli* [188, 232]. IgY also conferred protection against dental caries that are formed by *Streptococcus mutans* [170, 252]. Yang et al (1997) [275] showed that IgY could specifically recognize digestive system cancer, which indicates its potential use in cancer prevention. Such protective effects have increased interest in applying IgY to pharmaceutical and food products [233].

### 1.12.4 Diagnostic reagent

IgY has been used as an immunological reagent. Fertel et al (1981) [276] demonstrated the application of IgY in determining prostaglandin in serum using radioimmunoassay. Altscuch et al (1984) [277] reported that IgY against human antibody (IgG and IgM) was applicable to determining their concentration in biological fluid by the method of rocket-immunoeletrophoresis.

Since ELISA system was developed, IgY antibody was used as one of capture or detecting antibodies. Gardner and Kaye (1982) [278] prepared IgY specific to rotavirus, adenovirus, and influenza virus, demonstrated immunological detection of these viruses using the IgY as the first antibody, and fluorescein isothiocyanate (FITC)-conjugated rabbit IgG specific to IgY as the second antibody.

An immunological advantage of IgY as an immunological tool over using rabbit IgG is the sensitivity of hens to antigens originated from mammals. A number of proteins exist whose amino acid sequence are well preserved among mammals, and many of these proteins have no or little antigenicity toward mammals. Therefore, for preparing antibodies against those proteinous antigens, the hen is highly promising as an alternative animal, because of the immunological distance of hen from mammals. In fact, Carroll and

Stollar (1983) [279] succeeded in preparing IgY against RNA polymerase II which has not generated its specific antibody in mammals. Many researchers have succeeded in producing IgY specific to less immunogenic antigen against mammals. Tini et al (2002) [280] raised a polyclonal IgY antibody against the α-subunit of hypoxia inducible factor-1 (HIF-1α), a vital master regulator of oxygen homeostasis. Affinity-purified anti HIF-1a IgY antibodies allowed detection of HIF-1α protein in electrophoretic mobility shift assays (EMSAs), immunoblots, immunoprecipitation and immunofluorescence experiments using hypoxic cells from different mammalian cell lines. These results support the versatile application of IgY. High-sensitivity C-reactive protein (hs-CRP) is considered one of the most promising markers for cardiac risk assessment [281]. Many researchers have also demonstrated the ELISA application of IgY for determination of various important, but very minor biological substances, such as 1,25-dihydroxyvitamin D [282], hematoside (NeuGc) [283], human transferrin [205], human dimeric IgA [284], and high-molecular weight mucin-like glycoprotein-A(HAGP-A) [285].

Another use of IgY which is not limited to ELISA is often used in the histochemical assay. Antibodies have proved useful in both serological and immunohistochemical detection of cytosolic thymidine kinase (TK1) in patients with all types with of cancerous diseases [286]. Wu et al (2003) developed the anti- TK1 IgY antibody raised against a synthetic 31-amino acid peptide from the C-terminal of human HeLa TK1 enzyme and showed that this antibody provides a sensitive and beneficial probe of TK1 in both serological and immunohistochemical detection for an early prognostic marker and for monitoring cancer patients undergoing treatment. Specific IgY against different proteins or antigenic agents may be useful to study the functional or structural properties of these molecules.

Grass carp hemorrhage virus (GCHV), as a member of aquareovirus, is a pathogen causing hemorrhagic disease of grass carp, and was found to be the most pathogenic aquareovirus [287]. Qui et al (2001) [288] studied molecular characterization of GCHV by Western blotting using chicken anti-GCHV immunoglobulin (IgY).

To detect biological agents, an IgY based double antibody sandwich ELISA was developed. Santoro et al (2003) [289] reported that polyclonal anti-rabbit platelet factor 4 (PF4) IgY was a specific and sensitive probe that could be used for assaying PF4 in rabbit plasma samples. Polyclonal antibodies were raised and purified from chick egg yolk and rabbit serum. The cobra venom was sandwiched between immobilized affinity purified IgY and the rabbit IgG. The detection concentration of cobra venom was in the range of

0.1 to 300 ng [290]. Carbodiimide conjugation, flumequine was conjugated to cationized bovine serum albumin (cBSA-flumequine) and to cationized ovalbumin for the immunization of chicken, cOVA-flumequine used coating antigen in the immunoassay. In the indirect competitive assay, standard flumequine was incubated together with the anti-flumequine antibodies, giving the maximum residue level (50μg/kg) of flumequine detection in raw milk [291].The optimal conditions for competitive ELISA in determining BMALP (0.1-10 μg/mL) from ALPs in milk samples were using $10^3$ fold diluted crude IgY specific against BMALP as primary antibody and $10^3$-fold diluted goat anti-chicken IgG-ALP conjugate as the secondary antibody [292]. Anti-hemoglobin Bart's (Hb Bart's) IgY antibodies (Abs) for development into an enzyme-linked immunosorbent assay (ELISA) test for thalassemia diagnosis had strong reactivity in cases of pathologic thalassemic diseases and weak reactivity in cases of nonpathologic thalassemic diseases [293]. Two haptens synthesized preserving the major structural features of PNMCs, by a novel synthetic pathway by coupling the first hapten with bovine serum albumin and the second with thyroglobulin, from porcine thyroid glands, produced IgYs exhibiting a high binding capacity to carbaryl, trimethacarb, metolcarb, aminocarb, and promecarb [294]. IgY based Elisa system showed quantitative results to detect markers in foods. The developed immunoassay can be used as a semi-quantitative tool to confirm the presence or absence of a group of important allergic proteins in foods such as indirect immunoassay for the quantification of peanut proteins that could be detected in foods at least down to a 1 ppm level [295]. The detection of hidden hazelnut protein, using purified antibody extract and a coating antigen concentration of 10 μg/ mL, giving minimum detection limit of 10 μg/L, with an $IC_{50}$ of 618 μg/L. The cross reactivity testing shows a high specificity for hazelnut proteins and various foods and food additives were found to be non-reactive except beans, sunflower seed or poppy seed [296]. The indirect competitive ELISA showed that the detection limit of artificially synthesized antigens citrinin was 10 ng/mL, with a good linearity ranging 20-640 ng/mL [297]. Strip-based immunochromatographic assay was developed for rapid detection of morphine in urine samples by IgY type antibody against morphine, generated by immunizing chickens with well-characterized monoacetyl morphine-protein conjugate, showing the average $IC_{50}$ values of morphine as low as 9.45 ng/mL, the detection range of 1-1000 ng/mL and the lowest detection limit 2.5 ng/mL under optimal conditions of analysis [298].

We have also developed highly sensitive quantitative [299] and qualitative [300] detection systems for gliadin in food.

### 1.12.5 Ligand of an immunoadsorbent

Immuno-affinity chromatography has been applied as a useful method for purification of proteins (antigens). Rabbit IgG has been conventionally used as a ligand to be immobilized to the absorbent, such as cellulose or agarose. However, several disadvantages of this affinity chromatography have been pointed out when rabbit IgG was used as a ligand, because acidic pH values less than pH 2 are necessary for dissociation of the protein immunological bound to the rabbit IgG on the immunoadsorbent. Therefore, the dissociated protein is often denatured depending on its nature. Moreover, production of rabbit IgG in large amount is generally expensive. The immuno-affinity chromatography using rabbit IgG as a ligand has thus been applied for isolation of only certain proteins.

It was demonstrated that IgY is an effective alternative antibody as a ligand for an immunoadsorbent. In the experiment, IgY and rabbit IgG specific to mouse IgG were immobilized on Sepharose 4B, respectively, in order to compare its dissociation efficiency for the purification of mouse IgG. Mouse serum was applied on immunoadsorbents, and the adsorbent was eluted with the buffer solution of pH 4.0 and 2.0 stepwise. The mouse IgG dissociated at pH 4.0 was only half of that applied, and the remaining IgG was eluted with pH 2.0 buffer solution in an immunoadsorbent using rabbit IgG as a ligand. On the other hand, 97% of the mouse IgG was dissociated even at pH 4.0 on the immunoadsorbent using IgY as a ligand [170].

Lemamay et al (1999) [301] obtained high affinity polyclonal antibodies raised against purified human mannose-6-phosphate/insulinlike growth factor-II receptor (M6P/IGFII-R) prepared from hen's egg yolks. These antibodies allow M6P/IGFII-R quantification in frozen tissue sections, [301], and in formalin-fixed paraffin sections [302].

Therefore, for the sakes of cost and efficiency, the use of a polyclonal antibody such as IgY appeared to be practical in immunoaffinity chromatography to purify and/or isolate limited components with bioactivity in foodstuffs.

## 1.13 Rationale

Recent findings estimate about 2 million people in North America have CD. CD is an autoimmune disorder that occurs when the body reacts abnormally to gluten, a protein found in wheat, rye, barley, and in some patients, oats.

In gluten-sensitive CD patients, gluten peptides (for example, gliadin a glutamine- and proline-rich peptide from dietary gluten in wheat) readily enter intestinal cells,

activate host T cell receptors, stimulate cytokine release/activity and trigger an inflammatory reaction. Consuming gluten causes an immunological (allergic) reaction within the inner lining of the small intestine, which damages the tissues and results in impaired ability to absorb nutrients from foods. The inflammation and inability to absorb nutrients creates wide-ranging problems in many systems of the body including gastrointestinal problems, dermatitis herpetiformis, anemia and osteoporosis. There is no cure for CD. The only treatment is a strict gluten-free diet that must be followed for life.

### 1.14 Hypothesis

The expected function of gluten-binding egg yolk antibodies (IgY) as "Oral Passive Antibody Therapy" is to neutralize these gluten peptides before they enter the small intestine, and so to preempt the cascade of inflammatory events.

I hypothesize that IgY against gluten peptides in food bind and prevent these toxic peptides from initiating and perpetuating the immunopathological events which cause the changes in serology, histology, as well as symptoms and complications which develop in persons with CD.

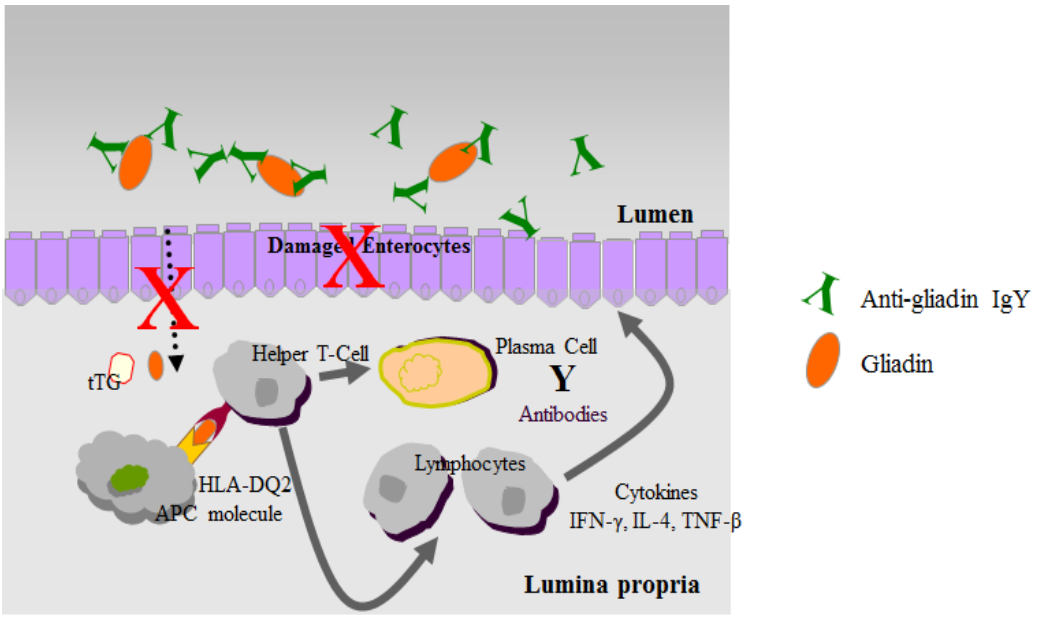



**Figure 1-9** Anti-gliadin IgY research hypothesis

## 1.15 Objectives

To produce and verify the efficacy of anti-gliadin IgY in neutralization of toxic gliadin protein both *in-vitro*, *in-vivo and ex-vivo*. The gliadin-antibody complex will prevent the absorption of free gliadin in the small intestine after ingestion, hence preventing the manifestation of CD caused due to ingestion of gliadin.

## 1.16 Specific objectives

### 1.16.1 Therapeutics approach

- To extract prolamins from four grains;
- To produce highly specific IgY against prolamins peptides causing celiac disease;
- To isolate the specific IgY from egg yolk using an economical method;
- To verify the efficacy of IgY in human cell culture system.
- To determine the dose of IgY for binding gliadin in complex meal conditions;
- To improve IgY survival by developing an encapsulation technology that will be resistant in the harsh environment in gastrointestinal tracts;
- To test *in vitro* and *in vivo* binding efficacy of IgY to gliadin;

### 1.16.2 Diagnostic approach

- To develop Biotinylated Double Sandwich ELISA for the quantification of gliadin in processed food sample.
- To develop Immunoswab and Immunustrip for qualitative determination of gliadin in food samples.

## 1.17 Study plan

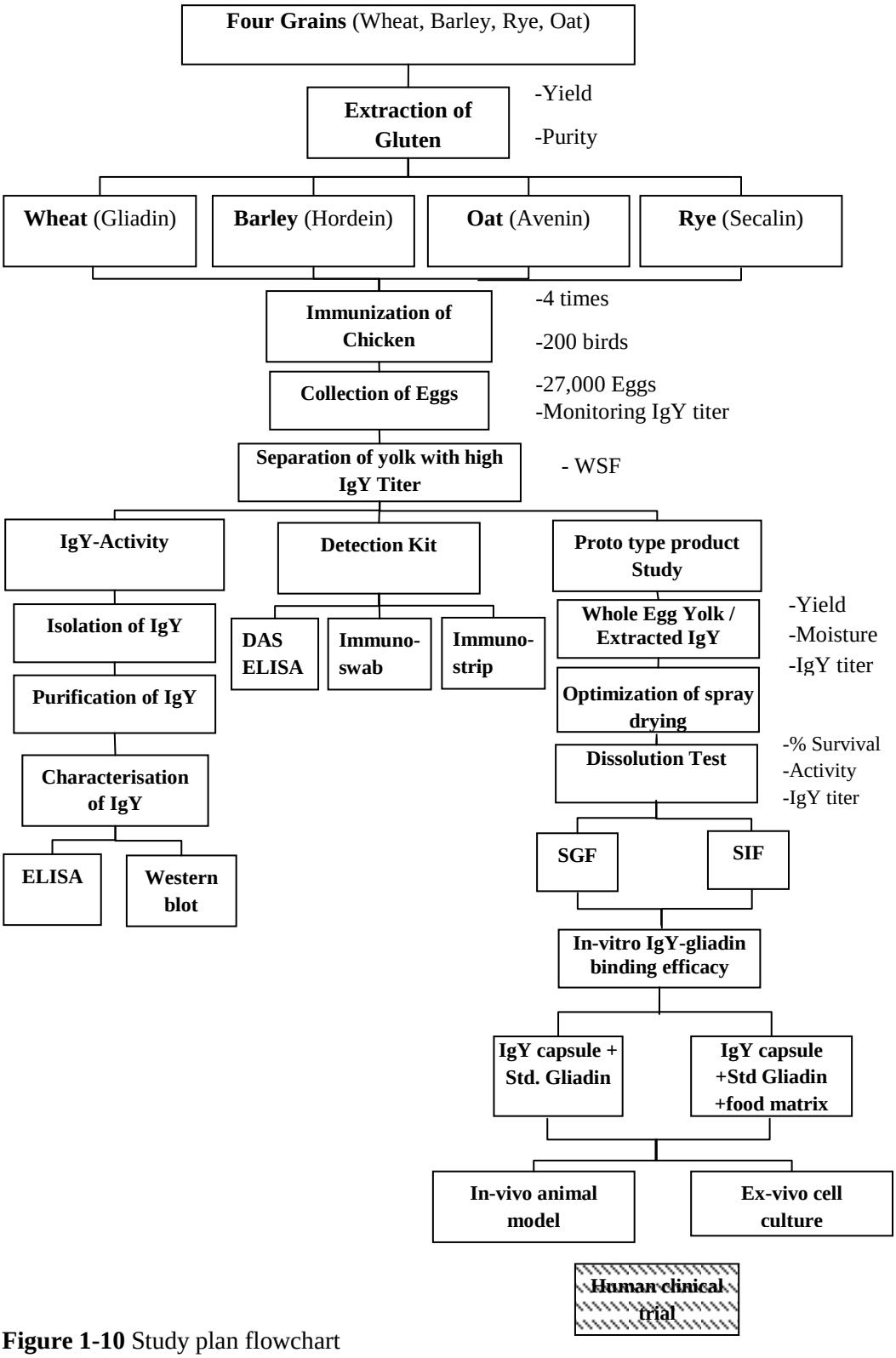



**Figure 1-10** Study plan flowchart

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# CHAPTER 2: Preparation, purification and characterization of IgY antibody for neutralizing dietary gluten

A part of this chapter is submitted to *Journal of Cellular Biochemistry*:
Gujral N, Lovlin R, Lobenberg R, and Sunwoo HH. Effect of specific anti-gliadin IgY antibody on epithelial intestinal integrity and inflammatory response induced by gliadin

## 2.1 Introduction

Celiac disease (CD) is a genetically predisposed food intolerance, induced by ingestion of gluten in grains (wheat, barley, rye and possibly oats). Gluten is a protein that composites prolamins and glutelins. Prolamins are known as gliadins in wheat, in barley as hordeins, in rye as secalins, and in oats as avenins are the main triggering factor in CD [1]. Due to high content of proline and glutamine, prolamins are resistant to gastrointestinal proteolytic enzymes. Upon oral administration, pepsin and trypsin resistant prolamins, named PT-gliadin, are penetrated from the intestinal lumen to the gut mucosa through transcellular and paracellular pathways [2], causing the damage of intestinal villous structure. Destruction of the villi hinders the absorption of nutrients from food into the bloodstream, leading to malabsorption syndrome [3], and symptoms such as nausea, diarrhea, weight loss and malnutrition problems [4].

As CD patients show various tolerant levels to gluten ingestion, a safe daily intake of gluten cannot be set. Every CD patient should avoid ingestion of food containing gluten for a life-time, because all of the disturbances revert when a strict gluten-free diet is established [5]. Gluten is, however, a common food ingredient in the human diet, presenting a big challenge for CD patients to avoid**.** The official limits described in the Codex Draft Revised Standard (2000) are 20 ppm for foodstuffs naturally gluten-free and 200 ppm for foodstuffs rendered gluten-free. As part of the Food Allergen Labeling and Consumer Protection Act of 2004, the US Food and Drug Administration issued a final rule in 2009, defining a gluten-free food for the food containing < 20 ppm gluten [6]. Persistent gluten intake by CD individuals has been shown to predispose symptomatic patients to cancer (especially small-bowel lymphoma) [7].

Due to the fact that antibodies can bind gliadin [8, 9], we use laying hens to produce IgY specific to gliadin. The use of laying hens as potent antibody producers has numerous advantages over mammalian polyclonal antibody production because of the high antibody content, the relative ease of using eggs versus serum, the low cost of animal maintenance, and large quantities of eggs availability. Laying hens usually lay about 250 eggs (approximately 4000 g of egg yolk) in a year. On the other hand, the serum collected from a rabbit is about 40 mL. One gram of egg yolk laid by the immunized hen contains about 10 mg of IgY whereas 1 mL of rabbit serum yields about 35 mg of IgG [10, 11]. The use of IgY may improve the gliadin binding efficacy because IgY is polyclonal, and therefore able to bind to multiple epitopes on various fractions of gliadin. In addition, there is an

economical advantage by replacing the expensive monoclonal antibody with inexpensive chicken egg yolk IgY.

We propose that gliadin specific IgY antibody may bind and prevent the gliadin from the intestinal permeation in the gastrointestinal tract.

The objectives of this study is to produce gliadin specific IgY egg yolk antibodies by immunizing chickens with PT-gliadin, to purify IgY antibodies by separating water soluble IgY from egg yolks without using harmful chemicals, and to characterize their binding activities and cross reactivity with other prolamins from barley, rye and oat, by using western blot and ELISA techniques

## 2.2 Materials and methods

### 2.2.1 Materials

Wheat (*Tricum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereal*), oat (*Avena sativa*), rice (*Oryza sativa*) and corn (*Zea mays*) flour were obtained from commercially available sources. Crude Sigma gliadin (G-3375), pepsin (P-7000; 800–2,500 units/mg protein), trypsin (P-8096, activity, 4x USP specifications), Freund's Incomplete Adjuvant, purified chicken IgG, rabbit anti-chicken IgG and rabbit anti-chicken IgG conjugated with horseradish peroxidase (HP) were purchased from Sigma (St. Louis, MO, USA). HYB-314 monoclonal antibody (mAb), chicken anti-mouse IgG conjugated with HP and BCA protein assay kit were purchased from Thermo Fisher Scientific Canada (Burlington, ON, Canada). Blue dextran was purchased from Pharmacia Biotech Inc., (Baie-d'Urfe, QC, Canada). 3,3′,5,5 Tetramethylbenzidine (TMB) liquid substrate for membrane and 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrates were purchased from KPL (Frederick, MD, USA). Sephacryl S-300 gel filtration column was obtained from GE Healthcare (Piscataway, NJ, USA). Bradford protein assay kit and Mini-Protein III electrophoresis kit were purchased from Bio-Rad Laboratory (Mississauga, ON, CA). Microtiter 96-wells plates were purchased from Costar Inc. (Cambridge, MA, USA). The ELISA Vmax kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA).

### 2.2.2 Prolamin extraction

Extraction of prolamins from four grains (wheat, barley, rye and oat) was modified from the method previously described [12]. In brief: 10 volumes of butanol were used to extract fat from each of the flour, stirred vigorously for 1 hr followed by

centrifugation at 3000 x g for 30 min. The pellets of barley, rye and wheat are then collected and further processed by removing other globular protein with 10 volumes of 0.5 M NaCl followed by similar mixing and centrifugation. A 10 volume aqueous solvent containing 1% acetic acid, 2% methanol and 50% propanol was then added to the residual pellet and mixed for 1 hr. After centrifugation the supernatant was collected, 2 volumes of 1.5 M NaCl added, kept at 4 $^{o}$C overnight followed by centrifugation to collect the barley, rye and wheat gliadin pellets. Whereas for oat (containing relatively high contents of lipid- and salt-soluble albumins and globulins), after the defatting process, the pellets were mixed with 50% ethanol for 1 hr and centrifuged. The collected pellets were then mixed with 10 volumes of solvent (1% acetic acid, 2% methanol and 50% propanol) for 1 hr and centrifuged. Two volumes of 1.5 M NaCl were added to the supernatant collected, kept at 4 $^{o}$C overnight followed by centrifugation to collect the oat avenin pellets.

### 2.2.3 Pepsin-trypsin-gliadin (PT-gliadin) preparation

PT-gliadin was prepared according to the method previously described [13], with minor modifications. Briefly, 50 g Sigma gliadin was dissolved in 500 mL 0.2 N HCl for 2 hr at 37 °C with 1 g pepsin. The resultant peptic digest was further digested by addition of 1 g after pH adjusted to 7.4 using 2M NaOH. The solution was stirred vigorously at 37 °C for 4 hr, and then boiled for 30 min, lyophilized, and then stored at -20 °C until used. PT gliadin was freshly prepared by suspending it in phosphate buffered saline (PBS) to a final concentration of 1 mg/mL.

### 2.2.4 Protein content

The BCA protein assay of gliadin extracted samples diluted in 6 M urea were performed, using Sigma gliadin (10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/mL) as reference. Bradford protein assay kit was used to quantify protein content in IgY water soluble fraction (WSF), using standard IgY (80, 60, 40, 20, 10 and 5 µg/mL)

### 2.2.5 Production of IgY antibody

Laying hens were cared for in accordance with the guidelines of animal warfare of the Canadian Council on Animal Care (CCAC 2000, Protocol Number #097, University of Alberta). Sigma gliadin, wheat gliadin, and PT-gliadin fractions [12] (500 µg of protein/mL) was suspended in sterilize PBS (pH 7.2) and emulsified with an

equal volume of Freund's Incomplete Adjuvant. Ten 23-weeks-old Single Comb White Leghorn chickens were subcutaneously injected with the PT-gliadin emulsions. Booster immunizations (500 µg of protein/mL) were given after 2 and 6 weeks of the initial immunization. Eggs were collected daily and stored at 4 °C until the extraction of the antibodies.

### 2.2.6 Purification of IgY antibody

The egg yolks from hyperimmunized hens were physically separated from the egg white and first mixed gently with eight 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 5.0-5.2 and incubated at 4 °C for 12 hr. The WSF was obtained by centrifugation at 3,125 x 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 of high titre was further purified by ammonium sulphate precipitation (60%) followed by Sephacryl S-300 gel chromatography. A portion (1 mL containing 10 mg protein) of WSF was fractionated by using a 1.0 x 110 cm column of Sephacryl S-300 which was equilibrated and eluted with PBS (0.15 M NaCl, 0.0027 M KCl, 0.0081 M disodium phosphate and 0.0015 M monopotassium phosphate, pH 7.2) at a flow rate of 3 mL/hr. Blue dextran and titrated water were used to determine void volume (Vo) and total volume (Vt) of the column, respectively. The partition coefficient was calculated from the formula: Kav = (Ve - Vo) / (Vt - Vo), in which Ve represents the volume of the peak fraction. The eluates (1 mL) were analyzed for IgY activity at 405 nm by ELISA. The eluates of IgY were pooled, freeze-dried and analyzed for protein content, total IgY and specific IgY. All chromatography data presented in this report represent the average of three experiments.

### 2.2.7 Quantitative ELISA for total IgY

Unless indicated otherwise, all incubations were performed at 37 °C with four times washing by PBS in each step. Microtiter plates were coated with 150 µL of rabbit anti-chicken IgY at a final concentration of 2.5 µg/well and incubated at 4 °C overnight. Non-specific binding sites were blocked with 200 µL of 3% BSA solution (w/v) in PBS for 45 min. The BSA solution was then discarded and the wells were washed three times with PBS. The IgY diluted in PBS) containing non-specific IgY and two-fold serial dilutions of purified chicken IgY in PBS (0.5 to 0.031 µg/mL) was added to each well (150 µL per well) for 1 hr. Plates were subsequently with 150 µL of rabbit anti-chicken IgY conjugated with horseradish peroxidase (HRP) (1:3,000 dilution) for 1 hr. After washing, 150 µL of freshly prepared ABTS substrate was added for color development. Optical density reading at 405 nm was taken after 30 min using an ELISA Vmax kinetic microplate reader (Molecular Devices Corp, Sunnyvale, CA). The ELISA value of antibody activity was determined by relative measurements of total IgY to standard concentration curve.

### 2.2.8 Quantitative ELISA for anti-gliadin IgY

Unless indicated otherwise, all incubations were performed at 37 °C with four times washing by PBS-T in each step. Microtiter plates were coated with 100 µL of gliadin (10 mg/mL of 60% ethanol) for 1 hr. Non-specific binding sites were blocked with 120 µL of 3% BSA solution (w/v) in PBS-T for 45 min. To each well, 100 mL of WSF (diluted 1:1,000 in PBS-T) or column fraction (diluted 1:3,000 in PBS-T) was added as a specific IgY, and non-immunized IgY prior to incubating for 1 hr. Plates were subsequently added with 100 mL of rabbit anti-chicken IgY conjugated with HRP (diluted 1:5,000 in PBS-T) and incubated for 90 min. After washing, 100 µL of freshly prepared substrate solution, ABTS in 0.05M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide was added. Optical density reading at 405 nm (OD 405) was taken after 30 min using an ELISA Vmax kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the control antibody from that of specific antibody.

### 2.2.9 Cross reactivity of anti-gliadin IgY to other prolamins

Unless indicated otherwise, all incubations were performed at 37 °C with four times washing by PBS-T in each step. Microtiter plates were coated with 100 µL of Sigma gliadin, prolamins extracts (wheat, barley, rye, oat), rice and corn (10 mg/mL of 60% ethanol) and incubated for 1 hr. Non-specific binding sites were blocked with 120 µL of 3% BSA solution (w/v) in PBS-T for 45 min. To each well, 100 µL of anti-wheat gliadin IgY WSF (diluted 1:1,000 in PBS-T) or column fraction (diluted 1:3,000 in PBS-T) was added and incubated for 1 hr. Plates were subsequently incubated with 100 µL of rabbit anti-chicken IgY conjugated with HRP (diluted 1:5,000 in PBS-T) for 90 min. After washing, 100 µL of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) in 0.05M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide was added. Optical density reading at 405 nm (OD 405) was taken after 30 min using an ELISA Vmax kinetic microplate reader. The ELISA value of anti-wheat gliadin IgY reactivity to other grains was determined by subtracting the OD of non-immunized IgY from that of specific IgY of each coating antigen. Percent cross reactivity was calculated based on reference to 100% reactivity between anti-wheat gliadin IgY antibody and wheat gliadin.

### 2.2.10 Electrophoresis of prolamin

The Mini-Protean III apparatus was used for 8.3cm x 6.4 cm x 1 mm electrophoresis gel. Sigma gliadin and extracted prolamins from wheat , barley, rye and oat were subjected to sodium dodecyl sulfate – Urea polyacrylamide gel electrophoresis (SDS-Urea PAGE). Separating gels contain 10% total acrylamide with 0.8% cross-linkage and stacking gel contain 5% acrylamide, with an addition of 5 M urea in both separating and stacking gel. Each well was loaded with 10 µg of protein in 6 M urea in a volume of sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 50% glycerol, 0.002% bromophenol blue). Fresh running buffer (pH 8.3; 0.025M Tris, 0.192 M glycine, 0.1% SDS) was used for each electrophoretic run and pre-cooled prior to use. Electrophoresis was carried out in 70 min with a constant voltage of 200. Gels were fixed in 12% trichloroacetic acid for 30 min and then stained with 0.5% Coomassie Brilliant Blue R-250 aqueous solution for 2 hr. The staining solution was then poured off and the gel was agitated overnight in destaining solution (7% acetic acid and 5% methanol in an aqueous solution).

PT-gliadin was subjected to SDS-PAGE under the same conditions as mentioned above except no addition of urea in sample buffer, separating gel and stacking gel.

### 2.2.11 Western blot

After electrophoresis run, the proteins fractionated on the gel were blotted onto 0.45 μm nitrocellulose membrane. After briefly soaking the nitrocellulose membrane, gel and filter paper in transfer buffer (0.025 M Tris base and 0.2 M glycine), the gel and nitrocellulose membrane were sandwiched with filter paper and fiber in the gasket, and run at 100 V for 1 hr in transfer buffer. The blots were then washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (buffer A) after each incubation step. All the reactions were performed at room temperature. The blots were incubated for 1 hr in buffer A/3% BSA to prevent non-specific reaction. The blots were then washed 5 times in buffer A and incubated for 2 hr with anti-wheat gliadin IgY (1:600 dilution in TBS).The blots were washed three times with buffer A and incubated with peroxidase-labelled rabbit anti-chicken IgG (1:1,000 dilution in TBS). After 1 hr the blots were washed three times with buffer A, followed by the addition of TMB substrate for membrane. The color developed within 5 min on the nitrocellulose membrane. The blots were then rinsed with water and dried between filter papers before photographing.

### 2.2.12 Statistical Analysis

The student t-test (one-tailed t-test) was used to analyze for significant differences ($p < 0.05$) between the control (zero antigen) and samples.

## 2.3 Results

### 2.3.1 Characterization of prolamin extracts

Contents of fat and prolamin in wheat, barley, rye and oat flours were quantified as shown in Table 2-1. Oat contained the highest fat content of 11% whereas wheat, barley and rye had lower fat content of 7-8%. Among the four grains, wheat contained the highest prolamin content of approximately 9.4 %, followed by barley 5.4%, rye 3.7% and oat 1%.

**Table 2-1** Fat, and prolamin content in 1 g of wheat, barley, rye and oat flour

| Flour | Fat Content (g) | Prolamin content (g) |
|---|---|---|
| Wheat | 0.074 ± 0.012 | 0.094 ± 0.006 |
| Barley | 0.083 ± 0.013 | 0.054 ± 0.005 |
| Rye | 0.084 ± 0.007 | 0.037 ± 0.002 |
| Oat | 0.112 ± 0.005 | 0.010 ± 0.003 |

Values are the mean of quadruple samples ± SD (on a dry matter basis)

Molecular weights of gliadin (Sigma), prolamins extracted from four grains (wheat, barley, rye and oat) and PT-gliadin were analyzed by using SDS-Urea PAGE and SDS PAGE, respectively. Sigma gliadin (SG) and wheat gliadin (WG) showed similar molecular weight of major protein bands range from 30 -70 kDa) on SDS-Urea PAGE (Figure 2-1A). Prolamin from barley (hordein) protein distribution was in the range of 40-60 kDa; Rye secalin (45 and 100-130 kDa); and oat avenin (23-35 kDa). PT-gliadin contained 1 peptide bands at 22 kDa on SDS PAGE (Figure 2-1B).

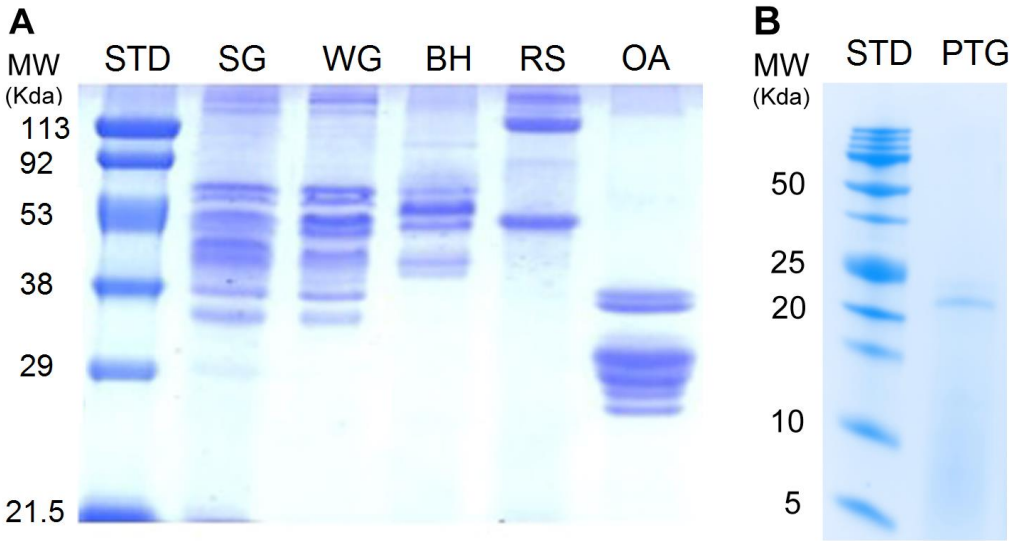


**Figure 2-1** (**A)** SDS-Urea PAGE of prolamin fractions from Sigma gliadin: SG; Wheat: WG; Barley hordein: BH; Rye secalin: RS; Oat avenin: OA; and; **(B)** SDS-PAGE of pepsin-trypsin digested Sigma-gliadin (PTG)

### 2.3.2 Production of anti-gliadin IgY

The anti-gliadin IgY antibodies obtained from egg yolks of chickens immunized with Sigma gliadin, wheat gliadin or PT-gliadin were weekly titrated by the indirect ELISA (Figure 2-2). The titre of anti-gliadin IgY was undetectable on day 0, rapidly increased ($p < 0.05$) from week 2 to week 4, and thereafter remained relatively constant ($p > 0.05$) during week 4-8 period. Among the three gliadin antigens, wheat gliadin elicited chickens to produce the highest anti-gliadin IgY.

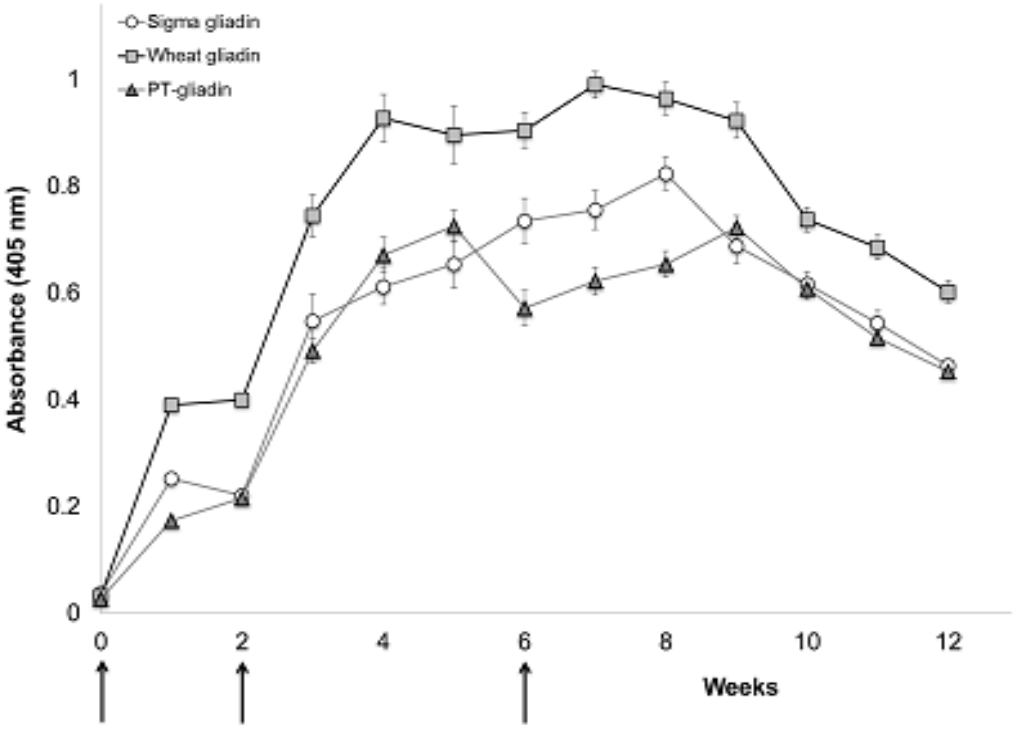


**Figure 2-2** Specific IgY antibody ELISA values in the egg yolk from chickens immunized with Sigma gliadin, wheat, and pepsin-trypsin resistant gliadin (PT-gliadin) (500 μg/mL protein) in PBS, emulsified with Freund's incomplete adjuvant. Booster immunizations were given at 2 and 6 weeks after the initial immunization. Values are the mean of quadruple samples, with vertical bars indicating the standard deviation.

The egg yolks during the 4-10 weeks of immunization period were pooled and quantified for protein, total IgY and gliadin specific IgY content. Table 2-2 shows that both protein and total IgY concentrations were similar regardless of the immunisation ($p > 0.05$). The gliadin specific IgY concentration were significantly higher in eggs of all the gliadins immunized chickens than in the non-immunized chickens ($p < 0.05$). Egg yolks of chickens immunized with PT-gliadin, Sigma-gliadin and wheat gliadin contain approximately 8% of specific anti-gliadin IgY in total IgY.

**Table 2-2** Concentrations of protein, total IgY, and specific IgY in purified IgY solution by ultra-centrifuge filtering

| IgY | Concentration (mg/g) | | | Specific IgY / Total IgY (%) |
|---|---|---|---|---|
| | Protein | Total IgY | Specific IgY | |
| Anti-Sigma gliadin | 150.1 ± 1.5 | 136.4 ± 2.9 | 10.77 ± 0.05 | 7.9% |
| Anti-wheat gliadin | 152.0 ± 1.4 | 136.9 ± 3.3 | 11.05 ± 0.09 | 8.1 % |
| Anti-PT gliadin | 149.0 ± 1.2 | 135.8 ± 3.0 | 10.41 ± 0.07 | 7.7 % |
| Non-specific | 140.5 ± 1.7 | 127.5 ± 4.4 | 0.79 ± 0.005 | 0.01% |

Values are the mean of quadruple samples ± SD.

### 2.3.3 Purification of IgY antibody

IgY from egg yolks were further extracted and purified by water soluble dilution method and ammonium sulfate (60%) followed by Sephacryl S-300 gel chromatography. elution profile of the IgY were determined by Indirect ELISA to determine total IgY content (explained above) and yielded one peak at $OD_{405}$ of 0.52. The fraction corresponding to Kav 0.2-0.24 was pooled and then lyophilized for further uses (Figure 2-3).

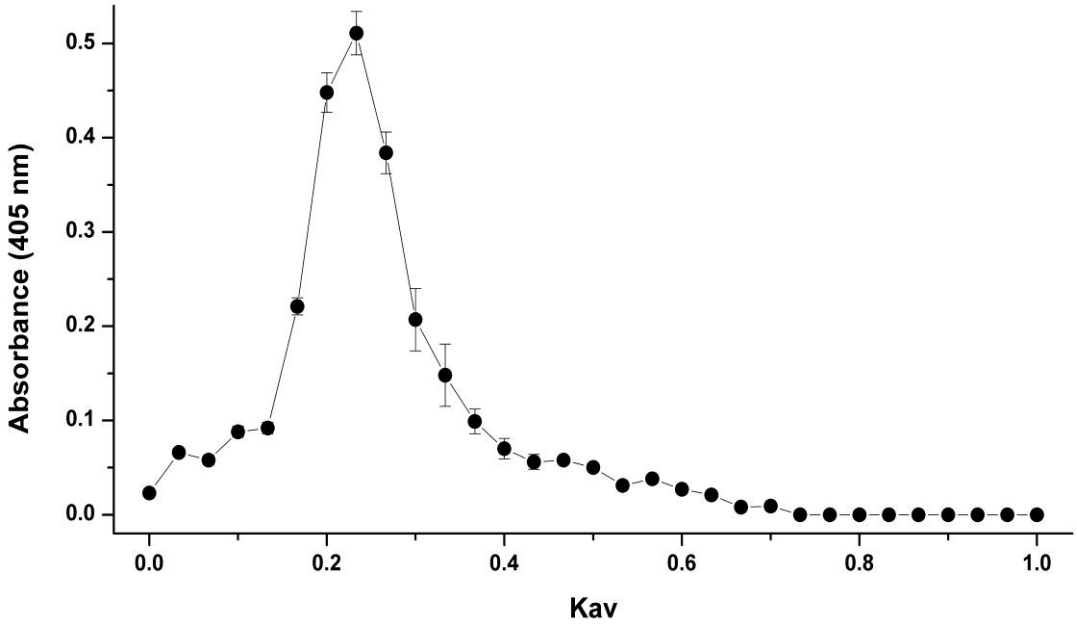


**Figure 2-3** IgY purification by Sephacryl S-300 chromatography. Each value of absorbance was determined by an indirect ELISA. Flow rate: 3 mL/hr, Column: 1.0 x 110 cm.

### 2.3.4 Western blot of anti-wheat gliadin IgY to prolamins

The binding efficacy of anti-wheat gliadin IgY to prolamins from wheat, barley rye, and oat or PT-gliadin was determined by western blot. Figure 2-4A shows that specific anti-wheat gliadin IgY antibody reacted with prolamins that were electrophoresized (SDS-Urea PAGE shown in Figure 2-4A) in the range of 30 -70 kDa , indicating specific anti-wheat IgY showed a cross-reaction with other prolamins from barley, rye and oat.

Anti-wheat gliadin IgY was also bound to the PT-gliadin fraction (22 kDa) (Figure 2-4B), showing that anti-wheat gliadin IgY bound to pepsin-trypsin digested gliadin peptide fraction.

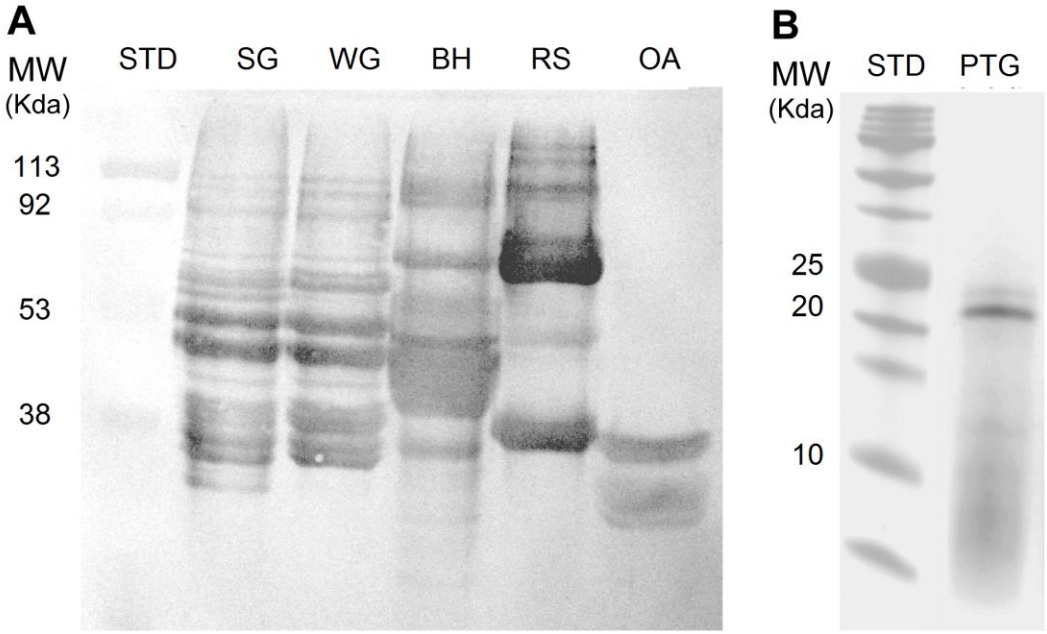


**Figure 2-4** Western blot of anti-wheat gliadin against: **(A)** Prolamins from Sigma gliadin: SG; Wheat: WG; Barley hordein: BH; Rye secalin: RS; Oat avenin: OA; **(**B) Pepsin-trypsin digested Sigma-gliadin (PTG)

### 2.3.5 Cross reaction of anti-gliadin IgY to other prolamins

Anti-wheat gliadin IgY cross-reactivity to Sigma gliadin, prolamin fractions from four grains (wheat, barley, rye and oat), PT gliadin, rice and corn was determined by ELISA (Table 2-3). The cross-reactivity of anti-wheat gliadin IgY was highest to Sigma gliadin (99.3%), followed by prolamin containing grains barley (91.3%), rye (80.2%) and oat (42.7%). Ant-wheat gliadin showed high binding affinity (98%) to PT-gliadin, whereas for non-prolamin containing grains the affinity was < 1%.

**Table 2-3** Cross reactivity of anti-wheat gliadin IgY to prolamins, PT-gliadin, rice and corn determined by indirect ELISA

| Prolamins | Anti-Sigma Gliadin IgY OD (405 nm) | Percentage % |
|---|---|---|
| Sigma | 1.144 ± 0.041 | 99.3 |
| Barley | 1.050 ± 0.082 | 91.3 |
| Rye | 0.924 ± 0.051 | 80.2 |
| Oat | 0.492 ± 0.038 | 42.7 |
| PT-gliadin | 1.129 ± 0.038 | 98. 0 |
| Rice | 0.011 ± 0.003 | 0.95 |
| Corn | 0.009 ± 0.002 | 0.78 |

Values are the mean of quadruple samples ± SD.

The cross reactivity of anti-wheat gliadin was calculated based on reference to 100% reactivity of anti-wheat gliadin IgY antibody and wheat gliadin ($OD_{405}$ = 1.152)

## 2.4 Discussion

Triticeae seed proteins contain albumins (hypotonic solutions soluble), globulins (isotonic solutions soluble), prolamins (aqueous alcohol soluble) and glutelins (dilute acids or bases, detergents, chaotropic or reducing agents soluble). Among these proteins, prolamins are exceptionally resistant to enzymatic degradation due to their high proline and glutamine content. The prolamins of wheat (gliadins), barley (hordeins), rye (secalins) and oats (avenins) constitute complex mixtures of homologous proteins which can be classified into several groups according to their molecular structure relationship. This classification establishes three groups of prolamins: the sulfur-rich (comprises α- and γ-type), the sulfur-poor (comprises C hordein and ω-type [gliadins, secalins]), and the high molecular weight (HMW) prolamins [14]. It is generally accepted that wheat gliadins, barley hordeins, rye secalins and oat avenins constitute the toxic gluten protein components which provoke the damage of the small intestine in celiac patients [15]. Four distinct groups, found corresponding to α, β, γ, and ω gliadins in wheat, were designated as α, β, γ, and ω monomeric prolamins [16].

Complying with literature, our electrophoresis results show that fractions of α-, β-, ω-, γ- gliadins ranged 30-78 kDa and glutelins (low molecular weight (LMW) ranged 30-45 kDa, intermediate molecular weight (IMW) ranged 50-78 kDa and high molecular weight (HMW) ranged > 90 kDa) [17]. When comparing the contents of these amino acids for the four prolamins, from Figure 2-1A, it was found that wheat, rye and barley are comparable, whereas oats expressed a different protein band pattern. The explanation for this difference is due to the difference in proline content. Oat gluten fraction has the same glutamine content but only half of the proline content of the other prolamins [18]. PT-gliadin is the pepsin and trypsin enzyme resistant peptides with the molecular size of 22 kDa (Figure 2-1B), which includes the 33-mer and its split product peptide fragments known to be harmful for celiac patients [19-21]. The anti-wheat gliadin IgY reacts with epitopes of toxic gliadin residue 57-89 in the mer-33 peptide sequence as follows: LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF [22]. The area of reactivity was observed to be α-gliadin 36 kDa in wheat, barley, and rye [23]. The celiac toxic prolamins at 20-30 kDa in oat avenins is separated from the remaining components of gliadins, secalins and hordeins that overlap within the 30–40 kDa region. The non-denaturing conditions of urea soluble prolamin fractions with LPYPQPQ, occurs in γ-gliadins, barley hordeins and rye secalins.

Laying hens immunized with gliadin fractions from Sigma gliadin, wheat gliadin and PT-gliadin produced a high level of anti-wheat gliadin IgY during the immunization period (Figure 2-2). It seems that gliadin (consisting of 689 or 691 amino acids) is a highly immunogenic antigen, to produce highly specific egg yolk antibodies, IgY.

The proportion of specific IgY antibody in the total IgY isolated from egg yolk has been investigated elsewhere in literature. IgY against the proliferating cell nuclear antigen (PCNA) contains 3.2% of IgY specific to the antigen [24]. The proportion of bovine proteoglyan-specific IgY in the total egg yolk IgY was 9.0% [25]. The percentage of specific IgY in total IgY from egg yolks ranged from approximately 7 to 16% against whole bacteria Fl cells including *Salmonella enteritidis, Salmonella typhimurium* [26], *E. coli O157:H7* [27], *E. coli 987P* [28], and *Clostridium perfringens* [29]. The percentage of specific antibody in total IgY ranged from 5 (anti-insulin antibody) to 28% (anti-mouse IgG antibody) [30]. Our results showed 8% of the gliadin-specific IgY concentration in total IgY determined by ELISA (Table 2-2). The western blot and ELISA cross-reactivity results show that anti-wheat gliadin IgY bound to all prolamin containing grains as well as the CD toxic gastrointestinal resistant gliadin peptide (PT-gliadin) (Figure 2-4).

To our knowledge, this study is the first to successfully produce polyclonal egg yolk antibody against prolamin peptide fractions (ranging between 30-70 kDa) from all possible grains containing gluten. The antibody produced in the egg yolk contained high titre and affinity against all four grains (wheat, barley, rye and oat). Hence, this anti-gliadin IgY has potential to be used as a diagnostic reagent to detect gliadin in foods as well as to neutralize enzyme resistant gliadin in the gastrointestinal tract as a therapeutic oral antibody for CD.

The anti-gliadin IgY antibody produced in this study has been evaluated further in *in vitro, in vivo*, and *ex-vivo* cell culture experiments to determine its potential use as an oral passive antibody therapy to treat CD. The purified anti-gliadin IgY has been used as a reagent in the development of immunological detection systems for gliadin in food.

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**CHAPTER 3: Effect of specific anti-gliadin IgY antibody on epithelial intestinal integrity and inflammatory response induced by gliadin**

A part of this chapter is submitted to *Journal of Cellular Biochemistry*:
Gujral N, Lovlin R, Lobenberg R, and Sunwoo HH. Effect of specific anti-gliadin IgY antibody on epithelial intestinal integrity and inflammatory response induced by gliadin

## 3.1 Introduction

Celiac disease (CD) is one of the most common autoimmune diseases, occurring in 1 out of 100–300 people worldwide [2]. CD is driven by an abnormal immune response to the ingestion of gluten in genetically (HLA DQ2 / DQ8) predisposed individuals. Among these gluten proteins, gliadin is exceptionally resistant to enzymatic degradation due to its high proline and glutamine content, that provokes the damage of the small intestine in celiac patients [3]. The pepsin and trypsin resistant gliadin (PT-gliadin) crosses the enterocyte as a consequence of increased release of zonulin leading to impaired mucosal integrity upon gliadin binding to CXCR3 receptor, or via transcytosis, or retrotranscytosis of secretory IgA through transferrin receptor CD71 [4]. It is the p31–43/49 peptides that triggers the innate immune response [5], leading to production of inflammatory cytokines [interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α)] and impaired mucosal integrity [6]. The 33-mer (p56-89) gliadin passages into the lamina propria then triggers the (T-helper cell mediated) adaptive immune response [7], contributing to the ongoing inflammation in CD small intestine [8].

Currently, there is no pharmacological therapy available to CD patients. Gluten-free diet (GFD) remains the only available treatment option. For a better quality of life in these patients, complementary therapeutic option is required. Hence, increasing efforts are being made to prevent gut immune trigger upon gluten ingestion in CD. Potential therapeutic options include the hydrolysis of toxic gliadin by exogenous enzymes [9]; the modification of gliadin-derived peptide pattern by Bifidobacteria [6]; the prevention of gliadin absorption by polymeric binders [10]; the inhibition of tight junction opening by zonulin antagonist [11]; the blockage of selective deamidation of specific glutamine residues by tissue tranglutaminase inhibitors [12]; the restoration of immune tolerance towards gluten by vaccine [13]; the modulation of immune response to gliadin by NKG2D/MICA blocker [14] and the neutralization of gliadin *in vivo* by IgY antibody [15]. All these therapeutic candidates are promising but still further studies are warranted.

Among these, oral passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the gastrointestinal tract (GIT) [16]. 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. IgY antibody has been proved to neutralize disease causing pathogens i.e., *Rotavirus* [17], *E. coli O157:H7* [18], *Salmonella enteritis* [19], *Clostridium perfringens*

[20].However, limited information is available about IgY antibody for neutralizing toxic gliadin in an intestinal epithelium culture system. In this study, Caco-2 cell culture was used to evaluate the effect of anti-gliadin IgY efficacy to inhibit gliadin induced impaired intestinal integrity, the gliadin absorption and the inflammatory response induced by gliadin. This cell line has been used in several studies as an *ex vivo* model of CD intestinal epithelia for initial testing of novel CD treatment options [21-23].

The objectives of this study is to produce anti-gliadin IgY antibody by immunizing chickens with gliadin, to purify IgY antibody by gel chromatography, and to characterize its reactivity to gliadin by western blot and ELISA techniques. The anti-gliadin IgY antibody is then tested for its efficacy to prevent gliadin induced impaired intestinal integrity and inflammatory response in Caco-2 cell culture, used as an *ex vivo* model for CD.

## 3.2 Materials and methods

### 3.2.1 Materials

Sigma gliadin (G3375), pepsin (P-7000; 800–2,500 units/mg protein), trypsin (P-8096, activity, 4x USP specifications), Freund's Incomplete Adjuvant, purified chicken IgG, rabbit anti-chicken IgG and rabbit anti-chicken IgG conjugated with horseradish peroxidase (HP) were purchased from Sigma (St. Louis, MO, USA). Pancreatic digested (PD)-casein was purchased from BactoTryptone (Sparks, MD, USA). Chicken anti-mouse IgG conjugated with HP were purchased from Thermo Fisher Scientific Canada (Burlington, Ontario, Canada). Tetramethylbenzidine (TMB) liquid substrate for membrane and 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrates were purchased from KPL (Frederick, MD, USA). Caco-2 cells were obtained from ATCC (Rockville, MD, USA). Cell culture media were purchased from Life Technologies Inc. (Burlington, ON, Canada). Electrophoresis Mini-Protean III, western blot apparatus, nitrocellulose membrane, and BCA protein assay and reagents were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Ontario, Canada). Gluten ELISA kit was purchased from Crystal Chem, Inc. (Downers Grove, IL, USA). Human IL-1β (Cat no: 88-7010) and TNF-α (Cat no: 88-7346) ELISA system were purchased from eBioscience (San Diego, CA, USA). Sephacryl S-300 gel filtration column was purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ, USA). Epithelial voltohmmeter was purchased from World Precision Instrument (Haven, CT, USA). BD Falcon HTS 24-multiwell insert system (1.0 μm) and 6-well culture plates were purchased from BD

Bioscience (Mississauga, ON, CA). Microtiter 96-wells plates were purchased from Costar Inc. (Cambridge, MA, USA). The ELISA $V_{max}$ kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA).

### 3.2.2 Preparation of pepsin-trypsin resistant gliadin (PT-gliadin)

PT-gliadin was prepared according to the method previously described with minor modifications [11]. Briefly, 50 g Sigma gliadin was dissolved in 500 mL 0.2 N HCl for 2 hr at 37 °C with 1 g pepsin. The resultant peptic digest was further digested by addition of 1 g trypsin after pH adjusted to 7.4 using 2M NaOH. The solution was stirred vigorously at 37 °C for 4 hr, boiled to inactivate enzymes for 30 min, lyophilized, and then stored at -20 °C until used. PT gliadin was freshly suspended in sterilize phosphate buffered saline (PBS, pH 7.2) to a final concentration of 1 mg/mL. Pancreatic digested (PD)-casein was used as a negative control in the experiment.

### 3.2.3 Preparation and purification of anti-gliadin IgY

Wheat gliadin (500 µg of protein/mL) was suspended in sterilized PBS (pH 7.2) and emulsified with an equal volume of Freund's Incomplete Adjuvant. Anti-gliadin IgY was prepared according to the method previously described with minor modifications[24]. Briefly, ten 23-weeks-old Single Comb White Leghorn chickens were subcutaneously injected with the PT-gliadin emulsions. Booster immunizations (500 µg of protein/mL) were given after 2 and 6 weeks of the initial immunization. Eggs were collected daily and stored at 4 °C until the extraction of the IgY. The egg yolks were diluted with acidified water to make the final dilution of 1:10 and adjusted to a pH 5.0-5.2 and incubated at 4 °C for 12 hr. The water soluble fraction (WSF) was obtained by centrifugation at 3,125 x g at 4 °C for 20 min. The supernatant was collected as the IgY rich WSF and titrated by indirect ELISA using PT-gliadin as a coating antigen. The IgY of high titre was further purified by ammonium sulphate precipitation (60%) followed by Sephacryl S-300 gel chromatography.

### 3.2.4 Quantitative ELISA for gliadin-specific IgY

Microtiter plates were coated with 100 µL of PT-gliadin (10 mg/mL in PBS (pH 7.2) and incubated at 37 °C for 1 hr. The plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-T), filled with 120 µL of 3% BSA solution

(w/v) in PBS-T for each well, and incubated at 37 °C for 45 min. The BSA solution was then discarded and the wells were washed four times with PBS-T. To each well, 100 µL of WSF (diluted 1:1,000 in PBS-T) or column fraction (diluted 1:3,000 in PBS-T) was added as a specific IgY, and non-immunized IgY as a control prior to incubating at 37 °C for 1 hr. After washing the plates with PBS-T for four times, 100 µL of rabbit anti-chicken IgY conjugated with HP (diluted 1:5,000 in PBS-T) was added to incubate at 37 °C for 90 min. The plates were then washed again four times with PBST to receive 100 µL of freshly prepared substrate solution, ABTS in 0.05M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. Optical density reading at 405 nm (OD 405) was taken after 30 min using an ELISA Vmax kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the non-immunized IgY from that of specific IgY.

### 3.2.5 Preparation of Caco2 cell culture system

Caco-2 cell culture was performed as previously described [25]. Caco-2 cells (passages 20-24) were maintained at 37 ºC in Dulbeco's Modified Eagle's Medium (DMEM) complemented with 4 mM glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids, 10% heat inactivated fetal calf serum, in an atmosphere of 90% air and 10% $CO_2$. Cells were cultured on 24-well culture plates with polyethylene terephthalate membrane inserts (pore size 1.0 µm) at a density of $3 \times 10^5$ cell/$cm^2$. The medium was changed three times a week and maintained for 21 days in medium for complete differentiation.

### 3.2.6 Transepithelial electric resistance (TEER) of Caco2 monolayer

The integrity and permeability of the monolayer was determined by measuring the TEER value using Epithelial voltohmmeter, according to manufacturer's instructions. The final values are expressed as Ω x $cm^2$ on the basis of the following equation: TEER = (R - Rb) x A, where R is the resistance of filter insert with cells, Rb is the resistance of the filter alone and A is the growth area of the filter in $cm^2$. The transepithelial electrical resistance values obtained in the absence of cells was considered as background measurements. All experiments were started based on the TEER of the monolayer when it reached 300-400 Ω $cm^2$ at 21 days after seeding cells on the transwell inserts.

### 3.2.7 Phenol red apparent permeability in Caco2 monolayer

In all experiments, phenol red was included in the apical chamber to estimate paracellular diffusion through the Caco-2 cell monolayer. The percentage of phenol red transported into the basal chamber was calculated as previously described [26]. In brief, 500 μM phenol red was subjected to the apical chamber. Aliquots of 100 μL were removed from the basal chamber after 2 hr incubation at 37 °C and added with 1 M NaOH. The optical density of the basal chamber contents was measured at 558 nm to detect any leakage of the phenol red through the intercellular spaces.

### 3.2.8 Neutralization of PT-gliadin by IgY in Caco2 cell culture

Caco2 cell culture was used to determine the PT-gliadin neutralization effect of anti-gliadin IgY. After formation of Caco2 monolayer, the 24-well Caco2 culture medium in the basal chamber was removed and replaced with 1200 μL phenol red free Hank's balanced salt solution (HBSS). Cells on the transwell inserts were also washed with phenol red free HBSS and replaced with 300 μL phenol red free HBSS. Caco2 cells were then subjected to 100 μL of 7 different experimental conditions (15 replicates): A) Control: PBS; B) Negative control: PD-casein (60 μg); C) PT-gliadin (60 μg); D) anti-gliadin IgY (40 ng); E) anti-gliadin IgY (10 ng) and PT-gliadin (60 μg) [1:6,000]; F) and anti-gliadin IgY (20 ng) and PT-gliadin (60 μg) [1:3,000]; G) anti-gliadin IgY (40 ng) and PT-gliadin (60 μg) [1:1,500].

After 1 hr, 2 hr and 4 hr incubation, the basal media was recovered, and quantified for diffusion of phenol red and PT-gliadin content by measuring the absorbance at 558 nm and enzyme-linked immunosorbent assay (ELISA; described below), respectively. The apical volume was maintained at 400 μL and basal volume at 1200 μL. The volume of aliquoted basal media at each time point was compensated by fresh phenol red free HBSS media. After each experiment, the TEER values were measured in all inserts to determine the effect on the intestinal integrity of each experimental condition (A-G).

### 3.2.9 Quantification of gliadin

A commercially available quantitative immune-based ELISA kit was used to quantify gliadin in apical and basal media of the Caco2 cell culture system according to the manufacturer's instructions. The analyses were performed in the apical and basal media after 4 hr incubation.

### 3.2.10 Quantification of inflammatory markers

Caco-2 cells (passages 20-24) were seeded at 50,000 cell/$cm^2$ on 6-well plates and grown with DMEM, culture medium was changed every 2 days under the condition as mention above. Experiments were performed 5 days post-seeding. PT-gliadin and/or anti-gliadin IgY was exposed to cells for 24 hr. Supernatants of Caco2 cells cultures of each experimental condition was used to quantify TNF-α and IL-1β by eBioscience ELISA system according to the instruction of the manufacturers with a linear range of 4-500 pg/mL. The result of this assays was expressed as pg/mL of media. The experiments were performed in triplicate on 3 different plates.

### 3.2.11 Statistics

The data are presented as the arithmetic mean for each experimental point ± SD. Statistical calculations were performed using one-way analysis of variance (ANOVA). Differences among groups were examined using Tukey post hoc test. Statistical significance at p value of 0.05 or less were considered significant. Statistical analyses was performed with the use of the SPSS-PC (SPSS, Chicago).

## 3.3 Result

### 3.3.1 Measurement of intestinal integrity

At 21 days, after seeding cells on the transwell inserts, the TEER value of Caco2 monolayer was maintained in the range of 305-310 Ω $cm^2$. The achieved TEER value was mirrored by minimal passage of phenol red from the apical to basal chamber across the cell monolayer of less than 1%.

### 3.3.2 IgY neutralization of toxic gliadin

To evaluate the neutralizing effect of anti-gliadin IgY on the deteriorating effects of PT-gliadin on intestinal Caco-2 monolayer integrity, Caco-2 cells were exposed to the different ratio of anti-gliadin IgY and gliadin. At 21 days, after seeding cells on the transwell inserts, the TEER values were in the range of 305-310 Ω $cm^{2,}$ indicating well-formed Caco-2 monolayers. Caco-2 monolayers have non-significant changes in TEER values after 4 hr in PBS, PD-casein and non-specific IgY (control conditions) ($p > 0.05$). Upon 4 hr exposure to PT-gliadin there is a significant decrease in TEER value of 52%, as compared to exposures to negative control conditions (85%). Basal TEER value of Caco2 monolayers at time zero is considered to have TEER value of 100%. However,

there was no significant TEER value change with exposure to anti-gliadin IgY and gliadin at a ratio of 1: 6,000 [anti-gliadin IgY (10 ng) and PT-gliadin (60 μg)] for 4 hr, ($p$ > 0.05) (Figure 3-1), indicating that anti-gliadin IgY neutralized the toxic gliadin and prevented gliadin-induced impairment of intestinal integrity. Anti-gliadin IgY at higher ratio (1:3,000 and 1:1,500) showed similar effects to Caco2 monolayers exposed to anti-gliadin IgY and gliadin at a ratio of 1: 6,000) (data not shown).

Maintained monolayer integrity was determined TEER in the range of 305-310 Ω $cm^2$ which is mirrored by < 1% passage of phenol red from the apical to basal chamber. Phenol red was added to the apical chamber to determine its permeability into the basal chamber after 4 hr of exposure. Upon 4 hr incubation with PBS and PD-casein as controls, < 6% phenol red permeated into the basal chamber and < 10% reduction in TEER value (Figure 3-1), indicating minimal disturbed intestinal integrity (Figure 4). After incubation for 4 hr with PT-gliadin, there was a significant permeation of 28.8% phenol red into the basal chamber (compared to negative controls, < 6%) and relative TEER value of approximately 52% (compared to negative control, 85%) ($p < 0.05$). Incubation with anti-gliadin IgY alone did not affect the intestinal integrity. When the monolayers were exposed to three different ratios of anti-gliadin IgY and gliadin for 4 hr, there was minimal phenol red permeation of 5.0-5.6% ($p > 0.05$), indicating that anti-gliadin IgY successfully prevented gliadin induced impairment of intestinal integrity at a ratio of 1:6,000.

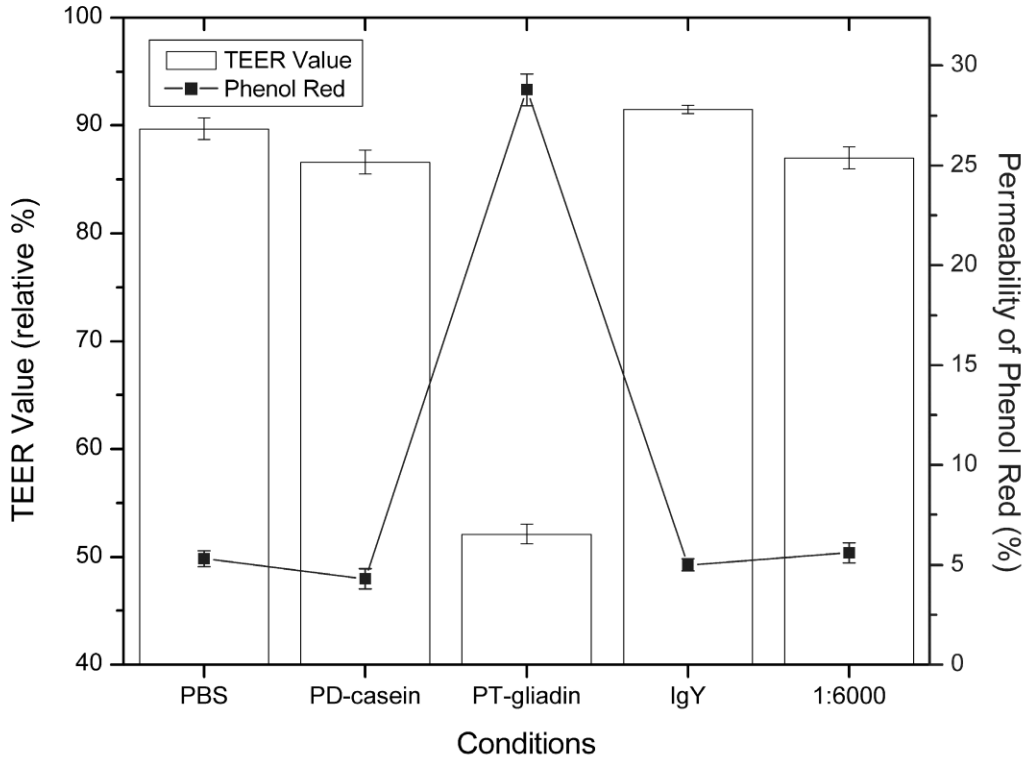


**Figure 3-1.** Relative transepithelial electric resistance (TEER) and phenol red permeation measurement to determine the effects of anti- gliadin IgY in Caco-2 cells.

Pancreatic digested casein (PD-casein) (60 μg);

PT-gliadin (60 μg);

Non-specific IgY (40 ng);

Anti-gliadin IgY (10 ng) and PT-gliadin (60 μg) [1:6,000]

† indicates statistically significant decrease in TEER value ($p < 0.05$)

‡ indicates statistically significant permeation of phenol red ($p < 0.05$)

Values are shown as mean ± SD. Analysis of each group was done in triplicates per plate. Five plates were repeated (n=15).

### 3.3.3 Effect of anti-gliadin IgY for gliadin permeation in Caco2 monolayer

PT-gliadin was added to the apical chamber with / without anti-gliadin IgY to determine its permeability from the apical chamber into the basal chamber. Upon Caco-2 monolayer exposure to PT-gliadin for 4 hr, significant decrease in Caco-2 monolayer

integrity was observed, resulting in PT-gliadin translocation of 14.8% of total PT-gliadin subjected to the apical chamber into the basal chamber ($p < 0.05$). However upon exposure to three different ratios of anti-gliadin IgY and PT-gliadin for 4 hr, the passage of gliadin into the basal chamber was inhibited leading to undetectable levels of PT-gliadin in the basal chamber. Table 3-1 showed that approximately 94% of PT-gliadin was recovered in the apical chambers of monolayers exposed to three different ratios of anti-gliadin IgY and PT-gliadin for 4 hr. However, the gliadin was not detected in basal chamber, indicating that the rest of gliadin (approximately 6%) seems to be located in the epithelial cells. Thus, anti-gliadin IgY neutralized PT-gliadin in the apical chamber, blocking gliadin absorption through the Caco-2 monolayer.

**Table 3-1** Gliadin content quantified in the apical and basal chamber of Caco-2 cell cultures exposed to gliadin digested with / without anti-PT gliadin

| Condition | % Gliadin | | | | | |
|---|---|---|---|---|---|---|
| | Apical | | | Basal | | |
| | 1 hr | 2 hr | 4 hr | 1 hr | 2 hr | 4 hr |
| A | ND | ND | ND | ND | ND | ND |
| C | 98 ± 3 | 90.3 ± 3 | 76.3 ± 3† | ND | 2.5 ± 0.5 | 14.8 ± 1‡ |
| D | ND | ND | ND | ND | ND | ND |
| E | 96.2 ± 1 | 93.5 ± 1 | 94.2 ± 1 | ND | ND | ND |
| F | 94.0 ± 1 | 93.7 ± 1 | 94.9 ± 1 | ND | ND | ND |
| G | 97 ± 1 | 94.9 ± 1 | 94.5 ± 1 | ND | ND | ND |

A: Phosphate buffered saline (PBS)

C: Pepsin-trypsin digested gliadin (PT-gliadin)

D: anti-PT-gliadin

E: PT-gliadin : anti-gliadin IgY (1:6,000)

F: PT-gliadin : anti-gliadin IgY (1:3,000)

G: PT-gliadin : anti-gliadin IgY (1:1,500)

Data from a representative experiment performed are shown as mean ± SD (n=15).

† indicate statistically significant lower gliadin content in apical chamber ($p < 0.05$)

‡ indicate statistically significant higher gliadin content in basal chamber ($p < 0.05$)

ND: Not detectable

### 3.3.4 Quantification of inflammatory markers

Caco-2 cells subjected to PT-gliadin for 24 hr has been shown to stimulated cytokines IL-1β and TNF-α production (Figure 3-2). The production of cytokine TNF-α was 6-7 folds higher than IL-1β. When a mixture of PT-gliadin was incubated with anti-gliadin IgY, cytokines IL-1β and TNF-α production were significantly ($p < 0.05$) reduced. Production of TNF-α was detectable in the anti-gliadin IgY and gliadin combination group at 1:6,000, indicating the ratio of IgY and gliadin was not enough to neutralize gliadin in Caco-2 cell cultures. However, in the other two groups with high anti-gliadin IgY of 1: 3,000 and 1:1,500 there are undetectable levels of TNF-α. On the other hand, IL-1β levels remained undetectable with anti-gliadin IgY co-incubation as low as 1:6,000 ratio. No cytokines were produced in cultures exposed to control PBS, PD-casein and IgY.

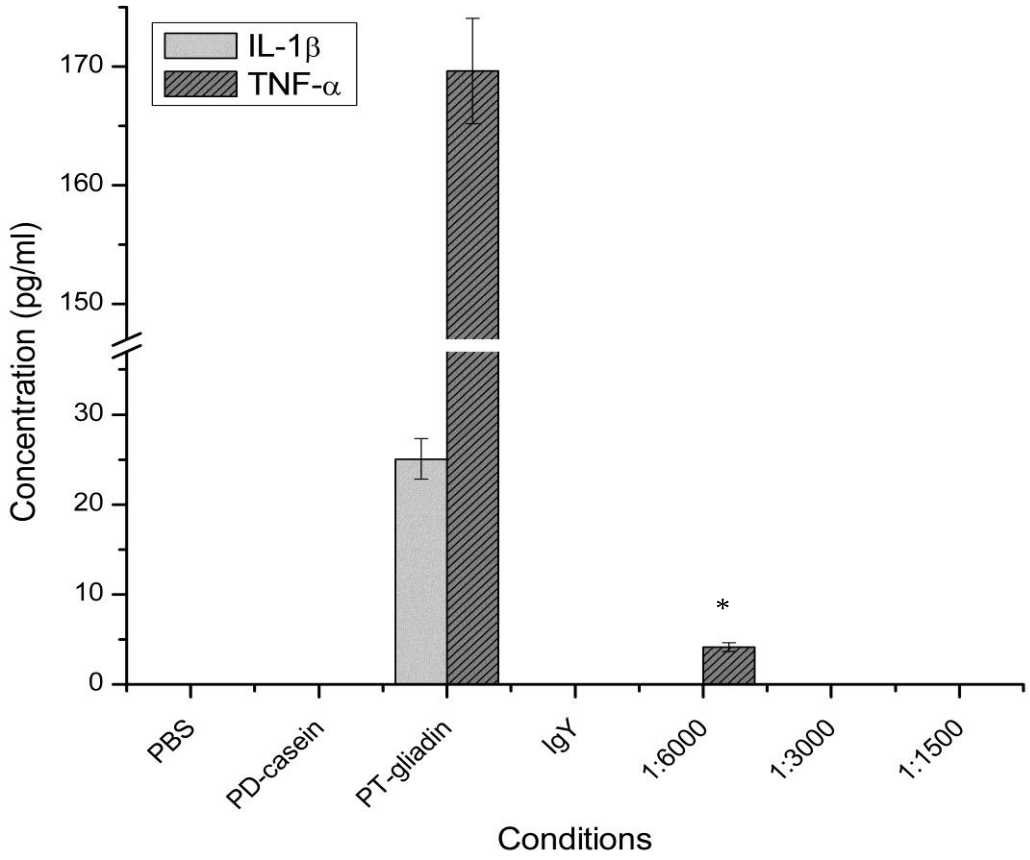


**Figure 3-2.** Pro-inflammatory cytokine (IL-1β and TNF-α) in Caco-2 cell.

Results are expressed as mean ± SD (n=9)

* indicates statistically significant decrease in TNF-α production ($p > 0.05$) compared to PT-gliadin

### 3.4 Discussion

CD is caused by pepsin and trypsin resistant gliadin (PT-gliadin) including 12 -mer (p31-43) and 33-mer (p56-89) peptides [27-29] found in gluten. PT-gliadin translocates through transcellular and paracellular passage of absorption from the intestinal lumen to the gut mucosa [30], causing the pathophysiologic processes in CD and its clinical presentations.

Several therapeutic approaches have been attempted to neutralize and/or prevent gliadin absorption which will lead to inhibition of the initial step of gliadin-induced toxicity in CD individuals. ALV003, a mixture of glutenase and endoprotease was reported to hydrolyse toxic gliadin peptide by enzymes [9]. These enzymes may hydrolyse other peptides in the GIT other than gliadin. Another candidate being extensively studied is the prevention of toxic gliadin peptide absorption by inhibition of zonulin by Larazotide [11]. This drug candidate inhibits the paracellular route of gliadin absorption through tight junctions, which is not the only mechanism of gliadin absorption. Indeed, gliadin may gain access to the mucosa through transcellular pathways in addition to paracellular route. Hence, this strategy might be best exploited in combination with other treatments. P(HEMA- co -SS) is another intersting polymer reported to attenuated gliadin-induced changes in permeability and inflammation [10]. Further investigation of the mechanisms of action and its interaction with human tissues is required. All these therapeutic candidates are promising but still further studies are warranted. Among these, oral passive antibody has potential as a therapeutic option for CD

Oral antibody passive immunotherapy is of significant value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the GIT [16]. IgY has also been tested against gastrointestinal pathogens such as *Salmonella enteritidis, Salmonella typhimurium* [19], *E. coli O157:H7* [31], *E. coli 987P* [32], and *Clostridium perfringens* [20]. Due to the fact that antibodies can bind gliadin [24, 33], we use laying hens to produce IgY specific to gliadin. The use of laying hens as potent antibody producers has numerous advantages over mammalian polyclonal antibody production because of the high antibody content, the relative ease of using eggs versus serum, the low cost of animal maintenance, and large quantities of egg availability. Laying hens usually lay about 250 eggs (approximately 4000 g of egg yolk) in a year. On the other hand, the serum collected from a rabbit is about 40 mL. One gram of egg yolk laid by the immunized hen contains about 10 mg of IgY whereas 1 mL of rabbit serum yields about 35 mg of IgG [34, 35]. The use of IgY may improve the gliadin binding efficacy because IgY is polyclonal, and

therefore able to bind to multiple epitopes on various fractions of PT-gliadin. In addition, there is an economical advantage by replacing the expensive monoclonal antibody with inexpensive chicken egg yolk IgY. Our previous *in vivo* mice feeding study has also proved that anti-gliadin IgY prevented > 99% gliadin absorption in the GIT of mice fed with gliadin and anti-gliadin IgY [15]. Hence, this anti-gliadin IgY produced has potential to be used as a therapeutic oral antibody for CD to neutralize enzyme resistant gliadin in the GIT.

Small-bowel mucosal biopsy organ culture system has been used extensively to clarify the pathogenesis of CD. Since, they contain a number of different cell types, the investigation in the system are numerous, all the way from the epithelium to the mucosal lamina propria. CD researchers have used this model to study the immune mechanisms [5], as well as the contribution of different cytokines [36]. However, the toxic effect of gliadin is detectable with biopsy samples from active CD patients and short-term treated (less than 3 years on GFD) CD patients who are likely still to have mucosal IgA deposits present. If the CD mucosa is taken from CD patients on GFD for > 4 years, the biopsy samples do not secrete autoantibodies to the supernatant due to overall absence of plasma cells and helper T cells as well as the failure of memory B cells to become activated [37, 38]. This method has been widely used in studies aiming to clarify the pathogenesis of CD, but it is not widely used in studies related to novel treatment developments. In this study, we employed Caco-2 epithelial cell instead, in order to determine the ability of anti-gliadin IgY to inhibit gliadin absorption at epithelial level. Hence there is no additional benefit to use the model with underlying celiac pathogenesis.

In literature, Caco-2 monolayers have been used to study inflammatory response induced by gram-positive and -negative bacterial cell surface polysaccharides, teichoic acid, protein A, peptidoglycans, lipid A-associated proteins, lipoproteins and deoxyribonucleic acid, to regulate cytokine synthesis [39]. Peptidoglycans, lipoproteins, lipoteichoic acid, lipopolysaccharide, flagellin and unmethylated CpG dinucleotides in bacterial DNA are also reported to bind Toll-like receptors and induce cytokine synthesis in Caco-2 cells [40, 41].

Caco-2 monolayers is an ideal *ex vivo* model of CD intestinal epithelium since it has been reported to have decreased electrical resistance, increased absorption, induced intestinal permeability [11], exerted pro-apoptotic activity [22, 23], and stimulated release of proinflammatory cytokines (such as TNF-α and IL-1β), upon PT-gliadin stimulation [6]. Caco-2 cell cultures have been employed to study the effects of potential CD treatments

such as zonulin antagonist (a tight junction modulator) [11], Bifidobacteria [6], germinating cereal enzymes [9], and polymeric binders of gliadin [10]. Hence, in this study Caco-2 monolayers are used to evaluate the efficacy of anti-gliadin IgY to neutralize gliadin at intestinal epithelium level.

Our results show that PT-gliadin subjected to the apical chamber leading to the flow of the toxic peptides from the apical compartment to the basal compartment of the Caco-2 cultures. However, the aforementioned flow of peptides has been inhibited in presence of anti-wheat-gliadin and PT-gliadin co-incubated cultures. This is particularly important since the cytoskeleton rearrangement upon gliadin stimulation has been interrupted in the presence of anti-gliadin IgY. In this context, the minimum ratio of anti-PT gliadin IgY and PT-gliadin (1:6,000) contributed to maintain the intestinal barrier integrity.

Li and colleagues have reported dramatically drop of Caco-2 monolayer integrity when the culture medium was changed since essential amino acid glutamine is important for intestinal barrier function, as a result of a catastrophic loss of electrical resistance but started to recover after 1 hr and it takes up to 2-4 hr for protein synthesis to recover [42]. Hence in this study we incubated PT-gliadin and/or anti-gliadin IgY for 4 hr to evaluate their effects on intestinal integrity. Other researchers have reported impaired intestinal integrity of Caco-2 monolayers after 6 hr [27], and 3 hr [11] exposure to PT-gliadin.

In the present study, PT-gliadin has also shown to trigger the production of pro-inflammatory cytokines (TNF-α and IL-1β), explained by the activation of pro-inflammatory pathways (NF-kB). NF-kB is known to be activated in small intestinal mucosa of CD patients [43], and gluten peptides have been shown to upregulate the expression of cytokines such as TNF-α [44] and IL-1β [45], related to the innate immune response. TNF-α content was detectable in the presence of anti-gliadin IgY with intestinal digests of gliadins of 1:6,000 ratio indicating that anti-gliadin IgY was not enough to neutralize gliadin in the Caco-2 cell cultures. The presence of anti-gliadin IgY with intestinal digests of gliadins as low as (1:3,000) completely abolished the TNF-α and IL-1β production. The latter is explained by inhibition of NF-kB induction due to the gliadin neutralizing efficacy of anti-gliadin IgY. NF-kB (subunit proteins: p65 and p50) is made inactive by Ik-B in the cytosol. Exposure to gliadin prompts phosphorylation and results in destruction of Ik-B [46]. After destruction of IkB, NF-kB enters the nucleus and binds with DNA-activating genes that encode for the increased inflammatory mediators (cytokines) production, leading to cellular dysfunction and tissue destruction [47].

TNF-α together with IL-1β are the important cytokines that involved in NOS activation, which act as a mediator to facilitate the interaction of intraepithelial lymphocytes and intestinal epithelial cells promoting tissue inflammation [48]. In addition, TNF-α also has a positive effect on attraction of neutrophils, which cause perpetuation of inflammatory responses, cell damage, and eventually epithelial barrier dysfunction [6].

There attempts reported in literature to treat CD by inhibiting inflammatory responses induced by gliadin at intracellular or submucosal level, such as zonulin antagonist [11], tissue tranglutaminase inhibitors [12], peptide vaccine [13], and NKG2D/MICA blocker [14].

The anti-gliadin IgY antibody used in this study has proved its potential use as oral antibody to neutralized intestinal digests of gliadin peptides (PT-gliadin), inhibiting gliadin-induced cytotoxic and pro-inflammatory responses at intra-luminal level, before they enter the small intestine, and so the cascade of gliadin induced inflammatory events in CD is inhibited. Hence, oral administration of anti-gliadin IgY may contribute to maintaining healthy and normal intestine by masking the ingested gliadin peptides. The reported data extend the spectrum of beneficial effects that IgY antibody might exert on intestinal epithelial cells function in other gastrointestinal immune function triggered by antigens other than gliadin.

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# CHAPTER 4*: In-vitro* and *in-vivo* binding activity of chicken egg yolk immunoglobulin y (IgY) against gliadin

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## 4.1 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 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 titre 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 review of the literature reveals that IgY has been used as an 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], 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 non-toxic and non-irritant. 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 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 anti-gliadin 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*.

## 4.2 Materials and methods

### 4.2.1 Material

Wheat (*Triticum aestivum*) flour was obtained from 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 (Frederick, MD, USA). D-mannitol, sorbitol and microcrystalline cellulose were purchased from PCCA (London, ON, CA). BCA protein assay kit was purchased from Thermo Scientific, Fisher Canada (Burlington, ON, Canada). Bradford protein assay kit and Mini-Protein III electrophoresis kit were purchased from Bio-Rad Laboratory (Mississauga, ON, CA). Complete protease inhibitor was purchased from Roche (Rockville, MD, USA). Pepsin (P-7000), pancreatin (P-8096), gliadin (G-3375), rabbit anti-chicken IgG Peroxidase, chicken IgG, TMB substrate for membrane and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Microtiter 96-wells plates were purchased from Costar Inc. (Cambridge, MA, 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, USA).

### 4.2.2 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 hr prior to centrifugation at 3,000 x 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 hr. After centrifugation at 3,000 x 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 x 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.

### 4.2.3 Production of anti-gliadin IgY antibody

Ten 23-weeks-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 hr. 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.

### 4.2.4 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 µL of WSF (diluted 1:1,000 in PBS-T) of hyperimmunized egg yolk or samples from dissolution test at each time point and non-specific IgY (diluted 1:1,000 in PBS-T) as a control were added to the plates. After washing, 100 µL of rabbit anti-chicken IgY-horse radish peroxidase (HP) (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 titre was determined by subtracting the value of the non-specific IgY.

### 4.2.5 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 anti-chicken 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.

### 4.2.6 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 air-tight containers. Egg yolks without protectants were also dried as a reference. Four EYP formulations were capsulated into gelatin capsules (“00” size) at 500 mg EYP per capsule.

### 4.2.7 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 air-tight container protected against light at RT.

### 4.2.8 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 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.

### 4.2.9 ELISA-gliadin inhibition assays of EYP

A 1.6 mg/mL EYP-M was preincubated with serial dilutions of Sigma gliadin (ranged from 0 to 25.6 mg/mL) at 37 °C for 1 hr in SGF or simulated intestinal fluid (SIF, containing 10 mg/mL pancreatin (trypsin, chymotrypsin, elastase and 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 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 hr. 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 were added to the wells and incubated at 4 °C overnight. After washing the plates four times with PBS-T, 150 µL of rabbit anti-chicken IgY conjugated

with HP (diluted 1:3,000 in PBS) was added to each well and incubated at 37 °C for 1 hr. 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 anti-gliadin IgY without gliadin ($A_0$). The standard curve was normalized by expressing experimental absorbance values (A) as $A/A_0$ X100.

### 4.2.10 *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 standard chow. A day before the treatment all the mice were kept on a 24 hr 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 water; Group B: EYP-M 4 mg and sigma gliadin 100 mg powder reconstituted in 0.2 mL PBS; Group C: EYP-M 20 mg and sigma gliadin 100 mg powder reconstituted in 0.2 mL PBS; Group D: EYP-M 100 mg reconstituted in 0.2 mL PBS. The formulations were orally administered to the fasted mice by a micropipette. After 12 hr, 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 PBS neutralized containing Complete Protease Inhibitor, mixed well under continuous shaking for 1 hr, and centrifuged at 10,000 x g, 15 min. The supernatant was collected and analyzed for residual gliadin by heterosandwich ELISA mentioned below.

### 4.2.11 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 KLQPFPQPELPYPQPQ 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 was blocked with 120 µL 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 hr. Then, 100 µL gliadin-specific IgY (diluted 1:2,000 in PBS-T) was added and incubated for 1 hr. After washing the plates, 100 µL chicken anti-mouse IgG conjugated with HP (1:5,000 in PBS-T) was added to each well and incubated for 1 hr. Each well was incubated with 100 µL of freshly prepared TMB substrate followed by optical density reading at 650 nm by 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.

### 4.2.12 Statistical analysis

The analysis of variance (ANOVA) and the student 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), between IgY formulations with 0, 12.5, 25, 37.5, 50% mannitol, sorbitol or MCCP, long-term stability of EYP formulations, IgY survival during dissolution test in SGF as well as % gliadin absorbed between different *in vivo* feeding conditions. The differences were considered statistically significant at $p < 0.05$.

## 4.3 Results

### 4.3.1 Production of IgY

Table 4-1 illustrates the anti-gliadin antibody (IgY) titre from egg yolks collected from hyperimmunized chicken. The activity of anti-gliadin IgY detected on day 0, gradually increased at week 2 after the first immunization. The 1st booster immunization was given at week 2, illustrating a rapid increase of IgY titre with a peak at week 4. The 2nd booster was given at week 4 to maintain the high titre 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.

**Table 4-1** Anti-gliadin IgY antibody titre 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 titre[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-weeks-old laying hens were immunized with gliadin (500 $\mu$g of protein/mL) at week 0, 2, and 4 and eggs were collected daily for measuring daily egg production. [b] Values of IgY titre are the mean of optical density $\pm$ SD (n = 10).

### 4.3.2 Optimum spray dry condition

Table 4-2 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 ℃ inlet and 60 ℃ outlet, or 100 ℃ inlet and 80 ℃ outlet, since they resulted in minimal IgY degradation.

**Table 4-2** Total IgY content and specific IgY activity of egg yolk powder without protectants at different spray dry temperatures

| Items | Inlet/Outlet temperatures (°C) | | | |
|---|---|---|---|---|
| | 140/110 | 120/100 | 100/80* | 80/60* |
| 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 |

*Significantly high IgY content as compared to higher temperatures ($p < 0.05$).

### 4.3.3 Effect of protectants

Table 4-3 illustrates IgY content in EYP with protectants. For the spray dry processing, protectants (mannitol, sorbitol, 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) as 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.

**Table 4-3** Total IgY content in egg yolk powder with different concentrations of three protectants

| | IgY content (mg/g) | | | |
|---|---|---|---|---|
| Protectants | 12.5% | 25% | 37.5%* | 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 |

*Significantly higher IgY content at protectants 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.

### 4.3.4 Long-term stability of EYP formulations

Figure 4-1 illustrates the long-term stability of EYP formulation 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.

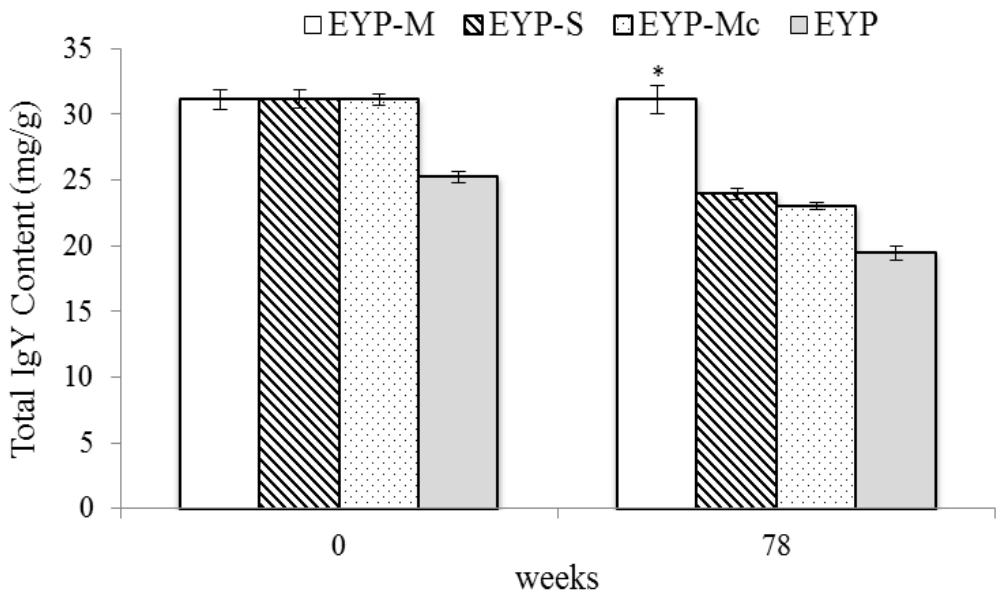


**Figure 4-1** Long-term stability of EYP capsules without and with protectants (EYP, EYP-M, EYP-S and EYP-Mc) at 37.5% (on dry basis) after 78 weeks storage. EYP, egg yolk powder; EYP-M, egg yolk powder mixed with mannitol; EYP-S, egg yolk powder mixed with sorbitol, EYP-Mc, egg yolk powder mixed with microcrystalline cellulose powder. * EYP-M formulation was significantly stable after 78 weeks ($p < 0.05$).

### 4.3.5 Dissolution test of EYP capsule under simulated gastric conditions

Figure 4-2 illustrates survivability of IgY in EYP, EYP-M, EYP-S and EYP-Mc in dissolution apparatus I, SGF 500 mL medium, paddle speed 100 rpm at dissolution time 0, 15, 30, 45, 60, 90 and 120 min**.** Gastro-resistance, and the release of IgY under simulated gastric condition was assessed by USP dissolution testing of the EYP capsule formulations in SGF for 2 hr. At each time interval, the samples were neutralized to inhibit pepsin activity, and IgY activity was determined by indirect ELISA (Figure 4-2).

After 2 hr 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 hr in SGF.

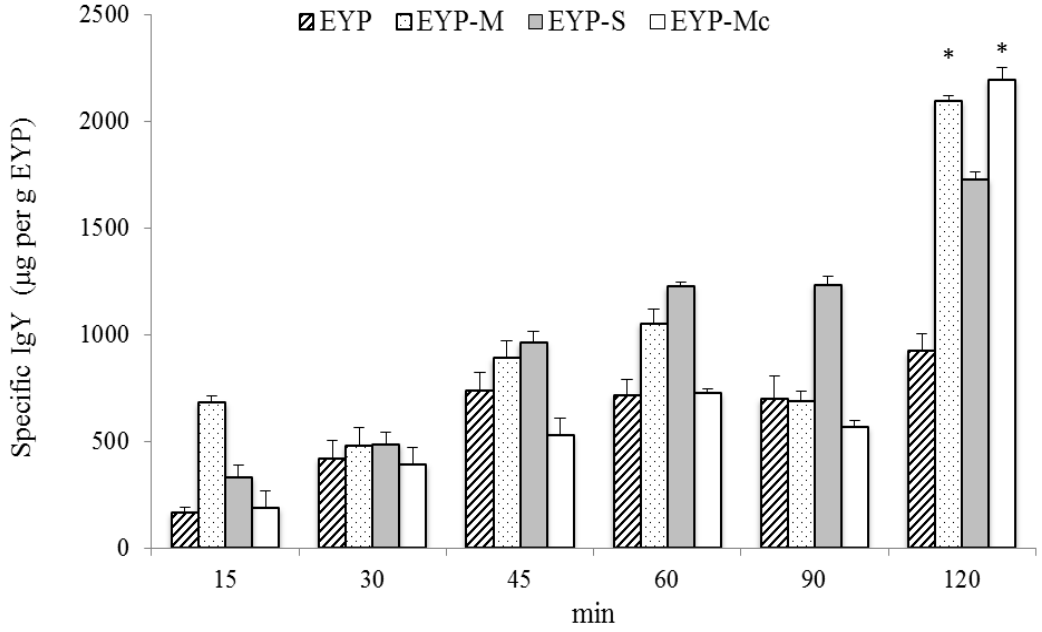


**Figure 4-2** Survivability of IgY in EYP, EYP-M, EYP-S and EYP-Mc in dissolution apparatus I, SGF 500 mL medium, paddle speed 100 rpm at dissolution time 0, 15, 30, 45, 60, 90 and 120 min. * EYP-M and EYP-Mc formulations had significantly protective effects on IgY antibodies in 120 min under simulated gastric fluid conditions ($p < 0.05$).

### 4.3.6 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. Figure 4-3 and Figure 4-4 illustrates *in-vitro* binding study determined by competitive ELISA of EYP-M containing anti-gliadin IgY antibodies to gliadin ranging from 0-1.28 g after 1 hr 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 amount 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 4-3a), while in the presence of food the EYP-M bound to 10.5 mg/mL gliadin was bound to

(Figure 4-3b). Under SIF condition, the same amount of EYP-M bound to 10 mg/mL gliadin, regardless of a food matrix (Figure 4-4).

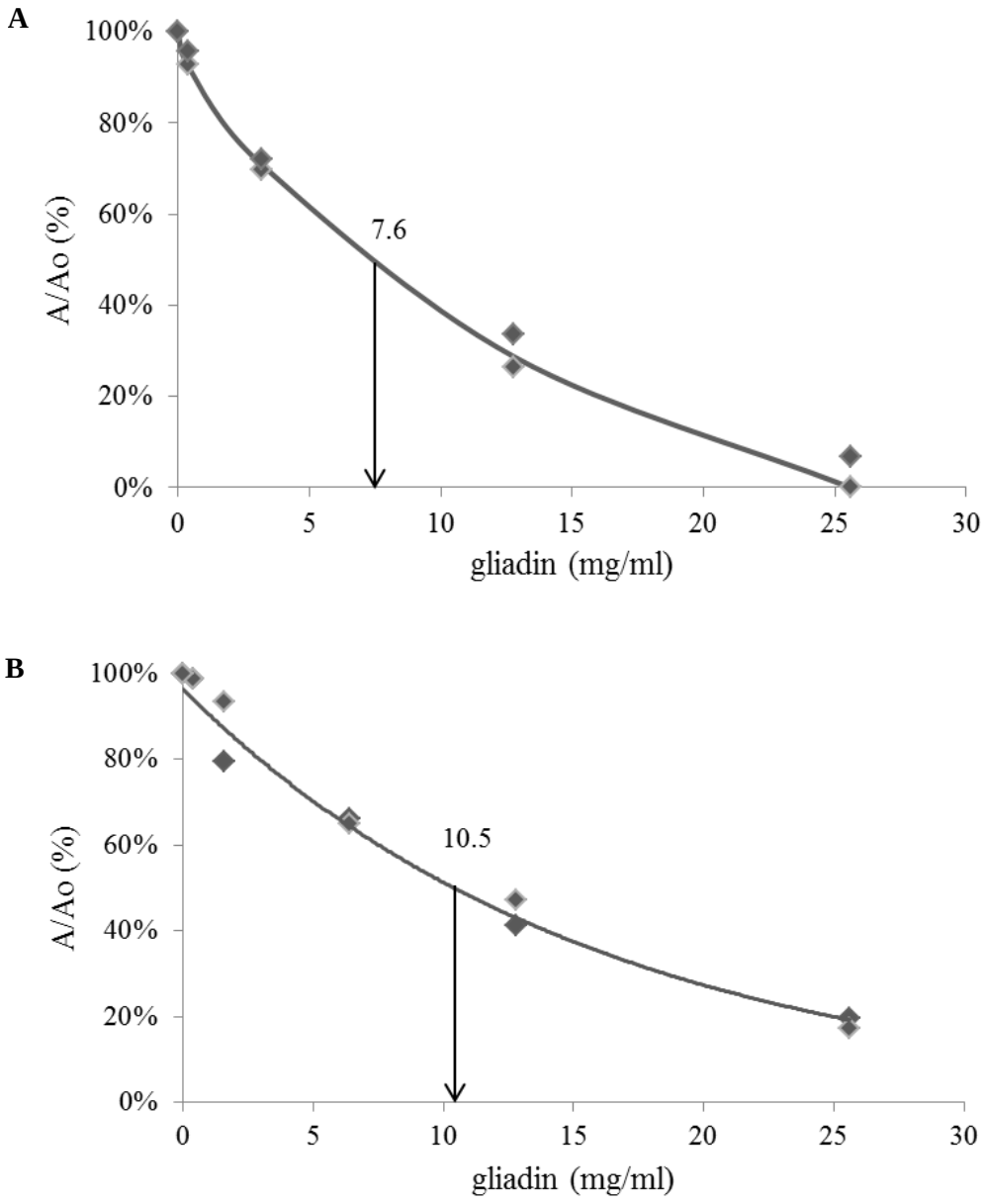


**Figure 4-3** *In-vitro* binding study determined by competitive ELISA of 0.08 g of EYP-M containing anti-gliadin IgY antibodies to gliadin ranging from 0-1.28 g after 1 hr exposure in SGF at 37 °C under **(A)** without food and; **(B)** with food conditions. A: absorbance of sample containing antibodies and gliadin; Ao: absorbance of control containing antibodies only. The arrows indicate 50% inhibition of control ($IC_{50}$).

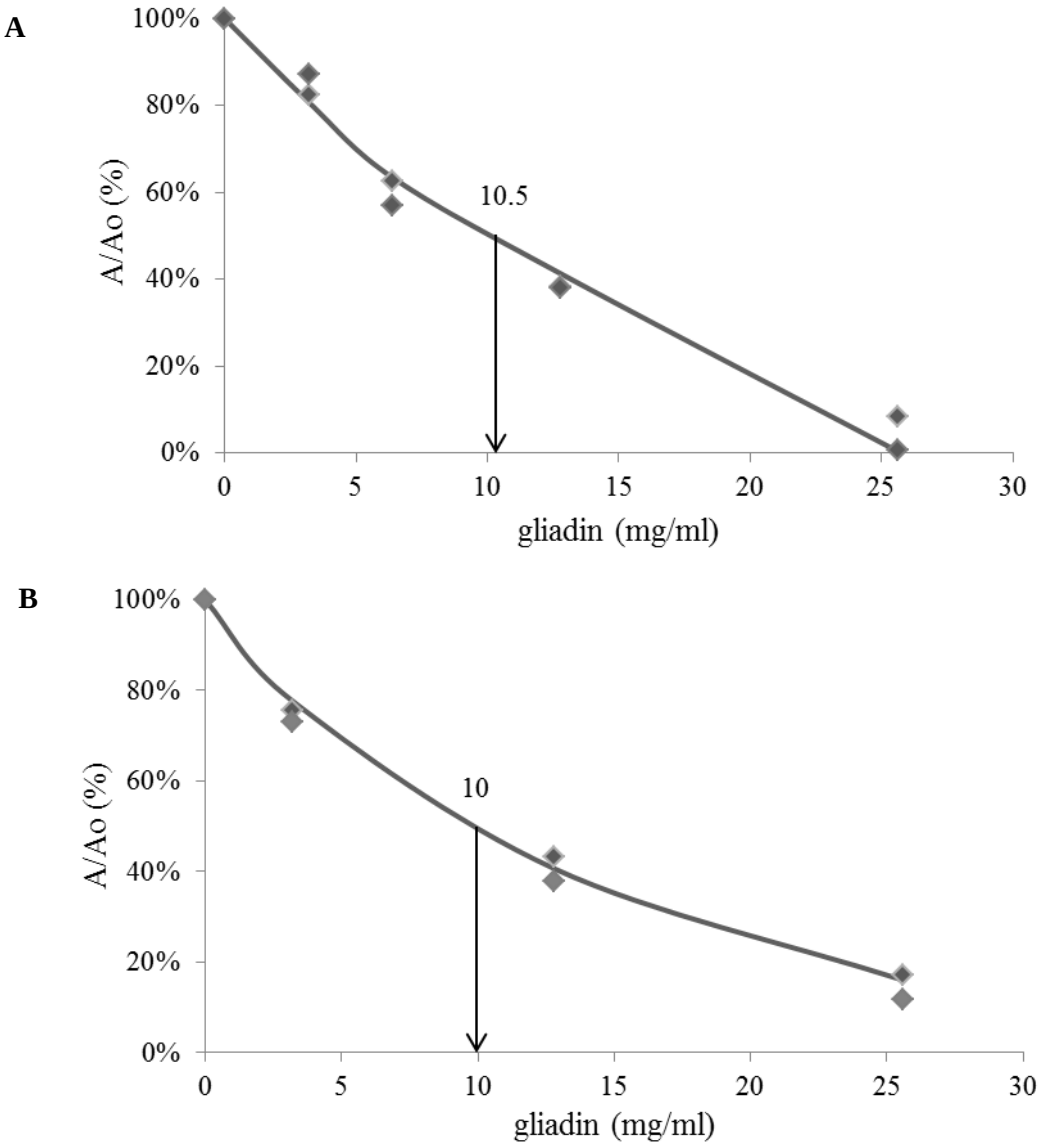


**Figure 4-4** *In-vitro* binding study results determined by competitive ELISA of 0.08 g of EYP-M containing anti-gliadin IgY antibodies to gliadin ranging from 0-1.28 g after 1 hr exposure in SIF at 37 °C under **(A)** without food and; **(B)** with food conditions. A: absorbance of sample containing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

### 4.3.7 *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 hr of feeding. Residual gliadin upon ingestion was determined at different sites along the GIT (stomach, small intestine and large intestine). Table 4-4 illustrates gliadin content in gastrointestinal tract after 12 hr 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.

**Table 4-4** Gliadin content in gastrointestinal tract after 12 hr feeding (the measurement of gliadin was assayed by heterosandwich ELISA)

| Groups | Feeding conditions | | Average gliadin remaining after 12 hr feeding (mg) | | | Residual gliadin (%) |
|---|---|---|---|---|---|---|
| | EYP (mg) | Gliadin (mg) | stomach | small intestine | large intestine | |
| 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* |
| D | 100 | - | N/A | N/A | N/A | N/A |

*Significantly reduced absorbed gliadin as compared to conditions A and B ($p < 0.05$)

Throughout the GIT, significantly more gliadin was retained in the lumen by increasing amount of EYP-M fed. The mice 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 1:25 and 1:5 to gliadin, significantly lower amount of gliadin was found to be residual in the GIT at 15.7% and 0.7%, respectively.

## 4.4 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 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 titre 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 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 long-term stability of 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 ºC for 52 weeks [21]. In another study [23], sorbitol was used during the spray dry 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 properties of IgG and not the actual antibody activity during the storage condition. In this study, IgY antibody content was determined during the 78 weeks of storage at room temperature. Among the 3 protectants, mannitol best stabilized the IgY antibody during 78 weeks of storage.

In order to effectively use anti-gliadin IgY as an oral immunotherapy to prevent gliadin absorption in the small intestine region, it needs to be active in the stomach and

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 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 enteric coating of IgY [16]. High concentration of sugar (30-50%) [13], or sorbitol (30% or more) [15], have 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 assure 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 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 hr 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 non-extracted egg yolk contains phospholipids and other proteins, which may have also contributed to additional gastroprotection effect.

For the first time the binding efficacy of anti-gliadin 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, 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 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 those of human of 20-30 hr [29]. The absorption of gliadin in experimental mice was found to achieve peak values between 2-3 hr after ingestion [30]. The transit time through the stomach is highly variable with an average time of 1 to 1.5 hr [31], and an average of 10 hr 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 hr 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 1:25 and 1:5 to gliadin, prevented 63.3% and 98.4% gliadin absorption, respectively, as compared to 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 co-administered with gliadin contained food. Further *in-vivo* studies need to be verified to prove this concept.

## 4.5 References


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# CHAPTER 5: Quantitative double antibody sandwich ELISA for the determination of gliadin in food

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## 5.1 Introduction

Glutens are plant storage proteins found in the seeds of cereal grains such as wheat, barley, rye, and oat. The cereal grains have been used as human food since ancient times. However, the ingestion of gluten causes celiac disease (CD) in genetically susceptible individuals. Due to the CD prevalence of one in 100-200 people in North America and European countries, CD is considered one of the most common gastro-intestinal diseases.

The only treatment available for CD individuals is a strict life-long gluten-free diet [1]. In reality, total avoidance of gluten intake is extremely difficult, due to hidden gluten because of contamination [2]. The threshold of prolonged gluten ingestion in CD individuals was determined to be lower than 50 mg per day [3]. The presence of hidden gliadin in contaminated food products represents an imminent risk, due to long term effect of regular ingestion of small amounts of gliadin [4].

Sensitive methods are needed to detect and quantify the presence of gliadin and other prolamins in foods. Currently, the enzyme-linked immunosorbent assay (ELISA) technique is the most commonly used method for food analysis. Various ELISA-based methods have been developed for the detection of trace amounts of gluten in foods [5-9]. Although monoclonal antibody (mAb) is extensively used in ELISA systems, the use of single mAb is not accurate due to the lack of two necessary epitopes when a peptide is hydrolyzed during the processing [10]. The peptide can be detected by exploiting the polyclonal antibody for its higher sensitivity due to its ability of binding to multiple epitopes. Whereas polyclonal antibodies have been traditionally developed in rodent and other mammalian species, the antibody raised in the chicken has unique advantages. Avian polyclonal antibody raised against different antigens produces chicken egg yolk IgY in abundant amounts compared to mammalian and rodent species. The IgY can also be obtained by non-invasive methods by collection of eggs. Thus chicken egg yolk IgY polyclonal antibody is alternatively very attractive and economical for the development of immunological detection assay [11], with limited information on the production of IgY antibody against gliadin.

The aim of this study was therefore to develop and validate a sensitive, specific and quantitative double antibody sandwich ELISA (DAS-ELISA) using polyclonal IgY as capture antibody and biotinylated mAb as detecting antibody. The DAS-ELISA was evaluated for detecting gluten content in Canadian gluten-free and wheat based food products.

## 5.2 Material and methods

### 5.2.1 Materials

All solvents and chemicals used in the present study were of analytical grade. Sigma gliadin (G-3375), bovine serum albumin (BSA), Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG, rabbit anti-chicken IgG conjugated with horseradish peroxidase (HP), NHS-LC-Biotin, and streptavidin-HP were purchased from Sigma (St. Louis, MO, USA). Electrophoresis Mini-Protean III, western blot apparatus, nitrocellulose membrane, DC and BCA protein assay and reagents were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Ontario, Canada). HYB-314 mAb (against p58-73 KLQPFPQPQLPYPQPQ of α-gliadin) and chicken anti-mouse IgG conjugated with HP were purchased from Thermo Fisher Scientific Canada (Burlington, Ontario, Canada). Tetramethylbenzidine (TMB) and 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrates were purchased from KPL (Frederick, MD, USA). Amicon Ultra-15 Centrifugal Filter Units (molecular weight cut-off, MWCO, 100 kDa) were purchased from Millipore (Billerica, MA, USA). Microtiter 96-wells plates were purchased from Costar Inc (Cambridge, MA, USA). The mechanical blender was purchased from Retsch GmbH & Co (Haan, Germany). The ELISA $V_{max}$ kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA).

### 5.2.2 Purification of anti-gliadin IgY antibody

Polyclonal egg yolk IgY antibody was raised against Sigma gliadin (used as gliadin standard in the ELISA) in chicken as previously described [11]. The anti-gliadin IgY antibody in the egg yolk was purified by salt precipitations using sodium sulfate 19% and 14%, followed by Amicon Ultra-15 Centrifuge filter units (MWCO, 100 kDa) to desalt and discard permeates. The purity of IgY was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduction conditions. Fractions were tested for binding activity using indirect ELISA, and total protein was determined by the Lowry method (DC protein assay). The purified IgY antibody was stored at 4°C until used.

### 5.2.3 Labeling of anti-gliadin monoclonal antibody

The mAb was biotinylated by using NHS-LC-Biotin conjugation kit according to the manufacturer's instructions. The proteins (500 µg) in phosphate buffered saline (PBS, pH 7.2) was covalently coupled to NHS-activated biotin (8 µg/100 µL in distilled water,

DW) and then incubated at room temperature (RT) for 4 hr. Unreacted biotin was removed by using ultracentrifuge filter units (MWCO, 10 kDa). The biotinylated mAb was stored at 4 °C until used.

### 5.2.4 Prolamin extraction and food sample preparation

Four grains (wheat, barley, rye and oat) and processed food samples purchased from local stores in Edmonton, Canada, were homogenized in a mechanical blender. Homogenized samples (1 g) were extracted with 2.5 mL cocktail solution (250 mM 2-mercaptoethanol, 2 M guanidine hydrochloride in PBS) for 1 hr in a shaking water bath. A 7.5 mL aliquot of 80% (v/v) ethanol was added to the solution. The mixture was vortexed and subsequently rotated at RT for 30 min. Extracts were centrifuged at 2,500 × *g* at RT for 10 min. The supernatants were analyzed for protein content by BCA protein assay with BSA as the reference at the absorbance of 562 nm.

### 5.2.5 Western blot assay

The Mini-Protean III apparatus was used for electrophoretic separation of prolamin extracts from four grains by SDS-Urea electrophoresis, according to the method of LaemmLi [12]. Prolamin extracts and Sigma gliadin were solubilized in 6 M urea and applied in equal amounts (10 µg). Separation was performed at 200 V for 1 hr, in pre-cooled running buffer (pH 8.3, 0.025 M Tris, 0.192 M glycine, 0.1% SDS), using 10% separating gel. Samples were prepared with four volumes of sample solution in 6 M urea and one volume of sample buffer (pH 6.8, 0.0625 M Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% Bromophenol blue).

The proteins were electrophoretically transferred from gel onto a nitrocellulose membrane for 1 hr at 100 V with transfer buffer (pH 8.5, 120 mM glycine, 120 mM Tris). The membrane was blocked with 3% BSA in Tris buffer (pH 8.0, 5 mM Tris-HCl, 0.15 M NaCl,) at RT for 1 hr. After blocking, the membrane was incubated with anti-gliadin IgY (10 mg/mL, 1:1,000 dilution in Tris buffer with 0.05% Tween-20, Tris-T) at RT for 2 hr. The membrane was then washed 5 times with 0.5% BSA in Tris at RT for 30 min. The primary antibody bound on the membrane was incubated with either rabbit anti-chicken IgY conjugated with HP (1:2,000 dilution in Tris-T) or chicken anti-mouse IgG HP (1:4,000 dilution in Tris-T) at RT for 1 hr, respectively. After washing the membrane, TMB substrate was applied on the membrane to develop a color. The reaction was stopped by washing the membrane with ultra-purified water.

### 5.2.6 Biotinylated double antibody sandwich ELISA

Unless indicated otherwise, all incubations were performed at 37 °C with four times washing by PBS-T in each step. Wells were coated with 100 µL of anti-gliadin IgY (10 µg) in PBS at 4 °C for 12 hr. Non-specific binding sites were blocked with 120 µL of 5% BSA for 45 min. One hundred µL/well of a 2-fold serial dilution of the Sigma gliadin was added in a concentration range from 0.625 to 80 ng/mL. Standards, buffer blanks and samples extracts were incubated for 1 hr in triplicates on each plate. Bound gliadins were detected by adding 100 µL/well of biotinylated mAb (1:5,000 dilutions in PBS) after incubation for 1 hr. Plates were subsequently incubated with 100 µL/well of streptavidin-HP conjugate, diluted 1:5,000 for 1 hr. After washing, 100 µL/well of freshly prepared TMB substrate was then added to each well and incubated for 5 min. Absorbance was read at 650 nm on ELISA reader.

### 5.2.7 Assay validation

All extracts from wheat, barley, rye, oat, corn and rice were 1:20 ELISA assay buffer, which corresponding to a portion of 100% in a typical food matrix, prior to analysis with the DAS-ELISA.

The accuracy of the method was evaluated by performing recovery studies. Three different gluten-free food samples (pasta, bread, and rice) were spiked with Sigma gliadin standard at levels of 1 µg, 10 µg and 100 µg per gram sample in a total volume of 10 mL extraction buffer, as previously described. For the determination of recovery rates, the extractions were done in triplicates and analyzed by the DAS-ELISA, and the mean values for the recovery were calculated. Intra-assay (within plate) precision was determined as the mean coefficients of variation (CV) based on 10 replicates. Inter-assay (between plates) precision was determined as the mean CV on the basis of triplicate analyses on 10 plates.

The limit of detection (LOD) was calculated from 3 times standard error of the predicted Y-value for each X in a regression divided by slope. Additionally, the LOD in three gluten-free food samples was determined in the same way, on the basis of five experiments. Linearity and range of detection were determined from serially diluted Sigma gliadin (1-80 ng/mL) standard curve plot.

## 5.3 Results

### 5.3.1 Production of IgY antibody

Chickens were immunized with Sigma gliadin for the production of IgY antibody. The Anti-Sigma gliadin IgY activities were at plateau during week 5 to 7, giving high titre of anti-gliadin IgY antibody. Pooled eggs with high titre were partially purified with water dilution method, followed by salt precipitation and ultracentrifugation. The purified IgY fraction was subjected to purity determination and quantification of specific IgY titre. Total protein content of the purified IgY was 10% higher than the total IgY content, hence the IgY fraction showed 90% purity. Total IgY was found to be approximately 1.1 times greater in the immunized chickens than in the non-immunized chickens (Table 5-1). The following results also showed that both protein and total IgY concentrations were similar between the anti-gliadin and non-specific IgY. As a result, the proportion of gliadin-specific IgY in the total egg yolk IgY was 7.9% and 0.6% in the gliadin IgY and non-specific IgY, respectively (Table 5-1).

**Table 5-1** Concentrations of protein, total IgY, and specific IgY in purified IgY solution by ultra-centrifuge filtering

| IgY | Concentration (mg/g) | | |
|---|---|---|---|
| | Protein | Total IgY | Specific IgY |
| Anti-Sigma gliadin | 150.1 ± 1.5 | 136.4 ± 2.9 | 10.77 ± 0.045 |
| Non-specific IgY | 140.5 ± 1.7 | 127.5 ± 4.4 | 0.79 ± 0.005 |

Values are the mean of quadruple samples ± SD.

### 5.3.2 Detection of prolamins from four grains

The polyclonal anti-gliadin IgY antibody was used to determine the IgY binding prolamin bands in the extracts of four grains (wheat, barley, rye, and oat) using western blot analysis (Figure 5-1).

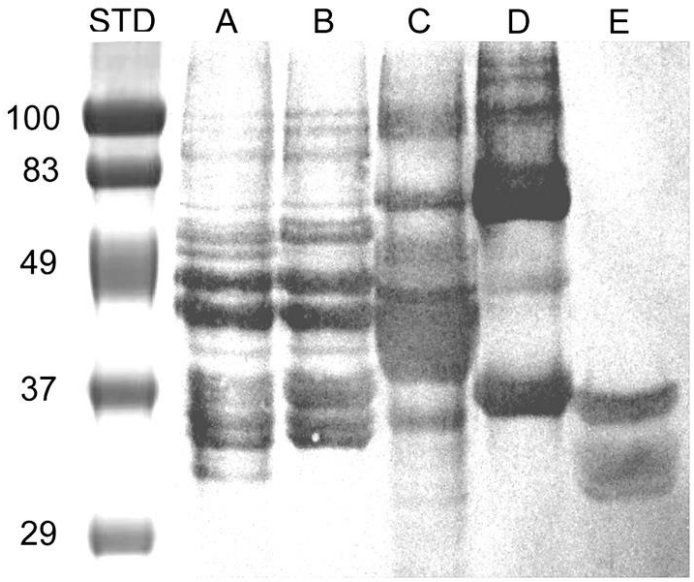


**Figure 5-1** Western blot analysis of prolamin extracts of four grains using purified polyclonal anti-gluten IgY antibody. Lane STD: molecular weight marker, protein sizes (kDa) are indicated on the left side of the paper. Lane A: Sigma gliadin; Lane B: wheat gliadin; Lane C: barley hordein; Lane D: rye secalin; Lane E: oat avenin.

Major IgY-binding bands were found at 34-38 kDa and at 42-48 kDa in wheat gliadin (Figure 5-1, lane B), corresponding to Sigma gliadin (Figure 5-1, Lane A). Similar IgY-binding bands with 38-48 kDa were found in barley (Figure 5-1, Lane C). Two different bands with 35-38 kDa and 60-74 kDa were detected by IgY in rye (Figure 5-1, Lane D). Lower MW IgY-binding bands with 30-34 kDa were found in oat (Figure 5-1, Lane E). Whereas IgY could react as prolamin-binding antibody in four grains, the mAb used in this study was found to strongly react with 34-38 kDa in wheat, barley, and rye.

### 5.3.3 Specificity

The cross-reactivity of anti-gliadin IgY was examined by indirect ELISA (Table 5-2) According to the OD value of the indirect ELISA, there was cross-reactivity of anti-gliadin IgY to barley hordein, rye secalin and oat avenin at 91%, 54% and 43%, respectively. Anti-gliadin IgY showed significantly low cross-reactivity (<1%) against rice and corn. The mAb reacts with prolamins from wheat, barley and rye, showing no cross-reaction to oat.

**Table 5-2** Cross reactivity of anti-sigma gliadin IgY to prolamins by indirect ELISA

| Grains | Anti-Sigma IgY | Percentage |
|---|---|---|
| Wheat | 1.144 ± 0.041 | 99.3 |
| Barley | 1.050 ± 0.082 | 91.32 |
| Rye | 0.624 ± 0.051 | 54.17 |
| Oat | 0.492 ± 0.038 | 42.71 |
| Rice | 0.011 ± 0.003 | 0.95 |
| Corn | 0.009 ± 0.002 | 0.78 |

Values are the mean of quadruple samples ± SD.

Percent cross reactivity was calculated by reference to 100% reactivity between Anti-Sigma gliadin IgY antibody and Sigma gliadin.

### 5.3.4 Double antibody sandwich ELISA standard curve

Sigma gliadin was used in a standard curve with concentrations from 1 ng/mL to 80 ng/mL. The working range of the assay was the linear part of the curve with a squared correlation coefficient ($R^2$ = 0.98). The six-point calibration curves as shown in Figure 5-2 ranged from 4 ng/mL to 40 ng/mL.

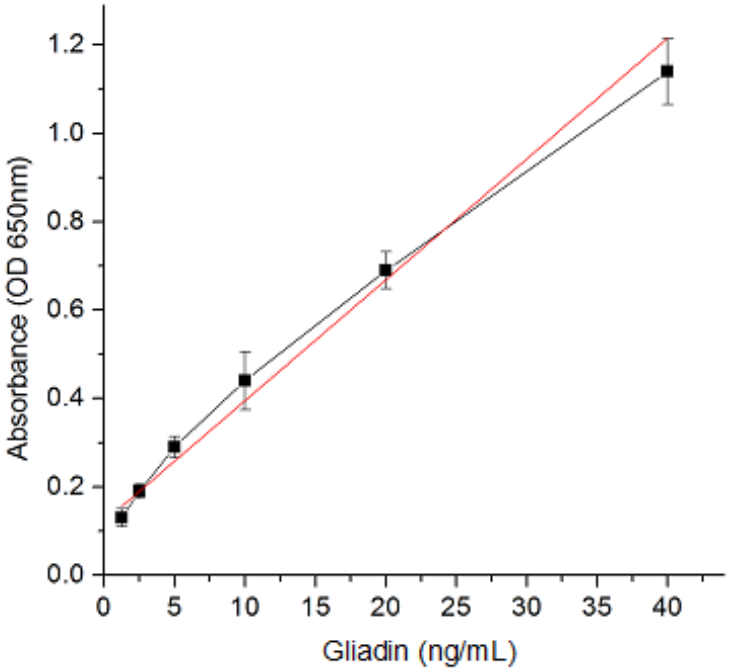


**Figure 5-2** Biotinylated DAS-ELISA using anti-gliadin IgY as a capture antibody and biotinylated mAb as a detection antibody. This assay was performed in ten replicates. Vertical bar indicates standard deviation. Straight line indicates the linear fit at "y = 0.0274x + 0.1215, $R^2$ = 0.98".

For determination of gliadin concentrations in foods, extracts from food samples were diluted accordingly to obtain OD values closest to the midpoint of the linear part of the standard curve. The OD values were used to calculate the gliadin concentrations.

### 5.3.5 Accuracy

Blank gluten-free food samples, spiked with three different amounts of Sigma gliadin before sample extraction, were used to determine the recovery rates as shown in Table 5-3. The recovery of gliadins in the gluten-free food samples was between 82% and 98%, independent of spiking level. The blank gluten-free food extracts without gliadin spikes contained less than 0.5 µg gliadin/g food sample (0.5 ppm).

**Table 5-3** Recovery (%) of gliadin from gluten-free samples spiked with 1 µg, 10 µg, and 100 µg/g of sigma gliadin protein

| Gluten-free foods | Recovery (%) of Sigma gliadin spiked in µg/g | | |
|---|---|---|---|
| | 1 | 10 | 100 |
| Bread | 82 $\pm$ 15 | 90 $\pm$ 11 | 95 $\pm$ 11 |
| Pasta | 83 $\pm$ 19 | 92 $\pm$ 10 | 96 $\pm$ 9 |
| Rice | 85 $\pm$ 19 | 95 $\pm$ 8 | 98 $\pm$ 7 |

Values represent the average of three spiking experiments and are reported as mean $\pm$ standard error of the mean (SEM).

### 5.3.6 Intra- and inter-assay precision

Grains granular bar, whole wheat bread, chocolate chip muffin, instant chicken noodle, spaghetti, and all-bran flakes were analyzed by the DAS-ELISA to determine the content of gliadin ranged from 3.2 to 36 ppm. These products were used for the determination of the intra-assay precision and inter-assay precision of the DAS-ELISA (Table 5-4). The intra-assay expressed as percentage of coefficients of variation (%CV) was 7.25% average of six food samples. The inter-assay precision was 9.51 % in food samples.

**Table 5-4** Intra- and inter-assay variances (%CV) determined for the gliadin DAS-ELISA using gliadin containing foods

| Foods | Gliadin (μg/g) | Intra-assay variance (% CV) | Inter-assay variance (% CV) |
|---|---|---|---|
| Grains granola bar | 7.5 | 8 | 11 |
| Whole wheat bread | 7.4 | 7 | 9 |
| Chocolate chip muffin | 5.7 | 8 | 12 |
| Instant chicken noodle | 36.0 | 9 | 13 |
| Spaghetti | 10.3 | 9 | 11 |
| All bran flakes | 3.2 | 9 | 9 |

### 5.3.7 Limit of detection

The LOD of the DAS-ELISA corresponds to 4 ng/mL Sigma gliadin standard in PBS, equivalent to 0.8 μg/g sample (0.8 ppm), considering sample dilution factor of 200. At lower concentrations a reproducibility and repeatability of gliadin content was not validated.

## 5.4 Discussion

Celiac disease is an autoimmune disease of the small intestine caused by the ingestion of gluten proteins from widely prevalent food sources such as wheat, rye, and barley. In many human leukocyte antigen (HLA) DQ2 (or DQ8)–positive individuals, exposure of the small intestine to gluten induces an inflammatory response, leading to destruction of the villous structure of the intestine [13]. The ingestion of gliadin causes malabsorption syndrome [14], clinical symptoms of an autoimmune attack [15], and skin reactions [16]. Among gliadin peptides, a 33-mer peptide from α-gliadin was responsible for initiation of the inflammatory response for CD patients [17]. In this study we developed heterosandwich ELISA with the use of both mouse mAb and chicken IgY for the achievement of a highly sensitive detection system for gluten in foods, targeting the gliadin antigen. The toxic gliadin is highly immunogenic to chickens and induces the production of greatly specific anti-gliadin IgY in the yolk. The IgY antibodies present

2% in egg yolk, indicating that the rest of the component includes α and β-livetins, lipoproteins and fatty acid molecules, which could reduce the sensitivity of ELISA. To accomplish this, highly purified egg yolk IgY was obtained by elimination of fat and other proteins and used as a reagent for western-blot assay and DAS-ELIA.

In our previous study, the IgY base qualitative Immunoswab assay developed was as sensitive as 1.25 μg/mL, which was obtained from 60% ethanol solution. In this study, we targeted on the development of quantitative detection for gliadin in grains and gluten-free and contained processed foods by specific and sensitive DAS-ELISA. The ELISA was successfully used to quantify extracted gliadin, in both gliadin-spiked gluten free foods and in various commercial foods known to contain wheat gluten. The DAS-ELISA was constructed using purified polyclonal IgY as the capture antibody and biotin labeled mAb (HYB 314) as detecting antibody. The anti-gliadin IgY recognized α/β/γ-gliadin at 30-45 kDa and ω-gliadin at 39-55 kDa [18, 19]. There are several reports regarding the proportion of specific IgY in total IgY when chickens were immunized with various antigens. The proportion of bovine proteoglycan-specific IgY in the total IgY was 9.0% [20]. Similarly, the proportions of bovine and human lactoferrin-specific IgY in the total IgY were 8.7% and 9.2%, respectively [11]. In this study, our results showed 7.9% of the specific anti-gliadin IgY concentration in total IgY by quantitative ELISA. The use of purified specific anti-gliadin IgY antibody could have resulted in higher specificity by maximizing the detection of gliadin in complex food matrixes. A single mAb based ELISA has the drawback that it may not detect altered or destroyed epitopes during the food processing. This remains to be evaluated for gliadin, but in general, it is accepted that the combination of mAb and polyclonal antibody is favorable for gliadin analysis and resulted in higher specificity. The observed cross-reactions of anti-gliadin IgY antibody by indirect ELISA are obviously due to similar epitopes on the different four grains, which are highly found in barley hordein and less in rye secalin and oat avenin. Western blot analysis using the anti-gliadin IgY antibody was performed to confirm the ELISA data. The possibility of cross-reaction with other less commonly used grass family, sorghum, millet, wild rice, etc., cannot be excluded and remain to be examined. A rabbit polyclonal IgG antibody used to detect wheat gliadin cross-reacted with oat, rice and maize prolamins [21]. On the other hand, the specificity of the mAb was also tested against four grains by ELISA and western blot assay (data not shown). The mAb (HYB 314) used in this study recognizes residue p58-73 (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) of 33-mer peptide of

prolamins from wheat, barley and rye in the range of 34-38 kDa, demonstrating no cross-reaction to oat avenin. PN3 [6], CDC5 [8], and G12 [9], mAbs react with prolamins in wheat, barley, rye and oat, whereas R5 reacts with prolamins from wheat, barley and rye only [7].

The key parameters and characteristics of the DAS-ELISA were further subjected to in-house validation. The accuracy of the method was studied with recovery experiments, by adding varying amounts of Sigma gliadin standard to three different blank gluten-free food matrixes such as bread, pasta and rice cake. The variances were highest at the lowest amounts of spiked Sigma gliadin (1 µg/g), probably because of poor sample homogeneity at this low level. The recovery of Sigma gliadin in matrixes was almost complete at higher dose of Sigma (100 µg/g), whereas it was reduced by 15% from lowest amount of spiked Sigma gliadin. The results agree with the common assumption that food matrix components may interfere with either the extraction or the immunoassay procedures leading to suboptimal recovery rates. A full validation of the extraction procedure remains to be performed. The recovery of the gliadin in DAS-ELISA was considered to be satisfactory. The intra-assay and inter-assay precision results for DAS-ELISA were obtained from gliadin contents of six relevant gliadin-containing wheat grain based foods, ranging from 3.2-36.0 ppm. The repeatability of the DAS-ELISA as measured by intra-assay precision was 7.25%, and the reproducibility as measured by interassay precision was 9.51%. The performance of the present DAS-ELISA is 0.8 ppm gliadin in terms of sensitivity, which is lower than the US Food and Drug Administration limit (August 2011) of <20 ppm gluten (equivalent to 10 ppm gliadin) for gluten-free foods. The three Canadian gluten-free labeled products quantified, containing less than 10 ppm gliadin, met the proposed FDA rule for gluten-free labelling. The developed DAS-ELISA showed less sensitivity to detect gliadin in foods compared to other ELISA systems. For instance, mAb to the α-gliadin motifs QQQPFP of 33-mer shows a LOD of 1.56 ppm gliadin [7]. Competitive ELISA systems using PN3 mAb antibody showed LOD of 1.2 ppm gliadin [22]. Whereas homosandwich ELISA using mAb as both capture and detection antibodies may not detect hydrolyzed gliadin in processed foods due to single targeting of the gliadin fractions, heterosandwich ELISA with polyclonal antibody and mAb detected the gliadin at LOD of 3.3 - 15 ng/mL [23, 24]. In this study we have significantly lowered the detection limit to 0.8 ppm, which should allow detection of trace amount of gliadin in various foods including gluten-free foods by using a combination of IgY and mAb.

The DAS-ELISA for the quantification of gliadin in foods was developed using anti-gliadin IgY as a capture antibody and biotinylated mAb (HYB 314) as a detection antibody. It is capable to detect trace amounts of gliadin as low as 0.8 ppm in food. The use of IgY as a capture antibody reduces the cost as compared to mAb coated on ELISA plates. Therefore, the combination of anti-gliadin IgY and biotinylated mAb (HYB 314) provides a broad range of detection, increases sensitivity, and reduces cost of reagents used in the detection system, making it suitable for the use as a quality control tool in laboratories, food manufacturing industries and by regulatory agencies to perform detection of gliadin in both gluten-free and gluten-contained food products.

## 5.5 References


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# CHAPTER 6: Qualitative detection of gliadin in food: Rapid Immunoswab and Immunostrip assays

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## 6.1 Introduction

Celiac disease (CD) individuals have loss of villous structure in their proximal small intestine mucosa upon consumption of gluten. Destruction of the villi, hinders the absorption of nutrients from food into the bloodstream, leading to malabsorption syndrome. The only practical treatment so far is to avoid all foods that contain wheat, rye, barley and possibly oats [1].

As every CD individual has different tolerance levels to gluten ingestion, a safe daily intake of gluten cannot be set. Thus, a testing system for identifying wheat gliadin (as well as the other cereal prolamins) in a gluten-free diet is needed to ensure a safe diet for CD patients who present a broad range of sensitivity to gluten intake, with clinical manifestations to minimal amounts of gliadin reported [2].

The European Union, World Health Organization and Codex Alimentarius (Revised Standards for gluten-free foods In Report of 25th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses, 2003) require reliable measurement of gliadin, rather than all wheat-derived proteins, which include albumins, globulins and starch granule proteins. The official limits described in the Codex Draft Revised Standard (2000) are 20 ppm for foodstuffs naturally gluten-free and 200 ppm for foodstuffs rendered gluten-free. As part of the Food Allergen Labeling and Consumer Protection Act of 2004, the US Food and Drug Administration issued a final rule in 2009, defining a gluten-free food for the food containing < 20 ppm gluten [3].

Immunochemical tests are reliable for the detection of gliadin in food since they provide specific, sensitive recognition of gliadin, based on mammalian polyclonal and monoclonal antibodies. Accordingly, many immunoassays were developed for gliadin detection based on antibody and antigen interaction such as Enzyme Linked Immunoassay (ELISA) [3-8], and competitive ELISA methods [9-12].

While mammalian antibodies against gliadin are extensively used in immunoassays, chicken egg yolk antibodies (IgY) are very attractive and economical for the development of immunological test systems. Immunized hens transfer high amount of IgY (200 mg) into their eggs, which can then be non-invasively obtained by simple collection methods [13]. However, there is no report on the immunization of chickens with gliadin peptides although chickens can produce more specific antibodies against gliadin than mammalian species.

This study was therefore undertaken to examine the use of antibodies raised in the chicken as a reagent to detect gliadin based on immunoassay technique. In this study

the chicken IgY antibody was used for the development of Immunoswab and Immunostrip assays using IgY as a capture antibody and /or detection antibody. The new detection systems were then evaluated for their reliability and sensitivity for a gluten intolerance health purpose.

## 6.2 Materials and methods

### 6.2.1 Materials

Crude Sigma gliadin (G-3375), Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG and rabbit anti-chicken IgG conjugated with horseradish peroxidase (HP), tetrachloroauric acid (gold chloride) were purchased from Sigma (St. Louis, MO, USA). HYB-314 monoclonal antibody and chicken anti-mouse IgG conjugated with HP were purchased from Thermo Fisher Scientific Canada (Burlington, Ontario, Canada). Blue dextran was purchased from Pharmacia Biotech Inc., (Baie-d'Urfe, QC, Canada). Tetramethylbenzidine (TMB) and 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrates were purchased from KPL (Frederick, MD, USA). Bio-Rad protein assay kit was purchased from Bio-Rad Laboratory (Mississauga, ON, CA). Sephacryl S-300 gel filtration column and CNBr-activated Sepharose 4B were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ, USA). Microtiter 96-wells plates were purchased from Costar Inc. (Cambridge, MA, USA). The ELISA $V_{max}$ kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA).

### 6.2.2 Preparation of antigen

The isolation of gliadin was performed according to the method previously described [14]. Briefly, ten grams of crude Sigma gliadin were defatted with butanol under stirring condition for 1 hr. After centrifugation (5000 rpm for 15 min), the residual solvent was removed by evaporation in a vacuum drier at room temperature. The step was repeated once. Albumins and globulins were extracted three times with 40 mL of 0.4 M NaCl from the defatted crude Sigma gliadin. The suspension was stirred for 20 min, centrifuged (4,500 rpm) for 30 min at room temperature, and subjected to gliadin extraction twice with 40 mL 60% (v/v) aqueous ethanol from the sediment. One mg of purified gliadin was diluted to 10 mL with deionized water. The solutions containing purified gliadin were then analyzed for protein content by the method of Bio-rad protein

assay with bovine serum albumin (BSA) as a standard at the absorbance of 595 nm. This extracted gliadin was used for the immunization of chickens and further studies.

### 6.2.3 Immunization of chickens

Laying hens were handled in accordance with the guidelines of animal welfare of the Canadian Council on Animal Care approved by the Animal Care and use Committee of University of Alberta (Protocol Number 098/10/10). Sigma gliadin (200 µg/mL protein) was suspended in sterilize phosphate buffered saline (PBS, pH 7.2) and emulsified with an equal volume of Freund's incomplete adjuvant. Eight 23-weeks-old Single Comb White Leghorn chickens were intramuscularly injected with the emulsified saline, with or without gliadin at four different sites (0.25 mL per site) in the breast muscles (two sites per left and right breast muscle). A booster immunization was given after two weeks and four weeks of the initial immunization. Eggs were collected daily and stored at 4 ºC until the extraction of the antibodies.

### 6.2.4 Purification of IgY antibody

Polyclonal egg yolk IgY against the purified gliadin was produced in laying hens during the immunization period. 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 hr. 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 of high titre was further purified by ammonium sulfate precipitation (60%) followed by Sephacryl S-300 gel chromatography. A portion (1 mL containing 10 mg protein) of water soluble fraction was fractionated by using a 1.0 × 110 cm column of Sephacryl S-300 which was equilibrated and eluted with phosphate buffered saline (PBS, 0.15 M NaCl, 0.0027 M KCl, 0.0081 M disodium phosphate, and 0.0015 M monopotassium phosphate, pH 7.2) at a flow rate of 3 mL/hr. Blue dextran and titrated water were used to determine void volume (Vo) and total volume (Vt) of the column, respectively. The partition coefficient was calculated from the formula: Kav = (Ve-Vo) /

(Vt-Vo), in which Ve represents the volume of the peak fraction. The eluates (1 mL) were analyzed for protein at 280 nm absorbance and IgY activity by ELISA. The eluates of IgY were pooled, freeze-dried, and analyzed for protein content, total IgY, and specific IgY. All chromatography data presented in this report represent the average of 3 experiments.

The fraction containing 180 kDa was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels under non-reducing conditions (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and stained with Coomassie Brillant Blue.

### 6.2.5 Indirect ELISA for anti-gliadin IgY quantification

Microtiter plates were coated with 100 µL of gliadin (10 µg/mL of 60% ethanol) and incubated at 37 °C for 1 hr. The plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-T), filled with 120 µL of 3% BSA solution (w/v) in PBS-T for each well, and incubated at 37 °C for 45 min. The BSA solution was then discarded and the wells were washed four times with PBS-T. To each well, 100 µL of WSF (diluted 1:1,000 in PBS-T) or column fraction (diluted 1:1,000 in PBS-T) was added as a specific IgY, and non-specific IgY as a control prior to incubating at 37 °C for 1 hr. After washing the plates with PBS-T for four times, 100 µL of rabbit anti-chicken IgY conjugated with HP (diluted 1:5,000 in PBS-T) was added to incubate at 37 °C for 90 min. The plates were then washed again four times with PBS-T to receive 100 µL of freshly prepared substrate solution, ABTS in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. Optical density reading at 405 nm (OD 405) was taken after 30 min using an ELISA Vmax kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the control antibody from that of specific antibody.

### 6.2.6 Isolation of anti-gliadin IgY

The enzymatic digestion of gliadin was carried out essentially as described by Bronstein et al [15]. One gram of gliadin was dissolved in 10.0 mL 0.1 mol/L HCl, 20 mg pepsin was added and after digestion (2 hr, 37 °C) pH was adjusted to 8-0 by 5.0 mol/L NaOH. The gliadin was then further digested with 20 mg trypsin (4 hr, 37 °C) at constant stirring. Inactivation of trypsin was achieved by heating (90 °C, three minutes). Insoluble

material was removed by centrifugation (10,000 x g, 30 minutes). The PT-gliadin was prepared within 24 hr before use and kept at room temperature.

One gram of sepharose is pretreated and washed several times with 200 mL 1 mM HCl for 15 min on a sintered glass filter (porosity G3). A total amount of 15 mg protein (PT-gliadin dialysed for 16 hr at 4 °C against the coupling buffer (0.1 M $NaHCO_3$ (pH 8.3), 0.5 M NaCl) was coupled to the pretreated CNBr-activated Sepharose 4B resin in 5 mL coupling buffer by gentle mixing for 1 hr at RT or overnight at 4 $^{o}$C, then washed away excess ligands with 25 mL of coupling buffer. The remaining active groups are blocked with 0.1 M Tris pH 8.0, let it stand for 2 hr, and then washed 3 times with acid and base alternating pH: 0.1 M NaAc (pH 4.0), 0.5 M NaCl and 0.1M Tris (pH 8.0), 0.5 M NaCl. After washing the beads with 10 mL PBS, the freeze dried IgY fraction from gel chromatography was dissolved in 5 mL PBS at 5 mg/mL concentration, filtered through 0.22 µm, added to the beads and rolled overnight at 4 ºC. The beads are washed with 10 mL PBS and packed in a 1.5 cm x 1.3 cm column and carefully washed with a 50 mmol/L Tris/HCl (pH 8.0), 0.15 mol/L NaCl buffer. The antibody is eluted with 0.1 M glycine pH 2.4 in 1 mL fractions, immediately added to 30 µL 3 M Tris pH 8.8 and 20 µL 5 M NaCl. The IgY fractions containing reactivity against PT-gliadin were pooled and stored at 4 °C after the addition of sodium azide. The final IgY concentration was determined spectrophotometrically from the absorbance at 280 mm.

### 6.2.7 Preparation of bread sample stock solution

Wheat bread (1 gram) was blended in a food blender with 500 mL of 60% ethanol for 5 min at room temperature. The mixtures were centrifuged for 10 min at 2,500 × g at room temperature. One mL of the supernatant was serially diluted with PBS and 60% ethanol into eppendorf tubes for DAS-ELISA and Immunoswab assay, respectively.

### 6.2.8 Double antibody sandwich ELISA for gliadin detection

Unless indicated otherwise, all incubations were performed at 37 °C with four times washing by PBS-T in each step. The assay was carried out with the purified gliadin-specific IgY as a capture antibody and gliadin-specific mAb HYB-314 as a detection antibody. The mAb was raised against a synthetic peptide corresponding to residues KLQPFPQPELPYPQPQ of alpha gliadin peptide (58-73). Wells were coated with 100 µL of gliadin-specific IgY (100 µg/well) in PBS at 4 °C overnight. Nonspecific binding sites were blocked with 120 µL of 5% BSA for 45 min. The 100 µg of purified

gliadin was dissolved in 1 mL of 60% ethanol. One hundred µL aliquot of each serially diluted sample (1.28 µg – 0.625 ng) in PBS was added to triplicate wells and incubated at room temperature for 1 hr. Then, 100 µL of gliadin specific mAb (diluted 1:2,000 in PBS-T) was added to each well and incubated at 37 °C for 1 hr. After washing, 100 µL of chicken anti-mouse IgG conjugated with HP (diluted 1:5,000 in PBS-T) was added to each well and incubated at 37 °C for 1 hr. Finally each well was incubated with 100 µL of freshly prepared TMB peroxidase substrate for 5 min at room temperature. Optical density reading at 650 nm was taken by an ELISA Vmax kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of the control antibody from that of specific antibody.

### 6.2.9 Assay quality control

Within-plate and between-plate precision profiles were constructed using, respectively, six replicate standard curves on one plate, and six replicate plates, each with triplicate standard curves. The performance of assay has been evaluated by precision studies determining the coefficient of variation.

### 6.2.10 Immunoswab assay for gliadin detection

Different concentrations of gliadin antigens were spiked in 60% ethanol and aliquoted in eppendorf tubes (50 µL). The tagged calgiswab was mixed with 50 µL of gliadin specific IgY capture antibody (100 µg/mL) in PBS pH 7.4 to incubate and dried for 10 and 5 min, respectively, at room temperature. The mixture was then fixed with 50 µL of 95% ethanol for 1 min and dried for 5 min at room temperature. Swabs were then blocked with 5% BSA (50 µL) for 10 min at room temperature and washed with PBS (0.05% BSA) at pH 7.4 for 5 times by simple fill and aspiration steps in a test tube. The washed swabs were then incubated for 5 min with different concentrations of gliadin antigen spiked in ethanol solution (60%). The swabs were then incubated with gliadin specific mAb antibody (HYB 314) (10 µg/mL) for 5 min in a separate eppendorf tube (50 µL) at room temperature. The detection of the chicken IgY bound to antigen was conducted by incubating the swabs in 50 µL of chicken anti-mouse IgG-HP (1:5,000 diluted in 1% BSA) for 5 min at room temperature. The swabs were then carefully washed as described above and incubated with 50 µL of TMB substrate for color development. Control swabs were also incubated at the same time in PBS solution. Between steps, swabs were washed with PBS for 5 min. A digital camera with high pixel

size and optical zoom was used to capture images of the blue color with and without eppendorf tubes.

### 6.2.11 Colloidal gold IgY preparation

A 10 mL solution of 0.01% tetrachloroauric acid (gold chloride) in Milli-Q water was brought to boiling on a hot plate. A 0.2 mL sodium citrate solution (1%, w/v) was rapidly added to the boiling gold chloride solution and allowed to boil for 10 min until it developed a typical bright wine red color of colloidal gold, stirred continually and allowed to cool down gradually. The pH of the colloidal gold solution was maintained at 7.4 by addition of dilute 10mM $Na_2CO_3$ and storage at 4 °C in a dark-colored glass bottle until used. A 10 mL of colloidal gold solution is then stirred with 2.5 mL anti-gliadin IgY antibody (4 mg/mL) for 30 min with slow stirring. A 1% (wt/vol) BSA was added to block excess reactivity of the gold colloid, stirred for 30 min, incubated for 1 hr at RT, followed by centrifugation at 12,000 rpm for 30 min to remove unconjugated antibody from the solution. The pellets obtained were washed three times with 10mM Tris (pH 8.0) containing 1% BSA under centrifugation at 10,000 rpm for 30 min to remove traces of unconjugated antibody. The pellets were then resuspended in 2 mL of 10% sucrose, 1% BSA, and 0.05% sodium azide in phosphate buffer (pH 7.4). The gold immunoprobe was stored at 4 °C for the further use.

### 6.2.12 Immunostrip preparation

Nitrocellulose (NC) paper was cut into strips of 3 mm x 50 mm and marked for lines C, T and G, as shown in Figure 6-1. A 2 μL/ line of 1 μg/mL gliadin specific mAb (HYB 314) and 100 μg/mL rabbit anti-chicken IgG (1:100) in PBS was dispensed along line T and line C on the NC membrane, respectively. The antibodies were then fixed with 2 μL ethanol. The nitrocellulose strips are left to dry at for 30 min at 37 $^{o}$C. The remaining part of NC membrane was then blocked with a mixture of 5% BSA, 5% sucrose and 5% PVP in PBS (pH 7.4) for 1 hr at RT, then incubated for 30 min at 37 $^{o}$C. A 2 μL/ line of IgY-immunogold conjugate was then applied to the G line and incubated for 30 min. The immunostrip is ready to use and dipped into samples dissolved in 40% ethanol below line G.

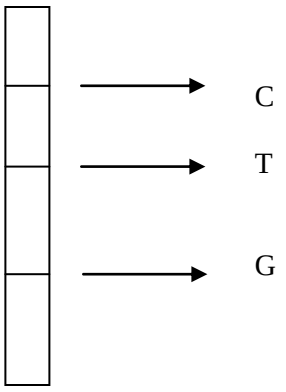


**Figure 6-1** Nitrocellulose paper marked with positions: C, control line; T, test line; G, immunogold line

### 6.2.13 Preparation of bread sample stock solution

Wheat bread (1 g) was blended in a food blender with 500 mL of 60% ethanol for 5 min at room temperature. The mixtures were centrifuged for 10 min at 2,500 × g at room temperature. One mL of the supernatant was serially diluted with 60% ethanol and 40% ethanol into eppendorf tubes for Immunoswab and Immunostrip assays, respectively.

## 6.3 Results

### 6.3.1 IgY antibody production

The changes of anti-gliadin antibody activity in the egg yolk from chickens hyperimmunized with gliadin peptides were determined by indirect ELISA (Figure 6-2). The activity of anti-gliadin IgY was detected on day 0, rapidly increased ($p < 0.05$) at week 2, and reached to the highest level at week 8 with a gradual reduction, thereafter.

The total IgY concentration in the egg yolk was fairly constant (9.2 ± 1.5 mg/mL) in the chickens hyperimmunized with gliadin during the whole experimental period. However, both protein and total IgY concentrations in the eggs of 4 – 10 weeks of immunization were similar regardless of the immunization. The titre of gliadin specific IgY pooled from egg yolks was 0.982 ± 0.045 OD. The titre is higher in the gliadin immunized chickens than in the non-immunized chickens ($p < 0.05$) shown at 0.078 ± 0.005 OD. In comparison, immunized chickens produced specific anti-gluten protein IgY that was approximately 13.6 times higher than that obtained from non-immunized chickens. The presence of specific IgY against gliadin in non-immunized chicken is expected from the commercial feed containing wheat grain as one of major feed ingredients in Canada.

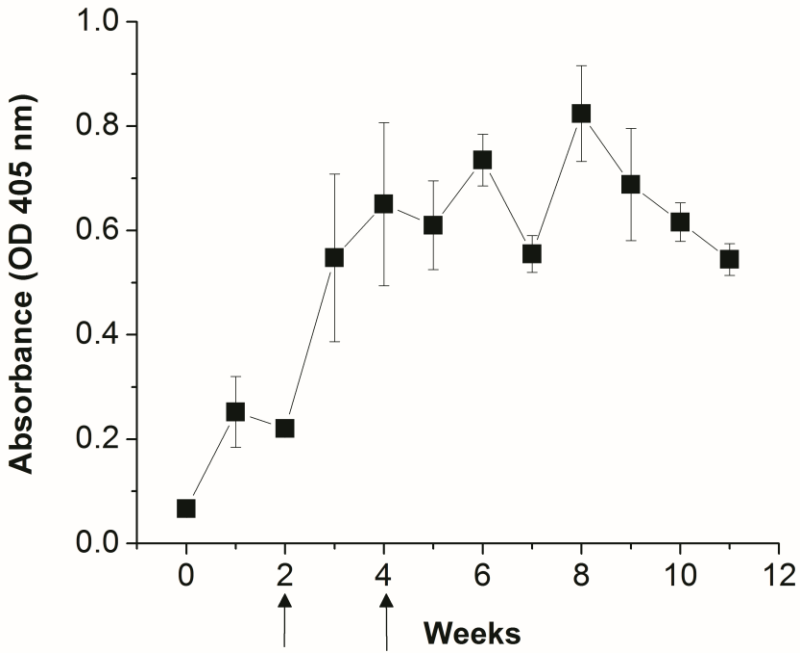


**Figure 6-2** Specific IgY antibody ELISA values in the egg yolk from chickens immunized with Sigma gliadin (200 µg/mL protein) in PBS, emulsified with Freund's incomplete adjuvant. Booster immunizations were given at 2 and 4 weeks after the initial immunization. Values are the mean of quadruple samples, with vertical bars indicating the standard deviation.

### 6.3.2 Purification of IgY antibody

For the development of the sensitive DAS-ELISA and Immunoswab assay in this study, crude IgY fractions from immunized hens were further purified by ammonium sulfate (60%) and Sephacryl S-300 gel chromatography. Both elution profile and ELISA values (OD 405 nm) of the IgY were determined (Figure 6-3). The peak fractions with molecular weight of 180 kDa at Kav 0.16 - 0.24 were pooled and then lyophilized for further uses. SDS-PAGE showed that the purity of IgY (180 kDa) at the peak of fractions was > 90% by electrophoresis (Figure 6-3).

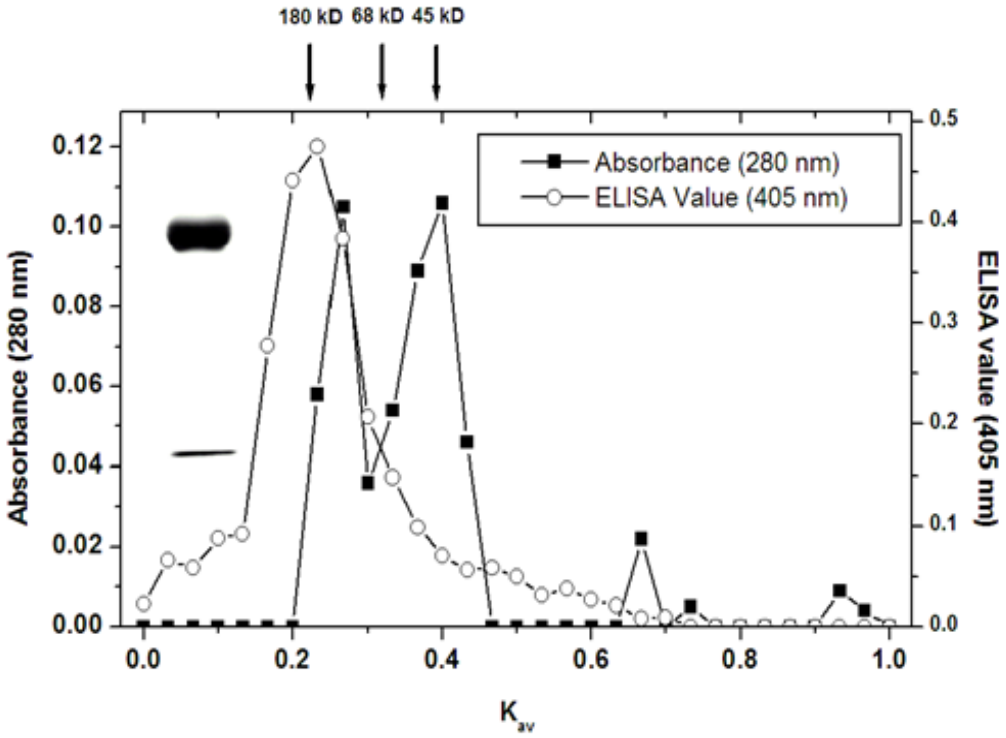


**Figure 6-3** The properties of water soluble egg yolk (absorbance at 280 nm) and IgY activity (ELISA) following fractionation by Sephacryl S-300 chromatography. Flow rate: 3 mL/hr, Column: 1.0 × 110 cm. The IgY fraction containing 180 kDa was verified by SDS-PAGE.

### 6.3.3 Double antibody sandwich ELISA for gliadin detection

The sandwich ELISA is a common enzyme immunoassay for the detection of certain proteins. In this study, the specificity and detection limit of DAS-ELISA were tested using anti-gliadin IgY and mAb (HYB-314) as capture and detection antibodies, respectively. For the test, different amounts (200-20 µg/well) of the IgY was coated on the plate and various dilutions (1:100-1:2,000) of mAb (HYB-314) was added and incubated at different times and temperatures. The pre-condition results aid in the development of the DAS-ELISA. The DAS-ELISA showed that the gliadin was detected as low as 5 ng/mL (Figure 6-4A) and the detectable range in the standard curve was between 10 and 80 ng/mL (Figure 6-4B). The low detection limit (10 ng/mL) could identify food samples containing as low as 1 mg gliadin per 100 g dry food, which is equivalent to 10 ppm of gliadin when diluted to 1:1,000 ratio.

To determine the reproducibility of the DAS-ELISA, the validity was examined further by precision testing. The intra- and inter-assay coefficient variations were 7.2% and 9.8%, respectively when they were evaluated by replicate measurements (n=6) of serially diluted gliadin fractions on a microtiter plate.

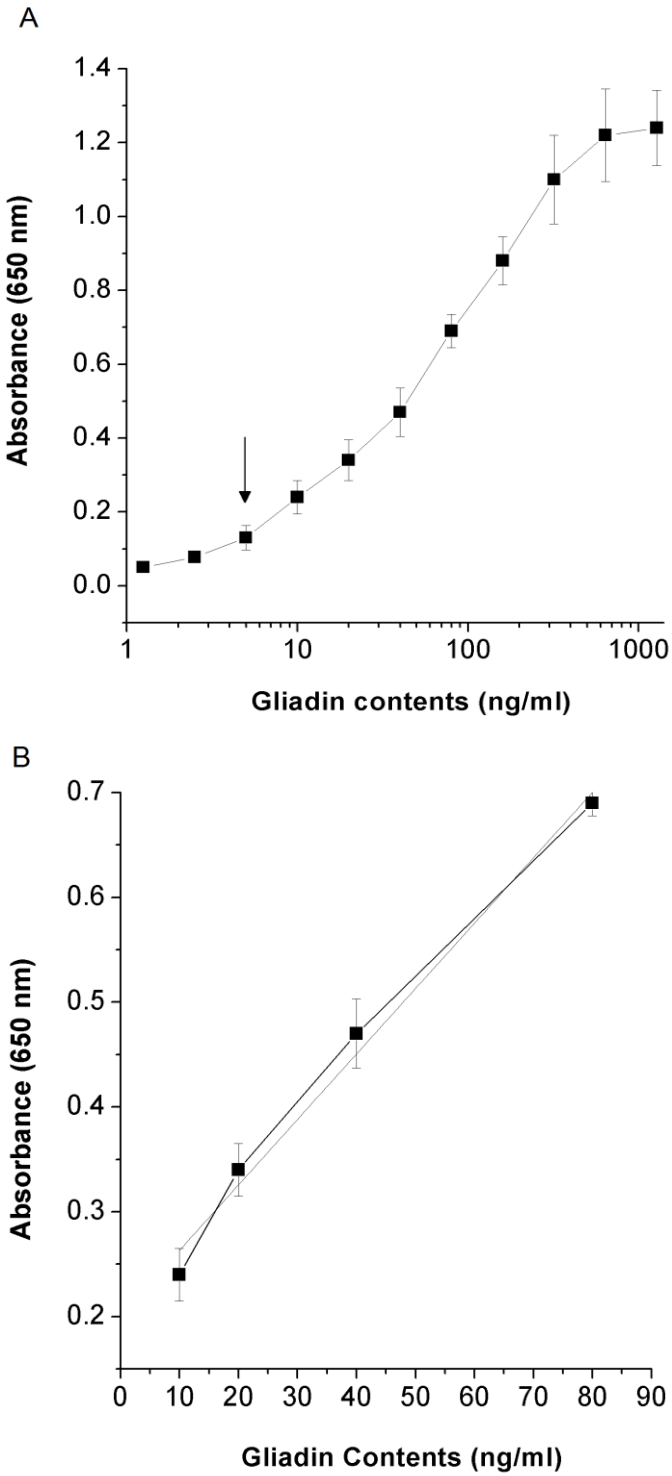


**Figure 6-4** Standard curve of DAS-ELISA for **(A)** the detection limit of 5 ng/mL of gliadin and; **(B)** the working linear range at 10 - 80 ng/mL of gliadin. Vertical bars indicate standard deviation. Straight line indicates the linear fit "$y = 0.006x + 0.201$, $R^2 = 0.99$".

Three bread samples were quantified with the DAS-ELISA for gliadin contents at dilutions of 1:512 to 1:8,192 from the stock sample preparation. Gliadin contents calculated based on reference to the standard curve, ranged from 23.65 ± 8.29 to 31.95 ± 5.96 mg/g of bread samples (Table 6-1), demonstrating 2.4-3.2% gliadin contents in these bread samples. Three gluten-free labelled samples were also quantified with the DAS-ELISA for gliadin contents at dilutions of 1:32 to 1:512 from the stock sample preparation. The gliadin content in the gluten-free products was determined to be lower than the limit of detection (5 ppm) of the DAS-ELISA developed.

**Table 6-1** Gliadin contents in bread samples at dilutions of 1:512 to 1:8,192 in PBS from the stock sample preparation by DAS-ELISA (ng/mL; mean ± SD).

| Samples | Dilutions | | | | |
|---|---|---|---|---|---|
| | 1:512 | 1:1,024 | 1:2,048 | 1:4,096 | 1:8,192 |
| Bread 1 | 96.2 ± 4.34 | 54.9 ± 9.33 | 31.2 ± 5.96 | 13.2 ± 5.48 | n/a |
| Bread 2 | 113.6 ± 10.24 | 60.4 ± 11.70 | 29.4 ± 6.87 | 11.3 ± 9.54 | 6.9 ± 4.93 |
| Bread 3 | 92.4 ± 8.29 | 57.2 ± 7.94 | 26.8 ± 5.72 | 12.8 ± 3.22 | n/a |

Note: n/a, OD value is out of range of standard curve.

### 6.3.4 Immunoswab assay

The development of antibody-based Immunoswab assay (Figure 6-5) involved 3 main steps: 1) sample application to the capture antibody (chicken egg yolk IgY) in coating swabs, 2) recognition of the captured gliadin by the detection antibody (monoclonal anti-gliadin IgG, HYB314-01), and 3) initiation of color reaction to the HP-labeled mAb (chicken anti-mouse IgG). The time required to perform the assay with both themonoclonal and polyclonal antibody was approximately 30 min excluding the time of coating IgY and blocking with BSA. The detection limit of Immunoswab was 1.25 µg/mL. The control swab without gliadin antigen showed no color in all the assays while the color intensity developed reflects the amount of gliadin present in the samples tested.

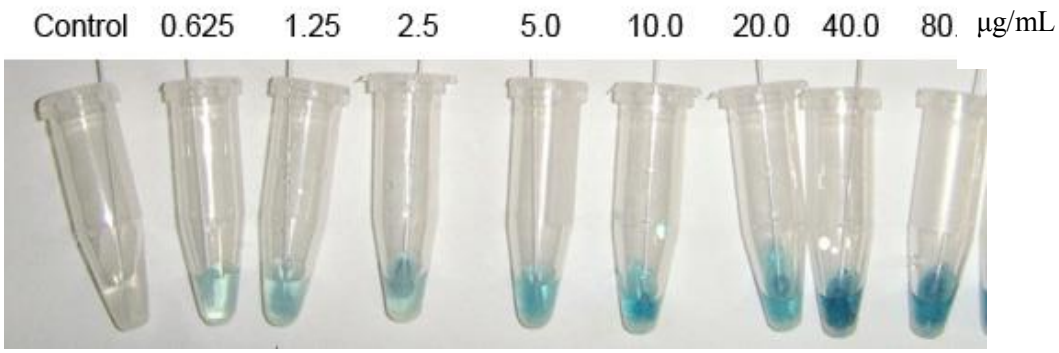


**Figure 6-5** Immunoswab assay for detection of gliadin diluted in 60% ethanol (0.625 – 80 µg/mL). Increased intensity of swab color observed with increasing gliadin concentration (from left to right) with the control swab (left side) tested in the absence of antigen.

Three bread samples were tested with the developed Immunoswab assay for detecting gliadin at dilutions of 1:2 to 1:32 from the stock sample preparation. Gliadin was detected in the assay and expressed by positive gliadin (+) (Table 6-2).

**Table 6-2** Gliadin detection in bread samples at dilutions of 1:2 to 1:128 from the stock sample preparation in 60% ethanol by Immunoswab assay.

| Samples | Dilutions | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 |
| Bread 1 | + | + | + | + | + | - | - |
| Bread 2 | + | + | + | + | + | + | - |
| Bread 3 | + | + | + | + | + | - | - |

Note: +, Positive gliadin; -, Negative gliadin

### 6.3.5 Immunostrip assay

The sensitivity of the test strip was determined by testing the sigma gliadin standard samples. The standards were prepared by dilution of the gliadin stock solution (1 mg/mL) with the 40% ethanol to reach the final concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 100 µg/mL. These standard samples were thereafter examined and judged by quantitative measurement of gliadin. The gliadin concentration was less than 0.1 µg/mL, if one band was visible as control line; and more than 0.25 µg/mL if two lines (test line and control line) were visible. For a gliadin concentration more than 100 µg/mL no band was observed at the test line (Figure 6-6).

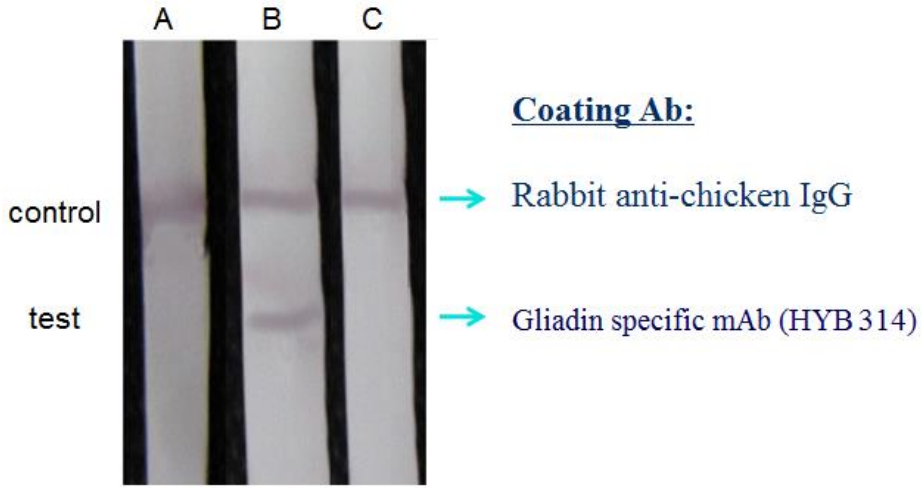


**Figure 6-6** Test results of working standards with different concentrations of gliadin in 40% ethanol using Immunostrips [**(A)** < 0.1 µg/mL; **(B)** 0.25 - 100 µg/mL; **(C)** ≥ 100 µg/mL].

The potential cross-reactivity with common grains was evaluated using wheat, barley, rye, oat, and rice flour. At appropriate dilutions the Immunostrip presents two bands (test and control) for all grains except for rice (Table 6-3)

**Table 6-3** Gliadin detection in flour samples at appropriate dilutions from the stock sample preparation in 40% ethanol by Immunostrip assay.

| Samples | Dilutions | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:5 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 |
| Wheat | + | + | + | + | + | - | - |
| Barley | + | + | + | + | + | + | - |
| Rye | + | + | + | + | | - | - |
| Oat | + | + | - | - | - | - | - |
| Rice | - | - | - | - | - | - | - |

Note: +, positive; -, negative

### 6.4 Discussions

Among gliadin peptides, a 33-mer peptide from α-gliadin was responsible for initiation of the inflammatory response for CD patients [16]. The development of immunoassays with chicken IgY could be a useful tool to detect gliadin in foods, the celiac disease protein. The gliadin is highly immunogenic to chickens and induces the production of greatly specific IgY in the yolk, from which the antibody can be partially purified by a simple dilution method [17]. In this study, highly purified egg yolk anti-gliadin IgY was obtained and used as a reagent for both Immunoswab and Immunostrip assays.

There are several reports regarding the proportion of specific antibody in total IgY when chickens were immunized with various antigens. The proportion of bovine proteoglycan-specific IgY in the total egg yolk IgY was 9.0% [18]. The percentage of specific IgY in total IgY from egg yolks ranged from approximately 7 to 16% against whole bacteria Fl cells including *Salmonella enteritidis*, *Salmonella typhimurium* [19], *E. coli* O157:H7 [20], *E. coli* 987P [21], and *Clostridium perfringens* [22]. The percentage of specific antibody in total IgY ranged from 5 (anti-insulin antibody) to 28% (anti-mouse IgG antibody) [23]. Our results showed 7.9% of the gliadin-specific IgY concentration in total IgY determined by the quantitative ELISA as previously reported [20].

The DAS-ELISA based Immunoswab and Immunostrip has been developed, comprising of capture and detection antibodies with high specificity due to the sandwich reaction of antibodies with an antigen. In some studies using mAb as both capturing and detecting antibodies, the detection of the gliadin is often poor due to single epitope targeting. For example, a homosandwich ELISA using a mAb to the α-gliadin 33-mer has a detection limit of 100 ng/mL gliadin [3], while the limit of hetero sandwich ELISA, with polyclonal antibody, was 15 ng/mL [4]. In the recent reports, using PN3 mAb antibody as competitive assays showed the detection limit of 1.2 ppm for Sigma gliadin [9]. The single antibody sandwich ELISA based on R5 mAb as both coating and detecting Ab showed the detection limit of 1.56 ppm gliadin [8].

Since IgY based DAS-ELISA showed the similar detection limit of gliadin ranged from 5 ng in our preliminary study, the specificity of IgY described here makes it well suited for simple and rapid determination of gliadin in foods. There is a growing desire for new gliadin assay that is easy to use and accessible on site compared to the cumbersome ELISA. Immunoswab and Immunostrip are faster and more cost-effective options in screening as compared to the traditional ELISA, requiring specific instruments such as microplate reader and washer.

The Immunoswab assay, developed with the IgY as coating antibody and HYB 314 as detection antibody showed the detection limit of 1.25 µg/mL. When tested with breads after diluting with 60% ethanol, the gliadin contents from 21.76 to 0.34 µg/mL. In Table 5.1, the presence of gliadin is expressed as positive gliadin (+) Immunoswabs for dilutions 1:2 to 1:32 (equivalent to 21.76 to 1.36 µg/mL by previously developed DAS-ELISA), which complies with the detection limit of the Immunoswab mentioned earlier.
The Immunostrip assay, developed with the HYB 314 as the coating antibody and IgY as the detection antibody showed the detection limit of 0.25 µg/mL. When tested with different types of flours after diluting with 40% ethanol, the gliadin contents from 89.09 to 0.54 µg/mL show positive results (2 visible bands). In Table 5.2, the presence of gliadin is expressed as positive gliadin (+) on the Immunostrip at their appropriate dilutions, which complies with the detection limit of the Immunostrip mentioned earlier. The Immunostrip showed no cross-reactivity with non-gliadin proteins.

The two detection systems (Immunoswab and Immunostrip) were developed with the combination of highly specific IgY antibody and mAb against gliadin. Since IgY can be simply produced in large quantity with a high titer, it was used to replace other sources of polyclonal antibodies or monoclonal antibodies which were conventionally used. IgY

egg yolk antibody can be an alternative antibody source of Immunoswab and Immunostrip by using as capturing or detecting reagent along with other mAbs (HYB, R5, PN3 or others) as capturing or detection antibody to effectively reduce the cost and limit of detection. Both, Immunoswab and Immunostrip detection systems have low detection limits of 1.25 μg/mL and 0.25 μg/mL, respectively, enabling gliadin detection in foods as well as foods rendered gluten free (1-200 ppm).

Celiac patients and restaurants can use this detection system to test gliadin contamination in food in order to prevent immunogenic reaction to susceptible persons. Industries can also employ the use of this rapid test for quality assurance of raw material as well as for monitoring gluten contamination throughout the food processing. Although the detection limit of the both assays are greatly influenced by the types of foods, the method generally detects gliadin as low as 1 ppm or less in foods.

## 6.5 References


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# CHAPTER 7: General discussion, conclusions, and future directions

## 7.1 General discussion

Recent findings estimate about 2 million of the population in North America have CD. CD is an autoimmune disorder in genetically susceptible individuals on gluten-containing diet. Gluten is protein found in wheat, rye, barley, and in some patients, oats. In gluten-sensitive CD patients, gluten peptides (for example, gliadin a small proline-rich peptide from dietary gluten in wheat) readily enter intestinal cells, activate host T cell receptors, stimulate cytokine release/activity and trigger an inflammatory reaction. Consuming gluten causes an immunological reaction within the inner lining of the small intestine, which damages the tissues and results in impaired ability to absorb nutrients from foods. The inflammation and inability to absorb nutrients creates wide-ranging problems in many systems of the body including gastrointestinal problems, dermatitis herpetiformis, anemia and osteoporosis [1]. To-date, there is no cure for CD. The only option for this group of individuals is a strict gluten-free diet (GFD) that must be followed for life.

Several therapeutic approaches have been attempted to neutralize gliadin and/or prevent gliadin absorption which will lead to inhibition of the initial step of gliadin-induced toxicity in CD individuals. ALV003, a mixture of glutenase and endoprotease was reported to hydrolyse toxic gliadin peptide [2]. These enzymes may hydrolyse other peptides in the gastrointestinal tract (GIT) other than gliadin. Another therapeutic candidate being extensively studied is Larazotide which is used to prevention of toxic gliadin peptide absorption by inhibition of zonulin [3]. This drug candidate inhibits the paracellular route of gliadin absorption through tight junctions, which is not the only mechanism of gliadin absorption. Indeed, gliadin may gain access to the mucosa through transcellular pathways in addition to paracellular route. Hence, this strategy might be best exploited in combination with other treatments to inhibit all routes of gliadin absorption. P(HEMA- co -SS) is another intersting polymer reported to attenuated gliadin-induced changes in permeability and inflammation [4]. Further investigation of the mechanisms of action and its interaction with human tissues is required. All these therapeutic candidates are promising but still further studies are warranted.

The realization of the use of antibody to neutralize toxins in the GIT gave rise to our laboratory to develop polyclonal egg yolk antibody to prevent CD manifestations induced by gastrointestinal absorption. Oral antibody passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the GIT [5]. 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 [6]. Chickens can produce high titre 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.). In literature, IgY antibody has been proved to neutralize disease causing pathogens (i.e., Rotavirus [7], *E. coli O157:H7* [8], *Salmonella enteritis* [9], *Clostridium perfringens* [10]) in the human GIT. In this context, we have recently explored a novel anti-gliadin IgY as an alternative to treat CD.

The main goal was to produce and formulate anti-gliadin IgY antibody formulation and to evaluate the potency and efficacy of anti-gliadin IgY as a CD alternative therapeutic agent in *in vitro*, *ex vivo* and *in vivo* systems.

In the first part of this work (chapter 2), we have shown the methodology of production and purification of anti-gliadin IgY in egg yolks of hyperimmunized laying hens. Gliadin peptides are components of a human diet, but are found to be highly immunogenic in laying hens [11], due to high phylogenetic differences between avian and mammalian species. In this study an 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 [12].

Since there is no animal model reproducing all features of CD [13], a colon carcinoma cell line (Caco-2) was used evaluate anti-gliadin IgY efficacy. In chapter 3, we have reported for the first time the evaluation of the anti-gliadin IgY efficacy in a Caco-2 cell culture system. Pepsin-trypsin treated gliadin (PT-gliadin) has been shown to trigger the production of pro-inflammatory cytokines (TNF-α and IL-1β), explained by the activation of pro-inflammatory pathways (NF-kB). NF-kB is known to be activated in small intestinal mucosa of CD patients [14], and gluten peptides have been shown to upregulate the expression of cytokines such as TNF-α [15] and IL-1β [16], related to the innate immune response. The presence of anti-gliadin IgY with intestinal digests of gliadin as low as (1:6,000) reduced the ability of gliadin peptides to activate TNF-α production, and completely abolished the IL-1β production. The latter is explained by inhibition of NF-kB (nuclear p65 subunit) induction due to the gliadin neutralization efficacy of anti-gliadin IgY.

To understand the mechanism by which anti-gliadin IgY is able to prevent the proinflammatory activity of gliadin peptide, it would be important to know if anti-gliadin IgY may inhibit the entrance of the gliadin peptide in the cell. In this regard, the penetration of gliadin peptide was evaluated in the presence or in the absence of anti-gliadin IgY. Our results strongly suggest that anti-gliadin IgY is an inhibitor of gliadin absorption in the cell, measured by ELISA.

In chapter 4, stability and efficacy of the formulations were determined. 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 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 [17, 18]. 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 [19], whereas another report showed that IgY titre was very stable under spray-drying temperatures of 140 to 170 °C with 30% Eudragit L30D-55 as protectant [20]. In this study, mannitol, sorbitol and MCCP at the concentration of 37.5% (on dry basis) significantly protected IgY from heat denaturation, while EYP (egg yolk powder) 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 long-term stability of 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 ºC for 52 weeks [21]. In another study [23], sorbitol was used during the spray dry 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 properties of IgG and not the actual antibody activity during the storage condition. 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 during long-term storage.

In order to effectively use anti-gliadin IgY as an oral immunotherapy to prevent gliadin absorption in the small intestine region, it needs to be active in the stomach and proximal part of the small intestine. Studies have shown that, purified IgY is extremely sensitive to gastric conditions, and is rapidly inactivated [24], 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 [25, 26]. 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 literature, such as microencapsulation with β-cyclodextrin and gum arabic and lecithin/cholesterol liposome [19], and the employment of the pH-sensitive methacrylic acid copolymer as enteric coating of IgY [27]. High concentration of sugar (30-50%) [17], or sorbitol (30% or more) [28], have 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 assure 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 stability of purified IgY from egg yolk without protectants against pepsin enzyme at lower enzyme/substrate ratios such as 1:20 [29], and 1:250 [27]. 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 (egg yolk power containing mannitol) and EYP-Mc (egg yolk power containing microcrystalline cellulose) capsule formulations retained over 80% activity after 2 hr 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 [30]. Also, changes in preferential solvation of protein molecules [31], and structural blockage of conformational changes of internal tryptophan residues when placed in sugar solutions may be partly responsible for added protection [17, 28]. The non-extracted egg yolk contains phospholipids and other proteins, which may have also contributed to additional gastroprotection effect.

The binding efficacy of anti-gliadin IgY was 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 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 those of human of 20-30 hr [32]. The absorption of gliadin in experimental mice was found to achieve peak values between 2-3 hr after ingestion [33]. The transit time through the stomach is highly variable with an average time of 1-1.5 hr [34], and an average of 10 hr through the intestine for mice [35, 36]. Hence, in this study we chose to determine the residual gliadin content in the mice GIT at 12 hr 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 1:25 and 1:5 to gliadin (equivalent to 1:16,340 and 1:3,268 anti-gliadin IgY to gliadin, respectively), prevented 63.3% and 98.4% gliadin absorption, respectively, as compared to 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 co-administered with gliadin contained food.

The anti-gliadin IgY produced was applied further as immunological diagnostic reagent for quantitative Double sandwich ELISA (DAS-ELISA) detection system in Chapter 5. The ELISA was successfully used to quantify extracted gliadin, in both gliadin-spiked gluten free foods and in various commercial foods known to contain wheat gluten. The DAS-ELISA was constructed using purified polyclonal IgY as capture antibody and biotin labeled mAb (HYB 314) as detecting antibody. The anti-gliadin IgY recognized α/β/γ-gliadin at 30-45 kDa and ω-gliadin at 39-55 kDa [37, 38].

There are several reports regarding the proportion of specific IgY in total IgY when chickens were immunized with various antigens. The proportion of bovine proteoglycan-specific IgY in the total IgY was 9.0% [39]. Similarly, the proportions of bovine and human lactoferrin-specific IgY in the total IgY were 8.7% and 9.2%, respectively [40]. In this study, our results showed 7.9% of the specific anti-gliadin IgY concentration in total IgY by quantitative ELISA. The use of purified specific anti-gliadin IgY antibody could have resulted in higher specificity by maximizing the detection of gliadin in complex food matrixes. A single mAb based ELISA has the drawback that it may not detect altered or destroyed epitopes during the food processing. This remains to be evaluated for gliadin, but in general, it is accepted that the combination of mAb and polyclonal antibody is favorable for gliadin analysis and resulted in higher specificity. The observed cross-reactions of anti-gliadin IgY antibody by indirect ELISA are obviously due to similar epitopes on the different four grains, which are highly found in barley hordein and less in rye secalin and oat avenin. Western blot analysis using the anti-gliadin IgY antibody was performed to confirm the ELISA data. The possibility of cross-reaction with other less commonly used grass family, sorghum, millet, wild rice, etc., cannot be excluded and remain to be examined. A rabbit polyclonal IgG antibody used to detect wheat gliadin cross-reacted with oat, rice and maize prolamins [41]. On the other hand, the specificity of the mAb was also tested against four grains by ELISA and western blot assay. The mAb (HYB 314) used in this study recognizes residue p58-73 (LQLQPFPQPQLPYPQPQLPYPQPQLP YPQPQPF) of 33-mer peptide of prolamins from wheat, barley and rye in the range of 34-38 kDa, demonstrating no cross-reaction to oat avenin. PN3 [42], CDC5 [43], and G12 [44], mAbs react with prolamins in wheat, barley, rye and oat, whereas R5 reacts with prolamins from wheat, barley and rye only [45].

The key parameters and characteristics of the DAS-ELISA were further subjected to in-house validation. The accuracy of the method was studied with recovery experiments, by adding varying amounts of Sigma gliadin standard to three different blank gluten-free food matrixes such as bread, pasta and rice cake. The variances were highest at the lowest amounts of spiked Sigma gliadin (1 µg/g), probably because of poor sample homogeneity at this low level. The recovery of Sigma gliadin in matrixes was almost complete at higher dose of Sigma (100 µg/g), whereas it was reduced by 15% from lowest amount of spiked Sigma gliadin. The results agree with the common assumption that food matrix components may interfere with either the extraction or the

immunoassay procedures leading to suboptimal recovery rates. A full validation of the extraction procedure remains to be performed. The recovery of the gliadin in DAS-ELISA was considered to be satisfactory. The intra-assay and inter-assay precision results for DAS-ELISA were obtained from gliadin contents of six relevant gliadin-containing wheat grain based foods, ranging from 3.2-36.0 ppm. The repeatability of the DAS-ELISA as measured by intra-assay precision was 7.25%, and the reproducibility as measured by interassay precision was 9.51%. The performance of the present DAS-ELISA is 0.8 ppm gliadin in terms of sensitivity, which is lower than the US Food and Drug Administration limit (August 2011) of <20 ppm gluten (equivalent to 10 ppm gliadin) for gluten-free foods. The three Canadian gluten-free labeled products quantified, containing less than 10 ppm gliadin, met the proposed FDA rule for gluten-free labelling. The developed DAS-ELISA showed less sensitive to detect gliadin in foods compared to other ELISA systems. For instance, mAb to the α-gliadin motifs QQQPFP of 33-mer shows a LOD of 1.56 ppm gliadin [45]. Competitive ELISA system using PN3 mAb antibody showed LOD of 1.2 ppm gliadin [46]. Whereas homosandwich ELISA using mAb as both capture and detection antibodies may not detect hydrolyzed gliadin in processed foods due to single targeting of the gliadin fractions, heterosandwich ELISA with polyclonal antibody and mAb detected the gliadin at LOD of 3.3 - 15 ng/mL [47, 48]. In this study we have significantly lowered the detection limit to 0.8 ppm, which should allow detection of trace amount of gliadin in various foods including gluten-free foods by using a combination of IgY and mAb.

The DAS-ELISA for the quantification of gliadin in foods was developed using anti-gliadin IgY as a capture antibody and biotinylated mAb (HYB 314) as a detection antibody. It is capable to detect trace amounts of gliadin as low as 0.8 ppm in food. The use of IgY as a capture antibody reduces the cost as compared to mAb coated on ELISA plates. Therefore, the combination of anti-gliadin IgY and biotinylated mAb (HYB 314) provides a broad range of detection, increases sensitivity, and reduces cost of reagents used in the detection system, making it suitable for the use as a quality control tool in laboratories, food manufacturing industries and by regulatory agencies to perform detection of gliadin in both gluten-free and gluten-contained food products.

In Chapter 6, DAS-ELISA based Immunoswab and Immunostrip has been developed, comprising of capture and detection antibodies with high specificity due to the sandwich reaction of antibodies with an antigen. In some studies using mAb as both capturing and detecting antibodies, the detection of the gliadin is often poor due to single epitope targeting. For example, a homosandwich ELISA using a mAb to the α-gliadin 33-mer has a detection limit of 100 ng/mL gliadin [49], while the limit of hetero sandwich ELISA, with polyclonal antibody, was 15 ng/mL [50]. In the recent reports, using PN3 mAb antibody as competitive assays showed the detection limit of 1.2 ppm for Sigma gliadin [46]. The single antibody sandwich ELISA based on R5 mAb as both coating and detecting antibody showed the detection limit of 1.56 ppm gliadin [45].

Since IgY based DAS-ELISA showed the similar detection limit of gliadin ranged from 5 ng in our preliminary study, the specificity of IgY described here makes it well suited for simple and rapid determination of gliadin in foods. There is a growing desire for new gliadin assay that is easy to use and accessible on site compared to the cumbersome ELISA. Immunoswab and Immunostrip are faster and more cost-effective options in screening as compared to the traditional ELISA, requiring specific instruments such as microplate reader and washer.

The Immunoswab assay, developed with the IgY as the coating antibody and HYB 314 as detection antibody showed the detection limit of 1.25 µg/mL. When tested with breads after diluting with 60% ethanol, the gliadin contents ranged from 21.76 to 0.34 µg/mL. The presence of gliadin is expressed as positive gliadin (+) Immunoswabs for dilutions 1:2 to 1:32 (equivalent to 21.76 to 1.36 µg/mL by previously developed DAS-ELISA), which complies with the detection limit of the Immunoswab.

The Immunostrip assay, developed with the HYB 314 as coating antibody and IgY as detection antibody showed the detection limit of 0.25 µg/mL. When tested with different types of flours after diluting with 40% ethanol, the gliadin contents from 89.09 to 0.54 µg/mL show positive results (2 visible bands). The presence of gliadin is expressed as positive gliadin (+) on the Immunostrip at their appropriate dilutions, which complies with the detection limit of the Immunostrip mentioned earlier. The Immunostrip showed no cross-reactivity with non-gliadin proteins.

The two detection systems (Immunoswab and Immunostrip) were developed with the combination of highly specific IgY antibody and mAb against gliadin. Since IgY can be simply produced in large quantity with a high titer, it was used to replace other sources of polyclonal antibodies or monoclonal antibodies which were conventionally used. IgY egg yolk antibody can be an alternative antibody source of Immunoswab and Immunostrip by using as capturing or detecting reagent along with other mAbs (HYB, R5, PN3 or others) as capturing or detection antibody to effectively reduce the cost and limit of detection. Both Immunoswab and Immunostrip detection systems have low detection limits of 1.25 μg/mL and 0.25 μg/mL, respectively, enabling gliadin detection in foods as well as foods rendered gluten free (1-200 ppm).

Celiac patients and restaurants can use this detection system to test gliadin contamination in food in order to prevent immunogenic reaction to susceptible persons. Industries can also employ the use of this rapid test for quality assurance of raw material as well as for monitoring gluten contamination throughout the food processing. Although the detection limit of the both assays are greatly influenced by the types of foods, the method generally detects gliadin as low as 1 ppm or less in foods.

## 7.2 Conclusion

In this study we report on the development of anti-gliadin IgY antibody

1. We have successful produced anti-gliadin antibody in egg yolks of hyperimmunized hens. Our result suggests that gliadin is highly immunogenic to hens and thus produce high antibody titre of gliadin-specific antibody, which can be used for development of oral passive immunotherapy as well as diagnostic reagent for gliadin detection.
2. We have performed *ex-vivo* proof-of-concept of anti-gliadin efficacy in human colon carcinoma cell (Caco2) cultures. The toxic gliadin showed to have significantly translocated from the apical compartment of Caco2 cells into the basal compartment and cytokines (IL-1β and TNF-α) production was significantly induced. The passage of gliadin into the basal compartment and cytokine production were significantly reduced in the presence of anti-gliadin IgY.
3. We have processed egg yolk IgY by spray dry. The optimization of spray dry conditions under variable inlet and outlet temperature settings was performed. Three carbohydrates were chosen as protectants against heat during the spray dry process and the protection effect was verified at different protectant concentrations and long-term stability test of the spray dried formulations were performed and stability tests were performed during the storage period. Of the three formulations, EYP-M had the highest long-term stability,
4. We have evaluated *in vitro* stability and release from gelatin encapsulated spray dry formulations with a dissolution apparatus (USP 27) under simulated gastric and intestinal conditions. The binding affinity of gliadin spiked with food/without food and anti-gliadin IgY formulation were also performed under the simulated gut conditions. EYP-M formulation remained resistant against harsh gastrointestinal conditions and effective in neutralization of gliadin under simulated gastric and intestinal conditions.
5. We have performed *in vivo* mouse and anti-gliadin IgY formulation feeding study with spiked gliadin to evaluate binding efficacy of IgY with gliadin. Our result suggests that anti-gliadin prevented > 99% absorption of gliadin.
6. Finally, we also developed highly sensitive quantitative ELISA-based detection systems as well as rapid qualitative detection systems for gliadin in food.

## 7.3 Future direction

In this study, we have demonstrated anti-gliadin IgY to be effective to neutralize gliadin in *in vitro, ex vivo* and *in vivo* systems. This anti-gliadin IgY formulation is proposed as a prophylaxis to CD patients who have a well-controlled GFD and wish to reintroduce gluten into their diet. Also to patients who are having persistent mild CD symptoms despite of being on a GFD (meaning that this group is being exposed to unknown sources or contaminated gliadin). Dosing is required depending on the amount of gliadin consumed. To prove this, a Phase I human clinical trial will be performed in CD patients having persistent CD-related symptoms despite of being on a GFD. Our anti-gliadin IgY formulation is aimed to reduce CD associated symptoms and improve clinical outcome in CD subjects exposed to unknown sources of gluten.

This approach of oral administration of anti-gliadin IgY with every meal will render these CD subjects a better quality of life due to restored intestinal epithelium. The absorption of nutrients will be regained. To further prove this concept, mild to moderate individuals with CD symptoms on a GFD will be recruited. The clinical trial will be undertaken to prove the hypothesis that anti-gliadin IgY antibody formulation can prevent the gastrointestinal symptoms, abnormal serology and cytokine markers in adult CD individuals exposed to unknown sources of gluten.

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